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Acute and chronic role of the 5-HT_3 neurotransmission on cholecystokinin tetrapeptide-induced panic symptoms in humans

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☙ *SUMMARY* ❧

Evidence suggests a role for cholecystokinin (CCK) in the neurobiology of panic disorder. Intravenous administration of cholecystokinin tetrapeptide (CCK-4) has been shown to induce panic attacks in healthy subjects and in panic disorder patients. The enhanced sensitivity to CCK-4 seen in panic disorder patients suggests anomalies in the CCK system. Results from investigations also show a role for serotonin (5-HT) in panicogenesis. Anxiolytic activity has been reported for 5-HT_3 receptor antagonists in various animal models and in humans. Moreover, experimental studies support a relationship between the 5-HT_3 and CCK systems.

The objective of this work was to evaluate the role of the 5-HT_3 system in CCK-4-induced panic-like symptoms. The effect of single and multiple oral doses of ondansetron, a selective 5-HT_3 receptor antagonist, on the CCK-4-induced behavioural and neuroendocrine changes was first examined in healthy male subjects. Then, the effect of ondansetron on total CCK (CCK_T) plasma concentrations was investigated in the same subject population. According to a double-blind design, 41 subjects were randomised to receive intravenous CCK-4 challenge test 60 minutes after a single (2 mg) and multiple (2 mg twice daily for 28 days) oral doses of either ondansetron or placebo.

Compared to placebo, subjects who received a single dose of ondansetron showed a significant decrease in the sum intensity of panic-like symptoms (iPSS) and in the anxiety score at peak effect of CCK-4. The mean basal plasma concentration of neuropeptide Y (NPY) was significantly higher and means of the maximal changes in cortisol, growth hormone (GH) and prolactin (PRL) secretion from baseline (Δmax) were significantly lower in the ondansetron group. There was no difference in Δmax for ACTH and NPY between groups. After chronic administration, there was no

difference in iPSS between groups. The mean basal NPY plasma level was significantly higher, whereas Δmax for NPY significantly lower in the ondansetron group as compared to placebo. The reduction in Δmax for GH and PRL observed in the ondansetron group as compared to the placebo group failed to reach significance. There was no difference between groups with respect to the Δmax for ACTH and cortisol.

There was no difference in either basal or stimulated vital sign profiles, CCK_T plasma levels, and CCK_T elimination rate constant between the ondansetron and placebo groups after the administration of either single or multiple doses.

Results suggest the following. First, decrease in behavioural and neuroendocrine responses provide strong evidence for an action of ondansetron single dose administration on CCK-4 and support a role for 5-HT_3 receptors in CCK-4-induced panic-like symptoms in healthy subjects. Second, single and multiple doses of ondansetron affect basal NPY and CCK-4-induced changes in NPY. Hence, one may suggest that 5-HT_3 may serve as an important regulator of basal and CCK-4-stimulated NPY release. Third, our results suggest a role for 5-HT_3 receptors in the neurobiology of anxiety and panic attacks through its interaction with CCK and NPY systems. Finally, the effect of ondansetron chronic administration on CCK-4-induced panic-like symptoms and on the different CCK component fractions still need exploration.

∞ *RÉSUMÉ* ∞

Bien que l'on comprenne mieux le trouble de panique, nos connaissances sur la maladie restent attachées à des notions hypothétiques. La coexistence de divers neurotransmetteurs de même que leur interaction rendent encore plus difficile l'accession à une meilleure compréhension de la neurobiologie sous-jacente à ce trouble. On croit que la panique pourrait résulter de perturbations de la fonction régulatrice au sein de divers systèmes de neurotransmetteurs se trouvant en interaction complexe. Actuellement, les preuves dont nous disposons indiquent qu'il pourrait s'agir d'un dysfonctionnement au niveau de la neurotransmission de la cholécystokinine (CCK) et de la sérotonine (5-HT).

La cholécystokinine est un neuropeptide présent dans le cerveau. Chez les rats, l'anxiété provoque une élévation des taux de CCK dans les régions limbique et corticale du cerveau. On sait que ces deux régions sont impliquées dans le traitement des stimuli externes et leur conversion en réactions émotionnelles. La modulation de la neurotransmission de la 5-HT peut également influencer le comportement animal dans les modèles d'anxiété expérimentaux qui sont mis à l'essai. Actuellement, le modèle animal caractérisant le trouble de panique chez l'humain reste à établir. En conséquence, des efforts considérables ont été consacrés à la recherche d'agents probants permettant d'étudier le trouble de panique chez l'humain. À cet égard, Bradwejn et ses collègues ont montré que le tétrapeptide de la cholécystokinine (CCK-4) pouvait constituer un exemple type adéquat pour l'étude de la neurobiologie de la panique. Récemment, ce groupe de chercheurs a découvert que les effets de l'administration intraveineuse de CCK-4 imitaient les symptômes de type panique chez l'humain. Les effets panicogènes sont reproductibles, fonctions de la dose et très proches de ceux qu'éprouvent les patients. Alors que la diminution aiguë des taux de tryptophane provoquait une intensification de l'effet panicogène, le traitement aux

inhibiteurs de recaptage de la 5-HT a eu pour résultat de prévenir l'effet panicogène induit par le CCK-4, ce qui justifie la théorie voulant que la complicité et l'interdépendance des systèmes 5-HT et CCK puissent être à la base de la panique. À la lumière de ces découvertes, il apparaît que la CCK endogène ayant été libérée en conséquence de l'anxiété puisse activer en retour le système central 5-HT. Toutefois, la classe de récepteurs de 5-HT qui pourrait être impliquée dans la médiation de l'effet panicogène de la CCK reste à déterminer.

Par conséquent, le principal but de ce travail a consisté à évaluer le rôle du circuit 5-HT_3 dans la panicogenèse. L'influence du blocage tant aigu que chronique des récepteurs 5-HT_3 sur les changements comportementaux et endocriniens induits par le CCK-4 a été évaluée chez des sujets masculins en santé. Le blocage des récepteurs a été réalisé grâce à l'administration orale d'ondansétron, un antagoniste sélectif des récepteurs 5-HT_3. L'évaluation de l'effet de l'administration d'une dose unique d'ondansétron, par rapport à une administration chronique, a fourni un aperçu d'un possible phénomène de neuro-adaptation pouvant survenir à la suite de l'administration chronique d'ondansétron. De plus, c'était la première fois que l'effet de l'ondansétron sur les concentrations plasmatiques totales de la CCK (CCK_T) chez les humains, après administration intraveineuse de CCK-4, faisait l'objet d'une investigation.

Conformément à la méthode de l'essai à double insu en parallèle, avec groupe placebo, 41 sujets ont été répartis au hasard dans l'un des deux groupes de traitement selon un schéma de répartition 1:2. Selon le groupe auquel ils appartenaient, les sujets ont été soumis au test de provocation au CCK-4 à raison d'une injection rapide de 50 µg, après avoir reçu par voie orale une dose unique (2 mg) et des doses multiples (2 mg, 2 fois par jour pendant 28 jours) d'ondansétron ou de placebo. Le CCK-4 était administré 60 minutes après la première et la dernière dose. L'intervalle entre les tests de provocation était de 28 jours.

Comparativement à ceux du groupe placebo, les sujets ayant reçu une dose unique d'ondansétron ont montré une diminution significative de l'intensité des symptômes de type panique (iPSS) et une diminution significative du sentiment d'anxiété, tel qu'évalué par les sujets sur l'échelle visuelle analogique (VAS) au pic de l'effet du CCK-4. Ces résultats ont été accompagnés d'une diminution, non significative toutefois, du nombre de symptômes de type panique (nPSS). Dans le groupe ondansétron, le taux moyen de base du neuropeptide Y (NPY) dans le plasma était significativement plus élevé alors que les taux maximaux moyens des changements (Δmax) au niveau de la sécrétion du cortisol, de l'hormone de croissance (GH) et de la prolactine (PRL) par rapport à la ligne de base étaient significativement plus bas. On n'a décelé aucune différence significative dans les Δmax de l'ACTH et du NPY, de même que dans les profils des signes vitaux de base ou dans ceux qui sont stimulés par le CCK-4 entre les deux groupes. Aucune différence significative n'a été relevée entre le groupe ayant reçu une dose unique d'ondansétron et le groupe placebo pour ce qui est des taux des concentrations plasmatiques de la CCK_T et de la constante de vitesse d'élimination de la CCK_T.

Après l'administration chronique, on n'a relevé aucune différence statistique au niveau des iPSS, nPSS et VAS entre les deux groupes. Dans le groupe ondansétron comparativement au groupe placebo, le taux moyen de base du NPY plasmatique était significativement plus élevé alors que le Δmax pour le NPY était significativement plus bas. On a remarqué dans le groupe ondansétron une diminution, non significative toutefois, dans les Δmax de la PRL et de GH. On a observé aucune différence significative dans les Δmax de l'ACTH et du cortisol entre les deux groupes. En ce qui a trait aux signes vitaux, aucune différence significative n'a été relevée entre le groupe ondansétron et le groupe placebo. Une interaction significative entre la première et la deuxième injection du CCK-4 a été observée dans chacun des deux groupes au niveau des mesures comportementales. Toutefois, l'effet de période sur les paramètres neuroendocriniens n'a été statistiquement significatif que dans le

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En conclusion, les résultats de cette étude permettent de supposer les éléments suivants: Premièrement, l'atténuation des réponses comportementales et neuroendocriniennes constitue la preuve manifeste que l'administration d'une dose unique d'ondansétron exerce une action sur le CCK-4, et elle permet en outre de confirmer le rôle que les récepteurs 5-HT_3 jouent dans les attaques de panique qui sont induites par le CCK-4 chez les sujets sains. Deuxièmement, l'ondansétron administré tant en dose unique qu'en doses multiples a un effet sur les taux de base du NPY et sur les changements enregistrés dans ces taux sous l'action du CCK-4. En conséquence, on peut en déduire que le 5-HT_3 pourrait posséder une importante action régulatrice sur la libération du NPY de base et du NPY stimulé par le CCK-4. Troisièmement, nos résultats permettent de supposer que les récepteurs 5-HT_3 jouent un rôle dans la neurobiologie des attaques d'anxiété et de panique, par le biais de son interaction avec les systèmes CCK et NPY. Finalement, l'ondansétron n'a aucun effet sur les concentrations plasmatiques de la CCK_T. Toutefois, l'effet de l'ondansétron sur les concentrations plasmatiques des différentes formes moléculaires de la CCK n'a pas été évalué dans le cadre de cette étude. Quant à l'effet chronique de l'ondansétron sur les changements comportementaux induits par le CCK-4, il reste à explorer.

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℘ LIST OF ABBREVIATIONS AND SYMBOLS ℘

ACTH.....	Adrenocorticotropic hormone
AUC.....	Area under the plasma concentrations as function of time curve
AUC_{0-12}.....	Area under the plasma concentrations as function of time curve from 0 to 12 hours
$AUC_{0-\infty}$.....	Area under the plasma concentrations as function of time curve from 0 to infinity
BID.....	Twice daily
CCK.....	Cholecystokinin
CCK_T.....	Total cholecystokinin plasma concentration
CCK-4.....	Cholecystokinin tetrapeptide
CCK-8.....	Cholecystokinin octapeptide
CCK-8s.....	Sulphated cholecystokinin octapeptide
Ci/mmol.....	Curie per millimole
Cl.....	Clearance
C_{max}.....	Maximal concentration
CNS.....	Central nervous system
CRF.....	Corticotropin releasing factor
CO_2.....	Carbon dioxide
C.V.....	Coefficient of variation
DBP.....	Diastolic blood pressure
Δ.....	difference from baseline
DSM-III-R...	Diagnostic and Statistical Manual of Mental Disorder - Revised 3rd edition
DSM-IV.....	Diagnostic and Statistical Manual of Mental Disorder - 4th edition
fmol.....	femtomole
GABA.....	γ-amino butyric acid

GAD.....	Generalised anxiety disorder
GH.....	Growth hormone
g/mol.....	gram per mole
HAM-A.....	Hamilton Anxiety Scale
HPA.....	Hypothalamo-pituitary-adrenal axis
HR.....	Heart rate
5-HT.....	Serotonin
5-HT_3.....	Serotonin subtype 3 receptor
5-HTP.....	5-hydroxytryptophan
iPSS.....	Sum intensity of symptoms on Panic Symptom Scale
i.c.v.....	Intracerebroventricular
I.S.....	Internal standard
i.p.....	Intraperitoneal
i.v.....	Intravenous
kg.....	kilogram
L/h.....	Liter per hour
LOQ.....	Limit of quantification
*m*CPP.....	*m*- chlorophenylpiperazine
MHPG.....	3-methoxy-4-hydroxphenyl-glycol
µg.....	microgram
µL.....	microliter
mg.....	milligram
mL.....	milliliter
min.....	minute
M.....	Molar (concentration)
ng/mL.....	nanogram per milliliter
nm.....	nanometer
NPY.....	Neuropeptide Y
NA.....	Noradrenaline

nPSS.....	Number of symptoms on Panic Symptom Scale
PSS.....	Panic Symptom Scale
pg/mL.....	picogram per milliliter
PRL.....	Prolactin
QC.....	Quality control
r.....	correlation coefficient
SCID.....	Structured Clinical Interview
SCL-90.....	90-item symptom checklist
SBP.....	Systolic blood pressure
s.c.....	Subcutaneous
TID.....	Three times a day
T_{max}.....	Time to reach maximal concentration
$t_{1/2}$.....	Half-life
UCCK-8.....	Unsulphated cholecystokinin octapeptide
VAS.....	Visual analogue scale
Vss.....	Volume of distribution at steady state
z.....	Elimination rate constant

À la mémoire de mon père
et
à ma mère que j'aime

∞ *PART ONE* ∞

INTRODUCTION

CHAPTER 1. UNDERSTANDING PANIC

Solomon has once said «Is there anything of which one can say, 'Look ! This is something new' ? It was here already, long ago ; it was here before our time» (Eccl. 1 :10). Panic disorder makes no exception. It has long been part of the turmoil of human life. Freud was first to describe the illness under the term *Anxiety Neurosis* in 1895.[1] Yet, it was only recently recognised as a separate diagnostic entity and remains, still today, mostly misdiagnosed.

1.1 THE ILLNESS

> ...I was just standing-up in line to get into the theatre when it suddenly happened. I became nauseous. My face felt clammy. Then, my heart started racing like crazy. I could barely speak; my throat was too dry. Breathing became a major challenge. I suffocated! I was swept into extreme anguish. I was losing my sanity. Surrounding noises were coming from so far away. I thought I was dying. Terrified, I got away from people. The drama finally ceased about 15 minutes later. Since, I live in constant fear and apprehension that an unpredictable attack of terror will occur again....

This case-scenario depicts many of the typical attributes of a panic attack. Various factors influence the assessment of true lifetime prevalence of the disorder in the population. First, fearing humiliation and rejection, people suffering from panic attacks may often not seek medical care and, hence, pass unnoticed. Second,

concurrent symptomatology such as depression and anticipatory anxiety may act as confounding effects making recognition of the illness even more difficult. Finally, the prevalence of panic disorder is further overlooked by the low sensitivity of the diagnostic tools used in epidemiological surveys. Nevertheless, it is currently estimated that about 9% of the population will suffer a panic attack in their lifetimes; 3.6% will experience recurrent panic attacks; and 1 to 3.8% will meet the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria[2] for panic disorder. The illness afflicts typically young adults mostly women.[3-10]

Panic attack is described by intense and terrifying anxiety sensation characterised by a sudden onset of fear during which four or more of the following symptoms develop: disturbance of respiratory function (dyspnea, choking), cardiac symptoms (palpitations, chest pain), sweating, hot flushes and cold chills, tremors, vertigo, nausea, diarrhea, insomnia, feelings of depersonalisation or derealization, apprehensiveness and/or discomfort, sense of impending doom, fear of dying, and fear of losing control or 'going' crazy. In general, panic attacks last from 5 to 20 minutes. Panic disorder is defined by recurrent panic attacks interrupted by periods of worries, anxiety in anticipation of an upcoming attack, and behavioural changes.[2, 11]

Panic disorder is associated with impairments in quality of life leading to serious consequences for the concerned individual as well as for the family and friends. Approximately 50% of patients develop some degree of social impairment such as agoraphobia. Affective disorders account for 50 to 75% of cases and drug-alcohol abuse for 27 to 36%. Suicide attempts have been reported in 7% of patients suffering of uncomplicated panic disorder and in more than 20% of patients if presence of comorbidities.[7, 11-17] Its chronic and debilitating course hamper the productivity and become a major handicap for the individual.

1.2 PATHOPHYSIOLOGY OF PANIC DISORDER

Despite an improved understanding of panic disorder, knowledge about the nature of the illness, in particular what causes it, remains firmly tied to hypothetical notions. Two notions stand-out and oppose their views: the psychological and neurobiological theories. Limitations associated to both hypotheses preclude definitive conclusions.

1.2.1 Psychological theory

Panic attack is claimed by some researchers[18-20] but not others [21, 22] to be dependent on a 'catastrophic' interpretation of anxiety symptoms such as palpitations and choking. Based on this theory, analogous to a domino reaction, a panic attack would be the endpoint of a rapid escalation in the intensity of anxiety and apprehension emerging from this cognitive disturbance. Trait anxiety and interoceptive conditioning have been incriminated as risk factors for panic.[23-25] However, panic attack does not necessarily involve catastrophic insights. For instance, panic attacks have been observed during non-REM sleep and relaxation session.[26-29] Although panic improves on placebo[30, 31], the theory is being questioned by the efficacy of medication in aborting a panic attack without influencing the anticipatory anxiety during which catastrophic thoughts presumably take place.[32, 33] It has been argued by cognitive theorists that manipulation of patient's beliefs about what to expect during testing may affect outcome. Once again, this argument was defended by some investigators[34, 35] and rejected by others.[36-39] One of the pitfalls raised is the difficulty to differentiate sensation from cognition.[40] Nevertheless, researchers agree with cognition constituting an important aspect of the disorder. The nature and the degree of the involvement of the psychological theory into the pathophysiology of panic disorder is yet to be defined.

1.2.2 Neurobiological theory

Currently, the illness is categorised according to whether or not there is agoraphobia associated to panic.[2] Central to Hippocrate's beliefs (460-377 BC) was the idea that careful observation of the symptoms specific to an individual and of that person's reaction to disease should be taken into account before decision making with regard to treatment.[41] Interestingly, in the recent years, panic disorder classification has been revisited based on symptom analysis.[38, 42-45] It has been observed that panickers can be divided into two groups. Respiratory symptoms predominated in one of the group, whereas cardio-autonomic symptoms were found in similar proportions in both groups.[45] This finding was further substantiated by the greater susceptibility of some patients with panic disorder to the hyperventilation produced by the carbon dioxide (CO_2) breathing challenge.[38] Likewise, pharmacological studies provided support for this modelling. Results indicated that imipramine, a reuptake inhibitor of both noradrenaline (NA) and serotonin, showed superior efficacy in the group with respiratory symptoms, whereas alprazolam, a γ-amino butyric acid (GABA) agonist, was more effective in the other group.[45] These observations agree with Snaith's view [46] which states that symptoms are the direct results of a disturbance in neurobiological systems and recapture the essence of Hippocrate's thoughts. Hence, diagnostic of panic disorder would regroup heterogeneous individuals, each of them experiencing a specific symptomatology which could reflect either the neurobiological system(s) in fault or the one(s) involved to compensate the failing system(s) or a mixture of both.

Anticipatory anxiety complicates the search for understanding the underlying neurobiology of panic disorder. Their respective symptomatology may partly overlap raising questions about the relative contribution of anticipatory anxiety to panic. Beside, anticipatory anxiety and panic may well evolve independently in the course of the disorder. It has been suggested that panic anxiety would be elicited by pathophysiological routes different from those involved in anticipatory anxiety or

generalised anxiety.[47-49] Most of the theories implicating neurotransmitters in the biological aetiology of panic disorder have resulted from observed effects of pharmacological agents. Currently, dysfunction within the noradrenergic, GABAergic, serotonergic and cholecystokininergic systems are being scrutinised for their role in the pathophysiology of panic disorder.

Evidence points to a defect in the noradrenergic (NAergic) neurotransmission as one basis for panic. Comparatively to normal subjects, patients suffering from panic disorder seem to behave differently to anxiogenic effects of isoproterenol, a ß-agonist, and yohimbine, an α_2-antagonist.[50-54] In a study conducted by Nesse et *al.*[50], patients suffering from panic disorder showed a blunted heart rate response to isoproterenol as compared to healthy subjects. The authors concluded that a down-regulation or a decrease in the peripheral ß-receptor sensitivity could be responsible for this effect. In a series of studies [51, 52, 54], Charney et *al.* have observed an increase in blood pressure and in the 3-methoxy-4-hydroxyphenyl-glycol (MHPG) plasma levels, the NA main metabolite, in panic disorder patients following yohimbine comparatively to controls. Dysregulation of α_2-receptors was further suggested after clonidine (α_2-agonist)-induced a blunted growth hormone response in panic disorder patients.[55-58] Unfortunately, neuroendocrine findings have been inconsistent. No change in growth hormone response was also reported after clonidine administration.[59, 60] Discrepancies in MHPG plasma levels and systolic blood pressure response have been noticed between studies. Some studies reported a significant increase in MHPG levels and in the systolic blood pressure.[55, 56] These results were not obtained in other trials.[57, 61] Charney et *al.*[54] distinguished a subgroup within their study population. This subgroup showed an increase in anxiety and MHPG levels post yohimbine and a blunted growth hormone response post clonidine.

Pharmacological studies have not been very supportive of the NAergic dysregulation theory. Albeit positive results obtained in open trials[62-64], specific NAergic uptake

inhibitors, such as desipramine and maprotiline, and NAergic ß-blocker propranolol were found to be no different than placebo in reducing the number of panic attacks in double-blind placebo-controlled trials.[65-67] The nature of the implication of the NAergic and serotonergic blockade by imipramine in panic remains ambiguous. Each system may equally contribute or mutually reinforce their individual action. Further investigations are required to understand the role of these two neurotransmitter systems in panic.

GABA has long been linked to anxiety states. Increase density of peripheral benzodiazepine receptors has been associated to acute stress, whereas a decrease has been observed after long-lasting stress.[68, 69] A literature review provides several lines of evidence for a role of the GABA receptors in panic disorder. Density of peripheral benzodiazepine receptors was found to be significantly lower in patients with panic disorder comparatively to normal subjects and obsessive-compulsive patients.[70] Flumazenil, a GABA antagonist, has been shown to induce panic-like attacks in patients suffering from panic disorder.[71, 72] Symptoms mimicked those of a natural panic attack with less respiratory components. In contrast, panic was not induced in patients with post-traumatic stress disorder. This finding tallies with the subgroup modelling defined by Briggs et *al.*[45]

In a set of experiments, saccadic eye movements to a moving target and catecholamine plasma levels were measured at baseline and post-benzodiazepine treatment in healthy subjects and in patients with panic disorder.[73-75] Baseline parameters did not differ between groups. However, comparatively to normal subjects, the results indicated a significant decrease in peak velocity and in catecholamine response denoting a lower sensitivity of patients with panic disorder to benzodiazepine treatment.

On a different line of thoughts, inverse agonists produced intense anxiety in humans.[47, 76] Based on this finding, the involvement of an endogenous inverse agonist in anxiety

disorders has been speculated. Higher urinary concentrations of tribulin, an endogenous inverse agonist yet to be confirmed, has been found in patients suffering of generalised anxiety disorder.[77] However, the hypothesis was refuted in panic disorder following failure of flumazenil in counteracting panic. The antagonist acted rather like an inverse agonist.[71] Conformational shift, disarrangement, and abnormal regulation of the benzodiazepine receptor were suggested to explain these findings.[71]

Alprazolam efficacy as anti-panic agent has been shown.[30, 31, 78, 79] At present, alprazolam remains the sole benzodiazepine officially approved in panic disorder in spite the efficacy of other congeners.[78-80] Open trials[81, 82] and one double-blind placebo cross-over trial[83] have shown that valproic acid, an anticonvulsant facilitating GABA transmission, has anti-panic efficacy. This data was supported by failure of lactate infusion to induce panic in 10 out of 12 patients pre-treated with valproic acid.[84]

Serum cortisol elevation has been observed after yohimbine (an α_2-antagonist), caffeine (an adenosine receptor inhibitor), and ß-carboline (a GABA-inverse agonist) challenges.[47, 52, 85] Compared to baseline values, sustained high cortisol levels were measured over a 4-hour interval after oral fenfluramine challenge in patients with panic disorder, whereas a significant reduction of cortisol was detected in depressive patients and healthy subjects.[86] In opposite, cortisol serum levels did not change during spontaneous panic attacks and sodium lactate- or CO_2 inhalation-induced panic.[48, 87-90] Surprisingly, cortisol serum levels increased in phobic patients when facing novelty but not when facing feared objects.[91] Recently, a so-called *uncoupling* of the NAergic system and the hypothalamic-pituitary-adrenal (HPA) axis was demonstrated in panickers. As opposed to healthy subjects, there was no significant correlation found between either baseline MHPG and baseline cortisol or maximal decrease in MHPG and maximal decrease in cortisol in patients suffering from panic disorder.[92] Based upon the above observations, it has been suggested that the increase

in cortisol levels was more akin to anticipatory or generalised anxiety than true panic.[91, 93-95]

These observations again illustrate the heterogeneity of the illness. There is no doubt that dysfunctional NAergic and GABAergic systems may be implicated in some ways in panic disorder. Yet, neither one of them can stand alone and account for the problem. Panic is no longer involving simple imbalances in the functioning of one or two neurotransmitters. Rather, the current thinking suggests that panic would originate from regulation disturbances in a variety of complexly interacting neurotransmitter systems. In view of the purpose of this work, serotonergic and cholecystokininergic systems will be discussed in more details under separate chapters.

CHAPTER 2. SEROTONERGIC NEURONAL SYSTEM

It was early 1868. Fortuitous discovery originating from research in the cardiovascular system highlighted a new page in medical history. Ludwig and Schmidt observed that perfusion with clotted blood caused increased vascular resistance.[96] The isolation of this endogenous substance languished in those early years. Nevertheless, data were gradually accumulated and in 1948 Page's group coined the word «serotonin». The mysterious molecular structure was finally identified as an indole derivative known as the 5-hydroxytryptamine (5-HT).[97-99] Meanwhile, Erspamer's group detected through their work on the enterochromaffin system a catechol derivative with an amine group present in the gastrointestinal mucosa. This substance was called 'enteramine'.[100-102] Eventually, research work from Page's and Erspamer's groups merged in 1952 where it was shown that the molecular structure of enteramine was identical to that of serotonin.[102, 103]

This pivotal finding marked a turning point in research from which transpired a rapid development in the serotonin adventure. Serotonin was granted its colour of nobility when identified as a neurotransmitter in 1957.[104] Since then, studies have concentrated on the biochemical function of serotonin and its interrelationships with other neurotransmitters. This chapter will briefly review the neuroanatomic organisation of the serotonergic neuronal system to carry on with a focus on a particular serotonergic receptor and the evidence of its contribution in panic disorder.

2.1 NEUROANATOMIC ORGANISATION

In spite of its recognition over more than a century ago it is still difficult to do proper justice to the complexity and the incredible flexibility of the serotonergic (5-HT) neuronal connections.

Serotonin is a biogenic amine largely distributed within the central nervous system. Cell bodies are mostly located in the raphe nuclei of the midbrain and rostral pons. The highest density is in the dorsal raphe nucleus. Anatomically speaking, the configuration of this nucleus involves the periaqueductal gray matter of the midbrain located behind the oculomotor nucleus and stretches out to the fourth ventricle at level of the locus coeruleus. The median raphe nucleus, which lies within the reticular formation, contains serotonergic cell bodies along with large quantity of non-serotonergic-containing neurons. Serotonergic cell bodies have also been identified in the tegmental reticular nucleus of the pons. Dorsal, median, and tegmental reticular nuclei project their axons to the forebrain and the limbic structures. Dorsal raphe nucleus projects mainly to the thalamus and the striatum, whereas the median raphe nucleus innervates the septal area and the hippocampus. Projections to these strategic central areas imply a potential activity of 5-HT in the regulation of mental states. Finally, other nuclei with smaller amount of cell bodies are spreading along the midline raphe. These nuclei send their projections to the intrinsic brainstem connections as well as to the spinal cord and, hence, control the autonomic, sensory, and motor functions.[105-109]

Mapping studies revealed that, in actual fact, the 5-HT system consists of a multiplicity of subsystems. Major features confirm the evidence of this heterogeneity. First, two types of axonal morphology have been described. One of the types originates from the dorsal raphe nucleus and shows fine axons with very small varicosities. Axons of the other type are coarse and possess large varicosities. Contrasting with the former, they

emerge from the median raphe nucleus and resist to the neurotoxicity of amphetamine derivatives.[110, 111] Second, diffuse terminal plexus of fine 5-HT fibers dominate in the cortex, whereas dense terminal plexus of coarse fibers prevail in the hippocampus, the lateral entorhinal area, the parietal and posterior cingulate cortex.[110, 112-114] Finally, the end-point result of the 5-HT neurotransmission depends upon the nature of the synaptic connections. By making synapse to both specific neurons and to a large variety of neurons, the 5-HT system may play a crucial role in regulating other neurotransmitters' synthesis, release, and action.

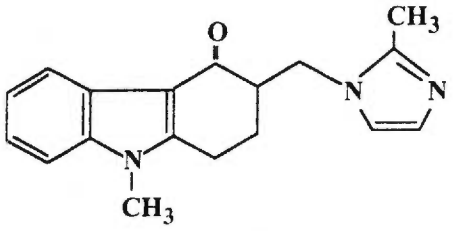
The first serotonin receptor was described in 1957.[104] Since, receptor-gene cloning works supported by operational and transductional studies have identified several subtypes of 5-HT receptors in the peripheral and the central nervous systems.[115, 116] Unlike the other 5-HT receptor subtypes, the 5-HT_3 receptor is an ion channel that produces rapid sodium-potassium membrane exchange when stimulated by an agonist. This rapid depolarisation results in a significant influx of extracellular calcium which then triggers the release of neurotransmitters.[117-121] Regional binding studies reveal that the peripheral distribution of 5-HT_3 binding sites include pre- and post-ganglionic autonomic, sensory, and enteric neurones.[122, 123] For many years, it was thought that the central distribution of 5-HT_3 receptors significantly differed between species. Recently, it was realised that this so-called variable pattern resulted from discordant terminology of the neuroanatomy.[124] There is now agreement that the highest density of 5-HT_3 binding sites is found in the nucleus tractus solitarius, the area postrema and the substantia gelatinosa of the brainstem. Nuclei located in the lower brain such as dorsal vagal complex and spinal trigeminal nucleus contain high density of 5-HT_3 receptors as well. Lower binding site density is found in the cortical areas and the limbic system, including the hippocampus and the amygdala.[124-131] Development of several agonist and antagonist agents led to speculations on the existence of 5-HT_3 receptor subunits and, possibly, subtypes between and within species. Recently, a human 5-HT_3 type A subunit has been cloned.[132-136]

Several animal studies provided evidence for the existence of serotonin-neuropeptide and serotonin-neurotransmitter interactions and coexistence. It has been shown in rats that the release of atrial natriuretic peptide secondary to volume expansion is mediated by 5-HT.[137] Serotonin makes synapse to vasoactive intestinal polypeptide, neuropeptide-Y (NPY), and enkephalin-containing neurons.[138-141] Co-localisation with calcitonin gene-related peptide, galanin, enkephalin, neurokinin A, neurotensin, somatostatin, substance P, and thyrotropin-releasing hormone has been identified.[142-149] Moreover, serotonin synthesis and/or release in the central nervous system may be modified by corticotropin-releasing factor, galanin, NPY, substance P, and cholecystokinin.[150-155] Besides neuropeptides, 5-HT_3 receptors are involved in the regulation of acetylcholine and dopamine neurotransmission. Serotonin facilitates dopaminergic transmission and reduces acetylcholine release from the cerebral cortex.[156-158] Finally, it has been suggested that GABAergic axon terminals could behave as presynaptic modulators of 5-HT neurons.[159-161]

Based on considerable evidence, the notion of one neuron - one neurotransmitter no longer stands. Current understanding of the neuroanatomy has expanded beyond such a simplistic approach. We now know that neurotransmitters cannot be considered in isolation. The 5-HT neuronal circuitry does not deviate from this thinking. Abundant 5-HT projections of different nature emerge from the raphe nuclei and make synapse with several peptide- and neurotransmitter-containing neurons. As a result, 5-HT_3 receptors may display a large scale influence on numerous peripheral and central functions.

2.2 ONDANSETRON

Recently, a selective serotonin antagonist, ondansetron, has been developed by the Glaxo Research Group in England and introduced in our medical practice. Its chemical structure is as follows.



Ondansetron

± 1, 2, 3, 9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazole-4-one

Ondansetron monohydrochloride dihydrate (ondansetron) is a weak base soluble in aqueous solutions. Its molecular weight is 293.37 with a pK_a of 7.4, hence, the compound is 50% unionised at physiological p*H*. The diffusion coefficient log D equals 2.2 and 0.6 at p*H* 10.6 and 6.0, respectively. Ondansetron possesses one asymmetric centre. The drug substance is a racemate (1:1).[162]

Human pharmacokinetic data come from studies carried out mostly in healthy subjects. Few studies include patients suffering from cancer. Following oral administration, ondansetron is completely and rapidly absorbed by passive diffusion via a transcellular pathway across the intestinal mucosa independently of the formulation used.[163, 164] Detected in plasma within 30 minutes after ingestion, the time to reach maximal concentrations (T_{max}) occurs between 1.0 to 2.1 hours.[165-167] Inter-individual variability of plasma concentrations is substantial. After a single 8-mg tablet, mean peak plasma concentrations (C_{max}) vary from 19.9 to 42.0 ng/mL.[165, 167-169] After administration of 8 mg thrice daily (TID) for 5 days and 8 mg twice daily (BID) for 21 days, mean C_{max} are 38.9 and 47.9 ng/mL, respectively.[165, 167] There is no evidence of accumulation.[166] Ondansetron undergoes presystemic metabolism (first pass-effect), which leads to a bioavailability of approximately 60%.[167, 170] Plasma concentrations seem to be dose-independent as shown

by a non-linear absorption of different doses ranging from 8 to 64 mg. This finding suggests saturation of the first-pass metabolism.[165, 171] The extent of ondansetron absorption as indicated by the area under the mean concentration-time curve (AUC) and the C_{max} are slightly higher in patients.[166, 168, 172, 173] The bioavailability is around 85-87% reflecting variations within the first-pass metabolism.[173] Food slightly enhances the extent of absorption but not the absorption rate of ondansetron.[174] Ondansetron is widely distributed into the body with a volume of distribution at steady-state (V_{ss}) of approximately 1.9 L/kg. Although not easily, it crosses the blood-brain barrier with a brain-plasma ratio less than 0.5.[175, 176] The percentage of ondansetron bound to human plasma proteins ranges from 70 to 76%.[166, 177]

In the liver, ondansetron is first converted to the 6-, 7-, and 8-hydroxyondansetron through oxidation at the 6-(<5%), 7-(<20%), and 8-(40%) positions, respectively.[175, 178] Multiple cytochrome P 450 (CYP) forms including CYP1A1, CYP1A2, CYP2D6 and CYP3A intervene in the biotransformation of ondansetron.[179] There is no evidence of CYP2D6 polymorphic metabolism. The 6- and 7-hydroxylated forms are less active, whereas the 8-form is equipotent to ondansetron.[180, 181] Although the hydroxyl metabolites possess some degree of 5-HT_3 antagonistic activity, these metabolites are unlikely to contribute to the therapeutic effect. In fact, none of the metabolites have been detected in plasma. It is suggested that the hydroxylation would be followed by glucuronide (45%) or sulphate (20%) conjugation before leaving the liver.[175] In humans, N-demethylation remains a minor metabolic route.[165, 178]

Elimination of ondansetron, mostly as metabolites, occurs through urine (60%) and faeces. Less than 5% of a dose is excreted in urine as unchanged drug. Total clearance (CL) ranges between 25 to 50.7 L/h and 16 to 32 L/h in healthy subjects and patients suffering from cancer, respectively.[175] This represents 40% of the total hepatic blood flow. Therefore, variations in ondansetron clearance may be expected in presence of very large changes in hepatic blood flow.[175] Although young healthy male subjects show a 4- to 5-

fold range in ondansetron clearance, there is no evidence of modality in the distribution.[175] Renal clearance equals 10 to 20 mL/min.[166, 167] Plasma clearance increases with increasing doses indicating a non-linear kinetics.[182] Plasma half-life increases with age ranging from 2.8 to 3.3 hours in the age group below or equal to 60, 4.7 hours in the age group of 61-74, and 5.5 hours in the age group greater or equal to 75.[166-168, 170]

Clearance decreases by half and the bioavailability increases to 80% in mild to moderate hepatic impairment. Patients with severe hepatic impairment have a mean plasma clearance reduced to 35% and a bioavailability of 98% suggesting a decrease in the intrinsic hepatic metabolism function.[183, 184] Comparatively to male subjects, weight-normalised CL and Vss are significantly lower, whereas C_{max} and AUC are significantly higher in females. Half-life remains constant. In the elderly, mean CL decreases while half-life, bioavailability, C_{max} and AUC increase. Inconsistencies have been observed in the Vss.[168, 170] Once again, inter-variability is considerable. Therefore, clinical significance of these findings remains trivial. The absorption phase of ondansetron is subjected to circadian variations as demonstrated in a study of 24 young healthy male subjects who received 8 mg TID at 07h00, 15h00, and 23h00 on three consecutive days. The steady-state AUC at 23h00 was 15% lower than that following the morning dose. C_{max} was significantly lower and T_{max} more prolonged. A diurnal variation in hepatic metabolism and gastric motility have been suggested as incriminating factors.[185]

Ondansetron binds with high affinity to 5-HT_3 receptors. It fails to interact with other neurotransmitter systems: 5-HT_1-like, 5-HT_2 and 5-HT_4 receptors; alpha- and beta-adrenergic receptors; dopaminergic receptors; nicotinic and muscarinic cholinergic receptors; GABA receptor complex; and histaminergic receptors.[186-188] The antagonist potency of the R- and S-isomers of ondansetron varied according to tissues. Although caution is required in data interpretation, this may support the existence of 5-HT_3 receptor subtypes.[186] Ondansetron is well known for its antiemetic activity.[189-191] Potential as anxiolytic[187, 192, 193], antipsychotic[194-196], enhancer of cognitive functions[197, 198], and anti-

craving agent[192, 199, 200] has been shown in animals and humans. However, further investigations are required before including these potential activities in its pharmacological profile. Ondansetron lacks of anticonvulsive and sedative activity.[187]

2.3 SEROTONIN AND PANIC DISORDER

Animal behavioural studies suggest that 5-HT receptors may play a role in the pathogenesis of anxiety. However, evidence accumulated to date are scattered with contradictory results with regard to the nature of the 5-HT activity shift. For instance, it was shown that increasing 5-HT neurotransmission at central receptors either by electrical stimulation of the median raphe nucleus or by a 5-HT agonist such as 5-hydroxytryptophan (5-HTP) favours behavioural suppression paralleling an anxiogenic effect.[201-203] Clomipramine, a presynaptic 5-HT uptake inhibitor, intensified the suppressed behaviour produced by electrical stimulation.[204] Conversely, depletion of brain 5-HT by neurotoxic lesions of the efferent projections or by a 5-HT synthesis inhibitor such as the para-chlorophenylalanine (PCPA) shows a disinhibitory effect on the behaviour.[205-209] Tryptamine antagonists, methysergide and cyproheptadine, antagonised the effect of electrical stimulation.[204] According to these observations, one could promptly conclude that increasing and decreasing 5-HT activity would lead to an anxiogenic and anxiolytic effect, respectively. However, such a conclusion is not supported by the separation-distress vocalisation animal model. Blockade of serotonin activity and isolation increase vocalisation which is interpreted as an anxiogenic effect. Stimulation of serotonin activity by quipazine administration, a non-specific 5-HT agonist, decreases separation-induced vocalisations denoting an anxiolytic effect.[210] Acute administration of clomipramine and paroxetine, both selective 5-HT uptake inhibitors, reduced distress calls.[211] In animals, administration of 5-HTP, the immediate precursor of serotonin, was reported to exert both anxiogenic and anxiolytic effects depending on the dose.[212] In light of these findings, animal studies have shown that decreasing or increasing 5-HT neurotransmission may result in anxiolytic

activity depending on the animal model examined. This may presuppose that animal models used in experimental studies may indeed measure different facets of anxiety such as generalised anxiety, anticipatory anxiety and panic-like behaviour. Likewise, through its different receptors subtypes, 5-HT system may be engaged in different stages of the anxiety pathogenesis or in different types of anxiety. Furthermore, it may exert a more complex role in the control of anxiety mediating both inhibitory and facilatory effects via its different receptor subtypes.

Anxiolytic activity has been reported for 5-HT_3 receptors antagonists in various animal models. Low oral doses of ondansetron (0.5 to 1 μg/kg) increased social interaction of rats under high light unfamiliar conditions, but not under low light unfamiliar conditions. Ondansetron was shown to be 500 times more potent than diazepam in this paradigm. At higher doses (0.1 and 1 mg/kg), there were variable results that were borderline to little or no effects.[187, 213] In a dose-dependent manner, 5-HT_3 receptor antagonists ondansetron, zacopride, ICS 205-930, and BRL 43694 increased the exploratory behaviour of mice and reduced the number of body postures adopted by marmosets in response to confrontation.[187, 214, 215] These results matched those obtained with diazepam. In the behavioural observation test, oral ondansetron 10 and 100 μg/kg reduced agitation of monkeys. The ondansetron effect was not dose-dependent. Comparatively to oral diazepam 2.5 mg/kg, ondansetron was less effective and has no significant effect on the startle response, motor coordination or alertness. In rats, ondansetron (0.05 to 1.6 mg/kg i.p.) failed to show anxiolytic activity in the water-lick conflict test which evaluates the anxiety level through punishment with electrical stimuli. As opposed to ondansetron, diazepam 5 mg/kg produced striking anxiolytic activity in this animal model.[187] Contrary to 5-HT_{1A} ligands, 5-HT_3 antagonists inhibited the anxiety induced by withdrawal from drugs of abuse and diazepam. This observation is of interest because sudden withdraw of ondansetron did not cause any anxiety rebound.[192, 199] Using the light/dark exploration test, injection of ondansetron into dorsal raphe nucleus and the amygdala reduced aversive behaviour. No effect was observed when injected into the median raphe nucleus, the

striatum, and the nucleus accumbens.[192] Even though anxiolytic activities were observed in animals with ondansetron, extrapolation of these results to anti-panic activity in humans remains hazardous.

Paralleling animal findings, there is growing evidence from clinical studies in support of a 5-HT hypothesis in panic disorder. 5-HTP, which stimulates serotonin synthesis, has been shown to possess some panic reducing properties in humans.[216, 217] Imipramine, a potent 5-HT reuptake inhibitor, was the first antidepressant to show improvement in panic symptoms.[218-220] Interestingly, a significant correlation was observed between clinical improvement in agoraphobia and plasma concentrations of imipramine. Desipramine, the N-desmethyl metabolite of imipramine, preferentially inhibits the noradrenaline (NA) reuptake. Its plasma concentration profile failed to show a correlation with phobic improvement.[218] In both cases, anti-panic effect did not correlate significantly with plasma levels. It was suggested that anti-panic activity would be observed at much lower doses than that required for anti-phobic activity.[218, 221] Clomipramine, the most potent 5-HT reuptake blocker, showed decrease in the number of panic attacks.[216, 221-223] As imipramine, clomipramine is desmethylated in a metabolite that preferentially blocks the NA reuptake.[224] Hence, imipramine and clomipramine mainly inhibit the serotonin reuptake and, through the desmethyl metabolite, the noradrenaline reuptake. Although the omnipresence of the NA participation, there is evidence that the effect in agoraphobia and panic be related to the serotonin activity.

Zimeldine, a specific 5-HT uptake inhibitor, was superior to imipramine in reducing the symptoms of patients suffering from agoraphobia with panic attacks.[225] In a double-blind study of 44 panic disorder patients with or without agoraphobia, fluvoxamine, a specific 5-HT reuptake inhibitor, was compared to maprotiline, a specific NA reuptake blocker.[226] After an initial worsening of symptoms, treatment with fluvoxamine resulted in a profound reduction in the number of panic attacks, anxiety symptoms, and avoidance behaviour

confirming previous findings.[222] Maprotiline had no effect on the number of anxiety symptoms and the panic attack rate.[226]

In a double-blind placebo-controlled study, the antipanic effect of ritanserin, a specific 5-HT_2 receptor antagonist, with that of fluvoxamine was assessed in 60 patients with panic disorder as defined by DSM-III-R[227] criteria. Although effective in generalised anxiety disorder (GAD)[228, 229], ritanserin was no different than placebo whereas fluvoxamine produced a significant reduction in the number of panic attacks.[230] Trazodone, primarily a 5-HT_2 antagonist, has too been found ineffective in a double-blind study, while alprazolam and imipramine were highly effective in decreasing phobic avoidance and frequency of panic attacks.[219]

m-Chlorophenylpiperazine *(m*CPP) is a metabolite of trazodone that binds with a descending rank order to 5-HT_{1C}, 5-HT_3, 5-HT_2, 5-HT_{1A}, 5-HT_{1D} and α_2.[231, 232] Although, considerable controversy still surrounds its anxiogenic potential[233-238], *m*CPP is being used as a probe for anxiety in animal and human studies. In animals, the probing agent reduced social interaction (anxiogenic effect) which is antagonised by 5-HT_3 antagonist.[239] Oral and intravenous administration of *m*CPP has been shown to cause panic-like symptoms in humans. Panic attacks were induced in 60% of panic disorder patients. In contrast, neither depressed patients nor healthy subjects experienced these effects.[233, 234, 240] These data led to the postulate that some panic disorder patients would have a hypersensitive postsynaptic serotonin receptor system. *m*CPP-induced anxiogenic effects were attenuated by ritanserin.[236] In healthy subjects challenged with *m*CPP, BRL 46470, a 5-HT_3 antagonist, showed no effect.[238]

Limited studies suggest that direct acting 5-HT_{1A} agonists seem to date ineffective in panic disorder although effective in GAD. The double-blind comparative efficacy of buspirone, a 5-HT_{1A} partial agonist, imipramine and placebo in panic disorder indicated the superiority of imipramine over placebo but failed to show statistical differences between buspirone and

placebo.[220] These findings concurred with those ensued from a another buspirone controlled study.[241]

Recently, an open label study conducted with ondansetron in 31 patients who met DSM-III-R criteria for panic disorder has been completed in the United States. Treatment was initiated with ondansetron 0.25 mg BID increased every 4 weeks for non-responders to 0.5 mg BID and 1 mg BID. Improvement was observed within the first 4 weeks of treatment. Results showed a significant reduction in the intensity of panic attacks and in the anticipatory anxiety score particularly after the 1 mg BID dose. Further investigations are required.[242]

In view of neurobiological responses, reports are inconclusive. Infusion of 5-HTP (60 mg) to seven women suffering from panic disorder with phobic avoidance and seven healthy women resulted in a significant increase in mean plasma MHPG levels in patients compared to normal controls. Significant increase in ß-endorphin and cortisol plasma levels was observed in both groups with no difference between them.[217] Fluvoxamine significantly increased plasma melatonine and slightly reduced the MHPG levels.[230] Fluvoxamine and fluoxetine increased ß-endorphin and cortisol.[243, 244] Interestingly, the endocrine effects produced by acute administration of fluvoxamine in rats were abolished after 4 days of repeated treatment suggesting neuro-adaptation.[245]

*m*CPP increased the release of prolactin along with adrenocorticotropic hormone (ACTH) and cortisol in animals and humans.[246] Kahn et *al.* have reported an increase of cortisol in panic disorder patients when compared to controls and patients with major depression after stimulation with *m*CPP.[247] In animals, the increase of prolactin was attenuated by pretreatment with metergoline, a 5-HT_1 and 5-HT_2 antagonist, whereas 5-HT_3 antagonist had no effect on the prolactin response.[248, 249] Ritanserin and metergoline diminished the *m*CPP-induced secretion of prolactin and cortisol in humans.[236, 250]

Ipsapirone is a 5-HT_{1A} full agonist in pre-synaptic and partial agonist in post-synaptic. A single oral dose (0.3 mg/kg) ipsapirone was compared to placebo in a double-blind randomised study of 14 panic disorder patients and matched controls. Mean baseline plasma ACTH and cortisol levels were not different between the two groups. However, panic disorder patients showed a significantly lower ipsapirone-induced increase in ACTH and cortisol levels than that observed in controls. Ipsapirone induced minimal anxiogenic and fearful effects in both patients and controls. It was suggested that these findings would reflect the hyposensitivity of pre- and postsynaptic receptors due to an hyper-serotonergic activity in panic disorder.[251]

The downfall of these clinical findings often points out to the type of patients included in studies. Several clinical studies mainly included patients suffering essentially from agoraphobia with or without panic attacks. Diagnostics of obsessive compulsive and generalised anxiety disorders were also included in some studies. In the hope of circumvent the difficulty to study panic disorder, the use of challenge tests, such as *m*CPP, was put forward to mimic the illness. However, the non-selectivity of the challenge agent rises further difficulty in the interpretation of the results. Nevertheless, based upon animal and clinical studies, 5-HT neuronal system certainly plays a role into the pathogenesis of anxiety and possibly in panic anxiogenesis. Whether a particular subset of receptors initiates the panicogenic process remain unsolved. Studies with selective agonists and antagonists are necessary to dissect the panicogenesis.

CHAPTER 3. CHOLECYSTOKININERGIC SYSTEM

The history of cholecystokinin presents analogies with that of serotonin. Observations made as early as mid-1800s led to evidence that an endogenous substance stimulated bile secretion and flow independently from the autonomic system. In 1928, Ivy and Oldberg were first to extract this substance from the small intestine and to show stimulation of the gallbladder contractions after intravenous administration of the substance in dogs. The name 'cholecystokinin' (CCK) was suggested from the point of both origin and functional connotations.[252] Shortly afterwards, in 1943, Harper and Raper isolated from the mucosa of the duodenum a substance that stimulated pancreatic enzyme secretion. Based upon its physiological action, the substance was called 'pancreozymin'.[253] But one will have to await until 1960s to combine these findings together. Mutt and Jorpes showed by purifying a CCK peptide as a 33 amino acid hormonal polypeptide that CCK included the amino acid sequence corresponding to pancreozymin.[254] Finally, CCK was identified in the brain by Vanderhaegen et al. in 1975.[255] The name CCK got entrenched because it was the first name given.

3.1 NEUROANATOMIC ORGANISATION

Cholecystokinin is a member of the heterogeneous gastrin family. At present, there is evidence of several molecular forms ranging from 4 to 83 amino acid residues.[256-261] Biologically active CCK fragments are synthesised in the rough endoplasmic reticulum from a 115-amino acid chain, called preproCCK.[262, 263] The exact preproCCK processing is yet to be established. However, it is proposed that the preproCCK first undergoes proteolytic cleavage by a signal peptidase in the Golgi apparatus producing

the proCCK polypeptide. Then, the CCK precursor is rapidly transported into small secretory vesicles along with various proteolytic enzymes. During the axonal transport, proCCK is converted into smaller active α-carboxyamidated forms of CCK which are subsequently stored into synaptic granules at the nerve endings.[261, 264, 265] The C-terminal amide portion of the peptide is a prerequisite for the biological activity.[266] Amino acid sequence of sulphated octapeptide (CCK-8s) and tetrapeptide (CCK-4) cholecystokinin is Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe and Trp-Met-Asp-Phe, respectively. CCK of various chain lengths is released by calcium (Ca^{++})-dependent neurosecretion.[267, 268] The fast entry of calcium into the cell in response to appropriate stimulation appears to be mediated through the L-type calcium channels.[269] Peripherally circulating CCK is rapidly cleaved by enzymatic activity through kidney, liver, brain, and gut vascular beds. Enzymatic proteolysis of CCK also takes place in the bloostream.[270-275]

CCK is present in the gut, the peripheral nervous system, and is found in large amounts in the brain where CCK-8s predominates.[256, 264, 276-280] CCK mRNA, CCK-like immunoreactivity (CCK-LI), and CCK binding site distribution varies throughout the brain. The cortical area and, to a lesser extent, the hippocampus and amygdala all express CCK mRNA.[281-284] Unlike CCK mRNA, CCK-LI is found in many brain areas. The highest levels are detected in the cerebral cortex (≥ 0.2 nmoL CCK-8 equivalent/g) with higher amount in the enthorhinal cortex than the neocortex. Rich immunoreactivity is also detected in the medial amygdaloid nucleus, olfactory lobe, basal ganglia (caudate nucleus and putamen), and hippocampus. In addition, the medial preoptic area of the hypothalamus shows numerous CCK nerves. Moderate levels are identified in the nucleus accumbens, septum, ventromedial thalamus, periaqueductal gray, substantia nigra, and area postrema. Low to negligible amounts of CCK-LI are detected in lateral thalamic nuclei, globus pallidus, median eminence, cerebellum, corpus callosum, and the internal capsule.[277, 285-289] In the brain, high densities of binding sites are identified in the cerebral cortex, the amygdaloid nuclei,

the olfactory lobe, and the cerebellum. Moderate to high densities are found in the basal ganglia (caudate nucleus, putamen, and nucleus accumbens), whereas moderate densities are detected in the claustrum and the hippocampus. Few binding sites are found in the internal capsule, the globus pallidus, and most thalamic and hypothalamic nuclei.[290, 291]

Two distinct CCK binding receptors have been identified by autoradiography and confirmed by molecular cloning.[292-298] CCK receptors are classified as CCK_A (for Alimentary) and CCK_B (for Brain) and belong to the G protein-coupled receptors.[296] The peripheral subtype receptors refer to the CCK_A receptors and are mainly localised in the gastrointestinal system (colon, pancreas) and the gallbladder. Centrally, CCK_A receptors have been found in very high densities in the area postrema and the nucleus tractus solitarius. Substantia nigra and basal ganglia (caudate nucleus and ventral putamen) show moderate to high levels of CCK_A binding sites. The supraoptic and paraventricular nuclei of the hypothalamus contain high levels of the receptor. Finally, presence of CCK_A receptors has been characterised in the vagus nerve, ventral tegmental area, interpeduncular nucleus, and posterior nucleus accumbens.[293, 294, 299-302] CCK_A receptors exhibit a higher affinity for CCK-8s than for unsulphated CCK-8 (UCCK-8), CCK-4 and pentagastrin.[299, 303]

Predominating in the CNS, CCK_B receptors, or central subtype also called CCK_B/gastrin receptors by its similarity to the gastrin receptor, are widely but not equally distributed within the brain and the vagus nerve.[292, 293, 302] In addition, these receptors have been found in human stomach and colon carcinomas and on peripheral lymphocytes and monocytes.[304-306] Relatively non specific, these receptors exhibit high and similar affinity for CCK-8s, UCCK-8, CCK-4 and pentagastrin.[292, 303, 307] The potential existence of CCK_B subsites has recently been raised based on the distinct actions of two highly selective CCK_B agonists, BC 197 and BC 264, in animal models of anxiety.[308] Two major findings resulted from this experimental study. First, it has

been shown that BC 197 is selective for B_1 sites, whereas BC 264 has the same affinity for B_1 and B_2 sites. Second, it appears that activation of B_1 and B_2 sites induced anxiogenic- and anxiolytic-like effects, respectively. In continuity with these results, Léna *et al.* have shown that BC 197 increases dopamine release in a dose-dependent manner from the anterior nucleus accumbens where CCK_B receptors prevail.[309] In contrast, low concentrations of BC 264 inhibit dopamine release, whereas high concentrations activate its release. Both effects of BC 264 were prevented by the CCK_B antagonist, PD 134308, but not by the CCK_A antagonist, L 364,718. In view of these animal findings, it has been suggested that CCK_B receptors may not be identical. At present, only one gene has been identified for each receptor.[296, 298] Therefore, variations in the affinity state of the receptor subtype have been incriminated for these different responses.[310]

The unique character of the CCK distribution profile suggests neuronal coexistence of CCK with other neurotransmitters and neuropeptides. CCK coexists with oxytocin[311, 312], dopamine[312-315], gamma-aminobutyric acid (GABA)[316, 317], glutamate[318], vasoactive-intestinal-polypeptide (VIP)[318], and corticotropin-releasing factor[319]. Neuronal co-localisation has been described with opioids[320] and serotonin[321-323]. The particular CCK receptor localisation determines the nature of their functions. Hence, in theory, the biological regulation of CCK as well as that of the other neurotransmitters, neurohormones and neuropeptides may be influenced by mutual interaction or modulatory effects.[324-336]

3.2 CCK BEHAVIOURAL PROFILE IN ANIMAL MODEL OF ANXIETY

The development of several animal models reflecting behavioural elements of anxiety has brought tremendous support in the role evaluation of endogenous substances in stress. Unfortunately, the relationship between a particular animal model and a

specific anxiety syndrome remains to be established. Moreover, the degree of sensitivity of a model to certain categories of substances differs denoting the modulation of a distinct underlying biological path which is not particularly emphasised by the model. Nevertheless, evidence collected from these paradigms suggests a functional role of CCK in anxiety.

The plus-maze anxiety model is of particular interest. Compared to group-housed rats, a 7-day-isolation period affected the rat behaviour on the plus-maze test showing a significant increase in the anxiety level of the animals reflected by reductions in the number of line-crossings and closed arm entries, and in the time spent in the open arms.[337] Subcutaneously (s.c.), intraperitoneally (i.p.), and intracerebroventricularly (i.c.v.) administered caerulein, a non-selective CCK_A/CCK_B receptor agonist, mimicked the isolation-induced anxiety-like behaviour in rats. Caerulein significantly attenuated the exploratory behaviour of rodents as illustrated by decreases in time spent in the open part and in the number of line-crossings and closed arm entries.[337-340] Likewise, animals showed an anti-exploratory behaviour after systemic injection of CCK-4.[341] Central, but not peripheral, administration of pentagastrin, a CCK fragment with high CCK_B binding selectivity, increased the level of anxiety of rats as shown by a dose-dependent decrease in the time spent in the open arms.[342] As opposed to the anxiogenic-like effects induced by caerulein, CCK-4 and pentagastrin, CCK-8 administration resulted in contradictory behavioural changes. Some authors indicated a decrease in open-arm entries after s.c. injection of CCK-8.[343] Others detected this activity profile with UCCK-8, whereas CCK-8s was inactive.[344, 345] Other CCK_B agonists such as the butylocarbonyl (BOC)-CCK-4, a N-acylated derivative CCK_B receptor agonist resistant to aminopeptidase activity, and the BC 197, but not BC 264, decreased exploratory activity.[308, 345, 346] The locomotion parameter remained unchanged in all testing.

In models reflecting more closely the natural behaviour of the animal such as defensive behaviour and the ultrasonic vocalisation (USV) tests, peripherally administered CCK-4

and BOC-CCK-4 respectively induced defensive behaviour and related stress emotions in African green monkeys and increased the number of distress calls in rats.[345, 347] In contrast, i.v. pentagastrin failed to alter the Rhesus monkey behaviour.[348] In rats, CCK-8s had no effect on the USV test.[345] Infusion of pentagastrin into the amygdala, but not in the striatum nor the nucleus accumbens, potentiated the acoustic startle response in rats denoting anxiety and fear.[349]

Using the black/white box model, the number of line crossings and the time spent in the illuminated (white) section decreased after i.p. BOC-CCK-4 and i.c.v. pentagastrin denoting an anxiogenic activity.[341, 345] Conversely, CCK-8s i.p. resulted in an anxiolytic-like action in increasing these two parameters.[345] The anxiolytic effect was further observed in the avoidance and open field models. Acute i.p. injection of CCK-8 lowered avoidance response. Interestingly, repeated injections did not produced any changes in the avoidance behaviour. This result may suggest a possible tolerance to CCK-8 action.[350] Central CCK-4 administration enhanced locomotion and rearing response in rats.[351] Administration of CCK-4 and BC 264 had no effect on the rat behaviour in the operant conflict paradigm.[352]

In the attempt to study more systematically the involvement of a particular receptor, the anxiolytic effects of CCK antagonists were evaluated in several of these animal models. In the plus-maze model, pretreatment with proglumide and lorglumide (i.p.), two rather unselective CCK_A and CCK_B antagonists, blunted and even blocked the anxiogenic response produced by caerulein and CCK-4[338, 341] but accentuated that of CCK-8 and suppressed motor activity.[343] High doses (1 mg/kg) of CCK_A antagonist, devazepide, were required to antagonise CCK-4-induced behavioural changes. In contrast, CCK_B antagonist L 365,260 reversed the CCK-4 action at low dose (10 μg/kg), whereas higher doses intensified the anti-exploratory activity of CCK-4.[341] However, this latter effect was contradicted by Rex *et al.* who showed that 100 μg/kg of L 365,260 antagonised BOC-CCK-4-induced anxious behaviour.[346] Otherwise

inactive in this model, BC 264 induced anxiety when co-administered with increasing doses of L 365,260 and showed anxiolytic characteristics in presence of CI-988, a selective CCK_B receptor antagonist.[308] CI-988, dose dependently, reduced the anxiogenic effect of pentagastrin and BC 197.[308, 340, 342] The CI-988 action was not blocked by flumazenil.[342] But the lack of effect of flumazenil on CI-988 requires further study.[344] In a dose-dependent manner, CI-988 and L 365,260 given alone increased the time that animals spent on the open arms.[340, 353] However, devazepide was only active at high doses.[340] Devazepide and L 364,718, both antagonists of CCK_A receptor, failed to block the anxiogenic effects produced by pentagastrin and BC 197.[308, 342] Peripherally injected L 365,260 diminished the acoustic startle response induced by pentagastrin after intra-amygdala administration in rats, whereas central PD 135158 administration, also a CCK_B receptor antagonist, completely blocked the response.[349]

The relative contribution of the CCK_B receptor in the mediation of the anxiogenic effect of CCK agonists seems unclear when using the black/white box model. Comparable to benzodiazepines, oral or i.p. administration in rats of CI-988 or s.c. administration in mice of PD 135158 increased the time spent in the white compartment and line-crossings denoting a decrease in the anxiety level of animals. Time spent in the black section decreased accordingly.[342, 353-355] In addition, it antagonised the effect of pentagastrin.[342] In mice experimentally made dependent to diazepam, behavioural studies revealed that CI-988 was an effective inhibitor of the withdrawal anxiety observed during the following days of treatment termination.[353] On the other hand, devazepide i.v., a CCK_A antagonist, increased or had no effect on the number of line-crossings, whereas L 365,260, a CCK_B antagonist, was inactive in this paradigm.[342, 356]

Peripheral administration of CI-988 showed a disinhibitory effect in the social interaction test similar to that observed with benzodiazepines.[342, 353, 354] Analogous to benzodiazepines but not in a dose-dependent manner, CI-988 increased, or tended to increase, the number

of shock taken in the electric conflict model in mice and in monkeys.[357, 358] Furthermore, CI-988 reduced the number of postures exhibited by the marmosets.[353] In the operant conflict paradigm, neither CI-988 nor L 365,260 showed behavioural changes.[352] Finally, pretreatment with LY 262691, a non-peptide CCK_B antagonist, showed no or an attenuating effect in the defensive behavioural response to CCK-4.[347, 352] Electrophysiological studies demonstrated that CI-988 blocked the increase in firing rate produced by CCK-8, pentagastrin, and cearulein.[353] In addition, the neuronal firing inhibition generated by CCK-8 in the nucleus tractus solitarius was prevented by previously preparing cells with CCK-4.[359]

Various conditions influence the density of CCK receptors in the CNS. Animal isolation was found to increase the number of CCK receptor in the frontal cortex.[337] Likewise, CCK receptor density in the frontal cortex was higher in animals having a low exploratory activity (anxious animals), whereas a decreased number was observed in the hippocampus.[360] Benzodiazepine withdrawal-induced anxiety was accompanied by an increase of CCK receptors in the frontal cortex and the hippocampus of rats. Increase preprocholecystokinin mRNA levels in these structures was detected as well.[361, 362] Benzodiazepines blocked the anxiogenic effect of CCK.[363, 364] Chronic treatment with dopamine antagonists significantly increased the number of CCK receptors in the cortex, possible consequence of a depletion in CCK-8 content.[330]

Taken as a whole, CCK agonists have been found to be anxiogenic agents in animal models related to novelty (plus-maze, black/white box, defensive burying), and to unconditioned/conditioned anxiety (USV test, defensive and avoidance behaviours). In general, it appears that CCK_B receptors are primarily involved in the mediation of the anxiogenic effects. Männistö *et al*[339] suggested that CCK_A and CCK_B exerted an opposite effect on the exploratory behaviour in rats. According to Männistö, activation of CCK_A would stimulate the exploratory behaviour (anxiolytic) while an inhibition (anxiogenic) would result from the activation of CCK_B. They also implied a possible involvement of the

vagus nervus, where are located both CCK_A and CCK_B binding sites. Hence, through the vagus nervus, peripherally administered CCK agonists could easily reach and stimulate CCK receptors in the brainstem areas which are mostly outside the blood-brain barrier.[365] The octapeptide molecular form does not penetrate the blood-brain barrier.[366] Recently, it has been suggested that systemic administration of CCK-4 could produce an anxiogenic response through a direct central action. Within two minutes of the i.v. injection of iodinated Bolton-Hunter CCK-4, radiolabelled CCK was identified in high concentrations in the rat amygdala and cortex. To a lesser extent, radiolabelled CCK was also present in some limbic areas, in particular the brainstem, caudate nucleus and the hippocampus.[367]

3.3 CCK IN HUMAN ANXIETY DISORDERS

Animal data provided substantial clues on the activity profile of CCK suggesting that anxiety disorders may originate from, or may involve, a disturbance in the neuropeptide network. Extensive human experience was gained from clinical studies mainly developed by Bradwejn and his colleagues.

Preliminary investigations provided insight into CCK changes under anxiety conditions. CCK receptor density was found to be higher in the frontal cortex of suicide victims as compared to subjects deceased from other causes. This change in density was linked to a despair-like situation in which the individual is unable to adapt.[368] A study conducted in 25 panic disorder patients and 16 healthy subjects showed a significant reduction in cerebrospinal fluid concentrations of CCK compared to those from controls.[369] Likewise, lower CCK levels were also measured in lymphocytes of patients with panic disorder compared to levels found in healthy subjects.[370] Significance of these findings is yet to be defined since, as of now, no correlation has been established between peripheral CCK levels and the clinical profile of the illness. Furthermore, it is unknown if abnormalities in the functional state of central CCK are detectable in periphery.

The outcome of intravenous CCK-4 and CCK-8s administrations in healthy subjects was first examined by de Montigny.[371] In this exploratory setting, ten subjects received 21 injections of increasing doses of CCK-4 over a 5-minute period. Various degree of gastrointestinal distress preceded feelings of fear. Panic-like attacks were induced in seven subjects after administration of 20 to 150 μg of CCK-4. CCK-8s was injected in two subjects who had previously experienced panic-like attack with CCK-4. Intense gastrointestinal symptoms prompted cessation of CCK-8s administration. Neither one of them presented psychological attributes of a panic attack. Unlike meprobamate and naloxone, pretreatment with lorazepam prevented the CCK-4-induced fear.

Three double-blind cross-over studies have been conducted in healthy subjects. In a first trial, 30 subjects were randomly assigned into one to two treatment sequence groups that determined the order in which they received 2 mg flumazenil or placebo. Two i.v. 50 μg CCK-4 boluses were administered one day apart, 15 minutes after study treatment. Flumazenil did not influence the anxiety response produced by CCK-4. This result led to the conclusion that CCK-4 effects were not mediated through GABA receptors.[372] However, flumazenil showed panicogenic properties in panic-suffering patients only.[71] Therefore, it was suggested that replication of this study in patients would be more enlightening on this particular hypothesis. Effects of single 100-mg dose of CI-988 on 50 μg CCK-4 were evaluated in 30 male subjects. Anxiogenic response was rated on the Panic Symptom Scale (PSS). This scale consists of symptoms set from the DSM-III-R for diagnoses of panic disorder. The sum intensity scores and panic attack rate were significantly lower in the CI-988 group as compared to the placebo group. Number of symptoms, and time to onset and duration of symptoms did not differ between the two groups.[373] Finally, effectiveness of 10 and 50 mg of L 365,260 to antagonise 0.3 μg/kg pentagastrin was assessed in 15 healthy subjects according to a five way cross-over design. Compared to saline injection, pentagastrin significantly increased the PSS and the anxiety scores marked on a visual analogue scale. Pretreatment with L 365,260 dose dependently

reduced the pentagastrin effects with complete abortion of the action following the highest dose.[374]

Compared to saline injection, CCK-4 (25-50 μg) and pentagastrin (0.6 μg/kg) consistently elicited panic-like attacks in patients with panic disorder as per DSM-III-R criteria.[375-379] However, some discrepancies exist about the incidence of panic-like attacks induced by the neuropeptide. Bradwejn *et al.* reported a panic rate of 100% in patients receiving a 50-μg dose of CCK-4. The rate fell to 91% following a 25-μg dose.[380] In contrast, van Megen *et al.* found that 25 μg and 50 μg of CCK-4 induced panic in 44% and 71% of patients, respectively.[377] To date, two controlled double-blind dose-finding trials with CCK-4 have been concluded. Thirty six healthy subjects received randomly either 9 μg, 25 μg, or 50 μg of CCK-4, or saline solution. Panic-like attack was experienced in none of the control group as compared to 11%, 17%, and 47% of subjects who received 9 μg, 25 μg, and 50 μg of CCK-4, respectively.[381] These results confirm previous findings.[380] In patients, the anxiety and panic responses appear to be dose dependent: 17% (10 μg), 64% (15 μg), 75% (20 μg), and 75% (25 μg). None of the patients panicked after injection of a saline solution.[382]

An open-labelled study was conducted to assess imipramine, a well known antipanic drug, in the CCK-4 anxiety model. A total of 11 patients diagnosed with DSM-III-R panic disorder with or without agoraphobia, who previously panicked in response to CCK-4, received imipramine. The imipramine doses administered were individualised and aimed to abort their usual panic attacks. Hence, patients received between 100 and 300 mg of imipramine daily for a 3- to 26-month duration. After 8 weeks of successfully blocked panic attacks, patients were re-challenged with 20 μg i.v. CCK-4. Imipramine significantly reduced the number and the sum intensity of symptoms produced by CCK-4 as evaluated on the PSS scale. Time to onset of symptoms was delayed and duration of the reaction was shortened. Panic attacks were induced in 18% of patients who all panicked after a first CCK-4 injection.[383] In a double-blind, placebo controlled, cross-over study, 29 patients

with panic disorder were challenged twice, a week apart, with 20 μg CCK-4 intravenously injected 90 minutes after administration of a single dose of L 365,260 [10 mg (n = 18), 50 mg (n = 15)] or placebo (n = 17). Number and sum intensity of symptoms were significantly lowered by both doses of L 365,260 compared to placebo. The 50-mg dose was superior to the 10-mg dose. The incidence of panic-like attack as defined by the DSM-III-R criteria was 33%, 0%, and 88% for patients receiving 10 mg of L 365,260, 50 mg of L 365,260, and placebo, respectively.[384]

In humans, anxiety or panic-like symptoms appear within 60 seconds of a CCK-4 injection.[376] Acute response to CCK-4 or pentagastrin lasts from 1 to 4 minutes and returns to baseline value within 10 to 20 minutes.[380, 382, 385] The panicogenic effects of CCK-4 mimic those experienced by patients during a spontaneous panic attack. However, symptoms take place in a precipitous manner instead of occurring according to the well described crescendo profile. The panicogenic effect of CCK-4 is more pronounced in patients than in healthy subjects.[380] Both pentagastrin and CCK-4 result in a marked increase in the heart rate and systolic/diastolic blood pressure during the induced panic-like attack most likely due to a sympathetic system activation.[371-373, 378, 382] To this effect, Abelson *et al.* have shown a very brief and repetitive surge of epinephrine release after pentagastrin infusion.[385]

Tolerance may develop as a result of continuous administration of CCK as well as repeated CCK receptor blockade.[386, 387] The anxiogenic response to CCK-4 intermittently administered appears to be reproducible as reported by Bradwejn *et al.* The reproducibility of the behavioural changes produced by CCK-4 was examined in 11 patients with panic disorder as defined in the DSM-III-R. The study consisted of injection of a saline solution followed upon recovery by a 25 μg CCK-4 administration. This treatment sequence was repeated 2 to 3 days later. As previously reported, CCK-4 evoked higher number and sum intensity of symptoms than saline solution. There was no difference as for the number and the sum intensity of symptoms between sessions. Although not significant, the panic rate

fell from 81.8% after the first session to 72.7% after the second. Nine percent of patients panicked in the placebo group on both sessions. Onset of symptoms was the only parameter that showed significant difference between sessions. The onset occurred earlier after the second CCK-4 injection.[388] In some occasions, animal and human responses were somewhat reduced suggesting a small, but non-significant, habituation effect.[372, 389] Similarly, repeated exposure of CO_2, a well documented anxiogenic agent, led to a reduced anxiogenic response.[390]

According to the above animal and human study outcomes, the place held by CCK-4 as anxiogenic paradigm is promising. Among other existing pharmacologically induced anxiety models[52, 234, 391-393], CCK-4 has been compared to single-breath inhalation of a gas mixture of 35% CO_2 and 65% O_2. In a small open trial, 14 healthy subjects received 35% CO_2 inhalation and 12 received 25 μg CCK-4 i.v. injection. Results indicated that both challenge tests were comparable with respect to the number of symptoms, whereas CCK-4 significantly stood out in producing symptoms of greater intensity. The number of panic attacks experienced by subjects as defined in the DSM-III-R showed a similar incidence that is, 17% after CCK-4 administration and 21% after CO_2 inhalation.[394] Applying the same study design to a 22-panic patient population, number and sum intensity of symptoms collected in the CCK-4 treatment group did not differ from those obtained in the comparison group. There was a trend toward a higher incidence in panic-like attacks in the CCK-4 group.[395] At present, CCK-4 has not been tested against other paradigms.

By their work, Bradwejn and colleagues have much contributed to the validation of CCK-4 as novel probing agent in anxiety disorders. At present, CCK-4 satisfies most of the criteria for an ideal model of anxiety put forward by Guttmacher *et al.*[396] Somatic and emotional components of a spontaneous panic attack and those evoked by i.v. CCK-4 administration are nearly identical as noted by the patients. It is a safe, reversible, and reproducible paradigm. Patients with panic disorder are more prone to react to CCK-4

than healthy subjects. Finally, well documented effective pharmacological treatment of panic disorder also antagonised CCK-4-induced panic-like action.

Towards the mechanistic aspects of CCK-4 as panic-provoking agent, Bradwejn *et al.* have hypothetically suggested the contribution of both central (limbic and cortical) and brainstem regions. The involvement of central regions became a potential mechanism of action with the demonstration of presence of radiolabelled CCK-4 in cortical and limbic regions after systemic administration of the neuropeptide in rats.[367] Conversely, brainstem regions which are poorly shielded by the blood-brain barrier offer the easiest way for CCK-4 to generate the anxiogenic-like process. Brainstem areas are rich in CCK immunoreactivity and CCK_B binding sites and communicate with higher neuronal levels through complex circuits. These brainstem circuits are composed of several interconnected nuclei enabling the peptide to stimulate central regions within milliseconds. Although the plausibility of these mechanisms, the exact neuronal path(s) borrowed by the neuropeptide remain(s) hypothetical.

CHAPTER 4. CCK-5-HT NEURONAL SYSTEM INTERACTIONS AND RESEARCH HYPOTHESIS

The complexity of the neuroanatomic circuitry of the serotonergic and CCK systems opens up the likelihood of potential interactions at various levels of physiological functions. Separate populations of CCK- and 5-HT-containing neuronal cells are found in the rat dorsal raphe.[321] To date, there is no evidence for coexistence of CCK and 5-HT in the same neurons. However, 5-HT axons reach structures which are rich in CCK neurons such as the limbic system and the hippocampus. Indeed, it is conceivable that CCK-containing neurons may receive serotonergic inputs which may influence the synthesis, release and action of the neuropeptide. Inversely, CCK-containing neurons may make synapse to serotonergic neurons and hence modify serotonin synthesis, release and action.

Experimental studies provided insights supporting the complicity and inter-dependence of the 5-HT and CCK systems. To this effect, it has been observed that i.c.v. injections of 10 to 100 ng of CCK-4 stimulated the metabolism of serotonin in the rat brain. Larger doses were less effective.[332] Similarly, central injections of CCK-8s reduced 5-HT content in some brain areas such as the hypothalamus.[397] Contrasting with the central injection, i.p. CCK-8s administration lowered 5-HT metabolism denoting a decline in the turnover of the neurotransmitter.[398] *In vitro* studies demonstrated that CCK-4 (10^{-8} M), but not CCK-8, decreased the affinity and increased the number of 5-HT_2 receptors.[399] Boden *et al.* have reported that CCK-8s excited 5-HT neurons through CCK_A receptors because L 364,718, a CCK_A antagonist, but not L 365,260, a CCK_B antagonist, opposed the 5-HT neuronal activation by CCK.[400] In the guinea-pig, i.p. administration of 10 μg/kg of BOC-CCK-4 did not affect the extracellular levels of cortical 5-HT at baseline. However, during exposure to the elevated plus-maze model of anxiety, BOC-CCK-4 amplified the rise in extracellular 5-HT normally observed in this test and produced anxiogenic effects.

Pretreatment with i.p. administration of L 365,260 (100 μg/kg) antagonised both effects. When administered alone, L 365,260 showed anxiolytic properties, decreased basal 5-HT levels, and prevented the rise in 5-HT induced by exposure to the elevated plus-maze test. Under these conditions, there was no change in serotonin metabolism.[346]

Alternatively, stimulation of endogenous 5-HT activity has been shown to influence the CCK effects. Vasar *et al*[193] have shown that pretreatment of rats with 10 μg/kg of ondansetron completely reversed the anti-exploratory effect of 5 μg/kg of caerulein, a non-selective agonist of CCK_A/CCK_B, indicating the involvement of serotonergic mechanisms in the regulation of emotional behaviour ; 0.1, 1, or 100 μg/kg of ondansetron did not produce any remarkable changes in the locomotor and exploratory activity of rats. As a result, the behavioural effects of ondansetron in animals were described by a bell-shaped dose-response curve. Series of animal studies conducted by Paudice and Raiteri[401, 402] indicated that serotonin enhanced the depolarisation-evoked release of CCK from synaptosomes of rat cerebral cortex or nucleus accumbens. Likewise, 1-phenylbiguanide, a $5\text{-}HT_3$ agonist, enhanced CCK release. This effect was not observed under basal conditions or after pretreatment with $5\text{-}HT_3$ receptor antagonists such as MDL 72222, ICS 205-930 and ondansetron. The antagonist of $5\text{-}HT_1/5\text{-}HT_2$ receptor, methiothepin, did not block the CCK release by serotonin indicating that the effect was most likely mediated by $5\text{-}HT_3$ receptors located on CCK-releasing nerve terminals. In two very small randomised, double-blind, cross-over pilot studies, pretreatment with ondansetron at 0.15-mg/kg and 2-mg doses infused over a 15- and 20-minute period, respectively, failed to prevent the pentagastrin-induced anxiety in panic disorder patients.[403, 404] Recently, it was shown in an 8-week double-blind study that the anxiogenic effects of CCK-4 were blocked by fluvoxamine, a selective serotonergic reuptake inhibitor (SSRI), administered as 150 mg daily dose to 26 panic disorder patients.[405]

These experimental findings together with the evidence supporting that disturbance in 5-HT and CCK neurotransmission may be associated with the state of panic may infer a link

between 5-HT_3 and CCK in the cascade of physiological events preceding a panic attack. However, the nature of the involvement of CCK and 5-HT in the development of a panic attack is yet to be defined. Therefore, the aim of the present work was to evaluate the role of the 5-HT_3 neuronal system in the development of the clinical and physiological features observed during a panic attack induced by intravenous CCK-4 administration. A selective serotonergic 5-HT_3 receptor antagonist, ondansetron, was selected to study this particular receptor subtype. The research work was divided into three parts. The first part consisted of the evaluation of the acute and chronic role of the 5-HT_3 neuronal system in the mediation of CCK-4-induced behavioural and neuroendocrine changes in healthy subjects by administration of oral ondansetron. The acute versus chronic approach was used to evaluate possible neuro-adaptation following chronic ondansetron administration. The second part of the work investigated for the first time the effect of a single and chronic administration of ondansetron on CCK plasma concentrations in humans. Finally, the pharmacokinetic profile of low-dose ondansetron at steady-state was determined by a novel methodological assay.

∞ *PART TWO* ∞

METHODOLOGY

STUDY DESIGN

This research work consisted of a 4-week prospective, double-blind, randomised, parallel-group, placebo controlled study. Multiple oral doses of ondansetron or placebo were administered twice a day at breakfast and bedtime to healthy male subjects for 57 ± 6 doses. Each subject underwent (1) a Screening Phase, (2) a Treatment Phase consisting of two Challenge Periods separated by a 27 ± 3-day interval, and (3) a Follow-Up Phase. A time and procedures schedule for the overall conduct of the study is outlined in Appendix 1.

The Screening Phase was performed for each subject on an outpatient basis with at least three days and a maximum of two weeks separating the time of screening and admission into the Treatment Phase. During the Treatment Phase, subjects were randomised to receive either ondansetron 2 mg or matching placebo twice daily for the study period. Subjects returned every week to the Psychobiology and Clinical Trials Research Unit in Anxiety Disorders of the Clarke Institute of Psychiatry (Toronto, Ontario, Canada) for assessment on an outpatient basis. On Study Days 1 and 29, subjects received the intravenous CCK-4 challenge test. The Follow-Up Phase was conducted on an outpatient basis 3 to 5 days after administration of the last study drug dose for clinical assessment.

STUDY POPULATION

Inclusion Criteria

1. Subjects were male between 18 and 55 years of age and within ± 20% of their ideal body weight determined as follows: 50 ± 2.3 kg / 2.5 cm of height higher or lower than 150 cm.

2. Subjects signed the informed consent form (Appendix 2).
3. Subjects had a clinically normal 12-lead electrocardiogram (ECG).
4. Subjects qualified as being healthy with regards to the physical examination, psychiatric status, vital signs, clinical laboratory tests, Structured Clinical Interview for DSM-III-R (SCID for non-patients), Hamilton Anxiety Rating Scale (HAM-A, Appendix 3), and Symptom Checklist-90 (SCL-90, Appendix 4).

Exclusion Criteria

1. Subjects with clinically significant condition of a disorder of the cardiovascular; neurological; respiratory; endocrine; genitourinary; renal; gastrointestinal; HIV infection or other chronic diseases requiring immediate treatment as determined by examining physician.
2. Subjects with any history of, or who currently met, Axis I DSM-IV criteria.
3. Subjects with condition which might have affected the absorption, distribution, metabolism, or excretion of drugs.
4. Subjects with active alcohol or substance abuse. Current abuse of alcohol was defined by an average consumption greater than 315 g/week, where 15 g = ½ pint of beer (340 mL) or 1 glass of wine (130 mL). Subjects with any history of alcohol or substance abuse or dependence (Axis I DSM-IV criteria) within 6 months of screening (Visit 1) were also excluded.
5. Subjects with evidence of clinically significant hepatic disease (including hepatitis B and C), as indicated by ALT > 3 times upper limit of normal range, or AST > 2 times upper limit of normal range.
6. Subjects with significant biochemical abnormalities.
7. Subjects having habitual and heavy consumption of xanthine-containing beverages and foods (e.g., more than three cups of coffee per day at the time of enrolment into the study).

8. Subjects who had multiple or severe allergies.
9. Subjects who had taken over-the-counter (OTC) medication (excluding vitamins and mineral supplements), or alcohol-containing beverages within 72 hours prior to Study Day 1.
10. Subjects who had taken prescription medication (other than psychoactive drugs) within one (1) week prior to Study Day 1.
11. Subjects who had taken prescription of psychoactive drugs.
12. Subjects with a history of life-threatening neoplasms (treated within the last 5 years prior to study entry) other than basal carcinoma of the skin.
13. Subjects who had received any investigational drug within three (3) months prior to the screening visit.
14. Subjects who previously received CCK-4 injection.
15. Subjects with history of hypersensitivity to ondansetron or other serotonin agents (e.g., Buspar®, Sansert®, Sandomigran®, Sandomigran DS®).
16. Subjects who had a positive drug urine screen at Visit 1.

NUMBER OF SUBJECTS

Since the sum intensity of symptoms (iPSS) and the number of symptoms (nPSS) are highly correlated ($r = 0.9057$), either variable can be considered to be the primary efficacy variable. The effect size calculated using Bradwejn and Koszycki[383] was 1.6. However, for a group of healthy subjects, the effect size was expected to be much less and, therefore, an effect size of 1.2 was chosen. For either iPSS or nPSS comparison with an effect size of 1.2, two-tailed α of 0.05 and a power of 90%, led to a sample size of 18 subjects per group for a total of 36 subjects. This power reduced to 88% when a 1:2 allocation scheme was used leading to a sample size of 12 subjects for the placebo group and 24 subjects for the ondansetron group. This was based on Cohen's sample size for comparing treatment.[406]

To ensure adequate clinical data from 36 evaluable subjects for the final analysis, it was intended that 42 subjects would be randomised to double-blind treatment consisting of 28 subjects in the ondansetron group and 14 subjects in the placebo group.

DRUGS AND TREATMENT

Materials

Test Drug and Control Agents

1. Test Drug

Ondansetron (GR38032F) supplied as 1 mg film-coated tablets.

2. Control Agent

Placebo tablets supplied as film-coated tablets identical in size, colour, and weight to active ondansetron tablets.

3. Challenging Agent

CCK-4 (50 μg in 2.5 mL of NaCl 0.9%) consisting of an amino acid chain of TRP-MET-ASP-PHE-NH_2.

Supply

Active ondansetron and placebo tablets were formulated as identical tablets and quality assured by Glaxo Wellcome Inc., Mississauga, Ontario, Canada. CCK-4 was synthesised by Peninsula (Belmont, CA, USA) and prepared by GIS médicament (Nantes, France).

Packaging and Labelling

All study drug were packaged into blister strips and labelled by Glaxo Canada Inc. Each subject's study drug consisted of a box of 10 strips: two (2) strips per treatment week plus 2 extra strips. A one-part label was affixed on each medication box and contained the following information:

- study number
- number of tablets
- subject number and space for subject initials
- dosage and storage instructions
- space for the investigator telephone number
- 'Investigational Drug, to be used by qualified investigators only' statement
- sponsor identification

Each strip contained 14 tablets of either ondansetron 1 mg or placebo. A label was affixed on each strip and contained the study number, the number of tablets, and the sponsor traceability number. The subject initials were recorded thereupon. Study drug were dispensed along with a leaflet containing the following information: dosage and storage instructions; space for the investigator telephone number; 'Investigational Drug, to be used by qualified investigators only' statement; and sponsor identification.

A code break envelope was provided by the sponsor. The envelope concealed the identity of the drug, the lot number, and the expiration date. This could be unblinded for emergency purposes only. If unblinding occurred, the investigator had to discontinue the study drug and to provide a written explanation on the Clinical Record Form (CRF).

Dose Regimen

Subjects who qualify for entry at Visit 2 were provided with three (3) strips of double-blind study drug. Subjects were instructed to take, orally, 2 tablets at breakfast (before 10:00 hours) and at bedtime (between 21:00 and 24:00 hours) everyday with approximately 150 mL of water. The first study drug dose was administered and ingested at the Research Unit.

At Visits 3, 4, and 5, study drug intake was reviewed for compliance evaluation. At the end of each visit, subjects were provided with an additional 2 strips of double-blind study drug.

At Visit 6, study drug was collected and compliance evaluated. The last study drug dose was administered and ingested at the Research Unit. No medication was dispensed at the end of this visit.

Randomisation

To minimise bias and to assure comparability of groups with respect to pertinent variables, a random code was generated by the sponsor to establish and maintain double-blind conditions. A blocking factor was used for the randomisation. The random code for a specific subject was written on all pages of the CRF. At Visit 2, subjects who met study entry criteria were assigned consecutively in a predetermined random order to receive either ondansetron 2 mg or placebo.

Storage of Drug and Return of Unused Medication

The study drug was kept between 15°C and 30°C for protection against environmental extremes, and in a locked storage facility to which access was only available to the investigator and designated associates. Upon delivery of study medication to the site, an inventory was performed to ensure receipt of the specified amount. Records of dispensing of clinical supplies were maintained throughout the study. At study conclusion, any unused study drug supplies were returned to the sponsor.

METHODOLOGY

Pre-Treatment Procedures

Observations and measurements

All Visit 1 evaluations were performed on an outpatient basis between 3 and 14 days prior to entry into the study. At this time, subjects had signed the informed consent and met the admission criteria. Additionally, subjects had to qualify as being healthy with no clinically significant laboratory abnormality for the screen to be successfully completed.

- Collection of demographic data including sex, date of birth, race, body weight (kg), height (cm), and smoking and drinking habits.
- A complete medical and psychiatric history including past medical history, previous medical and psychiatric diagnoses, concurrent medications, and prior medications were obtained.

- A complete psychiatric evaluation including mental status examination (SCID for non-patients), HAM-A, and SCL-90 were completed.
- A physical examination.
- A resting 12-lead ECG.
- Blood pressure and pulse in sitting position.
- Clinical laboratory tests as follows:

Haematology: Haemoglobin, haematocrit, total and differential leukocyte count, red blood cell count, and platelet count.

Routine urinalysis: Appearance, specific gravity, pH, protein, glucose, ketone, and microscopic examination (WBC, RBC, casts, etc.)

Blood Chemistry: Glucose, blood urea nitrogen (BUN), sodium, potassium, bicarbonate, chloride, creatinine, alkaline phosphatase, AST, ALT, calcium, inorganic phosphorus, albumin, total protein, uric acid, creatinine phosphokinase (CPK).

Drug urine screen.

Concomitant Treatment

The use of any medication were discouraged during study, starting with the screening visit and ending after all final assessments had been reviewed and judged acceptable by the investigator.

Subjects were instructed to consult the investigator before the start of any drug including OTC medication, unless such medication was for emergency use. In the event of such use, the name, time and dose of medicine were recorded, along with the reason it was taken and whether any adverse event occurred. The investigator had to withdraw the subject from the study if the medication taken by the subject caused a pharmacological interaction which might have interfered with the results

of the study. Vitamins, mineral supplements, acetaminophen, and psyllium intakes were allowed during the course of the study.

Instructions to Subjects

- Subjects were instructed to take orally, 2 tablets twice a day at breakfast (before 10:00 hours) and bedtime (between 21:00 and 24:00 hours) with approximately 150 mL of water.
- At Visit 1, subjects were notified of a weekly assessment visit.
- Subjects were instructed not to take the morning dose on Study Day 29, and not to eat and drink anything from 24:00 hours (midnight) the evening prior to Study Days 1 and 29. They were reminded the day before via telephone calls.
- Subjects were requested to bring with them all unused medication at each visit.
- Subjects were informed of any precautions or warnings, and medications that were not permissible.
- Subjects were asked to report any effects (physical and emotional changes) that might be experienced during the study after receiving the study medication.
- Subjects were instructed to keep the study drug at room temperature, in a safe place out of children's reach.

During Treatment Procedure

Acute Dosing Treatment Phase

Pre-challenge assessments were performed on Study Day 1 from -1 to 0 hour prior to the acute double-blind study drug dosing. The subject had at this time have successfully completed the Screening Phase. Vital signs were taken. Concurrent medications and adverse events/concurrent illnesses were recorded. Subjects

provided a urine sample prior to dosing. This sample was used as a drug urine screen. Upon completion of the pre-challenge assessments, subjects were assigned a random number.

Subjects were asked to sit in a reclining chair. A visual analogue scale (VAS) was marked by subjects to assess their anxiety level prior to the beginning of the experiment. The VAS score consisted of a distance in millimeters from the left-hand side of a 100-millimeter line to a perpendicular mark drawn by the subject. An intravenous catheter was installed into the antecubital vein of their right arm through which a NaCl 0.9% solution was slowly infused. The i.v. catheter maintained an open vein to allow CCK-4 injection and blood drawings. A blood pressure cuff with a pulse monitor attached to the Dinamap® (Critikon, Canada) sphygmomanometer was installed on the left arm. Thereafter, subjects were given two (2) double-blind study drug tablets with 150 mL of water. Sixty-minute postdosing, VAS assessment was repeated and CCK-4 50 μg injected in a bolus push (less than 5 seconds). During the CCK-4 Challenge Period the following tests were recorded.

- Vital signs prior to and 20, 40, 60, 80, 100, and 120-second post-challenge test.
- Description of any symptom experienced by the subjects following the CCK-4 injection, and time to onset and duration of symptoms. Immediately after symptoms abated, each symptom was evaluated with the Panic Symptom Scale (PSS, Appendix 5). All subjects were assessed as to whether they experience a panic-like attack as per DSM-IV criteria.
- Anxiety level at peak effect post CCK-4 challenge as marked on the VAS.
- Blood collection from the i.v. catheter for neurobiological hormone levels (adrenocorticotropic hormone [ACTH], cortisol, growth hormone, prolactin, and neuropeptide Y [NPY]) and CCK-4 plasma levels prior to and 2, 5, 10, and 15 minutes post CCK-4 injection.

Subjects were requested to limit communication concerning the study among themselves, if applicable. At the end of Visit 2, adequate amount of double-blind study drug supply was dispensed for the interval until the next scheduled visit.

Chronic Dosing Treatment Phase

Visit 3 was scheduled for one week after the subject had begun the double-blind study drug, and in no case might Visit 3 occurred less than 5 days or more than 10 days after randomisation. Subsequent visits were scheduled one week apart from each other, and in no case occurred less than 5 days or more than 10 days from each other. The following evaluations or tasks were performed during the double-blind treatment phase at the visits indicated.

- Vital signs (sitting blood pressure and pulse) were taken at Visits 3 through 6.
- A urine sample was collected at Visits 3 through 6. This sample was used as a drug urine screen.
- Occurrence of concurrent illnesses, adverse events, and concurrent medication intake at all visits was examined.
- Double-blind study drug was dispensed at Visits 3, 4, 5 and 6; and compliance checked from Visit 3 through Visit 6. Drug dispensed at the end of Visit 5 was collected at the start of Visit 6 (Study Day 29).
- On Visit 6, all assessments performed on Study Day 1 were repeated. In addition, blood was drawn from the i.v. catheter for a trough study drug plasma sample (Pre-dose sample) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 hours after ingestion of the study drug.

On Visit 6, subjects were requested to limit communication concerning the study among themselves, if applicable. Meals were served to the subjects at

approximately 4 hours post CCK-4 challenge test. Subjects remained in the Research Unit until all treatment assessments scheduled at 12 hours postdosing have been completed.

Post-Treatment Procedures

For subjects who discontinued prematurely during the double-blind phase because of an adverse event, a follow-up subject contact was required. Any subject with an abnormal laboratory value or a continuing adverse event was followed according to accepted medical standards for 30 days or until resolution of the abnormality or event.

Observations and Measurements

At Visit 7/Early Termination, the following evaluations were performed.

- A physical examination, including body weight.
- A brief psychiatric assessment.
- A resting 12-lead ECG.
- The blood pressure and pulse in sitting position.
- Clinical laboratory tests as for Visit 1, excluding drug urine screen test.
- Occurrence of concurrent illnesses, adverse events, and concurrent medication intake since last visit.

WITHDRAWALS AND DROPOUTS

Subjects might withdraw themselves or be withdrawn from the study at any time should it be felt that it was to the subject's detriment to continue. All cases withdrawn from the study were documented, including the reason for withdrawal and the final outcome, as these were included in the safety analysis of the results. The investigator had to make every effort to keep each subject in the study. However, if the investigator removed a subject from the study, a complete Visit 7/Early Termination evaluation was obtained. All remaining study drug was to be obtained from the subject.

Subjects who were removed from the study due to adverse events (clinical or laboratory) were treated and followed according to established medical practice. All pertinent information concerning the outcome of the adverse event was entered on the CRF. The investigator might choose to remove a subject from this study for any of a variety of reasons. The following is a list of possible reasons for removal of a subject. (This list is not meant to include all possible justifiable reasons).

- Intolerable adverse events.
- The subject was uncooperative or noncompliant with the protocol requirements. For example, failure to return for scheduled visits, non-compliance with the drug regimen, positive drug urine screen at any of the post-screening visits.
- The subject withdrew consent.
- The subject experienced any illness which made continued participation invalid.
- The subject was lost to follow-up.
- The subject died.
- Violation of the study protocol.
- In the investigator's judgement, withdrawal from the study was in the subject's best interest.
- The study was terminated by the manufacturer.

Additional subjects were enrolled in order to compensate for subjects dropped from the study. In such case, the subject is replaced with the next sequential available identifying number.

SAFETY

Due to the investigational nature of this study, subjects were carefully monitored. Any concurrent illness or adverse event experienced by a subject during any portion of the study was described in detail and fully evaluated. This full evaluation was provided regardless of the investigator's opinion as to whether the event was or was not causally related to the study drug or to participation in the study. Any pertinent information was recorded in the CRF and additional comments describing the course and outcome of these events were provided as appropriate.

DATA ANALYSIS

All significance tests were two-tailed with an overall significance level of 5%. The normality of the data distribution was tested with the Shapiro-Wilk statistic test. For continuous variables without repeated measurements that were normally distributed, unpaired t-tests (two-tailed) were used for comparison between the placebo and the ondansetron treatment. Otherwise, data were analysed using the Mann-Whitney *U* statistic test for two independent samples (two-tailed). For continuous variables with repeated measurements, repeated measures analysis of variance models were used. If applicable, adjustment for baseline differences between treatment groups was done using analysis of covariance (ANCOVA) models. For categorical variables, Fisher's exact or Chi-square tests were used. For all analyses, a p value below 0.05 was considered statistically significant. There was no interim analysis.

Subjects who took less than 80% of the prescribed dose or received wrong study drug at Visits 3,4, 5 or 6 were excluded from the analysis. All decisions to remove a subject from analysis were made and documented before the blinded study drug code was broken. Subjects who discontinued prematurely from the 12-hour pharmacokinetic study were not included in the pharmacokinetic analysis.

Primary Efficacy Variables

The primary outcome parameters were the mean change in the number and sum intensity of PSS symptoms experienced by the subjects after chronic treatment.

Secondary Efficacy Variables

Other outcome parameters were the mean change in the number and sum intensity of PSS symptoms after acute treatment. Changes in the time to onset and duration of symptoms, number of panic attacks, VAS scores, vital signs, and hormone levels (GH, cortisol, prolactin, ACTH, and NPY) were also looked at for both acute and chronic treatment.

The plasma concentrations of ondansetron were tabulated and graphically displayed. Principal pharmacokinetic parameters were area under the plasma concentration curve ($AUC_{0\text{-}12}$, $AUC_{0\text{-}\infty}$), peak plasma concentration (C_{max}), time required to reach maximal concentration (T_{max}), elimination rate constant (z), and half-life ($t_{½}$). Plasma concentration-time data for ondansetron were subjected to non-compartmental pharmacokinetic analyses. Areas under the curve were determined using the log-linear trapezoidal rule. $AUC_{0\text{-}\infty}$ was the trapezoidal area from time zero to the last measurable concentration extrapolated to infinite time by

addition of the area corresponding to the last measurable concentration. Pharmacokinetic parameters for ondansetron and data for CCK-4 were summarised.

Safety/Tolerability Variables

Safety was evaluated by measuring the frequency of adverse events, by monitoring vital signs, and by comparing physical examination and clinical laboratory results performed before and at the end of the study.

CONDUCT OF THE STUDY

The study conformed to Good Clinical Practice Guidelines and to the Declaration of Helsinki 1964, as modified by 41st World Assembly, Hong Kong, 1989 (Appendix 6). Approvals from the Institutional human experimentation committee of the University of Toronto and the Health Protection Branch of Health and Welfare Canada were obtained.

∞ *PART THREE* ∞

RESULTS

CHAPTER 1. ACUTE AND CHRONIC ROLE OF 5-HT$_3$ NEURONAL SYSTEM ON BEHAVIOURAL AND NEUROENDOCRINE CHANGES INDUCED BY INTRAVENOUS CHOLECYSTOKININ TETRAPEPTIDE ADMINISTRATION IN HUMANS

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1.1 ABSTRACT

The influence of single and multiple oral doses of ondansetron, a selective 5-HT_3 receptor antagonist, was evaluated against placebo on cholecystokinin tetrapeptide (CCK-4)-induced behavioural and neuroendocrine changes in humans. Compared to placebo, subjects receiving acute ondansetron treatment showed a significant decrease in the sum intensity of CCK-4-induced panic symptoms (iPSS). Pre-CCK-4 neuropeptide Y (NPY) plasma levels were significantly higher and maximal changes in cortisol, growth hormone and prolactin secretion from baseline (Δmax) significantly lower in the ondansetron group. After ondansetron and placebo chronic administration, there were no statistical differences in the iPSS between groups. Pre-CCK-4 NPY plasma levels were significantly higher, whereas Δmax for NPY significantly lower in the ondansetron group as compared to placebo. These results suggest a role for the 5-HT_3 receptor in the neurobiology of panic disorder through a possible interaction with CCK and NPY systems. Ondansetron chronic effect on CCK-4-induced behavioural changes needs further exploration.

KEY WORDS : Ondansetron; Cholecystokinin tetrapeptide (CCK-4); Panic disorder; ACTH; Cortisol; Growth hormone; Neuropeptide Y; Prolactin.

1.2 INTRODUCTION

Currently, evidence suggests a role for cholecystokinin (CCK) in the neurobiology of panic disorder. Intravenous (i.v.) administration of cholecystokinin tetrapeptide (CCK-4) has been shown to induce panic attacks in patients suffering from panic disorder and in healthy subjects. (Bradwejn et al. 1990, 1994b) In addition, panic disorder patients show an enhanced sensitivity to CCK-4 as compared to normal subjects suggesting anomalies in the CCK system. (Bradwejn et al. 1991) The role of CCK in panic disorder is further supported by the finding of enhanced CCK-4-induced intracellular calcium mobilisation in patients with untreated panic disorder as compared to healthy subjects. (Akiyoshi et al. 1997)

Investigations also suggest a role for the serotonin (5-HT) system in panic disorder. Anxiolytic activity has been reported for 5-HT_3 receptor antagonists in various animal models. (Jones et al. 1988; Costall et al. 1988) In panic disorder patients, administration of ondansetron, a selective 5-HT_3 receptor antagonist, has been shown to reduce the intensity of panic attacks. (Schneier et al. 1996) In contrast, comparative efficacy studies on buspirone, a 5-HT_{1A} partial agonist, fail to show statistical differences between buspirone and placebo. (Pohl et al. 1989) Likewise, antagonists of the 5-HT_2 receptor, ritanserine and trazodone, are ineffective. (Charney et al. 1986; den Boer and Westenberg 1990)

Experimental studies provide evidence supporting a relationship between the 5-HT and CCK systems. It has been observed that central injections of CCK-4 stimulate the metabolism of serotonin in the rat brain. (Itoh et al. 1988) In the guinea-pig, intraperitoneal (i.p.) administration of 10 μg/kg of butylocarbonyl (BOC)-CCK-4 amplifies the rise in extracellular 5-HT normally observed in the elevated plus-maze model of anxiety and produces anxiogenic effects. Pre-treatment with L 365,260, a CCK_B receptor antagonist, opposes both effects. When administered alone, L 365,260 shows anxiolytic

properties, decreases basal 5-HT levels, and prevents the rise in 5-HT induced by exposure to the elevated plus-maze test. (Rex et al. 1994)

Additionally, modulation of endogenous 5-HT_3 activity has been shown to influence the CCK system. Vasar *et al.* have shown that i.p. pre-treatment of rats with 10 μg/kg of ondansetron completely reverses the anti-exploratory effect of 5 μg/kg of caerulein in subcutaneous, a non-selective agonist of CCK_A/CCK_B, indicating the involvement of 5-HT_3 receptors in the regulation of anxiety. (Vasar et al. 1993) Animal studies indicate that serotonin and 1-phenylbiguanide, a 5-HT_3 agonist, enhance the depolarisation-evoked release of CCK from synaptosomes of rat cerebral cortex or nucleus accumbens. This effect is not observed under basal conditions or after pre-treatment with 5-HT_3 receptor antagonists such as MDL 72222, ICS 205-930 and ondansetron. In contrast, the 5-HT_1/5-HT_2 receptor blockade by methiothepin does not antagonise CCK release by serotonin indicating that the effect is most likely mediated by 5-HT_3 receptors located on CCK-releasing nerve terminals. (Paudice and Raiteri 1991; Raiteri et al. 1993) These animal findings further support the relationship between 5-HT_3 and CCK systems. In patients with panic disorder, chronically administered imipramine, a non-selective 5-HT/noradrenaline reuptake inhibitor, fluvoxamine and citalopram, two 5-HT reuptake inhibitors, prevent the CCK-4-induced panicogenic-like symptoms. (Bradwejn and Koszycki 1994a; Shlik et al. 1997; van Megen et al. 1997) Furthermore, it has been shown that acute tryptophan depletion intensifies the neuroendocrine changes produced by i.v. CCK-4 injection in healthy subjects. (Koszycki et al. 1996) These results provide evidence for a role of the 5-HT system in the CCK-4-provoked neurobiological changes.

Based upon these findings, it appears likely that the 5-HT_3 receptor might be a mediator of the panicogenic effect produced by CCK-4. The aim of the present study was to evaluate the role of the 5-HT_3 system in CCK-4-induced panic symptoms. Thus we studied the effect of a single oral dose (acute treatment) and multiple oral doses (chronic treatment) of ondansetron in the mediation of CCK-4-induced panic

symptoms in humans. The evaluation of the acute versus chronic effect of ondansetron was used to assess possible neuro-adaptation following chronic ondansetron treatment.

1.3 METHODS

STUDY DESIGN

The influence of ondansetron on CCK-4-induced behavioural and neuroendocrine changes was investigated in 36 healthy male subjects using a double-blind, randomised, parallel-group, placebo-controlled design. Subjects were randomly assigned into one of the two treatment groups according to a 1:2 allocation scheme. Depending on their assigned treatment group, subjects received i.v. CCK-4 challenge test after a single (2 mg) dose and multiple (2 mg twice daily for 28 days) oral doses of either ondansetron or placebo.

MATERIALS

As previously described, CCK-4, consisting of an amino acid chain of TRP-MET-ASP-PHE-NH_2, was synthesised by Peninsula (Belmont, CA, USA) and prepared by GIS médicament (Nantes, France) as intravenous solutions (50 μg in 2.5 mL of NaCl 0.9%). (Bradwejn et al. 1991) Ondansetron (1 mg calculated as free base) and placebo were formulated as film-coated pink tablets identical in size, colour, and weight. Ondansetron and placebo were prepared and quality assured by Glaxo Wellcome Inc., Mississauga, Ontario, Canada.

EXPERIMENTAL PROCEDURES

The study followed the guidelines of the Declaration of Helsinki of 1989. It was approved by the Institutional Human Experimentation Committee of the University of Toronto (Toronto, Ontario) and the Health Protection Branch of Health and Welfare Canada (Ottawa, Ontario). Subjects gave written informed consent.

Subject population

Male subjects between 18 and 55 years of age and within 20% of the ideal weight for height and body frame were allowed to participate in the study. The screening assessments were performed on an outpatient basis and included a medical history, physical and psychiatric examinations, vital signs, and a 12-lead electrocardiogram tracing. Structured Clinical Interview for DSM-III-R (SCID for non-patients), Hamilton Anxiety Rating Scale, and 90-point Symptom Checklist were completed to rule out any underlying psychiatric conditions. For the screening to be successfully completed, subjects had to be healthy as per the evaluating clinician and to show no clinically significant laboratory abnormality.

Behavioural and neuroendocrine evaluation

Two i.v. CCK-4 challenge test periods were performed 28 days apart. Subjects were instructed to maintain an overnight fast prior to each challenge period. Upon admission to the research unit in the morning of each period, subjects underwent a drug urine screen analysis and, immediately after, completed a visual analogue scale (VAS) to assess their anxiety level prior to the beginning of the experiment. Thereafter, an i.v. catheter was installed into the antecubital vein of the right arm through which a normal saline solution was slowly infused. The i.v. catheter maintained an open vein to allow CCK-4 injection and blood samplings. A blood

pressure cuff with a pulse monitor attached to a Dinamap® (Critikon, Canada) sphygmomanometer was installed on the left arm. Immediately after completion of these procedures, subjects received and ingested two tablets of study medication (either placebo or ondansetron) with 150 mL of water. Sixty minutes post dosing, subjects completed a second VAS assessment and, then, received a 50 μg bolus of CCK-4. Vital signs were recorded prior to and 20, 40, 60, 80, 100, and 120 seconds after CCK-4 administration. Subjects were instructed to describe any symptoms they experienced following the injection. The time to onset and duration of symptoms were recorded. Immediately after symptoms abated, the symptoms experienced by the subjects were evaluated with the Panic Symptom Scale (PSS). (Bradwejn et al. 1991, 1995; Bradwejn and Koszycki 1994a; Koszycki et al. 1996) This 23-item scale consisted of the criteria set for a panic attack described in the DSM-IV and included five panic-unrelated symptoms for validation. The intensity of symptoms was rated on a 5-point scale: 0 (not present) to 4 (extremely severe). All subjects were assessed as to whether they experienced a panic-like attack defined as per the DSM-IV criteria for a panic attack plus a score of two or more on the anxiety, fear and apprehension item of the PSS. Subjects were instructed to rate their anxiety on the VAS to reflect how they felt at peak effect after CCK-4 injection. At the end of the first challenge test period, subjects were dismissed from the research unit after instruction to continue taking study medication at breakfast and dinner times for the next 28 days at which time the second CCK-4 challenge test was performed sixty minutes after ingestion of the last ondansetron dosing. Compliance and adverse event assessments were performed weekly.

Adrenocorticotropic hormone (ACTH), cortisol (CORT), growth hormone (GH), neuropeptide Y (NPY), and prolactin (PRL) were measured as endocrine indicators for stress response. Blood samples were drawn from the indwelling cannula kept patent with normal saline solution prior to and at 2, 5, 10, and 15 minutes after each CCK-4 injection. Blood samples for NPY measurements were collected into ice-

chilled EDTA-containing test tubes. Immediately after blood collection, 0.3 mL of the enzyme inhibitor, aprotinin (10 000 KIU/mL, Sigma Chemical Company, St Louis, MO, USA), was added to test tubes. Tubes were then inverted to ensure good mixing of the blood with the enzyme inhibitor. Blood was centrifuged and plasma for ACTH and NPY levels was stored at -70°C until assayed, whereas serum for CORT, PRL, and GH was immediately analysed.

HORMONE LEVEL DETERMINATIONS

Plasma ACTH was determined at The Hospital-In-Common (Brampton, Ontario) by immunoradiometric assay (IRMA) using a commercially available kit (INCSTAR® Corporation, Stillwater, Mn, USA). The lowest level of detection and within-run coefficient of variation were 1.9 pmol/L and less than 10%, respectively. Cortisol, PRL, and GH serum levels were analysed at The Sick Childrens' Hospital (Toronto, Ontario). Cortisol serum levels were measured by competitive immunoassay with chemi-luminescent detection (Access®, Sanofi Pasteur Diagnostics, Chaska, Mn, USA). The lowest level of detection was less than 5.6 nmol/L. The within-run coefficient of variation was 10% at 75 nmol/L, 5% at 600 nmol/L, and 4% at 1000 nmol/L. Growth hormone serum levels were measured by monoclonal/polyclonal two-site immunometric assay with chemi-luminescent detection (Immulite®, Diagnostics Products Corporation, Los Angeles, CA, USA). The lowest level of detection was 0.003 µg/L. The within-run coefficient of variation was 5%. Prolactin serum levels were measured by enzyme immunometric assay with chemi-luminescent detection, with a lowest level of detection of less than 0.25 µg/L (Access®, Sanofi Pasteur Diagnostics, Chaska, Mn, USA). The within-run coefficient of variation was 5%. NPY plasma levels were analysed by radioimmunoassay in the Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, using an antiserum to h-neuropeptide Y and ^{125}I-labeled NPY as a tracer. The assay method used was a modification of the methodology developed by Mouri *et al.* (1992). The intra- and

interassay variations were 8% and 14%, respectively. The detection limit of the assay was 5 pg/mL of plasma.

STATISTICAL ANALYSIS

Since the sum intensity of symptoms (iPSS) and the number of symptoms (nPSS) are highly correlated ($r = 0.9057$), either variable can be considered to be the primary efficacy variable. The effect size calculated using Bradwejn and Koszycki (1994a) was 1.6. However, for a group of healthy subjects, the effect size was expected to be much less and, therefore, an effect size of 1.2 was chosen. For either iPSS or nPSS comparison with an effect size of 1.2, two-tailed α of 0.05 and a power of 90%, led to a sample size of 18 subjects per group for a total of 36 subjects. This power reduced to 88% when a 1:2 allocation scheme was used leading to a sample size of 12 subjects for the placebo group and 24 subjects for the ondansetron group. All other variables cannot be used in a confirmatory mode but rather as hypotheses generators. Protocol-defined secondary study end-points included the iPSS and nPSS symptoms experienced after acute treatment, time to onset and duration of symptoms, number of panic attacks, and change from baseline for VAS score, vital signs and neuroendocrine parameters for both acute and chronic treatment. The VAS score consisted of a distance in millimeters from the left-hand side of a 100-millimeter line to a perpendicular mark drawn by the subject. Change from baseline values (Δmax) was calculated by subtracting the baseline value from the maximal value observed after i.v. CCK-4 administration.

Data were analysed by using the SAS system for PC version 6.12 (Cary, NC, USA). The assumption of the normal distribution of data was tested with the Shapiro-Wilk statistic. For continuous variables without repeated measurements such as iPSS and nPSS scores, statistical comparisons were made by using a two-tailed t-test for unpaired samples. For continuous variables with repeated measurements such as VAS

scores, vital signs and neuroendocrine data, repeated measures analysis of variance models were used. Adjustment for baseline differences between treatment groups was done using analysis of covariance (ANCOVA) models. The Δmax values were analysed by a one way (treatment) ANCOVA. Mann-Whitney *U* statistic test for two independent samples (two-tailed) was used for continuous data without repeated measurements that are not normally distributed, such as onset and duration of symptoms. Chi-square test was used for categorical data. For cells with expected frequency less than 5, Fisher's exact test was used to analyse individual 2 x 2 tables, such as the number of panic-like attacks. For all analyses, a p value below 0.05 was considered statistically significant. Results are reported as mean ± SEM.

1.4 RESULTS

To ensure adequate clinical data from 36 subjects, a total of 41 subjects were randomised to one of the two treatment groups. One subject from the placebo group was discontinued due to protocol violation. From the ondansetron group, one subject was discontinued due to a severe allergic-type reaction to study medication and one withdrew his participation for reasons unrelated to the study. Therefore, 41 healthy male subjects completed the acute period of the study, 27 of whom received ondansetron treatment. Thirty eight subjects completed both the acute and chronic treatments, 25 of whom received ondansetron. Baseline characteristics of subjects are summarised in Table 1.1. There was no statistical difference in baseline characteristics between ondansetron and placebo groups.

PRIMARY STUDY END-POINTS

The primary outcome measures are summarised in Table 1.2. There were no statistical differences in the mean iPSS or nPSS symptoms experienced by subjects who received chronic treatment with ondansetron versus those who were administered placebo.

SECONDARY STUDY END-POINTS

Effect of acute treatment with ondansetron on PSS symptoms

The mean iPSS and nPSS symptoms experienced by subjects after a single oral dose of either ondansetron or placebo are shown in Table 1.2. A significant decrease in the mean iPSS score was observed in the ondansetron group compared to the placebo group ($p = 0.0306$). In line with this reduction, there was a decline in the mean nPSS score in the ondansetron group, which did not reach statistical significance ($p = 0.1577$). A student's t-test of the mean iPSS and nPSS measures between CCK-4 challenge periods within each treatment group showed significant differences within the ondansetron group (iPSS $p = 0.0192$, nPSS $p = 0.0211$) and the placebo group (iPSS $p = 0.0004$, nPSS $p = 0.0042$).

Effect of ondansetron on other behavioural measures

Individual PSS symptoms were further examined to identify those affected by the ondansetron treatment (Table 1.3). This statistical analysis was exploratory rather than confirmatory. Therefore, these results should be interpreted with caution. Nevertheless, the *post hoc* analysis revealed that, compared to placebo, the intensity of dyspnea, choking feeling, anxiety, fear and/or apprehension and fear of losing control was significantly reduced after acute treatment with ondansetron. There were no

differences between individual PSS symptoms after chronic treatment with ondansetron. Neither CCK-4 nor placebo treatment affected the five panic-unrelated symptoms, namely earache, itchy nose, stuffy nose, low back pain, and itchy feet, included in the PSS to evaluate response bias.

Although not statistically significant, there was a reduction in the number of panic-like attacks experienced by subjects who received a single dose of ondansetron compared to those who received a single dose of placebo (11/27 [41%] vs 9/14 [64%], $p = 0.1526$). The number of panic-like attacks was similar after chronic administration of either treatment (6/25 [24%] vs 4/13 [31%], $p = 0.7092$). The time to onset of symptoms showed a delay in the occurrence of symptoms in the ondansetron group after acute treatment but the difference failed to reach statistical significance (23 vs 20 sec, $p = 0.0747$) ; there was no difference after chronic treatment (19 vs 16 sec, $p = 0.2167$). There were no significant differences between ondansetron and placebo groups with respect to the duration of symptoms after either acute (136 vs 156 sec, $p = 0.2263$) or chronic (102 vs 95 sec, $p = 0.6334$) treatment.

Mean VAS anxiety scores are displayed in Table 1.2. Mean VAS anxiety scores at baseline and prior to CCK-4 administration, that are prior to and 60 minutes after study drug administration, respectively, were similar in both treatment groups after either acute or chronic exposure. There was no difference between mean VAS scores at baseline and mean pre-CCK-4 VAS scores. This allowed the use of mean pre-CCK-4 VAS values as the baseline in evaluating changes due to the CCK-4 challenge tests. Subjects rated themselves as significantly less anxious at peak effect of CCK-4 after acute treatment with ondansetron as compared to placebo ($p = 0.0274$). Likewise, mean change in the CCK-4-induced VAS score (Δmax) from VAS score measured prior to CCK-4 administration significantly differed between the acute ondansetron treatment group and the acute placebo treatment group ($p = 0.0298$). Once again, there was no difference between treatment groups after chronic

administration. A student's t-test of the VAS scores between CCK-4 challenge periods within each treatment group showed no significant differences with the exception of the post CCK-4 VAS score within the placebo group only ($p = 0.0008$).

Effects of ondansetron on cardiovascular measures

Table 1.4 shows the influence of ondansetron on mean basal vital signs (systolic/diastolic blood pressures and heart rate) and on intravenous CCK-4-induced maximal vital sign mean changes from baseline (Δmax). After acute treatment, there was no significant difference in mean basal and in Δmax vital signs between ondansetron and placebo groups. After chronic treatment, there was a reduction, which did not reach statistical significance, in basal systolic blood pressure in subjects who received the ondansetron treatment ($p = 0.0557$), whereas Δmax for any vital signs did not significantly differ between groups. There was no significant change in vital signs over time with the exception of the heart rate at the first CCK-4 challenge period ($p = 0.0108$). A repeated measures analysis of covariance of the changes in vital signs over time showed no significant differences between treatment groups with respect to systolic/diastolic blood pressures and heart rate at either CCK-4 challenge periods. A student's t-test of the vital signs between CCK-4 challenge periods within each treatment group showed a significant difference in the basal systolic blood pressure within the placebo group only ($p = 0.0280$).

Effects of ondansetron on neuroendocrine measures

Mean basal plasma hormone levels and CCK-4-induced neuroendocrine mean changes from baseline after acute and chronic treatment with ondansetron and placebo are summarised in Table 1.5. Values below the limit of detection have been imputed using a value just below the limit of detection. The Shapiro-Wilk statistic test for normality indicated that the distribution of the neuroendocrine data violated the assumption of

normality. A repeated measures analysis of covariance of the change in hormones over time showed no significant differences between the ondansetron and the placebo groups. The baseline value was a significant covariate with the exception of ACTH and PRL.

After acute treatment, there was a significant increase in the mean NPY plasma levels at baseline in the ondansetron group as compared to the placebo group ($p = 0.0024$). Compared to placebo, there was a significant reduction in the mean maximal increase in cortisol ($p = 0.0485$), GH ($p = 0.0049$) and PRL ($p = 0.0323$) secretion from baseline (Δmax) in the ondansetron group after intravenous CCK-4 administration. There were no differences in Δmax ACTH and NPY between the acute ondansetron treatment group as compared to placebo after CCK-4 injection.

After chronic treatment, the mean basal NPY plasma level was still significantly higher in the ondansetron group as compared to the placebo group ($p = 0.0103$). After CCK-4 injection, there was a significant reduction in Δmax NPY in the ondansetron group ($p = 0.0097$). There was a decline in the Δmax GH and PRL in the ondansetron group compared to the placebo group, but the difference failed to reach statistical significance. There was no difference between the ondansetron and the placebo groups with respect to the Δmax ACTH and CORT.

The maximal rise in ACTH, PRL, and cortisol occurred 5, 10, and 15 minutes, respectively, after CCK-4 administration for both acute and chronic ondansetron- and placebo-treated subjects. GH reached peak concentrations 15 minutes after CCK-4 injection for acute and chronic ondansetron- and placebo-treated subjects. NPY profiles were described by smooth curves. The maximal rise in NPY occurred 10 minutes after CCK-4 administration in the acute placebo group, whereas NPY peak plasma levels were reached 2 minutes after administration of CCK-4 in the chronic

placebo group. The maximal rise in NPY occurred 2 minutes after CCK-4 administration for the acute and chronic ondansetron-treated subjects.

A student's t-test of the mean ACTH and NPY plasma levels and for CORT, PRL and GH serum levels measured at baseline between CCK-4 challenge periods within each treatment group showed significant differences in the ondansetron group only. Compared to acute treatment, there was a significant decrease in mean basal ACTH ($p = 0.0482$) and CORT ($p = 0.0007$) levels after chronic administration of ondansetron. A reduction in the mean basal PRL serum level was observed between challenge periods but the difference failed to reach statistical significance ($p = 0.0517$). Mean basal neuroendocrine plasma or serum levels were not significantly different between challenge periods within the placebo group ($p > 0.35$).

ADVERSE EVENTS

Regardless to relationship to treatment, 89% of subjects reported adverse events in the ondansetron group compared to 71% in the placebo group. Gastrointestinal symptoms, mostly constipation, diarrhea, and discomfort, were more frequently reported in the ondansetron group (67%) compared to the placebo group (36%). In contrast, neurological symptoms such as headaches, tremors, dizziness, confusion, and paresthesia, were more frequent in the placebo group (50%) than in the ondansetron group (37%). Reports of cardiovascular-, psychiatric-, and musculoskeletal-related events were of similar incidence. The intensity of all reported adverse events were mild to moderate except for the allergic-type reaction which was severe. The clinical symptoms of this reaction consisted of the swelling of feet with development of painful red nodules under the toes. Symptoms resolved upon discontinuation of study medication (ondansetron) without further intervention.

1.5 DISCUSSION

From a behavioural point of view, our results show that acute treatment with ondansetron significantly decreased the sum intensity of symptoms and the anxiety score at peak effect of CCK-4 on the VAS. This outcome was further supported by a reduction, although not significant, in the number of symptoms experienced by subjects of the ondansetron group as compared to those of the placebo group. The four symptoms induced by CCK-4 that have been identified by the *post hoc* analysis namely dyspnea, choking feeling, anxiety/fear/apprehension and fear of losing control deserve special attention. These particular findings indicate that ondansetron attenuates not only somatic symptoms but also anxiety and cognitive symptoms induced by CCK-4. Ondansetron also decreased CCK-4-induced dyspnea, a symptom central to panic attacks. (Klein 1993) Moreover, our findings show a significant reduction in the cortisol, GH and PRL secretion in response to CCK-4 injection after acute treatment with ondansetron as compared to placebo. These measures represent biological changes which coincide with the panicogenic effect of CCK-4. Hence, behavioural and biological results are strong evidence for an action of acute treatment with ondansetron on CCK-4 and support the role of the 5-HT_3 network in the mediation of the panicogenic effect of CCK-4.

The unexpected significant increase in pre-CCK-4 (basal) NPY plasma levels observed after both acute and chronic treatment with ondansetron is of interest. Similar effects have been observed in the frontal cortex and hypothalamus of rats after chronic oral administration of imipramine, a 5-HT and noradrenaline (through its metabolite) reuptake inhibitor. (Heilig et al. 1988a) As basal NPY plasma levels were measured just prior to intravenous CCK-4 administration, a contribution of anticipatory anxiety prior to receiving CCK-4 cannot be excluded. It has been shown that low concentrations of the C-terminal fragment $NPY_{13\text{-}36}$ increase, possibly through Y_2 receptors, the exploratory behaviour of rats which is interpreted as a decline in the

animal anxiety levels. (Heilig et al. 1988b) Based on these experimental findings, one may suggest that ondansetron might activate the NPY anxiolytic system leading to a rise in basal NPY plasma levels and, consequently, reducing the anxiety state in humans. The exact mechanism by which ondansetron increased NPY plasma levels cannot be elucidated by this study but may involve a previously postulated non-synaptic interaction with NPY neurons. (Guy et al. 1988) Whether the increase of NPY could suggest the existence of a presynaptic inhibitory regulation of NPY release by 5-HT is unknown. Nevertheless, the interaction may influence various physiological functions. First, substantial numbers of NPY neurons are found in the arcuate nucleus of the hypothalamus. (Bai et al. 1985) This nucleus is well innervated by 5-HT and send projections to the paraventricular nucleus, the dorsomedial nucleus, and the median eminence. (Descarries and Beaudet 1978; Bai et al. 1985) NPY inhibits the production of cAMP under both basal and forskolin-stimulated conditions through the Y_2 receptors which predominate in the hypothalamus. (Westlind-Danielsson et al. 1987) Hence, one may suggest that perturbation of the NPY/5-HT circuit by ondansetron could attenuate, at least in part, the hormonal response to stimulation. However, this possible role may be trivial since there was no difference in the hormonal response between ondansetron and placebo groups after chronic administration even though basal NPY plasma levels were significantly different. Second, we now know that serotonin release in the brain cortex is inhibited by NPY and is increased by CCK. (Schlicker et al. 1991; Kendrick et al. 1991) Hence, one may suggest the existence of a 'cross-talk' between the 5-HT_3, CCK, and NPY systems that might have a role in the neurobiology of panic attacks.

Interestingly, our results show no difference in the iPSS scores, and the cortisol, GH and PRL changes between the ondansetron and placebo groups after chronic exposure. This lack of effect contrasts with the significant difference observed in these parameters after single dose administration of ondansetron as compared to placebo. Our results show also that the iPSS and nPSS values between challenge periods within

each treatment group were significantly different. An increase in the iPSS and nPSS scores in the ondansetron group reaching those of the placebo group would likely reflect a neuro-adaptation phenomenon occurring after chronic ondansetron exposure. However, our results show opposite trends. The reduction in the iPSS and nPSS values was further accentuated after the second relative to the first challenge period within the ondansetron group (iPSS $p = 0.0192$, nPSS $p = 0.0211$). Within the placebo group, values for both PSS parameters and the post-CCK-4 VAS score significantly decreased after chronic administration as compared to acute administration reaching those observed in the ondansetron group (iPSS $p = 0.0004$, nPSS $p = 0.0042$, post CCK-4 VAS $p = 0.0008$). This profile is more compatible with a placebo response theory which is further substantiated by the absence of a difference in the neuroendocrine parameters between challenge periods within the placebo group.

A placebo response may not be the only explanation for this lack of effect. For instance, an habituation effect cannot be excluded. Results observed in the placebo group after the first CCK-4 injection are comparable with the previously described CCK-4-induced behavioural and neuroendocrine changes in healthy subjects. (Bradwejn et al. 1994b, 1995; Koszycki et al. 1996) A significant decline in iPSS and nPSS during the second relative to the first challenge injection has also been reported. (Bradwejn et al. 1994b) Our results show a similar decline between challenge periods within the placebo period. The 40% drop in the iPSS score noticed in the placebo group after the second relative to the first challenge period may have heavily influenced the outcome of the study. Therefore, summation of both a placebo response and an habituation effect along with other unknown confounding factors may have contributed to this lack of effect. Nevertheless, results from the acute treatment with ondansetron taken together with significant NPY plasma level changes and the significant period effect observed within the ondansetron group for the behavioural changes as well as for the ACTH ($p = 0.0482$) and cortisol ($p = 0.0007$) levels indicate that some of the acute action of ondansetron might be maintained over time.

There was no difference in CCK-4-induced ACTH secretion between groups after either acute or chronic treatment. With respect to cortisol, the time course of release remains to be established due to a continued increase beyond the collection time. Hence, no conclusive observations may be drawn from the cortisol profile. Circulating cortisol levels began rising after ACTH levels reached peak values. This observation may suggest that circulating cortisol levels followed, at least in part, the ACTH plasma level profile. The significant decline in ACTH ($p = 0.0482$) and cortisol ($p = 0.0007$) baseline values between challenge periods within the ondansetron group may suggest a decrease of anxiety in anticipation of the CCK-4 injection. These results can be contrasted with those observed in the placebo group that show no significant change in ACTH and cortisol baseline values between both challenge periods.

The maximal change in NPY secretion from baseline (Δmax) provides some indication of a significant treatment difference in favour of ondansetron after chronic treatment. Findings show a significant reduction in the NPY secretion in response to CCK-4 injection after chronic treatment with ondansetron as compared to placebo ($p = 0.0097$). The significant decline in the CCK-4-induced NPY release in the ondansetron group taken together with the significant increase in the pre-CCK-4 NPY release detected in the ondansetron group as compared to the placebo group may be viewed as paradoxical: ondansetron administered prior to CCK-4 challenge procedure significantly increases basal NPY plasma levels but antagonises CCK-4-induced NPY release. The underlying mechanisms for such effects are unknown. A series of potential interactions seem to occur in a very complex system. Therefore, many more observations are necessary to understand these findings. Based upon these findings, one may suggest that the ondansetron-induced NPY release may have attenuated the CCK-4-induced panicogenic symptoms promptly after CCK-4 injection. In contrast, the CCK-4-induced NPY release observed in the placebo group may have occurred to counteract the panicogenic effects induced by CCK-4 leading to a delayed reaction in the placebo group compared to the ondansetron group. One may argue against this

concept based upon the absence of difference in the behavioural response between ondansetron and placebo after chronic treatment in spite of the significant changes in NPY plasma levels between both treatment groups. Whether the time course or the intensity of the NPY response changes upon re-exposure to CCK-4 due to a sensitisation of the NPY neurons by the first CCK-4 injection is unknown but remains a concept worth investigating further. A complete NPY plasma level profile with closer time points covering the first 2 minutes following the CCK-4 injection is essential to evaluate this hypothesis.

Regulation of adenohypophyseal hormone secretion involves numerous transmitters which play controversial roles as both stimulatory and inhibitory effects have been reported. Experimental studies indicated the existence of complex interactions between CCK and dopaminergic D_2 receptors apparently controlled by the nature of the D_1 activation state. (Li et al. 1994) In addition, it has been shown that 5-HT_3 antagonist agents such as ICS 205,930 and ondansetron prevented the increase of drug-induced dopaminergic neuronal activities. (Christoffersen et al. 1988; Carboni et al. 1989) Based on these findings, GH and PRL responses observed after acute treatment with ondansetron may have resulted from an interaction between CCK, 5-HT_3, and dopamine networks. Moreover, part of changes in GH may be secondary to NPY changes. NPY has been shown to stimulate the basal secretion of somatostatin which inhibits the GH release. (Milgram et al. 1990) Therefore, the significant increase in the NPY plasma levels produced by ondansetron prior to CCK-4 injection could theoretically have promoted the secretion of somatostatin and, consequently, inhibited GH secretion. However, differences in PRL and GH secretion between groups failed to show significance after chronic exposure in spite of the significant increase in basal NPY plasma levels in the ondansetron group. Given the small sample size and the intra-subject variability, the apparent absence of an effect does not exclude possible changes. Further evidence on the existence of these hypothetical mechanisms are required to better assess the mechanistic involved in these findings.

With respect to the vital sign measurements, CCK-4 produced hemodynamic changes of similar magnitude in the ondansetron group as in the control group. This finding suggests that CCK-4-induced hemodynamic changes involved a 5-HT_3-independent neurotransmission pathway.

In conclusion, results from this study suggest the following. First, decrease in behavioural and neuroendocrine responses provide strong evidence for an action of acute treatment with ondansetron on CCK-4 and support a role for 5-HT_3 receptors in CCK-4-induced panic attacks in healthy subjects. Second, acute and chronic treatment with ondansetron affects basal NPY and CCK-4-induced changes in NPY. Hence, one may suggest that 5-HT_3 may serve as an important regulator of basal and CCK-4-stimulated NPY release. Finally, our results suggest a role for the 5-HT_3 receptor in the neurobiology of anxiety and panic attacks through its interaction with CCK and NPY systems. Chronic effect of ondansetron on CCK-4-induced panic symptoms still needs exploration.

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Table 1.1 Demographic data[a].

VARIABLE	ACUTE TREATMENT[b]		CHRONIC TREATMENT[c]	
	Placebo (n = 14)	Ondansetron (n = 27)	Placebo (n = 13)	Ondansetron (n = 25)
age[d] (yrs)	27.1 ± 1.4	28.2 ± 1.0	27.3 ± 1.4	27.6 ± 1.0
height[d] (cm)	176.1 ± 1.4	180.1 ± 1.1	176.3 ± 1.5	180.0 ± 1.5
weight[de] (kg)	73.5 ± 2.4	77.6 ± 1.9	74.6 ± 2.3	77.8 ± 2.1
ethnic origin (n)				
caucasian	9 (64.3%)	24 (88.9%)	9 (69.2%)	22 (88.0%)
black	2 (14.3%)	0	2 (15.4%)	0
oriental	2 (14.3%)	2 (7.4%)	2 (15.4%)	2 (8.0%)
Hispanic	1 (7.1%)	1 (3.7%)	0	1 (4.0%)
tobacco user (n)				
never	9 (64.3%)	17 (63.0%)	8 (61.5%)	16 (64.0%)
former	0	6 (22.2%)	0	5 (20.0%)
current	5 (35.7%)	4 (14.8%)	5 (38.5%)	4 (16.0%)
alcohol consumption[d] (unit/week)	3.7 ± 1.0	3.0 ± 0.6	3.8 ± 1.1	3.1 ± 0.6
sitting SBP[d] (mmHg)	123.1 ± 3.2	121.8 ± 2.5	124.8 ± 3.0	122.4 ± 2.7
sitting DBP[d] (mmHg)	70.4 ± 1.6	71.8 ± 1.0	70.5 ± 1.7	71.8 ± 1.0
sitting heart rate[d] (bpm)	68.4 ± 2.3	68.0 ± 2.0	68.8 ± 2.4	68.8 ± 2.1
HAM-A score[d]	1.4 ± 0.4	1.3 ± 0.3	1.5 ± 0.4	1.4 ± 0.3
SCL-90 score[d]	9.1 ± 2.0	6.9 ± 1.5	9.0 ± 2.2	7.4 ± 1.5

[a] There was no statistical difference between treatment groups for the demographic variables.

[b] 2 mg.

[c] 2 mg twice daily for 28 days.

[d] Data are mean ± SEM.

[e] Body weights were within 20% of ideal body weight.

SBP, systolic blood pressure; DBP, diastolic blood pressure; HAM-A, Hamilton anxiety scale; SCL-90, 90-point symptom checklist score.

Table 1.2 Effect of ondansetron on intravenous CCK-4-induced behavioural changes.

	ACUTE TREATMENT[a]			CHRONIC TREATMENT[b]		
Parameter	Placebo (n = 14)	Ondansetron (n = 27)	p-value	Placebo (n = 13)	Ondansetron (n = 25)	p-value
PSS scores						
iPSS	25.9 ± 2.7	18.4 ± 2.0	0.0306	15.7 ± 2.2	15.0 ± 1.6	0.8088
nPSS	11.3 ± 0.8	9.8 ± 0.6	0.1577	8.5 ± 0.9	8.5 ± 0.7	0.9870
VAS scores						
baseline	1.8 ± 0.4	1.6 ± 0.2	0.6076	1.1 ± 0.2	1.2 ± 0.2	0.6942
pre CCK4	1.9 ± 0.4	1.7 ± 0.2	0.7912	1.3 ± 0.2	1.4 ± 0.2	0.7673
post CCK4	6.7 ± 0.6	5.2 ± 0.3	0.0274	4.2 ± 0.5	4.7 ± 0.4	0.4027
Δmax	4.9 ± 0.8	3.5 ± 0.4	0.0298	2.8 ± 0.5	3.3 ± 0.4	0.4389

Data are mean ± SEM.

[a] 2 mg.

[b] 2 mg twice daily for 28 days.

PSS, Panic symptom scale ; iPSS, sum intensity of PSS symptoms ; nPSS, number of PSS symptoms ; VAS, Visual analogue scale ; baseline, VAS anxiety score before ondansetron or placebo administration ; pre CCK4, VAS anxiety score 60 minutes after ondansetron or placebo administration that is immediately before intravenous CCK-4 administration ; post CCK4, VAS anxiety score at peak effect after intravenous CCK-4 administration ; Δmax, post CCK4 - pre CCK4.

Table 1.3 Effect of ondansetron on intensity of PSS symptoms induced by CCK-4.

PANIC SYMPTOM SCALE	ACUTE TREATMENT[a]		CHRONIC TREATMENT[b]	
	Placebo n = 14	Ondansetron n = 27	Placebo n = 13	Ondansetron n = 25
dyspnea	2.4 ± 0.3 (92.8)	1.5 ± 0.2[c] (85.2)	1.2 ± 0.3 (69.2)	1.4 ± 0.2 (76.0)
dizziness	0.9 ± 0.2 (71.4)	0.8 ± 0.2 (55.6)	0.5 ± 0.1 (53.8)	0.6 ± 0.2 (32.0)
unsteady feeling	1.4 ± 0.3 (71.4)	1.1 ± 0.2 (66.7)	0.8 ± 0.2 (61.5)	0.9 ± 0.2 (60.0)
faintness	1.4 ± 0.4 (64.3)	0.7 ± 0.2 (44.4)	0.5 ± 0.2 (38.5)	0.6 ± 0.1 (48.0)
palpitations	2.2 ± 0.4 (78.6)	1.6 ± 0.2 (77.8)	1.6 ± 0.4 (76.9)	1.6 ± 0.2 (88.0)
trembling/shaking	0.8 ± 0.3 (50.0)	0.6 ± 0.2 (40.7)	0.4 ± 0.1 (38.5)	0.2 ± 0.1 (20.0)
sweating	0.6 ± 0.3 (28.6)	0.9 ± 0.2 (51.8)	0.6 ± 0.3 (38.5)	0.4 ± 0.2 (28.0)
choking feeling	1.8 ± 0.4 (71.4)	0.7 ± 0.2[d] (44.4)	1.4 ± 0.4 (53.8)	0.9 ± 0.2 (48.0)
nausea	1.9 ± 0.4 (78.6)	1.1 ± 0.2 (55.6)	1.8 ± 0.3 (84.6)	1.0 ± 0.2 (48.0)
abdominal distress	2.2 ± 0.4 (78.6)	2.2 ± 0.2 (92.6)	1.8 ± 0.4 (76.9)	2.2 ± 0.2 (92.0)
feeling unreal or detached	0.6 ± 0.3 (35.7)	0.5 ± 0.1 (37.0)	0.2 ± 0.2 (15.4)	0.2 ± 0.1 (20.0)
numbness/tingling	1.9 ± 0.4 (85.7)	1.7 ± 0.3 (70.4)	1.2 ± 0.3 (61.5)	1.3 ± 0.2 (64.0)
hot flushes or cold chills	1.8 ± 0.4 (64.3)	1.8 ± 0.2 (85.2)	1.0 ± 0.4 (38.5)	1.2 ± 0.2 (60.0)
chest pain or discomfort	1.9 ± 0.4 (71.4)	1.4 ± 0.2 (63.0)	1.6 ± 0.4 (61.5)	1.4 ± 0.2 (76.0)
anxiety, fear or apprehension	2.3 ± 0.3 (92.8)	1.2 ± 0.2[e] (74.1)	0.9 ± 0.3 (53.8)	1.0 ± 0.2 (72.0)
fear of dying	0.4 ± 0.2 (21.4)	0 ± 0	0 ± 0	0 ± 0
fear of losing control	1.1 ± 0.2 (71.4)	0.4 ± 0.1[f] (40.7)	0.2 ± 0.1 (23.1)	0.2 ± 0.1 (16.0)
fear of going crazy	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Data are mean ± SEM scores on a scale of 0 to 4; data in parentheses are percentages of subjects reporting symptoms.

[a] 2 mg

[b] 2 mg twice daily for 28 days

[c-f] unpaired t-test, two-tailed (*post hoc* analysis to be interpreted with caution because one out of 18 symptoms may be significant by chance alone): [c] $p = 0.010$; [d] $p = 0.025$; [e] $p = 0.004$; [f] $p = 0.011$.

Table 1.4 Effect of ondansetron on intravenous CCK-4-induced vital sign changes.

	ACUTE TREATMENT[a]			CHRONIC TREATMENT[b]		
Parameter	Placebo (n = 13)	Ondansetron (n = 27)	p-value	Placebo (n = 13)	Ondansetron (n = 25)	p-value
sitting HR[c]						
baseline	72.4 ± 3.4[d]	72.6 ± 2.7	0.9774	73.6 ± 3.0	70.0 ± 2.6	0.3855
ΔMAX	28.3 ± 4.8	29.8 ± 2.7	0.7791	28.7 ± 5.6	31.1 ± 1.6	0.6494
sitting SBP[c]						
baseline	118.9 ± 3.6[d]	115.7 ± 2.3	0.4451	126.3 ± 4.2	117.3 ± 2.5	0.0557
ΔMAX	17.0 ± 2.8	17.4 ± 2.4	0.8739	15.2 ± 4.5	14.0 ± 2.0	0.3062
sitting DBP[c]						
baseline	70.4 ± 1.7[d]	70.7 ± 1.4	0.8669	72.4 ± 2.1	70.3 ± 1.1	0.3398
ΔMAX	8.5 ± 2.5	6.9 ± 1.2	0.5269	9.2 ± 3.2	7.2 ± 1.5	0.2849

Data are mean ± SEM.

[a] 2 mg.

[b] 2 mg twice daily for 28 days.

[c] HR, heart rate in bpm ; SBP, systolic blood pressure in mmHg ; DBP, diastolic blood pressure in mmHg.

[d] n = 14.

ΔMAX, maximal change from baseline.

Table 1.5 Effect of ondansetron on intravenous CCK-4-induced neuroendocrine changes.

Parameter	ACUTE TREATMENT[a]			CHRONIC TREATMENT[b]		
	Placebo (n = 14)	Ondansetron (n = 27)	p-value	Placebo (n = 13)	Ondansetron (n = 25)	p-value
ACTH (pmol/L)						
baseline	9.5 ± 2.0[c]	6.8 ± 0.8	0.2146	8.6 ± 2.1	5.5 ± 0.5	0.1786
ΔACTH	19.2 ± 3.1[c]	13.4 ± 2.7	0.0911	13.8 ± 2.7	8.6 ± 1.4	0.1320
CORT (nmol/L)						
baseline	460.4 ± 51.5	447.3 ± 33.6	0.8281	405.2 ± 40.7	355.2 ± 28.6	0.3179
ΔCORT	176.3 ± 25.1	128.8 ± 18.6	0.0485	128.5 ± 35.4	128.9 ± 23.4	0.4123
GH (μg/L)						
baseline	0.34 ± 0.12	0.81 ± 0.27	0.1185	0.42 ± 0.19	0.66 ± 0.37	0.5802
ΔGH	1.88 ± 0.71	0.21 ± 0.16	0.0049	2.05 ± 1.40	0.09 ± 0.15	0.0724
NPY (pg/mL)						
baseline	17.2 ± 1.6	25.2 ± 1.9	0.0024	17.9 ± 1.9	25.6 ± 2.2	0.0103
ΔNPY	13.6 ± 3.2	8.4 ± 3.0	0.4136	14.4 ± 2.9	4.0 ± 1.8	0.0097
PRL (μg/L)						
baseline	8.6 ± 1.2	10.0 ± 1.1	0.4436	7.8 ± 1.0	8.3 ± 0.6	0.6705
ΔPRL	5.9 ± 1.2	2.1 ± 0.9	0.0323	5.4 ± 2.0	2.4 ± 0.6	0.0645

Data are mean ± SEM.

[a] 2 mg.

[b] 2 mg twice daily for 28 days.

[c] n = 13.

ACTH, adrenocorticotropic hormone ; CORT, cortisol ; GH, growth hormone ; NPY, neuropeptide Y ; PRL, prolactin ; Δ, maximal change from baseline.

CHAPTER 2. INFLUENCE OF ORAL ONDANSETRON ON TOTAL CHOLECYSTOKININ PLASMA LEVELS FOLLOWING CCK-4 PANIC CHALLENGE PROCEDURE IN HEALTHY MALE VOLUNTEERS

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2.1 ABSTRACT

The purpose of this study was to gain insight into whether ondansetron treatment induced changes in total cholecystokinin (CCK_T) plasma levels before and after administration of the cholecystokinin tetrapeptide (CCK-4) panic challenge procedure in healthy male volunteers. Thirty-eight volunteers received a 50 μg-bolus of CCK-4 sixty minutes after a single oral dose (acute treatment) and multiple oral doses (chronic treatment) of ondansetron or placebo. Results showed no difference in CCK_T plasma levels and CCK_T elimination rate constant between the ondansetron and the placebo groups after either acute or chronic treatment. In conclusion, results from this study suggest that total CCK plasma levels are not influenced by either acute or chronic treatment with ondansetron. However, the effect of ondansetron on the different CCK component fractions still needs exploration.

KEYWORDS : Oral ondansetron pharmacokinetics ; Cholecystokinin tetrapeptide (CCK-4) ; intravenous CCK-4 administration ; human CCK plasma levels.

2.2 INTRODUCTION

Cholecystokinin (CCK) is a gastrin-like neuropeptide with several molecular forms ranging from 4 to 83 amino acid residues. CCK is present in the gut, the peripheral nervous system, and is found in large amounts in the brain where the sulphated octapeptide (CCK-8s) fragment predominates. (Eberlein and others 1992; Larsson and Rehfeld 1979; Reeve and others 1984; Rehfeld 1978) In recent years, it has been shown that intravenous administration of CCK-4 produces anxiety in animals and mimics panic attacks in humans. These findings led to the use of CCK-4 as a human challenge paradigm in the study of panic disorder and the screening of pharmacological agents with antipanic properties. (Bradwejn 1993)

It has been observed that intraperitoneal injection of CCK-4 amplifies the rise in cortical extracellular serotonin (5-HT) normally observed after exposure to the elevated plus-maze model of anxiety in guinea-pigs. (Rex and others 1994) Alternatively, experimental studies conducted by Paudice and Raiteri (Paudice and Raiteri 1991; Raiteri and others 1993) indicate that central administration of either serotonin or 1-phenylbiguanide, a 5-HT_3 agonist, enhance the depolarisation-evoked release of CCK from synaptosomes of rat cerebral cortex or nucleus accumbens. This effect is not observed under basal conditions or after addition of a 5-HT_3 receptor antagonist such as ondansetron, tropisetron, MDL 72222 and ICS 205-930, to the superfusion medium prior to 5-HT administration. Addition of methiothepin, a 5-HT_1/5-HT_2 antagonist, to the superfusion medium does not block CCK release by serotonin indicating that the effect is likely mediated by 5-HT_3 receptors located on CCK-releasing nerve terminals. So far, there has been no indication that CCK release through the central 5-HT system induced parallel changes in CCK plasma levels.

There is evidence from these experimental findings that CCK and 5-HT affect each other's release in the central nervous system and may modulate each other's function.

However, it is unclear whether the interaction between the CCK and 5-HT_3 systems may affect the total CCK (CCK_T) plasma concentration profile. This particular aspect of CCK measurement has never been investigated. Therefore, the purpose of this study was to explore the influence of treatment with ondansetron on CCK_T plasma concentrations in humans before and after CCK-4 panic challenge procedure. Our approach consisted of evaluating the acute and chronic effects of ondansetron on CCK_T plasma levels at baseline and on the 15-minute-CCK_T plasma level profile following intravenous administration of CCK-4 in healthy male volunteers. This work was done during the evaluation of the role of the 5-HT_3 neuronal system on the CCK-4-induced panic symptoms in this subject population.

2.3 METHODS

Study design

The influence of ondansetron on CCK_T plasma concentrations was evaluated in healthy male subjects using a double-blind, randomised, parallel-group, placebo-controlled design. To ensure adequate clinical data from 36 evaluable subjects for the final analysis, it was intended to randomise 42 subjects. Subjects were randomly assigned into one of the two treatment groups according to a 1:2 allocation scheme. Random numbers were generated by using SAS procedure PLAN (SAS Institute Inc., Cary, N.C., USA). Depending on their assigned treatment group, subjects received intravenous (i.v.) CCK-4 challenge test 60 minutes after single (2 mg) and multiple (2 mg twice daily for 28 days) oral doses of either ondansetron or placebo. Challenge test periods were separated by 28 days.

Materials

Cholecystokinin tetrapeptide, consisting of an amino acid chain of TRP-MET-ASP-PHE-NH_2, was synthesised by Peninsula (Belmont, CA, USA) and prepared by GIS médicament (Nantes, France) as intravenous solutions (CCK-4 50 μg in 2.5 mL of NaCl 0.9%). Ondansetron and placebo were formulated as film-coated pink tablets identical in size, colour, and weight. Active tablets contained 1.003 mg of ondansetron calculated as free base (100.3%) which fell between the required target range of 95% to 105% of the labelled amount. The preparation and the quality of ondansetron and placebo tablets were assured by Glaxo Wellcome Inc., Mississauga, Ontario, Canada.

Experimental procedures

The screening assessments were performed on an outpatient basis. Male subjects were between 18 and 55 years of age and within 20% of the ideal weight for height and body frame. Subjects were considered to be healthy based on medical history, physical and psychiatric examinations, and on the evaluation of vital signs, 12-lead electrocardiogram tracing and clinical laboratory test results. Structured Clinical Interview for DSM-III-R (SCID for non-patients), Hamilton Anxiety Rating Scale, and 90-point Symptom Checklist ruled out any underlying psychiatric conditions. At study entry, all subjects met the admission criteria and gave written informed consent. The study followed the guidelines of the Declaration of Helsinki of 1989. It was approved by the Institutional human experimentation committee of the University of Toronto (Toronto, ON, Canada) and the Health Protection Branch of Health and Welfare Canada (Ottawa, ON).

Subjects were instructed to maintain an overnight fast prior to the i.v. CCK-4 challenge test periods. Subjects were admitted to the research unit in the morning of

each challenge test period. Upon admission, they underwent a drug urine screen analysis. Thereafter, subjects were asked to sit in a reclining chair. An i.v. catheter was installed into the antecubital vein of their right arm through which a normal saline solution was slowly infused. The i.v. catheter maintained an open vein to allow CCK-4 injection and blood samplings.

Two CCK_T plasma concentration profiles were obtained. Blood samples were drawn from the indwelling cannula kept patent with normal saline solution at -10 minutes prior to and at 2, 5, 10, and 15 minutes after each CCK-4 injection. On Study Day 1, the first profile was completed one hour post acute treatment with either ondansetron or placebo. Subjects received and ingested two tablets of study medication with 150 mL of water. Sixty minutes post dosing, CCK-4 50 μg was administered in a bolus push (less than 5 seconds). Subjects were dismissed from the research unit approximately 30 minutes later after instruction to continue taking study medication at breakfast and dinner times for the next 28 days. On Study Day 29, the second CCK_T plasma concentration profile was obtained after chronic treatment with study medication. Subjects were administered the last study medication dosing (two tablets) with 150 mL of water. CCK-4 was injected 60 minutes post-dosing. Subjects remained in the research unit for 12 hours to allow determination of ondansetron pharmacokinetics. The i.v. line was flushed with a normal saline solution for a 2-minute duration after both CCK-4 boluses.

Blood samples for CCK_T were collected into chilled EDTA-containing test tubes. Immediately after blood collection, an enzyme inhibitor mixture containing aprotinin (1000 IU/mL), bestatin (0.03 mM), and phenylmethylsulphonyl fluoride [PMSF] (10^{-5} M, Sigma Chemical Company, St Louis, MO, USA) was added to test tubes. Tubes were then inverted to ensure good mixing of the blood with the enzyme inhibitors. Afterwards, the blood was centrifuged and the plasma stored at -70°C until assayed.

On Study Day 29, blood samples for ondansetron were drawn immediately before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 hours after study medication dosing. Blood was anticoagulated with heparin lithium, centrifuged, and the plasma stored at -20° C until assayed. Meals were served to the subjects at approximately 4 and 10 hours after CCK-4 injection. Caffeinated beverages were not allowed. At all other times, subjects took their meals at their regular times. Subjects were not allowed to consume alcoholic beverages or to engage in any strenuous or athletic activities throughout the duration of the study.

Ondansetron analytical method

Ondansetron plasma levels were analysed by a new sensitive high performance liquid chromatographic technique (HPLC) developed in the Department of Pharmacology, Faculty of Medicine, Université de Montréal (Montréal, Québec, Canada). (Dépôt and others 1997) The assay was controlled by daily calibration and quality control samples according to FDA guidelines. Calibration samples were prepared with drug free plasma that was spiked with increasing concentrations of ondansetron (0.5 to 15.0 ng/mL). Inter-assay precision and accuracy were determined from quality control (QC) samples spiked with three different concentrations of ondansetron (1.5, 7.5, and 13.0 ng/mL). Inter-assay coefficients of variation (n=14) were less than 10% ranging from 2.6 to 5.7%. The accuracy ranged from 100.3 to 105.3% for all three concentrations. The intra-assay coefficients of variation (n=6) ranged from 1.4 to 3.8%. The accuracy ranged from 100.8 to 104.0%. The lower limit of quantification (LOQ) was 0.5 ng/mL. Linearity of the calibration curves was validated from 0.5 to 15.0 ng/mL and determined by weighted least squares regression analysis ($1/x^2$). Peak height ratios of ondansetron and internal standard were plotted versus their corresponding plasma concentrations. The square of the correlation coefficients varied from 0.9914 to 0.9988.

Cholecystokinin analytical method

Plasma was extracted using Sep-Pak C_{18} cartridges. Each extract was then directly assayed for CCK_T plasma concentrations, that is the summation of endogenous CCK_T plasma levels and exogenously administered CCK-4, using a previously described radioimmunoassay. (Merani and others 1997) The antibody recognises the carboxy terminal portion of cholecystokinin and is highly sensitive to CCK-4. The range of the standard curve was determined to be 0.4-400 pg CCK/mL of plasma (0.7-672 fmol CCK-4 equivalents/mL) with a detection limit of 0.7 fmol CCK-4 equivalents/mL of plasma. Recovery of iodinated Bolton-Hunter CCK-4 from one mL of assay buffer and human plasma was determined to be 89 and 87%, respectively. Intra-assay ($\geq$ 10 replicates per assay) and interassay coefficients of variation were calculated as 8 and 9%, respectively. Specific activity of the monoiodinated tracer was $\cong$ 1025 Ci/mmol. At a 1:10 000 antisera dilution, binding of the tracer in absence of standard (zero binding) was 30.6 $\pm$ 3.4%, with half-maximal displacement (ED_{50}) of 8.9 $\pm$ 1.7 fmol. Non-specific binding was calculated as 1.9 $\pm$ 0.02%.

Pharmacokinetic analysis and statistics

Plasma concentration data for CCK_T were analysed using the SAS system for PC, version 6.12. Mean CCK_T plasma concentrations were identified from the individual plasma concentration profiles at -10 minutes prior to and at 2, 5, 10, and 15 minutes after intravenous CCK-4 administration. Maximum CCK_T plasma concentrations (CCK_Tmax) were identified from the individual 15-minute CCK_T plasma level profiles. The CCK_T elimination rate constant (CCK_Tz) was determined by linear least-squares regression analysis of the logarithmically transformed data that were subjected to noncompartmental pharmacokinetic analyses.

Ondansetron data were also subjected to noncompartmental pharmacokinetic analyses. The parameters estimated by this model were the total area under the plasma concentration-time curve ($AUC_{0-\infty}$), the elimination rate constant, and the elimination half-life ($t_{½,z}$). The elimination rate constant (z) was determined by linear least-squares regression analysis with use of the logarithmically transformed data in the elimination phase. The elimination half-life was calculated as the (ln2)/z. The $AUC_{0-\infty}$ was determined by adding AUC from 0 to the last plasma level point determined by trapezoidal rule to the remaining area calculated by dividing the last plasma level fitted to the concentration at that time by the elimination rate constant. The maximum plasma concentration (C_{max}) and the time at which C_{max} occurred (T_{max}) were identified from the individual plasma concentration-time profiles.

Statistical comparisons were made by using the SAS system for PC version 6.12. The normality of the data distribution was tested with the Shapiro-Wilk statistic test. Given the small sample size, missing CCK_T plasma levels were imputed using overall mean values at each time point for a given treatment period (acute or chronic). Plasma CCK_T levels below the LOQ were assigned a value of 0.3 pg/mL for mean calculations, that is the maximal plasma level below the LOQ. This conservative approach allows data from all subjects to be included in the analyses of covariance. CCK_T plasma levels obtained at baseline and during the 15-minute CCK_T plasma level profile were analysed by a two way ANCOVA with repeated-measures with time as the within-subject effect and treatment as between-subject factors. The default statistical model included main effects for treatment with the baseline CCK_T value as a covariate. Interaction terms included in the repeated measures analysis of covariance were the interaction between time and treatment and the interaction between time and the baseline CCK_T value. A significant time x treatment interaction would indicate that the effect of time is significantly different between treatment groups, whereas a significant time x basal CCK_T interaction would indicate that the change in response over time is dependent on the baseline CCK_T plasma level. CCK_Tmax and CCK_Tz

were analysed by a one way (treatment) ANCOVA. The results are expressed as mean ± SEM in the original unit. The difference between mean values was considered significant when $p < 0.05$.

2.4 RESULTS

Forty one subjects participated in the study, 27 of whom received ondansetron. Three subjects dropped out before completion of the second CCK-4 challenge test period: from the placebo group, one subject violated the study protocol; from the ondansetron group, one experienced a severe allergic reaction to study medication and one withdrew his participation for reasons unrelated to the study. Therefore, 38 healthy male subjects completed both challenge test periods. Thirteen subjects received placebo and 25 received ondansetron. The average age and body weight of the placebo-treated subjects were 27.3 ± 1.4 years and 74.6 ± 2.3 kg, respectively. The average age and body weight of subjects who received ondansetron were 27.6 ± 1.0 years and 77.8 ± 2.1 kg, respectively. Body weights were within 20% of ideal body weight. There was no statistical difference in demographic characteristics between ondansetron and placebo groups. Of the 25 subjects who received the 28-day course of ondansetron, one did not complete the ondansetron pharmacokinetic part of the study because of difficulty in obtaining blood samples.

Table 1 outlines the breakdown of missing values by treatment groups. A total of 13 values were imputed for the acute treatment (6.8%) and 12 values were imputed for the chronic treatment (6.3%). $CCK_T max$ values affected by imputing of missing values were updated accordingly. The $CCK_T z$ data was unaffected by the imputing.

The Shapiro-Wilk statistic test for normality showed that the data for the acute treatment was heavily influenced by outlier values for three subjects : two subjects

from the placebo group and one subject from the ondansetron group. The test indicated that the distribution of the data was significantly different from a normal distribution. Omitting these three subjects from the dataset resulted in normal distributions or reduced the degree of non-normality. Distribution of the CCK_Tz data was compatible with a normal distribution. Based on these results, four outlier data points were imputed instead of deleting the entire dataset for the three subjects. Data for the chronic treatment was not normally distributed mainly due to a few influential observations at the various time points. However, these values were not as extreme as the outlier data identified in the acute treatment dataset. The CCK_Tmax and CCK_Tz data were normally distributed. No outlier data was removed from the chronic treatment dataset.

Mean CCK_T plasma concentrations after acute and chronic treatment with ondansetron and placebo are listed in Table 2. Raw mean CCK_T plasma concentration-time profiles after acute and chronic treatment with ondansetron and placebo are shown in Figures 1 and 2, respectively. Raw mean CCK_T plasma concentration-time profiles between treatment period within each treatment group are displayed in Figures 3A and 3B. Pharmacokinetic parameters for ondansetron are listed in Table 3. The mean plasma concentration-time profile of ondansetron during chronic administration is illustrated in Figure 4.

After acute treatment (Fig. 1), there was no significant difference in basal CCK_T plasma levels between treatment groups. Likewise, there was no significant difference in the CCK_Tmax between the ondansetron and placebo groups (532.0 ± 116.2 pg/mL vs 862.6 ± 161.2 pg/mL, $p = 0.1054$). A considerable variation was observed in CCK_Tmax in both treatment groups which negated an apparent difference of 300 pg/mL in favour of ondansetron. The baseline CCK_T plasma level was not a significant covariate. Finally, there was no significant difference in the CCK_Tz between the ondansetron and placebo groups (0.3351 ± 0.018 min^{-1} vs 0.3337 ± 0.027

min^{-1}, p = 0.9667). For this variable, the baseline CCK_T was a significant covariate (p = 0.0025).

After chronic treatment (Fig. 2), there was no significant difference in CCK_T plasma levels at any time point between the treatment groups. There was no difference in CCK_Tmax between the ondansetron group and the control group (465.4 ± 54.9 pg/mL vs 548.6 ± 76.3 pg/mL, p = 0.3828). Although not statistically significant, CCK_Tz may seem slower in the ondansetron group as compared to the placebo group (0.3125 ± 0.017 min^{-1} vs 0.3661 ± 0.022 min^{-1}, p = 0.0660). Once again, the baseline CCK_T was a significant covariate (p = 0.0259).

As expected, there was a significant decrease in plasma CCK_T levels over time as indicated by the significant time effect (p = 0.0001). The time effect was similar for each treatment group as indicated by a non-significant time x treatment interaction (p = 0.2587 for the acute treatment, p = 0.3351 for the chronic treatment). The interaction between time and basal CCK_T level was not significant for either acute (p = 0.2257) or chronic (p = 0.9525) treatment.

A student's t-test of the mean CCK_T plasma level at each time point between treatment period within the placebo group (Fig. 3A) and the ondansetron group (Fig. 3B) indicated that plasma levels were not significantly different. Basal CCK_T plasma levels were within 2 pg/mL between acute and chronic administration within each treatment group. In the ondansetron group, differences of less than 95 pg/mL at each time point between acute and chronic treatment were observed. In the placebo group, there was more variation at each time point (from 20 to 220 pg/mL) between acute and chronic treatment.

2.5 DISCUSSION

This study was performed to investigate whether ondansetron treatment could alter CCK_T plasma levels at baseline as well as those obtained after intravenous CCK-4 administration when used as probing tool in panic disorder in humans. In this study, basal CCK_T plasma levels reflected endogenous CCK_T present in peripheral circulation, whereas CCK_T plasma levels measured after CCK-4 injection reflected the endogenous CCK_T and the exogenous CCK-4 injected.

Our findings did not show any significant effect of acute and chronic treatment with ondansetron on basal CCK_T plasma levels as compared to acute and chronic treatment with placebo. As per Figure 1, the 15-minute CCK_T plasma level profile obtained after the acute treatment with ondansetron may be viewed as blunted as compared to that obtained after the acute treatment with placebo. However, the apparent treatment difference ($p = 0.0799$ at 5 minutes) based on the changes in CCK_T plasma levels over time was not as pronounced when outlier data were imputed ($p = 0.1154$ at 5 minutes). There were no differences between ondansetron and placebo in the 15-minute CCK_T plasma level profile after chronic treatment. With regard to CCK_Tz, there was some indication of a treatment difference in favour of ondansetron after chronic treatment ($p = 0.0660$) but not after acute treatment ($p = 0.9667$). Basal CCK_T was a significant covariate indicating that the elimination rate constant was dependent on the CCK_T value at baseline. Given the small sample size of the placebo group, the high intra-subject variability may account for the inability to see a treatment effect. The intra-subject variability may involve several factors such as CCK tissue clearance or the state of neuronal activity of the CCK system. The nature and the contribution of the confounding factors to CCK_T plasma levels remain to be determined.

Our results also indicated that the pharmacokinetic profile of low dose ondansetron measured at steady-state shows considerable inter-individual variability. This is in concordance with pharmacokinetic data previously reported with high dose ondansetron. (Simpson and Hicks 1996)

In conclusion, results from this study suggest that total CCK plasma levels are not influenced by either acute or chronic treatment with ondansetron. Based upon these preliminary findings, future work should be devoted to the determination of a complete kinetic profile of CCK_T with closer blood sampling time points. Such a profile would allow the calculation of the area under the curve, the metabolic clearance rate and the volume of distribution of CCK_T in humans. Furthermore, the apparent absence of an effect does not exclude possible changes in ratios between the different CCK component fractions. Based upon the increase in the CCK_T plasma concentrations after the injection of the CCK-4 50 μg dose, one may assume that the plasma levels measured after CCK-4 injection were mostly tetrapeptide fragments. However, as the CCK_T plasma levels declined the ratio CCK-4:CCK-8s would be expected to change accordingly. Therefore, knowledge about the CCK component fractions of different molecular size could provide more informative data on the plasma cholecystokinin response.

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Table 2.1 Breakdown of missing values by treatment groups.

Reasons	ACUTE TREATMENT[a]			CHRONIC TREATMENT[b]		
	Total	Placebo	Ondansetron	Total	Placebo	Ondansetron
below LOQ [c]	4.2 (8)	1.0 (2)	3.2 (6)	2.1 (4)	0	2.1 (4)
not done [d]	2.6 (5)	1.6 (3)	1.0 (2)	4.2 (8)	1.0 (2)	3.2 (6)
Total	6.8 (13)	2.6 (5)	4.2 (8)	6.3 (12)	1.0 (2)	5.3 (10)

Data are percentages of missing values; data in parentheses represent the number of missing values out of 190 observations.

[a] 2 mg.

[b] 2 mg twice daily for 28 days.

[c] missing values imputed using a value of 0.3 pg/mL that is the maximal value below LOQ.

[d] missing values imputed using overall mean values at each time point for a given treatment period.

Table 2.2 Mean total CCK (CCK_T) plasma concentrations.

Time	Mean CCK_T plasma concentrations (pg/mL)					
(min)	ACUTE TREATMENT[a]			CHRONIC TREATMENT[b]		
	Placebo	Ondansetron	p-value	Placebo	Ondansetron	p-value
-10	7.6 ± 3.8	9.4 ± 3.7	0.1388	9.4 ± 2.7	7.2 ± 2.4	0.5129
2	707.4 ± 140.4	532.1 ± 101.2	0.3183	488.9 ± 67.9	437.6 ± 48.9	0.5445
	(555.4 ± 62.4)	(451.2 ± 44.9)	(0.1838)			
5	364.7 ± 108.5	123.3 ± 78.2	0.0799	265.1 ± 62.7	160.8 ± 45.1	0.1862
	(185.1 ± 31.4)	(122.5 ± 22.6)	(0.1154)			
10	254.0 ± 112.5	62.4 ± 81.1	0.1763	69.1 ± 43.3	98.9 ± 31.2	0.5800
	(66.2 ± 25.1)	(62.6 ± 18.1)	(0.9093)			
15	36.4 ± 10.2	27.9 ± 7.3	0.5016	16.4 ± 22.2	46.9 ± 16.0	0.2741

Data are least squares means ± SEM ; data and p-values in parentheses represent ANCOVA results after the four outlier values were set to missing and then imputed.

[a] 2 mg.

[b] 2 mg twice daily for 28 days.

Table 2.3 Pharmacokinetic parameters of multiple oral doses[a] of ondansetron.

Parameter	Mean ± SE (n = 24)	Range
C_{max} (ng/mL)	7.05 ± 0.48	3.04 - 11.35
T_{max} (hour)	1.74 ± 0.13	1.00 - 4.00
AUC_t (ng x h/mL)	40.00 ± 2.74	19.57 - 68.09
AUC_∞ (ng x h/mL)	47.15 ± 3.38	23.09 - 81.98
$AUC_{t/\infty}$ (%)	85.37 ± 1.02	69.88 - 92.64
z (hour $^{-1}$)	0.1795 ± 0.0072	0.1112 - 0.2571
$t_{½,z}$ (hour)	4.02 ± 0.18	2.70 - 6.23

[a] 2 mg twice daily for 28 days.

AUC = Area under the plasma concentration-time curve; C_{max} = peak plasma concentration; t_{max} = time to reach peak concentration; z = elimination rate constant; $t_{½,z}$ = half-life.

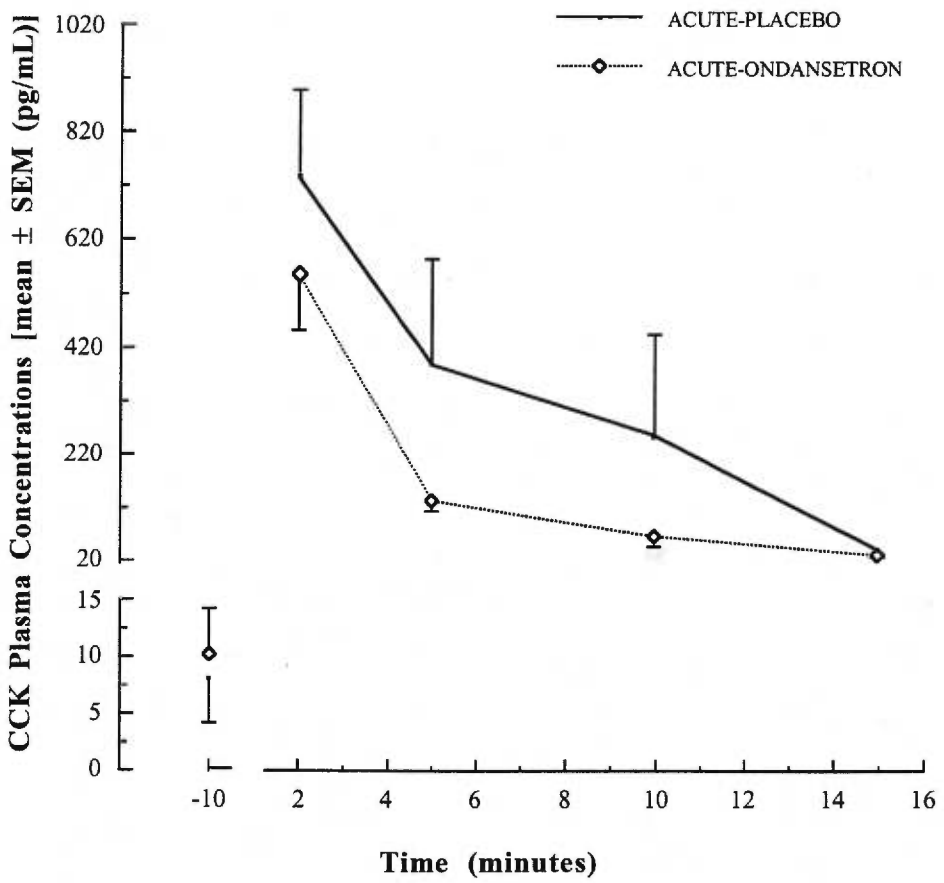

Figure 2.1 Mean CCK_T plasma concentration-time profiles after acute treatment with either ondansetron or placebo at -10 minutes prior to and at 2, 5, 10, and 15 minutes after CCK-4 challenge procedure.

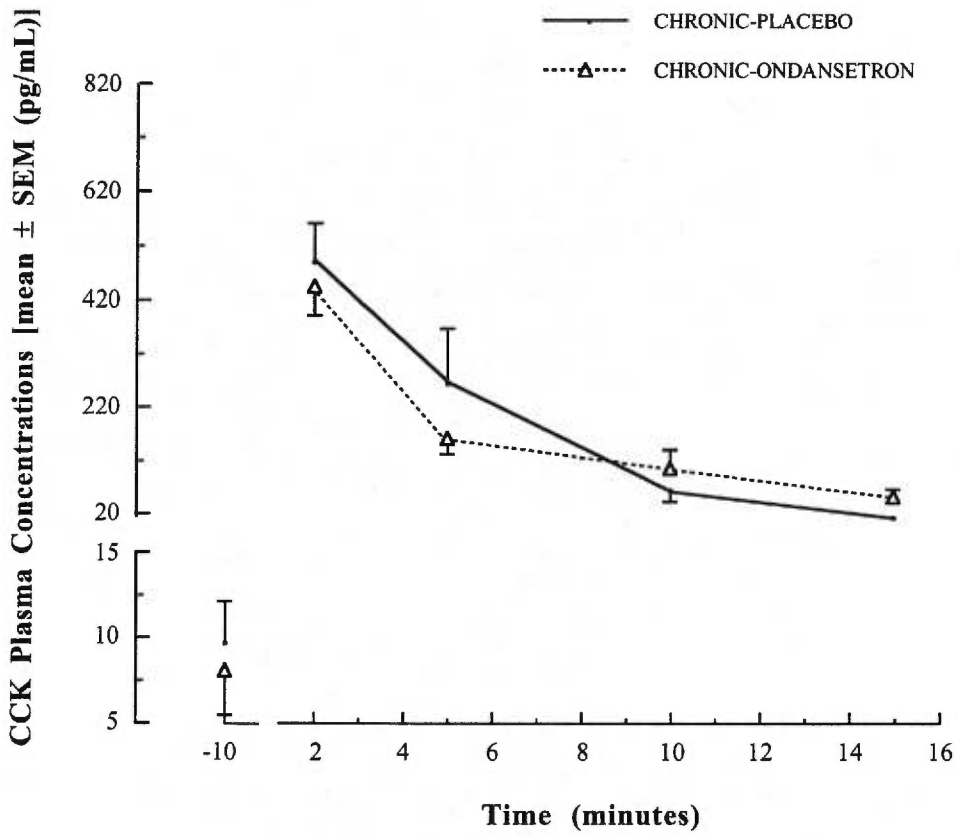

Figure 2.2 Mean CCK_T plasma concentration-time profile after chronic treatment with either ondansetron or placebo at -10 minutes prior to and at 2, 5, 10, and 15 minutes after CCK-4 challenge procedure.

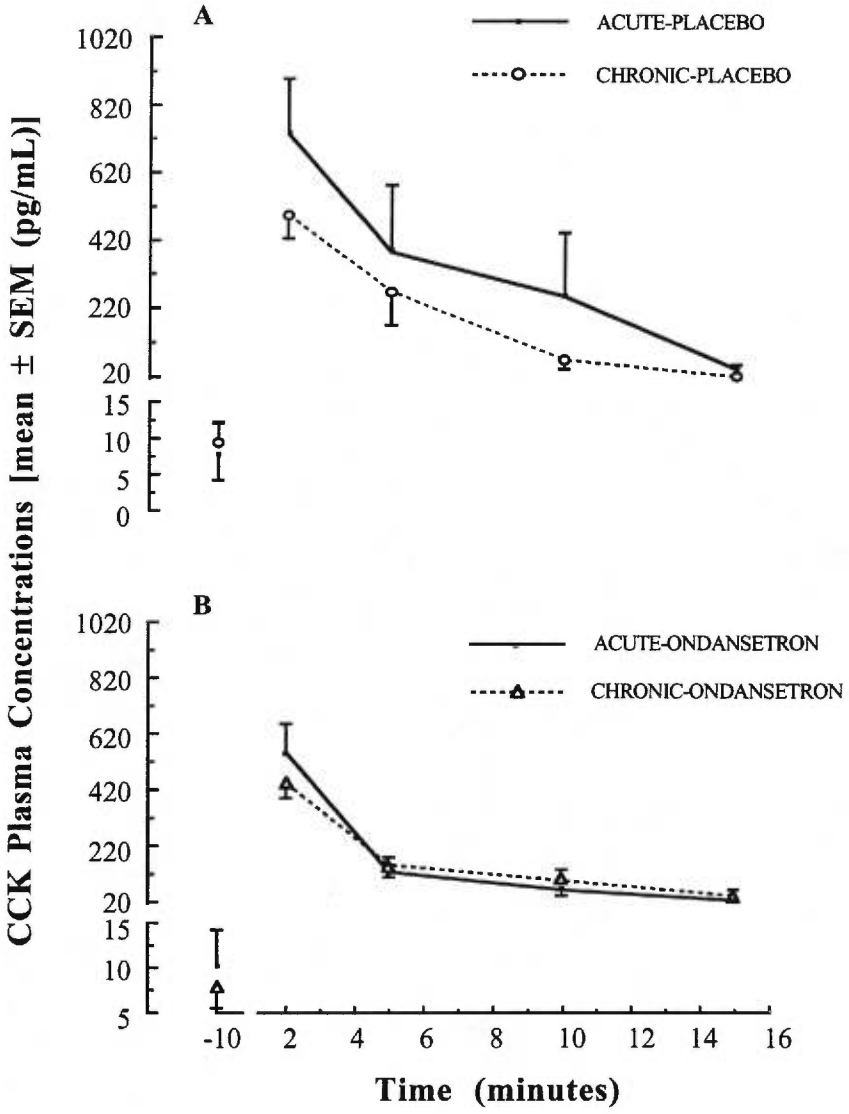

Figure 2.3 Mean CCK_T plasma concentration-time profiles between treatment period within the placebo (A) and the ondansetron (B) groups at -10 minutes prior to and at 2, 5, 10, and 15 minutes after CCK-4 challenge procedure.

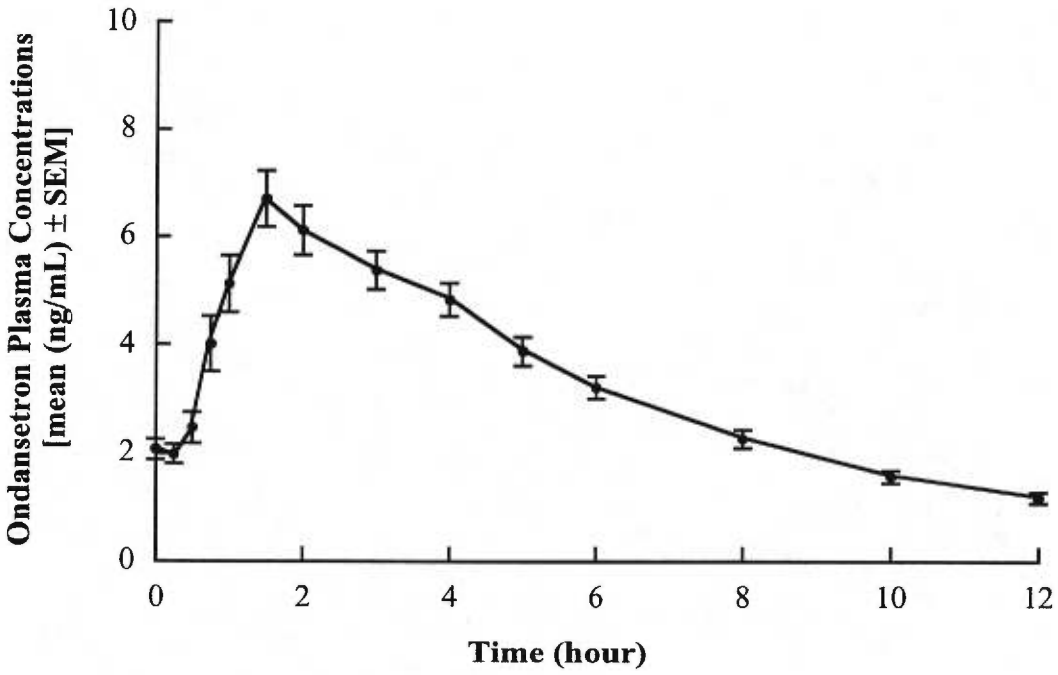

Figure 2.4 Mean plasma concentration-time profile of multiple oral doses of ondansetron. (n=24)

CHAPTER 3. ONDANSETRON ANALYSIS BY GAS CHROMATOGRAPHY/MASS SPECTROMETER TECHNIQUE.

Considering that capillary columns offer a better signal-to-noise ratio, ondansetron possible separation from biological samples was first examined by using high resolution gas chromatography attached to a mass spectrometer detector (GC/MS). This profiling approach is recognised as providing the most definitive information about a specific sample that is structural identification and quantification. In addition, there are no available reports on the ondansetron analysis using this technique.

Structural identification was accomplished by using the reference standard pure powder sample of ondansetron free base (Lot No. GR38032X) provided by Glaxo Research and Development (Middlesex, UK). The GC/MS consisted of a HP 5890 Series II GC attached to a 5971 MS detector. Ondansetron was separated on a HP-1 crosslinked methyl silicone gum capillary column (12 m x 0.2mm x 0.33 μm film thickness, Hewlett Packard, Kirkland, Québec, Canada). A 1 μg drug standard (1 μL of 1μg/μL in methanol) was injected in splitless mode ensuring delivery of the full sample to the column. Sample was analysed using a 50-400 scan range. Analytical analysis was performed under variable temperature conditions with the exception of the GC/MS interface temperature which was kept at 280 ° C.

Under these laboratory conditions, we were confronted with two difficulties. First, operating in the total ion mode, injection of the standard solution produced two peaks. An example of the elution profile is illustrated in Figure 3.1. Ondansetron eluted last. In the full-scan, the mass spectrum of ondansetron was dominated by four major fragments at masses 96, 198, 211, and 293 (Figure 3.2A). The first peak was mainly characterised by a fragment mass of 211 suggesting a degradation product of the parent compound (Figure 3.2B). Under any of the analytical conditions used, ondansetron was too thermally labile to elute without decomposition. The molecular structures that may potentially represent these fragments are shown on Figure 3.3. As expected, shorter retention times were observed with rising temperatures. Figures 3.1, 3.4, and 3.5 depict these observations. There was no consistency in the decomposition fragment fraction of the parent compound

produced at various temperature settings. The second problem related to tailing of the ondansetron peak leading to considerable loss in sensitivity.

To optimise resolution, the column was subjected to silylation techniques with injection of 10% solution of silane dimethyl-dichloride ($SiMe_2Cl_2$, Silyl-8™, Pierce, Rockford, Ill., USA) in methylene chloride. However, silylation did not solve the tailing problem. Based upon the high susceptibility of ondansetron to heat, the conversion of the parent molecule into its decomposition fragment by modulating the temperature settings was theoretically conceivable. Unfortunately, attempts to decompose ondansetron in its totality by using maximal temperature conditions permitted by the apparatus did not succeed. Furthermore, the 211/293 fragment mass ratio exhibited considerable variability independently of the analytical condition. Finally, in an effort to eliminate solute decomposition and thermally catalysed molecular rearrangements, the on-column-type injection was used (HP-5 crosslinked 5% Ph Me Silicone, 15 m x 0.25 mm x 0.25 μm film thickness). One particularity of this procedure lies on the fact that the sample is injected in a liquid state directly on the column. The initial temperature being near the boiling point of the solvent prevents pre-volatilisation of the solvent and consequently improved peak area. In spite of the absence of vaporisation of the sample, the elution profile remained unchanged.

In conclusion, ondansetron is being particularly prone to adsorption. The overall sensitivity of the GC/MS was then rapidly deteriorated mainly because of losing samples in the GC. Further attempts for optimisation, such as silanizing the injection port liner and column, setting various heating patterns and using on-column injection, failed to protect ondansetron from decomposition and rectify the tailing-profile of elution peak. In addition, ondansetron was barely detectable below the 50 ng/μL level. Therefore, we may conclude that, under these analytical conditions, GC/MS cannot be used for quantitative analysis of ondansetron.

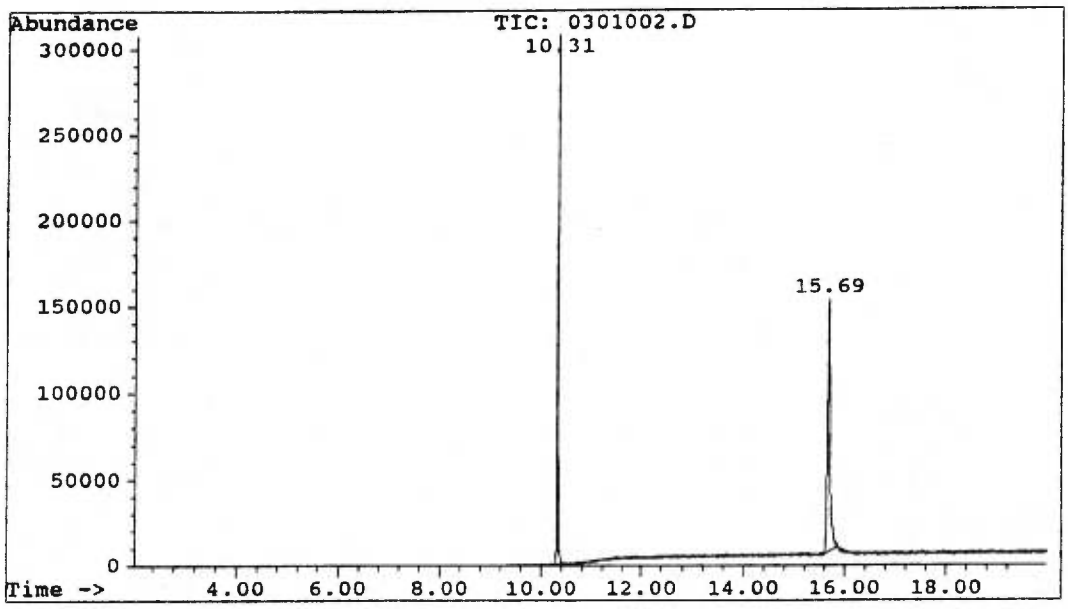

Figure 3.1 Chromatogram of ondansetron 100 ng after splitless injection. Injector temperature set at 250 °C; column temperature initially set at 100 °C increased by 20-degree increments reaching a final temperature of 280 °C.

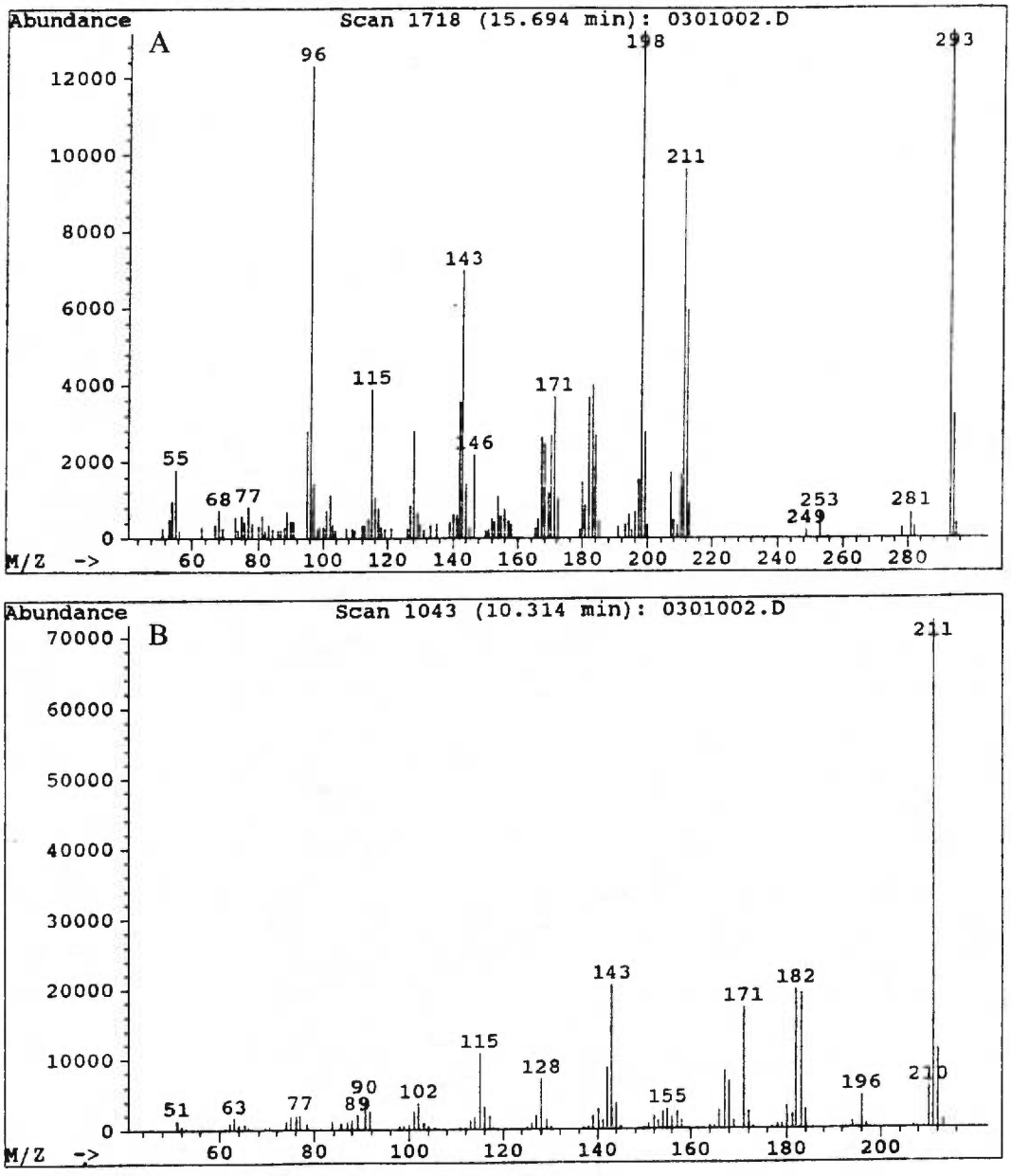

Figure 3.2 Individual peak spectrum. A : ondansetron. B : decomposition fragment.

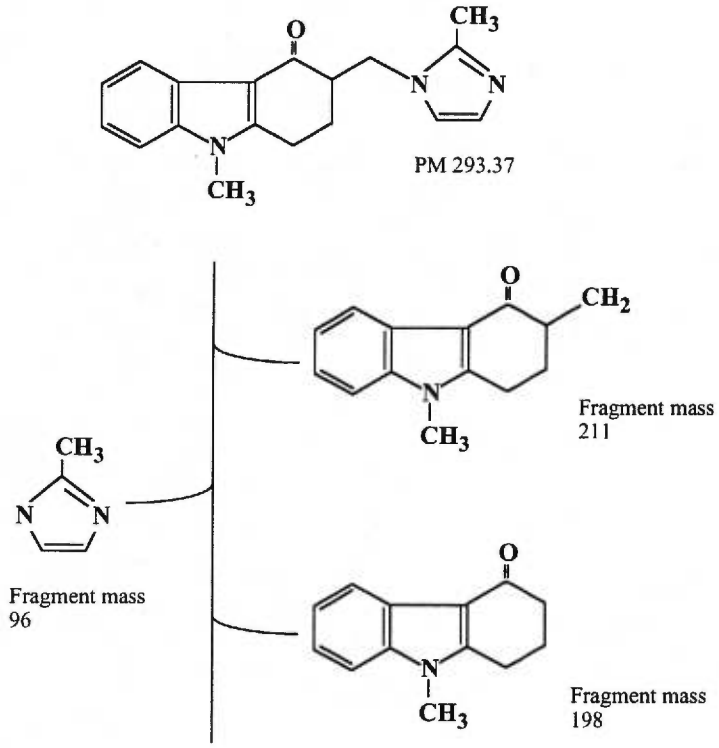

Figure 3.3 Proposed molecular structures of the major fragment masses composing the mass spectrum of ondansetron.

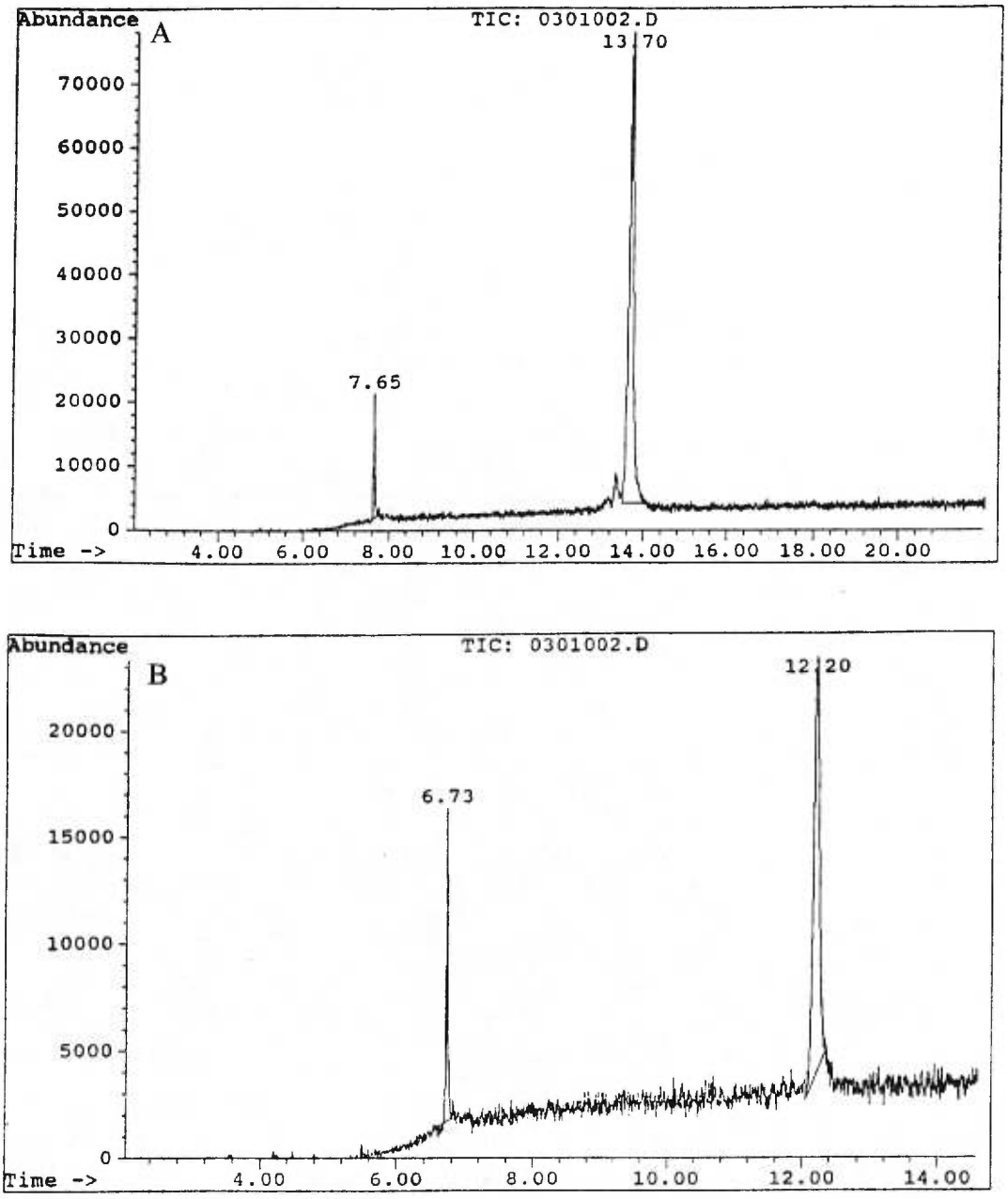

Figure 3.4 Chromatogram of ondansetron 500 ng after splitless injection. A : Injector temperature set at 150 ° C; column temperature initially set at 70 ° C increased by 70-degree increments reaching a final temperature of 280 ° C. B : Injector temperature set at 150 ° C; column temperature initially set at 100 ° C increased by 70-degree increments reaching a final temperature of 280 ° C.

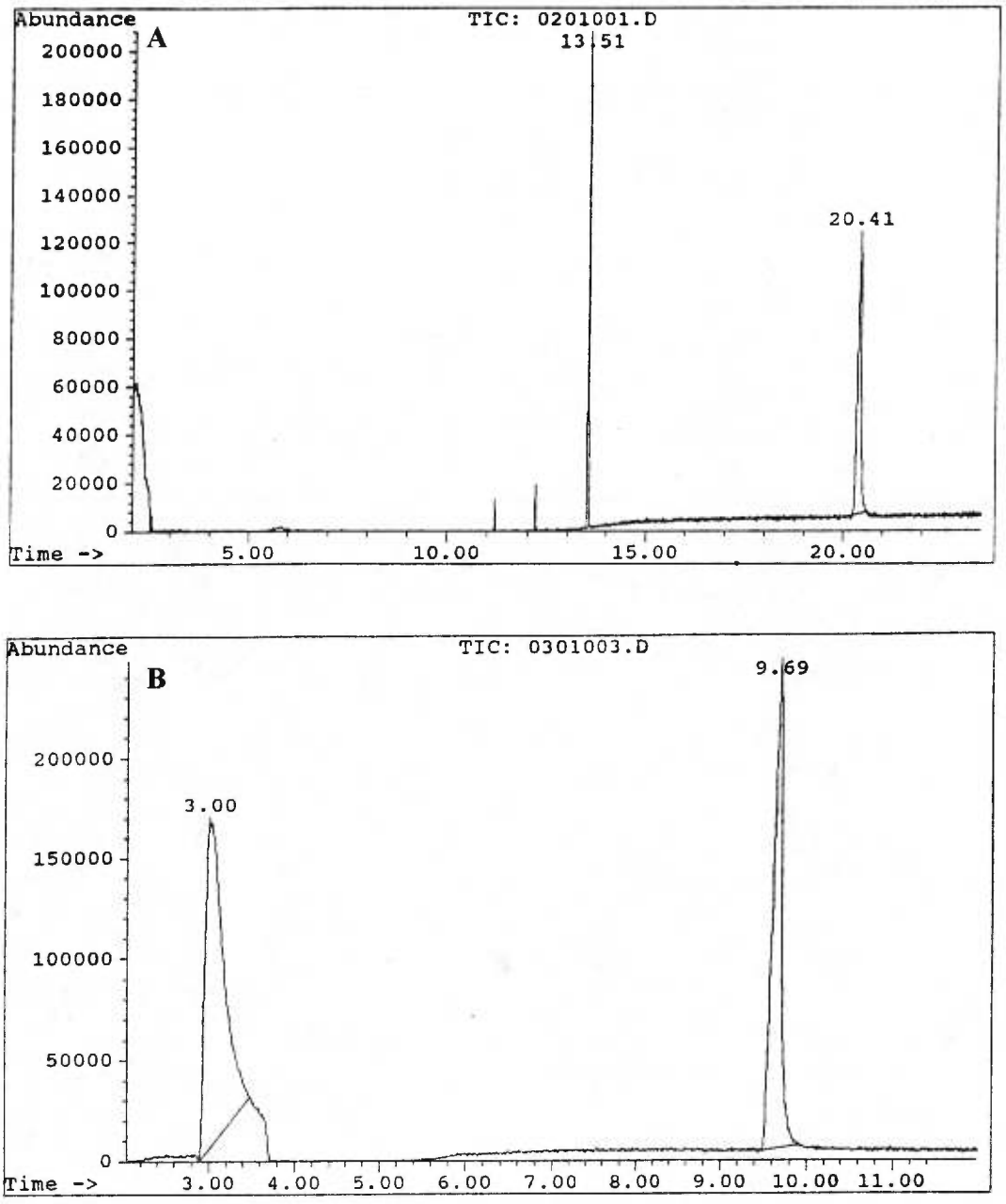

Figure 3.5 Chromatogram of ondansetron 100 ng after splitless injection. A : Injector temperature set at 300 ° C; column temperature initially set at 50 ° C increased by 20-degree increments reaching a final temperature of 280 ° C. B : Injector temperature set at 300 ° C; column temperature initially set at 240 ° C increased by 60-degree increments reaching a final temperature of 280 ° C.

CHAPTER 4. HIGH-RESOLUTION LIQUID CHROMATOGRAPHIC METHOD USING ULTRAVIOLET DETECTION FOR DETERMINATION OF ONDANSETRON IN HUMAN PLASMA

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4.1 ABSTRACT

This paper describes a simple technique for extraction and a sensitive high-performance chromatographic method for separation and quantitation of ondansetron in human plasma. The procedure involved liquid-liquid extraction of ondansetron from plasma, reversed-phase HPLC separation, and ultraviolet detection at 305 nm. The internal standard method was applied for quantitation. The recovery of ondansetron was > 85%. Linearity was good throughout the concentration range anticipated in human plasma from investigations in panic disorder (0.5-15 ng/ml, r^2 ranging from 0.9953 to 0.9988). This method was applied to the determination of plasma concentrations of ondansetron in humans.

KEYWORD: ondansetron

4.2 INTRODUCTION

Developed by Glaxo Research Group in England, ondansetron is a serotonin antagonist structurally related to serotonin (Figure 4.1). Since its introduction in our medical practice as an antiemetic, extensive pharmacological studies have indicated that ondansetron binds with high affinity to 5-HT_3 receptors and fails to interact with other neurotransmitter systems [1, 2].

There is growing evidence from animal and clinical studies in support of a 5-HT hypothesis in panic disorder [3-9]. As an antiemetic, the usual dose is in the range of 8 to 32 mg per day whereas preliminary results from clinical investigations in panic disorder support the use of doses as low as 2 to 4 mg per day. Consequently, the low plasma concentration range anticipated may require a very sensitive analytical method. HPLC analytical methods are available for analysis of ondansetron in human plasma [10-12]. Recently, a sensitive radioimmunoassay was developed to further enhance assay sensitivity [13]. These analytical methods all share a common extraction technique, a solid-phase extraction.

The objective of this work was to develop a rapid and simple analytical method using a novel extraction technique suitable for assay of ondansetron in human plasma. This paper describes a sensitive reversed-phase HPLC assay combined with a liquid-liquid phase extraction technique for the determination of ondansetron in human plasma. Data pertaining to the specificity, stability, reliable limit of detection, recovery as well as precision and accuracy are presented herein.

4.3 EXPERIMENTAL

4.3.1. Materials

A pure powder sample of ondansetron free base (Lot No. GR38032X) was provided by Glaxo Research and Development (Middlesex, UK). Loxapine (Lot No. 2C0790A, Figure 4.1), the acting internal standard, was supplied by Cyanamide Canada (Montreal, Canada). Acetonitrile, methanol and ethyl acetate were HPLC grade. The first two solvents were purchased from Fisher Scientific (Montreal, Canada). Ethyl acetate was purchased from Anachemia (Montreal, Canada). Sulfuric acid (H_2SO_4) was obtained from Baker Chemicals (Phillipsburg, NJ, USA). Certified buffer solution pH 9 (Lot No. SC5250211) was acquired from Fisher Scientific. All other chemicals were of analytical grade. Deionized water (Milli-Q® water purification system, Millipore, Bedford, MA, USA) was used throughout the study. Control human plasma was obtained from the Canadian Red Cross.

4.3.2. Standard Solutions

A 100 μg/ml ondansetron stock solution was prepared by dissolving 10 mg of ondansetron free base in 100 ml of methanol. Ondansetron powder was dissolved by 20-min sonication and Vortex-mixed. Dilutions to 1 μg/ml and 0.1 μg/ml of this stock solution were prepared in methanol. A 100 μg/ml internal standard (I.S.) stock solution was prepared by dissolving 10 mg of loxapine free base in 100 ml of methanol. The working I.S. solution was prepared by dissolving 600 μl of the stock solution in 100 ml of methanol. A final concentration of 0.6 μg/ml was obtained. All solutions (ondansetron and I.S.) were stored at 4 ± 1 °C.

The non-extracted ondansetron was prepared fresh by diluting the ondansetron stock

solution with 0.025 *M* H_2SO_4 for final concentrations of 1 μg/ml and 0.1 μg/ml. The non-extracted I.S. was also prepared fresh by diluting the I.S. stock solution with 0.025 *M* H_2SO_4 for a final concentration of 0.6 μg/ml. Analytical aliquots were prepared with these solutions. The final volume was completed to 200 μl with 0.025 *M* H_2SO_4. All ondansetron solutions were protected from direct light as per storage instructions provided by Glaxo.

4.3.3. Calibrant and quality control samples

The selectivity of the assay method was established with independent sources of plasma. Plasma samples obtained from the Canadian Red Cross were screened and those tested that demonstrated no interfering peaks at the retention times of ondansetron and the I.S. were pooled to constitute the matrix for calibrant as well as quality control (QC) samples. Figure 4.2 represents a chromatogram of an extracted human blank plasma.

Calibrant samples were prepared with drug free plasma that was spiked with increasing concentrations of ondansetron (0.5, 1.0, 2.5, 5.0, 10.0, and 15.0 ng/ml). Quality control samples were prepared with drug free plasma spiked with three different concentrations of ondansetron. Low, medium and high QC concentrations used were 1.5, 7.5, and 13.0 ng/ml, respectively.

4.3.4. Extraction procedures

Sample preparation comprised of a liquid-liquid extraction of ondansetron from control plasma samples spiked with standard samples (calibrant and quality control samples). In a 17 x 120 mm polypropylene screw cap conical tube (15 ml) purchased

from Sarstedt (Newton, NC, USA) was added 2.0 ml of plasma followed by 100 µl of a 0.6 µg/ml I.S. solution and 3.0 ml of certified buffer solution pH 9. After 15 s of vortex-mixing, 6.0 ml of ethyl acetate were added. The tubes were shaken for 15 min on an Eberbach shaker set at low speed and then centrifuged for 10 min at 1 300 *g*. The organic layer was transferred to a clean 15 ml polypropylene conical tube. Thereafter, 200 µl of 0.025 *M* H_2SO_4 was added. The organic layer was discarded after vortexing the tubes for 60 s and centrifuging for 5 min at 1 300 *g*. The excess of organic layer was then evaporated under a light stream of nitrogen for approximately 1 to 3 min at 45 °C. A 100 µl aliquot of the aqueous phase was injected into the HPLC system. This process was carried out at room temperature, unless specified otherwise, and under reduced daylight.

4.3.5. Instrumentation and chromatography conditions

Plasma samples prepared according to the extraction procedures described in Section 2.4 were analysed by HPLC. The HPLC consisted of a Waters Model 501 solvent delivery system attached to a Shimadzu autosampler (Model SIL-9A), along with a Kratos Model 757 Spectroflow detector and a Shimadzu Model C-R6A Chromatopac integrator. The ultraviolet detector was operated at a wavelength of 305 nm. Ondansetron and I.S. were separated on a 10 x 0.46 cm Sperisorb reversed-phase column packed with 10 µm C_{18} material (ID No. 069638, Chromatographic Sciences Company, Montreal, Canada). The mobile phase consisted of acetonitrile-0.02 *M* sodium phosphate monobasic buffer ($NaH_2PO_4 \cdot H_2O$) adjusted to pH 3 with phosphoric acid 85% (60:40, v/v). The mobile phase was pumped at a flow-rate of 1.5 ml/min [pressure of 450 p.s.i. (1 p.s.i.=6894.76 Pa)]. Chromatographic analysis was conducted at room temperature and protected from light.

4.4 RESULTS AND DISCUSSION

4.4.1. Optimisation of experimental conditions

Surface adhesion of ondansetron to glass represents a significant problem during the extraction process. Solid-phase extraction requires reconstitution of dry residue obtained from drying the organic phase under nitrogen. Ondansetron dry residue is also found on the sides of the extraction tubes and may hence troublesome the reconstitution. We have chosen a liquid-liquid extraction of ondansetron from plasma samples to circumvent this obstacle. Unlike ondansetron, the I.S. is a weaker base and required a more acidic solvent to ensure its solubility following extraction. Consequently, the optimum recovery of ondansetron and I.S. was achieved when the pH of the solvent was decreased from 3 to 1.8.

The mobile phase composition was optimised according to the ondansetron and I.S. retention times and the resolution of the chromatogram. We observed that the molarity of the buffer solution played a major role in the quality of the chromatogram. In fact, lower molarity caused significant noise of the baseline. Increasing the molarity from 0.005 to 0.02 *M* dramatically improved the signal-to-noise ratio.

An ultraviolet spectrophotometer scan showed detection of ondansetron at four wavelengths: 216, 245, 268, and 305 nm. Wavelength of 216 nm produced further sensitivity improvements of a factor approaching 2.5. However, more interfering peaks emerged from the plasma. As a consequence, we selected a detection wavelength of 305 nm. The molarity and the wavelength together gave an excellent signal-to-noise ratio. Under these experimental conditions, retention times were reproducible. Ondansetron and the I.S. were eluted at 3.9 and 5.6 min, respectively.

4.4.2. Precision and accuracy

Between-run precision and accuracy were determined from QC samples spiked with three different concentrations of ondansetron (1.5, 7.5, and 13.0 ng/ml). A total of twelve replicates of each QC concentration were assayed on six different days. The QC concentrations were determined from six different calibration curves which were assayed with the QC samples. Precision was expressed as coefficient of variation (C.V.), while accuracy was measured as the nominal percentage of the theoretical value obtained according to the following equation:

$$\text{Percentage of theoretical value} = (x / C_T) * 100$$

where X = mean determined concentration of a quality control pool and C_T = theoretical concentration.

Results are shown in Table 4.1. The lower concentration (1.5 ng/ml) showed higher coefficient of variation and lower accuracy. Coefficients of variation and accuracy for all three concentrations were very good.

The within-run precision and accuracy were determined similarly to the between-run precision and accuracy. A total of six replicates of the low, medium and high QC concentrations were assayed. Their corresponding back-extrapolated concentrations were all calculated from one calibration curve covering the 0.5 to 15 ng/ml concentration range. The within-run coefficients of variation were small (less than 5%). The within-run accuracy was also good (Table 4.2).

This assay displayed a lower limit of quantification (LOQ) of 0.5 ng/ml (Figure 4.3). Representative chromatograms of extracted ondansetron at low (1.5 ng/ml), medium (7.5 ng/ml) and high (13.0 ng/ml) QC concentrations are presented in Figure 4.4.

4.4.3. Calibration curves

Linearity of the calibration curves was validated from 0.5-15 ng/ml and determined by weighted least squares regression analysis ($1/x^2$) . Peak height ratios of ondansetron and I.S. were plotted versus their corresponding plasma concentrations. One calibration curve was assayed each day for six days (n=6). Calibration curves showed an average slope of 0.084 and an average y-intercept of 0.002. Precision and accuracy means for each concentration are shown in Table 4.3. The square of the correlation coefficients (r^2) varied from 0.9953 to 0.9988.

4.4.4. Extraction yields of ondansetron and I.S. from human plasma

Peak heights of ondansetron and the I.S. extracted versus non-extracted equivalent concentrations of drug were compared under identical chromatographic conditions. The low (1.5 ng/ml), medium (7.5 ng/ml) and high (13.0 ng/ml) QC concentrations were used. The absolute recovery of both compounds was evaluated on two separate occasions. On the first occasion, all three QC concentrations were assayed in 6 replicates. On the second occasion, the low QC concentration was assayed in replicates of 4 while the medium and high concentrations were assayed in replicates of 3. The overall extraction yields were determined on pooled data for both compounds. The overall extraction yields of ondansetron in plasma were 94.0 ± 14.7, 86.4 ± 10.1, and 89.7 ± 9.7% at 1.5, 7.5 and 13.0 ng/ml, respectively. The overall mean extraction yield of the I.S. was 81.8 ± 7.6%.

4.4.5. Short term stability of ondansetron in human plasma

The short term stability of ondansetron was assessed with three replicates of stability samples which were kept at room temperature (22 ± 4 °C) for 5 h versus two replicates of freshly thawed comparison samples. All three QC concentrations, that is low (1.5 ng/ml), medium (7.5 ng/ml) and high (13.0 ng/ml), were used. The coefficients of variation observed either on the peak height ratios or on the back-calculated concentrations varied between 0.7 to 5.2%. The lower QC concentration showed the highest coefficient of variation. The results demonstrate that the short term stability of ondansetron was not compromised under this condition.

4.4.6. Stability of extracted ondansetron and I.S. at room temperature

Stability of extracted ondansetron and the I.S. was evaluated by leaving the extracted samples at room temperature (22 ± 2 °C) for 21-24 h. QC samples freshly extracted were immediately injected (time 0) and then re-injected 21-24 h after sitting in the autosampler at room temperature. Evaluation involved all three QC concentrations of ondansetron. The coefficients of variation determined on the mean ondansetron ratios at the three QC concentrations were all less than 5% (values ranged from 1.0 to 3.6%). The coefficients of variation observed with the mean peak height for both products at the three QC concentrations tested were less than 15% (ranging from 7.0 to 11.9%). The difference between the mean peak height of the I.S. injected at time 0 and after 21-24 h was 2.0%. Therefore, the stability of extracted ondansetron and I.S. from plasma on the autosampler was not compromised after 24 h.

4.4.7. Freeze-thaw stability of ondansetron in human plasma

Freeze-thaw stability involved analysis of stability samples: thawed once, twice and thrice versus replicates of comparison samples that have been freshly prepared. One low (1.5 ng/ml), one medium (7.5 ng/ml) and one high (13.0 ng/ml) QC concentrations were used. All plasma samples originated from the same batch. The inter-freeze thaw coefficients of variation determined on the peak height ratios and back-calculated concentrations of ondansetron/I.S. ranged from 8.2 to 10.9% for the low QC concentration and from 1.2 to 2.8% for the medium and high QC concentrations. The nominal percent for ondansetron varied from 100.7 to 106.7%. In conclusion, ondansetron freeze-thaw variability is observed to be small (less than 15%) up to thawing three times.

4.4.8. Stock solution stability of ondansetron and I.S.

Stock solution evaluation involved analysis of ondansetron stock solution freshly prepared compared to two ondansetron stock solutions stored at 4 ± 1 °C for nine weeks and eight months. We observed little variability between ondansetron stock solutions kept under the specified conditions. The coefficient of variation determined on pooled data was 1.6%. A coefficient of variation of 6.7% was measured for the I.S. stock solution kept for eight months at 4 ± 1 °C compared to the freshly prepared solution.

4.4.9. Application of the method

The method was successfully applied to the determination of ondansetron plasma concentrations in five healthy male subjects. Each subject received ondansetron 2 mg

twice daily during a 4-week period, for a total of 56 oral doses. The plasma concentration-time profile for one of the subjects is illustrated in Figure 4.5. The pharmacokinetic characteristics derived from this profile show a mean plasma concentration of 5.4 ng/ml and a maximal concentration (C_{max}) of 9.8 ng/ml reached at 1.5 h (T_{max}). The elimination rate constant (z) was 0.1129 h^{-1} with a corresponding half-life of 6.1 h.

4.5 CONCLUSION

We described a reliable and reproducible analytical assay where the internal standard method is applied for quantitation of ondansetron. The chemical features of ondansetron complicate its extraction from plasma. The use of a liquid-liquid extraction technique resolves the problem of surface adhesion and provides a rapid way to extract many plasma samples. Results demonstrate that the analytical method described is precise and accurate. The rapid processing of numerous samples further supports the suitability of the method in pharmacokinetic studies.

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Table 4.1 Between-Run (n=12) precision and accuracy for ondansetron

Theoretical concentration (ng/ml)	Observed concentration (mean (ng/ml) ± S.D.)	C.V. (%)	Accuracy (%)
1.5	1.60 ± 0.08	5.0	106.7
7.5	7.45 ± 0.14	1.9	99.3
13.0	13.36 ± 0.23	1.7	102.8

Table 4.2 Within-Run (n = 6) precision and accuracy for ondansetron

Theoretical concentration (ng/ml)	Observed concentration (mean (ng/ml) ± S.D.)	C.V. (%)	Accuracy (%)
1.5	1.56 ± 0.06	3.8	104.0
7.5	7.56 ± 0.11	1.4	100.8
13.0	13.43 ± 0.20	1.5	103.3

Table 4.3 Calibration Curves (n = 6) for ondansetron

Theoretical concentration (ng/ml)	Observed concentration (mean (ng/ml) ± S.D.)	C.V. (%)	Accuracy (%)
0.50	0.50 ± 0.01	2.5	100.0
1.00	0.99 ± 0.07	7.2	99.0
2.50	2.54 ± 0.10	3.9	102.0
5.00	5.00 ± 0.13	2.7	100.0
10.00	9.69 ± 0.15	1.5	96.9
15.00	15.34 ± 0.19	1.3	102.3

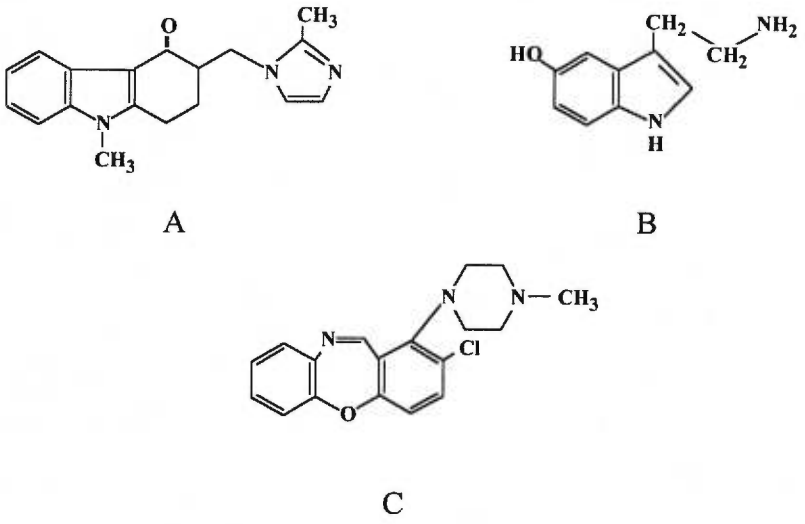

Figure 4.1 Chemical structures of ondansetron (A), serotonin (B) and loxapine (C).

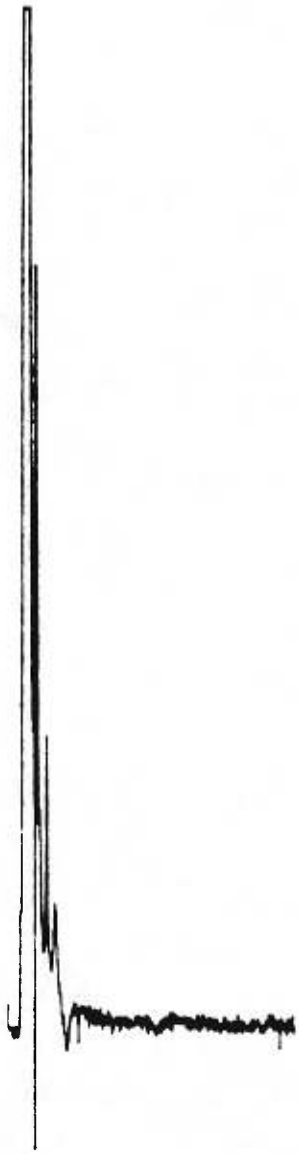

Figure 4.2 Chromatogram of an extracted human blank plasma.

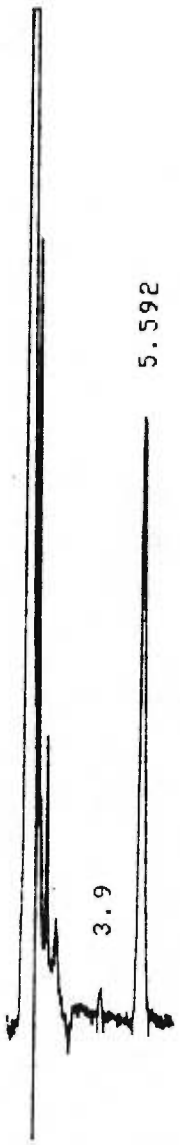

Figure 4.3 Chromatogram of ondansetron at lower limit of quantification (0.5 ng/ml).

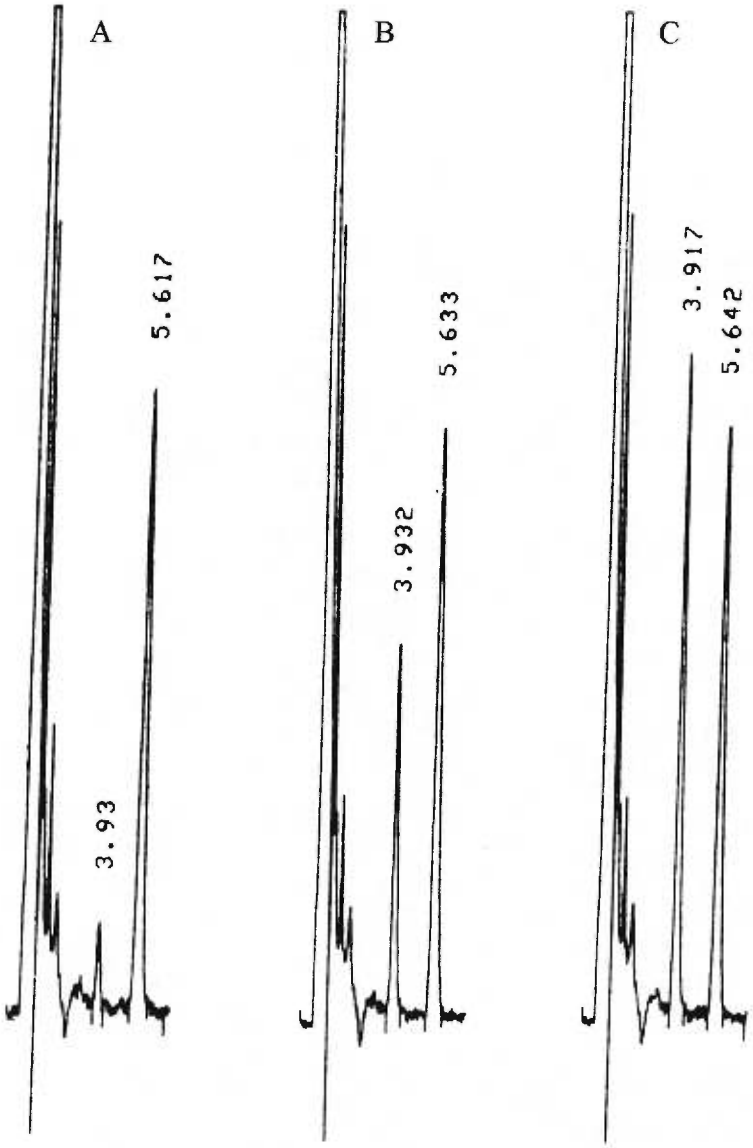

Figure 4.4 Chromatograms of extracted ondansetron at low (A, 1.5 ng/ml), medium (B, 7.5 ng/ml), and high (C, 13.0 ng/ml) QC concentrations.

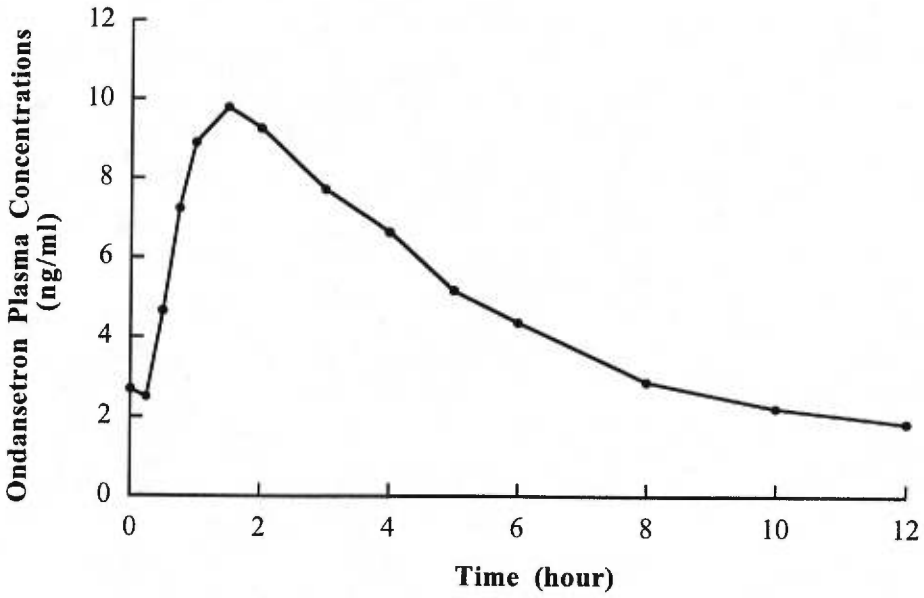

Figure 4.5 Plasma concentration-time profile of ondansetron in a male healthy subject after administration of multiple oral doses of ondansetron.

☙ *PART FOUR* ❧

DISCUSSION AND CONCLUSION

This research work was divided into three parts. The aim of the first part was to evaluate the role of the 5-HT_3 system in CCK-4-induced panic-like symptoms in humans. To do so, we studied the effect of a single and chronic administration of ondansetron in the mediation of CCK-4-induced panic-like symptoms in healthy male subjects. The evaluation of acute and chronic effects of ondansetron was used to assess possible neuro-adaptation following chronic ondansetron exposure. Compared to placebo, subjects receiving a single dose of ondansetron showed a significant decrease in iPSS symptoms and in the anxiety score at peak effect of CCK-4. The mean basal NPY plasma level was significantly higher and Δmax for cortisol, GH and PRL were significantly lower in the ondansetron group. There were no differences in Δmax for ACTH and NPY between the single dose administration of ondansetron and that of placebo. Vital signs did not differ between groups. After chronic administration, there was no difference in iPSS between both groups. The mean basal NPY plasma level was significantly higher, whereas Δmax for NPY significantly lower in the ondansetron group as compared to placebo. The reduction in Δmax for GH and PRL observed after chronic doses of ondansetron as compared to placebo failed to reach statistical significance. There were no differences in Δmax for ACTH and cortisol between groups. With regard to vital signs, there was no difference between groups. Decrease in behavioural and neuroendocrine responses provide strong evidence for an action of a single dose of ondansetron on CCK-4 and support a role for 5-HT_3 receptors in CCK-4-induced panic-like symptoms in healthy subjects. Also, results show that single and chronic administration of ondansetron affects basal NPY and CCK-4-induced changes in NPY. Hence, one may suggest that 5-HT_3 may serve as an important regulator of basal and CCK-4-stimulated NPY release. Finally, our results suggest a role for 5-HT_3 receptors in the neurobiology of anxiety and panic attacks through its interaction with CCK and NPY systems by a yet to be determined mechanism(s). Due to confounding factors, chronic effect of ondansetron on CCK-4-induced panic-like symptoms still needs exploration.

The purpose of the second part of the study was to gain insight into whether ondansetron administration induced changes in CCK_T plasma concentrations in humans before and after CCK-4-induced panic-like symptoms. This particular aspect of CCK measurement has never been investigated. Results showed no difference in CCK_T plasma levels and in the CCK_Tz between the ondansetron and the placebo groups after either single or chronic administration.

Finally, the last part of the study was devoted to determine the pharmacokinetic profile of low-dose ondansetron at steady-state by a novel methodological assay. Ondansetron was found to be particularly prone to adsorption and heat decomposition. Analytical attempts on the GC/MS apparatus failed to demonstrate its adequacy for quantitative analysis of ondansetron. In contrast, the HPLC analytical method provided excellent precision and accuracy in the determination of ondansetron plasma levels. The use of a liquid-liquid extraction technique provided a rapid way to extract many plasma samples. The rapid processing of numerous samples further supported the suitability of the method in pharmacokinetic studies.

This study also used the CCK-4 paradigm to further the understanding of the neurobiology of panic attacks and anxiety. The main findings of this study were that 5-HT_3 receptors interact with neurotransmission networks which show opposite behavioural action namely the CCK anxiogenic system and the NPY anxiolytic system. The mechanism by which ondansetron exerts its effect on CCK and NPY cannot be determined by this study. Therefore, the intention of the following discussion is to put forward hypotheses about possible mechanisms by which ondansetron might have influenced the ACTH, PRL, and GH responses to CCK-4 and to suggest further studies focusing on the mechanistic aspects of the 5-HT_3-CCK-NPY interaction. Limitations of the study are also discussed.

POSSIBLE MECHANISMS FOR ONDANSETRON EFFECTS ON ACTH, PRL, AND GH RESPONSES TO CCK-4

NPY is released in the blood during various stress conditions in rats and humans and is found in higher plasma concentrations in patients with panic disorder.[407-409] In agreement with these findings, CCK-4-induced anxiety significantly elevated NPY plasma concentrations in controls. In addition, CCK increases 5-HT biosynthesis and release in the central nervous system.[152, 400] Both neuropeptides, CCK and NPY, are known to regulate corticotropin releasing factor (CRF) secretion.[410, 411] Likewise, animal studies have shown that 5-HT and various direct-acting serotonin agonists stimulate the release of CRF through synaptic interaction between 5-HT nerve terminals and CRF-containing neurons.[412] Independently from the hypothalamic-pituitary-adrenal axis activation, it is suggested that CRF mediates anxiety-related symptoms. Behavioural effects caused by exposure to social stressors are reversed by administration of α-helical CRF 9-41, a competitive CRF antagonist.[413] Hence, the ACTH response induced by CCK-4 detected in the ondansetron and placebo groups may originate from the CRF response to the CCK-4 injection and the CCK-4-induced 5-HT and NPY releases. Since no significant difference was observed between the ondansetron and the placebo groups in the ACTH response, one may suggest that the 5-HT_3 circuit would have no or little involvement in the ACTH hormonal output. The net contribution of CRF as physiological mediator in the ACTH secretion and anxiety-related symptoms is unknown because the CRF plasma levels were not investigated in this study.

Our findings showed a significant reduction in the GH and PRL secretion in response to CCK-4 injection after single dose administration of ondansetron as compared to placebo. Regulation of adenohypophyseal hormone secretion involves numerous transmitters which play controversial roles as both stimulatory and inhibitory effects have been reported. Experimental studies indicated the existence of complex

interactions between CCK and dopaminergic D_2 receptors apparently controlled by the nature of the D_1 activation state. Hence, blockade of D_1 receptors facilitates the inhibitory regulation of the D_2 receptor affinity by CCK-8s through the activation of CCK_B receptors. In contrast, stimulation of D_1 receptors favours the CCK-8s-enhanced D_2 affinity via CCK_A and CCK_B receptors.[414, 415] The increase in dopamine levels after central administration of CI-988, a CCK_B antagonist, supports this concept.[416] Based on these findings, we may hypothesise a two-step dopaminergic modulation. The bolus injection of CCK-4 could initially cause a diminution in dopamine neurotransmission since CCK-4 has higher affinity for CCK_B than CCK_A receptors.[292] This assumption concurs with the capacity of CCK-4 to increase the amount of D_2 receptors.[399] The PRL response to CCK-4 challenge procedure could reflect a dopaminergic decline. Then, the tissue clearance of CCK-4 could restore the brain tissue dominance for CCK-8s leading to an increase in affinity for D_2 receptors. In addition, there is evidence that serotonin has considerable influence on dopamine neurons located in the arcuate nucleus.[417] Therefore, the GH response induced by CCK-4 could result from the summation of both stimulatory effects on the dopamine system by a dominant endogenous ratio CCK-8s/CCK-4 and CCK-4-induced 5-HT release. Since CCK-4 was injected in every subject, it is reasonable to anticipate a similar effect in both groups. However, results show a significant blunted response in the single dose ondansetron group compared to placebo ($p = 0.0323$ for PRL and $p = 0.0049$ for GH). It has been shown that 5-HT_3 antagonist agents such as ICS 205,930 and ondansetron prevent the increase of the drug-induced dopaminergic neuronal activity.[120, 418, 419] Hence, one may suppose that the action of CCK-4 on the dopaminergic activity together with the inhibition of dopamine release through 5-HT_3 blockade by ondansetron may have taken part in the PRL and GH responses observed in the ondansetron group after single dose administration. Moreover, part of the changes in GH may be secondary to NPY changes since coexistence of NPY, GH-releasing factor and somatostatin has been described in rat and human cortical neurons.[420, 421] NPY has been shown to stimulate the basal secretion of somatostatin

which inhibits GH release.[422] Therefore, the significant increase in the NPY plasma levels produced by ondansetron prior to CCK-4 injection could, in theory, have promoted the secretion of somatostatin. Yet, the reduction in the PRL and GH release observed after chronic exposure of ondansetron failed to reach statistical significance. Given the small sample size and the intra-subject variability, the apparent absence of an effect does not exclude possible changes. Nevertheless, further evidence on the existence of these hypothetical interactions are required to better assess the effect of ondansetron on the CCK-4-induced PRL and GH responses.

Based on the inhibitory action of CCK-4 on dopaminergic activity and that of ondansetron on dopamine release, a further increase in PRL secretion in response to CCK-4 would have been expected in the ondansetron group. On the contrary, our results showed a blunted PRL response ($p = 0.0323$) in the ondansetron group as compared to placebo after single dose administration ; whereas difference failed to reach significance after chronic administration of ondansetron ($p = 0.0645$). Therefore, we may likely assist in a series of potential interactions and would need many more observations to better evaluate the ACTH, PRL and GH responses to modulation of the 5-HT_3 receptor.

STUDY LIMITATIONS AND FUTURE STUDY WORKS

The importance of the response following chronic administration of placebo raises several questions. The contribution of a placebo response, an habituation effect and a sensibilisation counteraction has been previously discussed. Alternatively, it has been reported that administration of CCK-8 in rats produced anxiogenic-like effects only if rats were facing a novel environment.[423] The level of neuronal activity existing prior to CCK-8 administration has been suggested as a factor influencing the intensity of the anxiogenic effects produced by CCK-8. Based upon this finding, one may raise

concerns about the possibility that the notion of novelty would be negligible at the second CCK-4 bolus. In fact, the challenge session may no longer be perceived as novel because the event has been previously experienced by the subjects. Behavioural response to the second CCK-4 administration may then differ from that observed after the first injection. A significant decline in the iPSS and nPSS symptoms during the second relative to the first challenge injection has previously been reported.[372] Therefore, the notion of novelty should be carefully examined especially in healthy subjects for whom adaptation mechanisms are considered normal. One way to evaluate this hypothesis would be to repeat the study in two separate experiments. The first experiment would examine the effect of CCK-4 after a single dose administration of ondansetron in healthy subjects. In the second experiment, a group of healthy subjects different from the one who received the single dosing would be administered CCK-4 only after chronic exposure to ondansetron. Using such a design would keep the novelty criterion alike in both groups of subjects and could provide more informative data about the contribution of novelty in the model.

Results from the single dose administration of ondansetron reflect most likely true effects of an acute blockade of the 5-HT_3 receptors. However, chronic administration of ondansetron may not only reflect a chronic blockade of the 5-HT_3 receptors. Therefore, it is possible that modulation of receptors belonging to other 5-HT receptor subtypes and/or neuronal systems may have occurred after chronic exposure. Hence, results may represent the summation of inhibitory and stimulatory effects originating from these other neuronal systems. Contribution from these other networks to the final outcome is unknown and remains to be determined. Consequently, chronic effect of ondansetron on CCK-4-induced panic-like symptoms still needs exploration.

The other limitation of this study is related to the last blood sampling time chosen for the neuroendocrine measurements. Blood sampling was done only up to 15 minutes after CCK-4 challenge because of limits in blood volume collection. The 15-minute

time-concentration profile evaluated in the study proves to be insufficient to fully describe the neuroendocrine response to CCK-4. This is especially true for the cortisol and the GH responses which literally began 15 minutes after the CCK-4 injection. Although this evaluation has permitted the confirmation of previous results[424], future studies should extend the blood sampling times for the purpose of establishing a 60-minute profile of each hormone changes induced by CCK-4 administration. In addition, the neuroendocrine assessment could be completed by the measurement of other biological parameters such as the CRF and somatostatin plasma levels. This information could shed some light on the mechanistic aspects of the neuroendocrine changes produced by CCK-4-induced panic-like symptoms.

The role of NPY in the neurobiology of panic syndrome requires investigations in order to better understand the results of this study. In view of our results, interactions between 5-HT_3-NPY and CCK-NPY may be suggested in relation to anxiety in humans. With respect to the 5-HT_3-NPY interaction, the NPY plasma levels following the administration of ondansetron should be profiled. To determine whether ondansetron has unique specific effects on the NPY system, other compounds with similar molecular or pharmacological features should be evaluated. It is of particular interest that SSRI agents and those acting selectively on other 5-HT receptor subtypes, such as buspirone (a 5-HT_{1A} receptor antagonist) and methiothepin (a 5-HT_2 antagonist), be investigated. Furthermore, research should be directed towards the delineation of the role of Y_1 and Y_2 receptors in the mediation of the effects of ondansetron and in the interaction with CCK using various antagonists at NPY receptors. Knowledge about the NPY component fractions of different proteolytic fragments is also essential to generate mechanistic hypothesis.

Our design did not allow a complete evaluation of the CCK pharmacokinetic profile. Furthermore, the evaluation of the effects of ondansetron on CCK plasma levels was limited by the lack of knowledge about the CCK component fractions of different

molecular size. Hence, the effect of ondansetron on CCK plasma disposition still requires investigations which should include a full profile of CCK fragments. In addition, the study was conducted in healthy subjects. Therefore, the effect of ondansetron administration on the endogenous CCK disposition in patients during a panic attack could provide more informative data, although difficult to do.

We know, based upon the symptomatology profile presented by patients, that panic disorder may be an heterogeneous entity. In line with this thinking, a subgroup of panickers has been identified.[45] While cardio-autonomic symptoms are found in similar proportion between panickers, respiratory symptoms predominate in this subgroup. It has been reported that imipramine was more effective than GABA agonists in patients for whom respiratory symptoms constituted an important part of the syndrome.[45] Additionally, some patients suffering from panic disorder showed an enhanced sensitivity to the CO_2 provocation agent.[38] Whether panickers with respiratory symptoms and those with enhanced sensitivity to CO_2 are from the same subgroup population is unknown. Likewise, CCK-4 administration produced respiratory symptoms. Dyspnea is one of the four symptoms identified by the *post hoc* analysis of the individual PSS symptoms and may provide clues for future investigations. Our results suggest that ondansetron may attenuate the dyspnea experienced by the subjects following CCK-4 administration. Taken together, one may be inclined to hypothesise that the subgroup of panickers characterised by a dominance of respiratory symptoms may show greater sensitivity to CCK-4-induced panic and be affected more selectively by ondansetron treatment. To examine whether respiratory symptoms represent a variable that has bearing on the treatment outcome of CCK-4-induced panic-like symptoms, it may be of interest to include in future clinical studies with panic disorder patients a subdivision of the patient population into two different strata based on the symptom profile experienced during their panic attack.

The unique character of the 5-HT system is complex. Whether ondansetron influence the CCK-4-induced panic-like symptoms through a direct action $5\text{-}HT_3\text{-}CCK_B$ is unknown and cannot be elucidated by this study. However, the most remarkable element is that this unique circuit also exists in unity with other neuronal systems. Results suggest a series of potential interactions in a very complex system. The interaction between $5\text{-}HT_3$, CCK, and NPY neurotransmission proves to be somewhat involved in adaptive behaviours following CCK-4 administration in humans to maintain the homeostasis of the body.

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☙ *APPENDICES* ❧

APPENDIX 1. STUDY FLOW CHART: TIME AND PROCEDURE

Visit Study Day	1 *	2 1	3/4/5 8/15/22	6 29	7/ET **
Informed Consent	x				
Demographic Data Collection	x				
Medical and Psychiatric History	x				
Psychiatric Evaluation	x				x
Physical Examination	x				x
Haematology/Blood Chemistry/Urinalysis	x				x
Drug Urine Screen	x	x	x	x	
Pharmacokinetic Study Drug				x	
Neuroendocrine hormone/CCK-4 Levels		x		x	
12-lead ECG (resting)	x				x
SCID for non-patients	x				
Hamilton Anxiety Scale (HAM-A)	x			x	x
Symptom Checklist (SCL-90)	x			x	x
Panic Symptoms Rating Scale (PSS)		x[1]		x[1]	
Visual Analogue Scale (VAS)		x[2]		x[2]	
Eligibility Criteria for Randomisation		x			
Concurrent Medication	x	x	x	x	x
Vital Signs	x	x	x	x	x
Body Weight	x[3]				x
Record Adverse Events and Concurrent Illness		x	x	x	x
CCK-4 Administration		x		x	
Dispense Double-Blind Drug		x	x	x	
Compliance Check			x	x	

* Screening assessments performed between 3 and 14 days prior to entry into the study.

** Final assessments performed between 3 and 5 days after last dose of study drug.

ET Early Termination.

[1] PSS administered after CCK-4 administration as per protocol.

[2] VAS administered before and after study drug intake and CCK-4 administration as per protocol.

[3] Including height.

APPENDIX 2. INFORMATION AND INFORMED CONSENT FORM

I am invited to participate in a research study entitled 'EFFECTS OF ACUTE AND MULTIPLE ORAL DOSES OF ONDANSETRON ON CHOLECYSTOKININ TETRAPEPTIDE-INDUCED PANIC SYMPTOMS IN HEALTHY MALE SUBJECTS'. This research is done in partial fulfilment of requirements for a doctoral (Ph.D.) degree for Ms Michelle Dépôt. It will be conducted by Dr Jacques Bradwejn at the Clarke Institute of Psychiatry, a co-supervisor for the doctoral study with Dr Gilles Caillé of Université de Montréal. It is sponsored by GLAXO Canada Inc., Mississauga, Ontario, Canada. This research study involves 36 healthy male subjects between 18 and 55 years of age.

Background

Ondansetron (Zofran®) is a marketed medication prescribed for the treatment and prevention of surgery- and chemotherapy-induced vomiting. It has been administered orally and intravenously to approximately 20 000 people in Canada and in other countries. This medication is provided by GLAXO Canada Inc.

CCK-4 is a peptide (protein) that is naturally found in the human brain. This natural brain peptide has been postulated by Dr Bradwejn and other researchers to play a role in anxiety and panic attacks.

Purpose of the study

The primary objective of this study is to determine whether ondansetron 2 mg administered twice-a-day influences the panic-like symptoms and neuroendocrine changes induced by a CCK-4 injection. The secondary objective will determine the

extent to which ondansetron is distributed into the body and excreted from the body.

Study procedures and duration

1 Before the study begins, I will make an appointment at the research unit to receive a complete medical and psychological assessment which will take about 2 hours. This visit will involve:

i a complete medical and psychiatric (mental health) history;

ii a complete mental and psychological evaluation including mental status examination, anxiety and depression measurements;

iii a questionnaire about current smoking habits and caffeine consumption, any other problems and medications (including vitamins and mineral supplement). I understand that withholding information concerning previous or present medical problems or medications could be dangerous to my health;

iv a resting 12-lead electrocardiogram (heart tracing) to measure my heart function;

v a physical examination including vital signs (blood pressure and heart rate in sitting position), body weight and height;

vi a blood sample of about 30 mL will be drawn and a urine sample of about 50 mL will be collected for biochemical tests. If the results of these laboratory tests are not available because of technical problems or need to be re-checked, I agree to have this procedure repeated.

2 I authorise the research personnel to verify my participation on past clinical trials.

3 If the results of the above-mentioned procedures meet the requirements of the research study, I will be assigned a random number. This means that, among the 36 subjects participating in the study, I will be either one out of the 24 subjects receiving ondansetron 2 mg twice daily or one out of the 12 subjects receiving placebo (inactive medication) twice daily.

This study will last between 29 and 37 days. It involves initial (Visit 1) and final (Visit 7) assessments of about 1 to 2 hours each and 5 outpatient visits to the unit: Visit 2 of about 90 minutes or until clinical status returns to normal; Visits 3, 4 and 5 of about 15 to 30 minutes; and Visit 6 of about 13 hours. I will return to the unit within 3 to 14 days of Visit 1 for Visit 2; every following week for Visits 3, 4, 5, and 6; and finally 3 to 5 days after Visit 6 for my last appointment (Visit 7).

Visits 2 and 6 will involve:

i sitting blood pressure and heart rate;
ii series of psychological tests;
iii a questionnaire about any current problems and medications;
iv a urine sample of about 50 mL will be collected for biochemical tests;
v a catheter (small plastic tube) will be installed into a vein of my right arm through which a normal saline solution will slowly drip; this catheter will allow CCK-4 injection and facilitate blood drawings;
vi 2 tablets of the study medication will be administered to me with 150 mL of water;
vii a blood pressure cuff with a pulse monitor will be installed on my left arm; this will record my vital signs every 20 seconds for 2 minutes;
viii a 50-µg dose of CCK-4 will be injected through the catheter installed in my right arm;
ix blood samples will be drawn from my vein through the catheter of my right

arm to determine the hormone levels and the amount of CCK-4 and study medication in my blood. The total amount of blood taken from me during Visits 2 and 6 will not exceed 100 mL and 240 mL, respectively.

Visits 3, 4 and 5 will involve:

i sitting blood pressure and heart rate;
ii a urine sample of about 50 mL will be collected for biochemical tests;
iii a questionnaire about any current problems and medications.

Visit 7 will involve:

i a physical (including body weight) examination;
ii a mental and psychological evaluation;
iii a resting electrocardiogram to measure my heart function;
iv sitting blood pressure and heart rate;
v blood sample of about 30 mL will be drawn and a urine sample of about 50 mL will be collected for biochemical tests;
vi a questionnaire about any current problems and medications.

4 From Study Day 1 to Study Day 29, I will take 2 tablets (2 mg) twice-a-day, once at breakfast and once at bedtime, for a total of 4 mg per day. The usual dose is in the range of 8 to 32 mg a day. The study medication and research procedures will be provided free of charge. Since the study medications have been made to look alike, neither I nor the research personnel will know which of them I am taking but, in emergency, this information will be immediately available to the research physician or any other physician who needs to be attending me. I will return any unused study medication on each appointment visit.

I will be required to fast during the morning of Visits 2 and 6; this means no food or fluids for 8 hours before I receive the dose of the study medication. On the morning of Visit 6, I will not take my study medication; it will be administered to me by the research personnel. On visit 6, no fluids will be provided to any participant for at least 2 hours after and no food will be provided for at least 4 hours after taking the study medication; a regular diet will be provided thereafter except that no alcohol- or caffeine-containing food or beverages will be permitted. Smoking will not be permitted from 1 hour prior to dosing until 4 hours after dosing.

Risks and discomforts

As with any medication, undesirable side effects may occur carrying possible unexpected risks. I may develop an allergic reaction to the study medication. The following side effects have been observed during ondansetron treatment: headache; rare transient blurred vision; constipation; dizziness; numbness; tingling; muscle pains; sensation of flushing or warmth in the head or epigastrium; diarrhea; mild sedation; fatigue; abdominal pain and cramps; change in the muscular tone and in movement regulation.

CCK-4 has been administered to over 300 healthy subjects and patients. Over 150 of these subjects have received CCK-4 twice. It has not produced any serious and lasting side effects. Intravenous injection of CCK-4 may produce symptoms associated with anxiety and/or panic. These symptoms include: nervousness; dizziness; unsteady feeling or faintness; palpitations or accelerated heart; trembling or shaking; sweating; choking; nausea; diarrhea; abdominal distress; feeling detached from the body; feeling unreal; numbness or tingling; flushes or chills; chest pain or discomfort; fear of going crazy, of losing control, or a fear of dying. Three subjects had a brief period of

fainting. Other symptoms such as bitter taste or tickling of the throat have also been reported. I may or may not experience any of the above symptoms and it is unlikely that I will experience all of them. Symptoms usually last 2 to 5 minutes.

The total amount of blood taken from me during the study will not exceed 400 mL. For reference point, a standard Red Cross blood donation is 450 mL. The blood collection will be done by direct needle sticks or through a catheter which is a small plastic tube inserted in a vein of my arm. This plastic tube will be placed by an experienced person. The person collecting the blood will be experienced in this procedure, and I can expect some pain associated with inserting the needle (direct needle sticks). I understand slight bruising or inflammation can occur afterwards at the site the blood is taken from.

I agree to report any continuing or new reactions or sensations. Any condition experienced by me during this study will be fully evaluated by the research physician. I agree to return for follow-up examinations, if judged necessary by the investigator. I will be informed of any significant new findings or changes in the nature of the study or procedures, as they may occur. In such case, my consent will be re-obtained.

I have been informed that any serious medical event during the course of this study will be reported to GLAXO Canada Inc., the ethics committee supervising this study, the Health Protection Branch (HPB) of Health and Welfare Canada, Ottawa, and other regulatory authorities such as the Food and Drug Administration (FDA) of United States of America.

Prohibitions

i I agree to refrain from alcohol during the time that I am in the study.

ii I will not take any other medication without first consulting the research personnel, unless required for emergency use. When such medications are taken, the research personnel will document the name of medication, dose, and when it was taken. I understand my participation in the study may be terminated if, in the opinion of the research physician, the risk of an interaction is present, or use of the prescribed medication could interfere with the study.

iii I understand that I must not drink more than 3 cups of coffee or 5 cups of tea per day.

iv I understand that I must not be involved in other research projects while participating in this study.

v I understand that I should not give blood for 3 months before and after involvement in this study.

Use of any illicit drug is prohibited during the study and will terminate my participation in the study.

Benefits of the study

I understand that I will not receive any direct benefit from participating. The information obtained from this study may be useful to find treatment for panic disorder.

I will be paid $ 1 000.00 for completing the study. I understand that the data I will provide cannot be used unless I complete the entire study. For this reason, I will only receive a percentage of the full fee if I decide not to complete the study. Should this occur, I will receive a reduced fee according to the following schedule:

Visit (Study Day)	Experimental Day Stipend ($)	Total Payment ($)
2 (1)	50.00	50.00
3 (8)	100.00	150.00
4 (15)	100.00	250.00
5 (22)	150.00	400.00
6 (29)	400.00	800.00
7	200.00	1 000.00

If I decide not to complete the study, payment will not be made available any earlier than the end of the day upon which I would have completed the study had I remained in the study and completed it.

I may be withdrawn from the study by the investigator if it is thought to be in my best interest. Should this occur, full payment will be ordered at the time of discontinuation of my participation.

I understand that if I am uncooperative or noncompliant to the study requirements (e.g., fail to return for scheduled visits, non-compliance with the drug regimen, fail to return study medication, use of street drugs, unacceptable behaviour with the investigator or research personnel), I will be asked to leave the study without pay.

Voluntary nature of participation

I have been informed that I can withdraw from the study at any time I wish. I may be withdrawn from the study if it is thought to be in my best interest. If I withdraw from the study, I will come for a clinic visit at this time. Refusal to participate or discontinuation from the study will involve no loss of medical benefits to which I would otherwise be entitled and my refusal will not affect my selection for further studies.

Confidentiality

The data I will provide will be kept strictly confidential and secure to the extent permissible by law. All personal information concerning me (such as my name) will be held in confidence by the research personnel and GLAXO Group Research.

I have been informed that the results from the study will be available only to the researchers in this study and GLAXO Group Research. These data will be utilised only in connection with this study and will not be used for any other purpose or be disclosed to any party without my permission. I understand that results from this study will be submitted for publication in medical journals; published reports will refer to group data only. At no time my identity be disclosed.

I thereby authorise the release of my data dealing with this study to GLAXO. In such cases, my name will be removed from all documentation to ensure anonymity. I, as a study volunteer, permit GLAXO Group Research personnel to confirm the completion of this consent.

Contacts for information

I have been informed that members of the research team will answer any questions I may have concerning the study procedures, side effects of the study medication, discomfort or injury associated with the study, or my rights as a volunteer at any time during the study. In case of emergency, I will call Dr Bradwejn's office at 979-4735 as soon as practicable.

I HAVE READ THIS CONSENT FORM AND HAVE BEEN GIVEN A COPY.

I agree to participate in the study. This is to certify that this has been filled out voluntarily by me, without coercion. I understand that it is my responsibility to ask questions to clarify any points which I do not clearly understand. I have asked all such questions and have received answers to my satisfaction. I have not concealed or distorted any current condition, or any medical history information which might impair or affect my health or my participation in this study. I have answered all questions pertaining to my health accurately and truthfully. I am legally capable of giving informed consent.

_____

Print Name of Subject

_____ _____

Signature of Subject Date

_____

Signature of Witness

_____

Signature of investigator

CE DOCUMENT EST ÉGALEMENT DISPONIBLE EN FRANÇAIS. IL VOUS SERA REMIS SUR DEMANDE.

APPENDIX 3. HAMILTON ANXIETY SCALE (HAM-A)

		Circle the appropriate response for each item				
		none	mild	moderate	severe	very severe
ANXIOUS MOOD	Worries, anticipation of the worst, fearful anticipation, irritability	0	1	2	3	4
TENSION	Feelings of tension, fatigability, startle response, moved to tears easily, trembling, feelings of restlessness, inability to relax.	0	1	2	3	4
FEARS	Of dark, of strangers, of being left alone, of animals, of traffic, of crowds.	0	1	2	3	4
INSOMNIA	Difficulty in falling asleep, broken sleep, unsatisfying sleep and fatigue on walking, dreams, nightmares, night terrors.	0	1	2	3	4
INTELLECTUAL (cognitive)	Difficulty in concentration, poor memory	0	1	2	3	4
DEPRESSED MOOD	Loss of interest, lack of pleasure in hobbies, depression, early waking, diurnal swing	0	1	2	3	4
SOMATIC (muscular)	Pains and aches, twitching, stiffness, myoclonic jerks, grinding of teeth, unsteady voice, increased muscular tone.	0	1	2	3	4
SOMATIC (sensory)	Tinnitus, blurring of vision, hot and cold flashes, feelings of weakness, pricking sensation.	0	1	2	3	4
CARDIOVASCULAR SYMPTOMS	Tachycardia, palpitations, pain in chest, throbbing of vessels, fainting feelings, missing beat	0	1	2	3	4
RESPIRATORY SYMPTOMS	Pressure or constriction in chest, choking feelings, sighing, dyspnea	0	1	2	3	4
GASTRO-INTESTINAL SYMPTOMS	Difficulty in swallowing, wind, abdominal pain, burning sensations, abdominal fullness, nausea, vomiting, borborygmi, looseness of bowels, loss of weight, constipation	0	1	2	3	4
GENITO-URINARY SYMPTOMS	Frequency of micturition, urgency of micturition, amenorrhea, menorrhagia, development of frigidity, premature ejaculation, loss of libido, impotence	0	1	2	3	4
AUTONOMIC SYMPTOMS	Dry mouth, flushing, pallor, tendency to sweat, giddiness, tension headache, raising of hair	0	1	2	3	4
BEHAVIOUR AT INTERVIEW	Fidgeting, restlessness or pacing, tremor of hands, furrowed brow, strained face, sighing or rapid respiration, facial pallor, swallowing, belching, brisk tendon jerks, dilated pupils, exophthalmos, etc	0	1	2	3	4

APPENDIX 4. SYMPTOM CHEKLIST-90 (SCL-90)

Below is a list of problems and complaints that people sometime have. Please read each one carefully. After you have done so, please fill in one of the number spaces to the right that best describes how much that problem has bothered or distressed you during the past week including today.

HOW MUCH WERE YOU BOTHERED BY:	Not at all	A little bit	Moderately	Quite a bit	Extremely
Headaches	0	1	2	3	4
Nervousness or shakiness inside	0	1	2	3	4
Unwanted thoughts, words, or ideas that won't leave your mind	0	1	2	3	4
Faintness of dizziness	0	1	2	3	4
Loss of sexual interest or pleasure	0	1	2	3	4
Feeling critical of others	0	1	2	3	4
The idea that someone else can control your thoughts	0	1	2	3	4
Feeling others are to blame for most of your troubles	0	1	2	3	4
Trouble remembering things	0	1	2	3	4
Worried about sloppiness or carelessness	0	1	2	3	4
Feeling easily annoyed or irritated	0	1	2	3	4
Pains in heart or chest	0	1	2	3	4
Feeling afraid in open spaces or on the streets	0	1	2	3	4
Feeling low in energy or slowed down	0	1	2	3	4
Thoughts of ending your life	0	1	2	3	4
Hearing voices that other people do not hear	0	1	2	3	4

HOW MUCH WERE YOU BOTHERED BY:	Not at all	A little bit	Moderately	Quite a bit	Extremely
Trembling	0	1	2	3	4
Feeling that most people cannot be trusted	0	1	2	3	4
Poor appetite	0	1	2	3	4
Crying easily	0	1	2	3	4
Feeling shy or uneasy with the opposite sex	0	1	2	3	4
Feeling of being trapped or caught	0	1	2	3	4
Suddenly scared for no reason	0	1	2	3	4
Temper outbursts that you could not control	0	1	2	3	4
Feeling afraid to go out of your house alone	0	1	2	3	4
Blaming yourself for things	0	1	2	3	4
Pains in lower back	0	1	2	3	4
Feeling blocked in getting things done	0	1	2	3	4
Feeling lonely	0	1	2	3	4
Feeling blue	0	1	2	3	4
Worrying too much about things	0	1	2	3	4
Feeling no interest in things	0	1	2	3	4
Feeling fearful	0	1	2	3	4
Your feelings being easily hurt	0	1	2	3	4
Other people being aware of your private thoughts	0	1	2	3	4
Feeling others do not understand you or are unsympathetic	0	1	2	3	4
Feeling that people are unfriendly or dislike you	0	1	2	3	4
Having to do things very slowly to insure correctness	0	1	2	3	4

HOW MUCH WERE YOU BOTHERED BY:	Not at all	A little bit	Moderately	Quite a bit	Extremely
Heart pounding or racing	0	1	2	3	4
Nausea or upset stomach	0	1	2	3	4
Feeling inferior to others	0	1	2	3	4
Soreness of your muscles	0	1	2	3	4
Feeling that you are watched or talked about by others	0	1	2	3	4
Trouble falling asleep	0	1	2	3	4
Having to check and double-check what you do	0	1	2	3	4
Difficulties in making decisions	0	1	2	3	4
Feeling afraid to travel on buses, subways or trains	0	1	2	3	4
Trouble getting your breath	0	1	2	3	4
Hot or cold spells	0	1	2	3	4
Having to avoid certain things, places, or activities because they frighten you	0	1	2	3	4
Your mind going blank	0	1	2	3	4
Numbness or tingling in parts of your body	0	1	2	3	4
A lump in your throat	0	1	2	3	4
Feeling hopeless about the future	0	1	2	3	4
Trouble concentrating	0	1	2	3	4
Feeling weak in parts of your body	0	1	2	3	4
Feeling tense or keyed up	0	1	2	3	4
Heavy feelings in your arms or legs	0	1	2	3	4
Thoughts of death or dying	0	1	2	3	4
Overeating	0	1	2	3	4
Feeling uneasy when people are watching or talking about you	0	1	2	3	4

HOW MUCH WERE YOU BOTHERED BY:	Not at all	A little bit	Moderately	Quite a bit	Extremely
Having thoughts that are not your own	0	1	2	3	4
Having urges to beat, injure or harm someone	0	1	2	3	4
Awakening in the early morning	0	1	2	3	4
Having to repeat the same actions such as touching, counting, washing	0	1	2	3	4
Sleep that is restless or disturbed	0	1	2	3	4
Having urges to break or smash things	0	1	2	3	4
Having ideas or beliefs that others do not share	0	1	2	3	4
Feeling very self-conscious with others	0	1	2	3	4
Feeling uneasy in crowds, such as shopping or at a movie	0	1	2	3	4
Feeling everything is an effort	0	1	2	3	4
Spells of terror or panic	0	1	2	3	4
Feeling uncomfortable about eating or drinking in public	0	1	2	3	4
Getting into frequent arguments	0	1	2	3	4
Feeling nervous when you are left alone	0	1	2	3	4
Others not giving you proper credit for your achievements	0	1	2	3	4
Feeling lonely even when you are with people	0	1	2	3	4
Feeling so restless you couldn't sit still	0	1	2	3	4
Feelings of worthlessness	0	1	2	3	4

HOW MUCH WERE YOU BOTHERED BY:	Not at all	A little bit	Moderately	Quite a bit	Extremely
Feeling that familiar things are strange or unreal	0	1	2	3	4
Shouting or throwing things	0	1	2	3	4
Feeling afraid you will faint in public	0	1	2	3	4
Feeling that people will take advantage of you if you let them	0	1	2	3	4
Having thoughts about sex that bothered you a lot	0	1	2	3	4
The idea that you should be punished for your sins	0	1	2	3	4
Feeling pushed to get things done	0	1	2	3	4
The idea that something is seriously wrong with your body	0	1	2	3	4
Never feeling close to another person	0	1	2	3	4
Feelings of guilt	0	1	2	3	4
The idea that something is wrong with your mind	0	1	2	3	4

APPENDIX 5. PANIC SYMPTOM RATING SCALE (PSS)

Below is a list of symptoms that subjects might have experienced as a result of receiving the CCK-4 injection. The intensity/severity of each symptom experienced was evaluated on a scale from 0 to 4.

		INTENSITY/SEVERITY			
	NOT PRESENT	MILD	MODERATE	VERY SEVERE	EXTREMELY SEVERE
Feeling short of breath and/or smothering sensation	0	1	2	3	4
Dizziness	0	1	2	3	4
Unsteady feeling	0	1	2	3	4
Faintness	0	1	2	3	4
Palpitations and/or accelerated heart	0	1	2	3	4
Earache	0	1	2	3	4
Nose itching	0	1	2	3	4
Trembling and/or shaking	0	1	2	3	4
Sweating	0	1	2	3	4
Choking feeling	0	1	2	3	4
Stuffy nose	0	1	2	3	4
Nausea	0	1	2	3	4
Abdominal distress	0	1	2	3	4
Low back pain	0	1	2	3	4
Feeling unreal and/or detached from your body	0	1	2	3	4
Numbness and/or tingling in parts of your body	0	1	2	3	4

	NOT PRESENT	INTENSITY/SEVERITY			
		MILD	MODERATE	VERY SEVERE	EXTREMELY SEVERE
Hot flushes and/or cold chills	0	1	2	3	4
Itchy feet	0	1	2	3	4
Chest pain and/or discomfort	0	1	2	3	4
Anxiety, fear and/or apprehension	0	1	2	3	4
Fear of dying	0	1	2	3	4
Fear of losing control	0	1	2	3	4
Fear of going crazy	0	1	2	3	4

APPENDIX 6. DECLARATION OF HELSINKI

Recommendations guiding physicians in biomedical research involving human subjects
Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964
amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975
35th World Medical Assembly, Venice, Italy, October 1983
and the 41st World Medical Assembly, Hong Kong, September 1989.

INTRODUCTION

It is the mission of the physician to safeguard the health of the people. His or her knowledge and conscience are dedicated to the fulfilment of this mission.

The Declaration of Geneva of the World Medical Association binds the physician with the words: 'The health of my will be my first consideration' and the International Code of Medical Ethics declares that 'A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient'.

The purpose of biomedical research involving human subjects must be to improve diagnostic, therapeutic and prophylactic procedures and the understanding of the aetiology and pathogenesis of disease.

In current medical practice most diagnostic, therapeutic or prophylactic procedures involve hazards. This applies especially to biomedical research.

Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects. In the field of biomedical research a

fundamental distinction must be recognised between medical research in which the aim is essentially diagnostic or therapeutic for a patient and medical research, the essential object of which is purely scientific and without direct diagnostic or therapeutic value to the person subjected to the research.

Special caution must be exercised in the conduct of research which may effect the environment, and the welfare of animals used for research must be respected.

Because it is essential that the results of laboratory experiments be applied to human beings to further scientific knowledge and to help suffering humanity, the World Medical Association has prepared the following recommendations as a guide to every physician in biomedical research involving human subjects. They should be kept under review in the future. It must be stressed that the standards as drafted are only a guide to physicians all over the world. Physicians are not relieved from criminal, civil and ethical responsibilities under the laws of their own countries.

I. BASIC PRINCIPLES

1. Biomedical research involving human subjects must conform to generally accepted scientific principles and should be based on adequately performed laboratory and animal experimentation and on a thorough knowledge of the scientific literature.
2. The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol which should be transmitted for consideration, comment and guidance to a specially appointed committee independent of the investigator and the sponsor provided that this independent committee is in conformity with the laws and regulations of the country in which the research experiment is performed.

3. Biomedical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given his or her consent.
4. Biomedical research involving human subjects cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.
5. Every biomedical research involving human subjects should be preceded by careful assessment of predictable risks in comparison with foreseeable benefits to the subject or to others. Concern for the interests of the subject must always prevail over the interest of science and society.
6. The right of the research subject to safeguard his or her integrity must always be respected. Every precaution should be taken to respect the privacy of the subject and to minimise the impact of the study on the subject's physical and mental integrity and on the personality of the subject.
7. Physicians should abstain from engaging in research projects involving human subjects unless they are satisfied that the hazards involved are believed to be predictable. Physicians should cease any investigation if the hazards are found to outweigh the potential benefits.
8. In publication of the results of his or her research, the physician is obliged to preserve the accuracy of the results. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.
9. In any research on human beings, each potential subject must be adequately informed of the aims, methods, anticipated benefits and potential hazards of the study and the discomfort it may entail. He or she should be informed that he or she is at liberty to abstain from participation in the study and that he or she is free to

withdraw his or her consent to participation at any time. The physician should then obtain the subject's freely-given informed consent, preferably in writing.

10. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship to him or her or may consent under duress. In that case the informed consent should be obtained by a physician who is not engaged in the investigation and who is completely independent of this official relationship.
11. In case of legal incompetence, informed consent should be obtained from the legal guardian in accordance with national legislation. Where physical or mental incapacity makes it impossible to obtain informed consent, or when the subject is a minor, permission from the responsible relative replaces that of the subject in accordance with national legislation. Whenever the minor child is in fact able to give consent, the minor's consent must be obtained in addition to the consent of the minor's legal guardian.
12. The research protocol should always contain a statement of the ethical considerations involved and should indicate that the principles enunciated in the present Declaration are complied with.

II. MEDICAL RESEARCH COMBINED WITH PROFESSIONAL CARE (Clinical Research)

1. In the treatment of the sick person, the physician must be free to use a new diagnostic and therapeutic measure, if in his or her judgement it offers hope of saving life, re-establishing health or alleviating suffering.
2. The potential benefits, hazards and discomfort of a new method should be weighed against the advantages of the best current diagnostic and therapeutic methods.
3. In any medical study, every patient - including those of a control group, if any - should be assured of the best proven diagnostic and therapeutic method.

4. The refusal of the patient to participate in the study must never interfere with the physician-patient relationship.
5. If the physician considers it essential not to obtain informed consent the specific reasons for this proposal should be stated in the experimental protocol for transmission to the independent committee (I.2)
6. The physician can combine medical research with professional care, the objective being the acquisition of new medical knowledge only to the extent that medical research is justified by its potential diagnostic or therapeutic value for the patient.

III. NON-THERAPEUTIC BIOMEDICAL RESEARCH INVOLVING HUMAN SUBJECTS

(Non-clinical biomedical research)

1. In the purely scientific application of medical research carried out on a human being, it is the duty of the physician to remain the protector of the life and health of that person on whom biomedical research is being carried out.
2. The subjects should be volunteers - either healthy persons or patients for whom the experimental design is not related to the patient's illness.
3. The investigator or the investigating team should discontinue the research if in his/her or their judgements it may, if continued, be harmful to the individual.
4. In research on man, the interest of science and society should never take precedence over considerations related to the wellbeing of the subject.

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