An investigation into the anxiolytic properties of melatonin in humans.

By

Johannes Jacobus McCallaghan

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

OF RHODES UNIVERSITY.

DECEMBER 1998

ABSTRACT

The purpose of this project was to investigate the role of melatonin in the pathophysiology of anxiety in humans.

The literature study confirmed the intimate relationship between serotonin and melatonin. Melatonin is not only able to act as an agonist (in physiological concentrations) and an antagonist (at higher concentrations) on serotonin receptors but via control of brain pyridoxal kinase activity might have an effect on GABA, serotonin, dopamine and norepinephrine synthesis.

A clinical trial to investigate melatonin's effect on anxiety in humans was conducted as a pilot study. Thirty patients complaining of anxiety participated in a "N of 1" double blind placebo controlled trial. During the experiment each subject was thus exposed to melatonin and a placebo for a week at a time on two occasions.

During the first phase of the experiment, (Pair 1) patients showed a statistically significant reduction in their anxiety levels during the first period (P_1P_1), which was not the case during the second period (P_1P_2). The improvement however continued during the second phase of the experiment (Pair 2) so that there was also a statistically significant improvement during $P_2\ P_2$ (Period 2 / Pair 2) when placebo was administered.

It could not conclusively be shown that melatonin was responsible for the improvement in the patients' anxiety.

The explanation for these results suggests that the improvement was due to a:

1) placebo effect throughout,

2) psychotherapeutic effect due to contact with a clinician,

3) melatonin induced phase shift in the patient's endogenous melatonin response curve,

4) combination of all 3 options.

This pilot study lays the groundwork for a much more exhaustive study in which the melatonin of the patients is determined before melatonin is administered, the role of the clinician is clarified and the most appropriate time for melatonin administration is sought.

CONTENT

Acknowledgements

I gratefully acknowledge the support of the following people and organizations.

1) Professor Ben Potgieter for his guidance and supervision of the project.

2) My wife Karin for her support.

3) Lundbeck for financial assistance during 1996

4) Professor R Naidoo (The Department of Pharmacy, University of Port Elizabeth) for permission to use their laminar flow facilities.

5) Mr Mike Perkin (Senior lecturer/Pharmacist, Department of Pharmacy, University of Port Elizabeth) for the double blind packing of the melatonin and placebo's.

6) Dr Pieter Milne (Senior lecturer, Department of Pharmacy, University of Port Elizabeth) for analysis of the melatonin tablets

7) Pharmacare BPK for donating the starch used in the preparation of the placebo's

8) Mr J Bosma for his advice on the planning of the project and the statistical evaluations

9) Maggie Botes and Marietjie Sharp for sketches and typing

LIST OF TABLES, FIGURES AND ILLUSTRATIONS

CHAPTER 2

CHAPTER 3

LIST OF ABBREVIATIONS

ACTH	-	Adrenocorticotropic hormone
cAMP	-	Cyclic adenosine - $3^{l},5^{l}$ - monophosphate
cGMP	-	Cyclic guanosine - $3^{l},5^{l}$ - monophosphate
CNS	-	Central nervous system
DNA	-	Deoxyribonucleic acid
D S T	-	Dexamethasone suppression test
GH	-	Growth hormone
G R F	-	Growth hormone releasing factor
G A B A	-	Gamma - aminobutyric acid
IV	-	Intravenous
5HT	-	Serotonin (5- Hydroxytryptamine)
HPLC	-	High performance liquid chromatography
NREM Sleep	-	Non rapid eye movement sleep
O C D	-	Obsessive compulsive disorder
REM Sleep	-	Rapid eye movement sleep
S A D	-	Seasonal affective disorder
S C G	-	Superior cervical ganglia
S C N	-	Suprachiasmatic nuclei
TLC	-	Thin layer chromatography
Vd	-	Volume of distribution

CHAPTER 1: INTRODUCTION

Interest in the pineal gland began in 300 BC when it was discovered by Herophilus. Over the centuries, as scientific methods developed, the in depth study and accumulation of knowledge regarding the pineal gland intensified.(1)

Besides the fact that the pineal gland is instrumental in regulating the day/night cycle it is also implicated in a variety of psychiatric disorders.(1)(2) It is important to realise that there is not a simple relationship between the pineal gland (melatonin) and any of these disorders, but that it probably forms part of a complex dysfunctional process - there are thus good reasons why the pineal gland is called "the regulator of all regulators."(2)

The anxiolytic properties of melatonin are poorly documented - it has been demonstrated in mice that melatonin exhibits anxiolytic properties.(3)
Because melatonin is safe to administer to humans (4) and appreciating the suffering endured by humans due to anxiety, the anxiolytic effect of oral melatonin in humans is to be investigated.

An interesting extension of this study would be to investigate by means of a literature survey the possible interaction of melatonin with serotonin receptors and other neurotransmitter systems. The rationale of this is the comorbidity between anxiety and depression and the possibility that both may be due to dysfunctional $5HT_{1A}$ autoreceptors.(5)

This project is seen as a pilot study on the anxiolytic properties of melatonin. This study should add another dimension to the further clinical investigations into the pathophysiology of anxiety - that of melatonin phase response abnormalities.

The interactions of melatonin with other systems are presented in a flow diagram. The content and purity of "over the counter" melatonin in human research is also addressed by the analysis of melatonin tablets.

CHAPTER 2: THE HUMAN PINEAL GLAND

2.1 INTRODUCTION

The pineal gland was once described by Descrates as the seat of the soul. The human pineal gland is anatomically close to the third ventricle and the anatomical relationships are similar to those of the lower mammals.(1)

2.2 EMBRYOLOGY

The nervous system is ectodermal in origin and develops from the neural plate. From a hollow tubelike structure the central nervous system develops so that its caudal (tail) portion forms the spinal cord and the cephalic region the brain.
The brain is subdivided into 3 parts:

a) The prosencephalon (forebrain) which consists of:

- The telencephalon (rostral)
- The diencephalon (caudal)

b) The mesencephalon

c) The rhombencephalon which consists of:

- The metencephalon (pons & cerebellum)
- The myelencephalon (medulla).

See fig 1(a) and fig 1(b) (6)

The pineal gland develops from the diencephalon (caudal part of the roof plate) which also gives rise to the thalami, geniculate body, most of the hypothalamus, the roof of the third ventricle and the neurohypophysis.(7)

2.3 ANATOMY

The pineal gland is attached to the posterior roof of the third ventricle and lies between the posterior commissure and dorsal habenular commissure.(6)(7)(8) The human pineal is about 5 by 7 millimeter in size and weighs 100-150 milligram. It is covered by the pia mater which forms a capsule and sends septa inwards to form lobules.(8)

2.4 BLOOD SUPPLY

The posterior cerebral arteries, which are the two terminal branches of the basilar artery, branch into the posterior choroidal arteries(3); the medial posterior choroidal branches supply the pineal gland.(8)((9) See fig 2.(10)). The bloodflow is in the region of 4 millilitre/minute/gram, second only to the kidney. Arteries branch extensively in the capsule before entering the parenchyma. The extensive vascular bed contains vessels the size of capillaries.

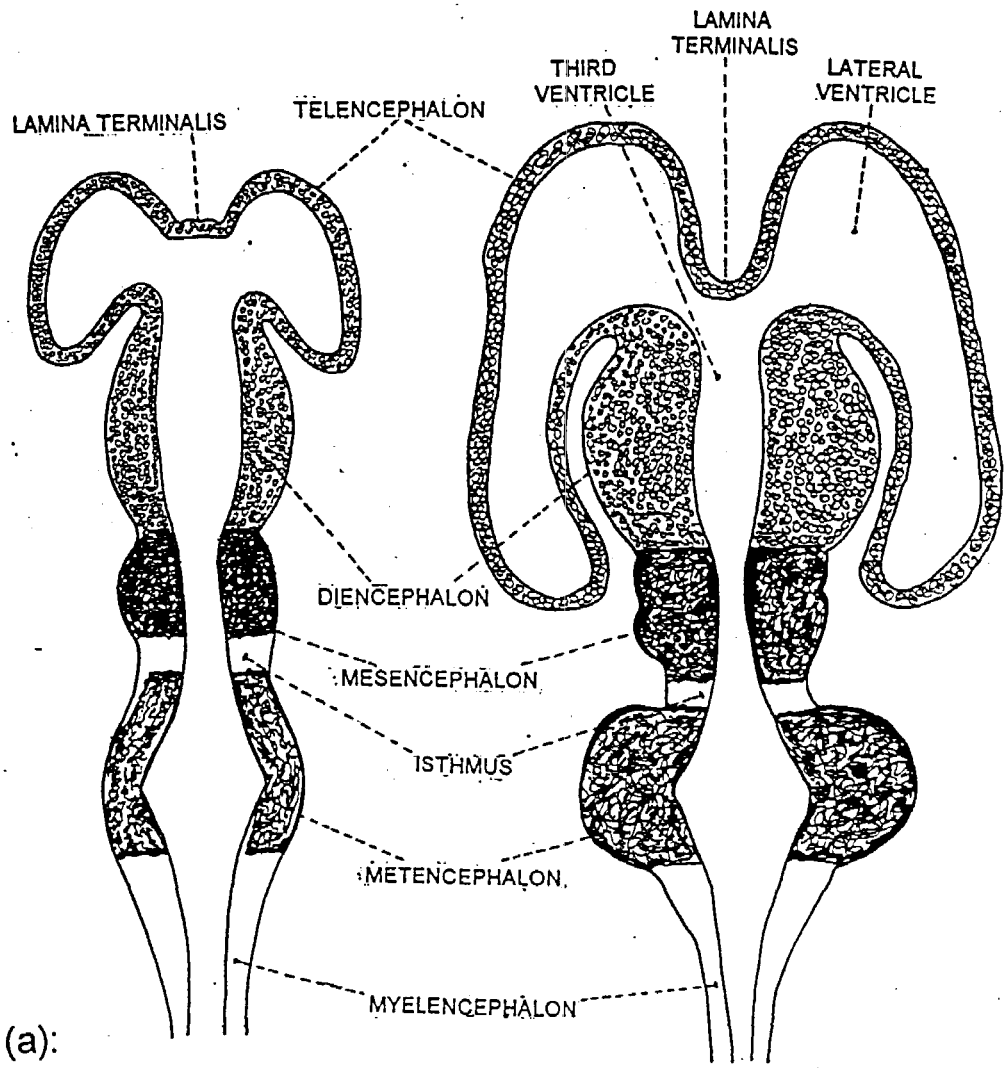


Fig 1(a):

Schematic presentation of the regional differentiation of the brain.

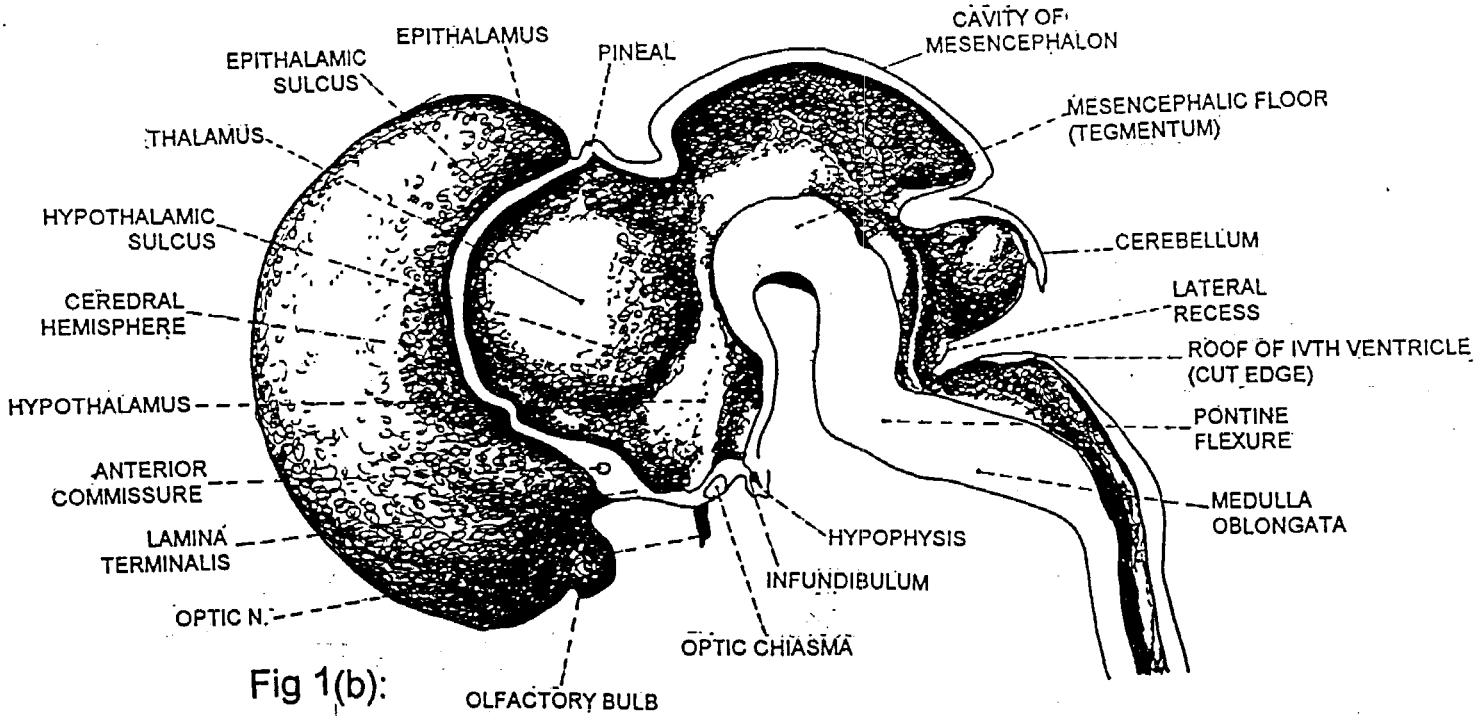


Fig 1(b):

Reconstruction of the structural relationship of the pineal gland in the brain of a 43mm human fetus.

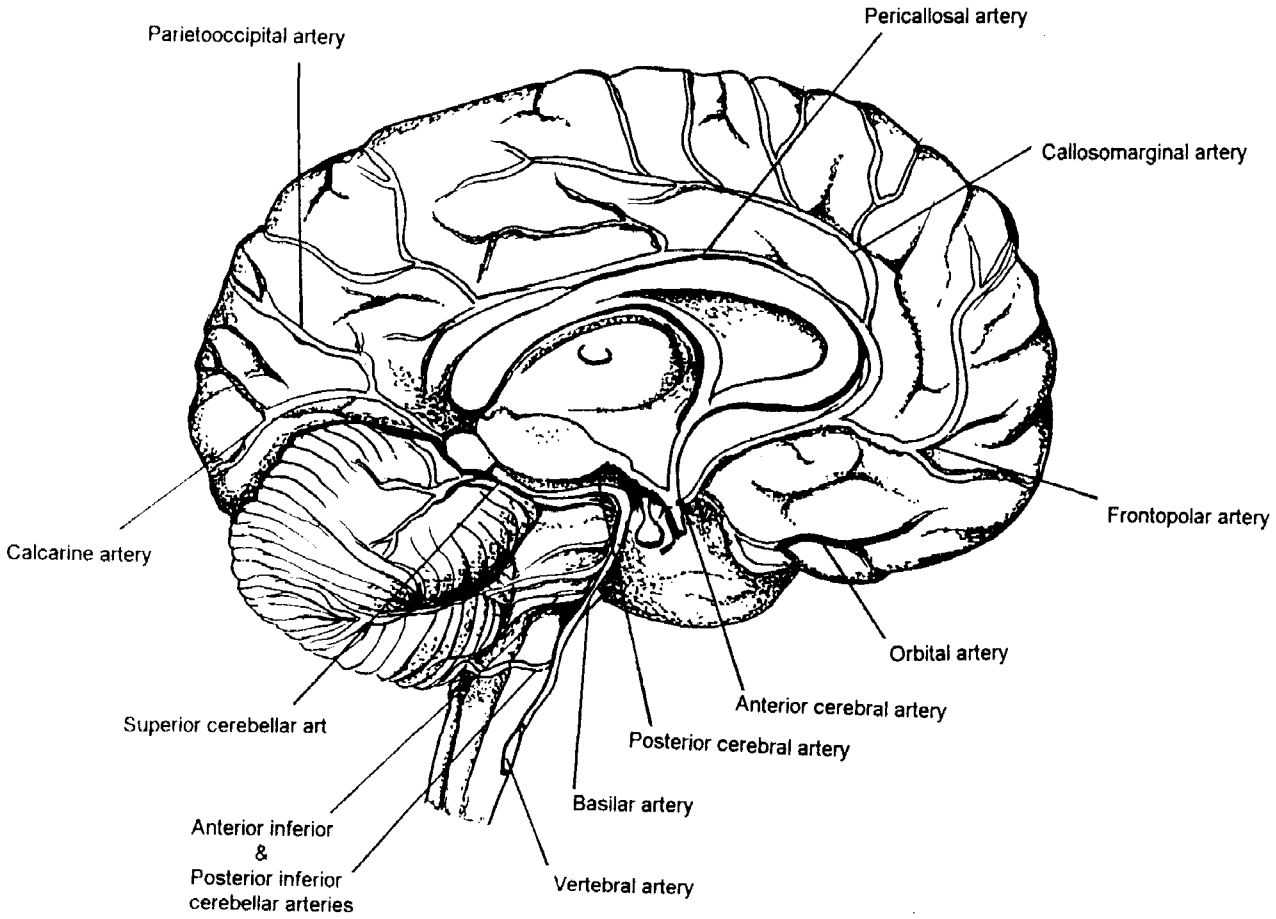
It seems that the cortical pineal tissue receives 1.2-2.0 times the blood flow of the medullary pineal. The blood supply of the pineal is also higher during the night. Removal of the superior cervical ganglia reduces the metabolic activity of, and the blood flow to, the pineal gland.(9) The pineal gland's capillaries are fenestrated with a high degree of permeability. It is thus one of the circumventricular organs (outside the blood brain barrier).(11)

2.5 VENOUS DRAINAGE

The pineal capillaries network empties in the great cerebral vein. In humans there is also drainage into the junction of the internal cerebral veins.(9) There appears to be a vascular resistance of intrinsic pineal vessels to venoreflux or backflow.(12)

2.6 NERVOUS SUPPLY

The nervous control of the pineal gland involves post-ganglionic sympathetic fibres from the superior cervical ganglion as well as input from the parasympathetic, commissural and peptidergic systems. The post-ganglionic fibres take their course via the internal carotid plexus (blood vessels) and form the nervi conarii before entering the gland. The nerve terminals do not form synapses with pinealocytes - they end in the vicinity of pinealocyte processes, around capillaries in many cases.(9)



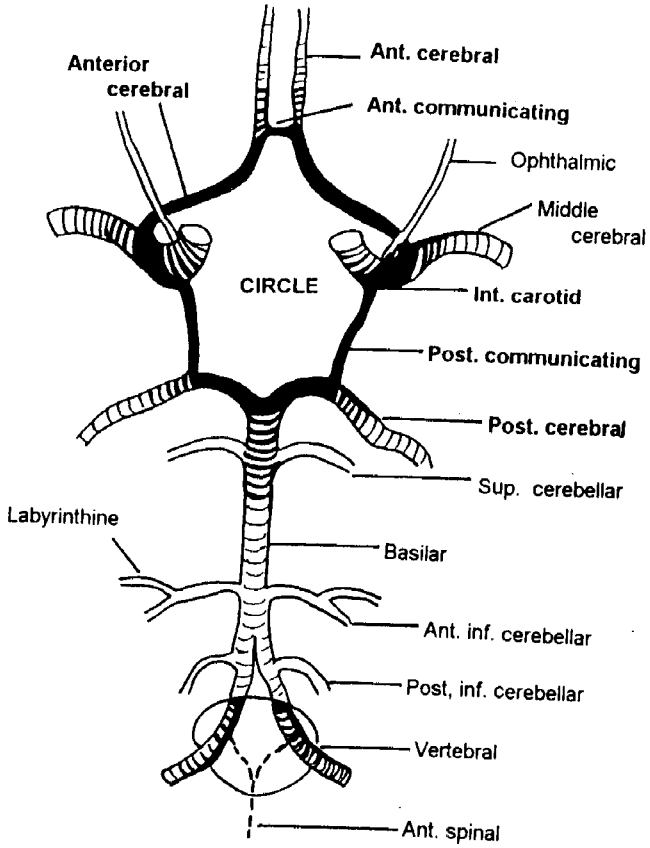


Fig 2 : The posterior cerebral artery's anatomical relationship.

The sympathetic innervation controls the production of melatonin- the major neurotransmitter being noradrenalin. The pineal gland is connected to the retina via the superior cervical ganglia (SCG) and the hypothalamic suprachiasmatic nuclei.(13) See fig 3.(9)

2.7 HISTOLOGY

The lobules of the pineal gland consist of pinealocytes and neuroglia. The pinealocytes which are best seen in silver preparations are irregular cells with long branching processes terminating in bulbous endings. The cytoplasm is noted for its extensive smooth endoplasmic reticulum. Brain sand (acervuli) is mainly in the capsule and septa.(14)

Calcification in the pineal gland was once regarded as a sign of degeneration. It has however been shown that the amount of calcification is not proportional to the age of the patient.(15)

2.8 PINEAL CONTENT

The pineal gland not only contains indoleamines but also polypeptides (example arginine vasotonin). (16)(17) Other indoles related to melatonin exist in the pineal. 5-Methoxytryptamine for example is an agonist on $5HT_{1A}$ receptors and may be involved in the modulation of serotonergic transmission.(18)

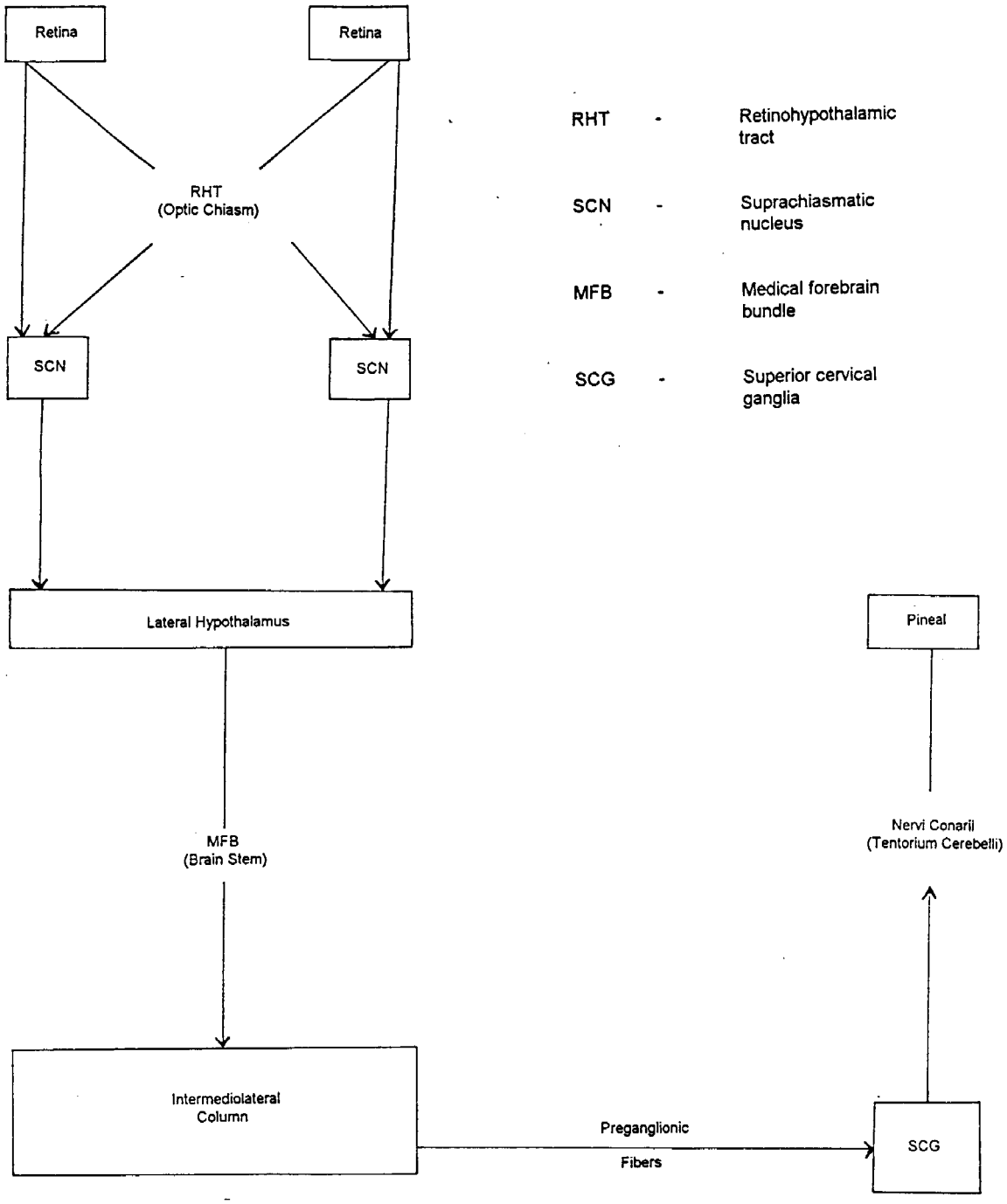


Fig 3 : Neural innervation of the mammalian pineal gland.

CHAPTER 3: MELATONIN

3.1 HISTORY

Melatonin was discovered by Lerner and his colleague in 1958.(18) This followed the interest in 5- Hydroxytryptamine during the 1950's which led to the development of the concept of a system that was able to synthesize biogenic amines - "Amine precursor uptake and decarboxylation system (APUD)."

Interestingly there is an inverse relationship of serotonin and melatonin concentrations in the pineal gland - serotonin levels being highest during the daytime.(19)

3.2 SYNTHESIS

Fig 4 Melatonin: synthetic pathway.(1)(19)

Melatonin is synthesized and secreted by the pineal gland during the dark phase of the day and is under noradrenergic control (post ganglionic sympathetic fibres.)(20)

The pineal gland takes up tryptophan from the circulation which is then hydroxylated and decarboxylated to serotonin.
N - acetyltransferase (NAT) (with 70-100 fold increased activity at night) is in most circumstances the rate limiting enzyme in

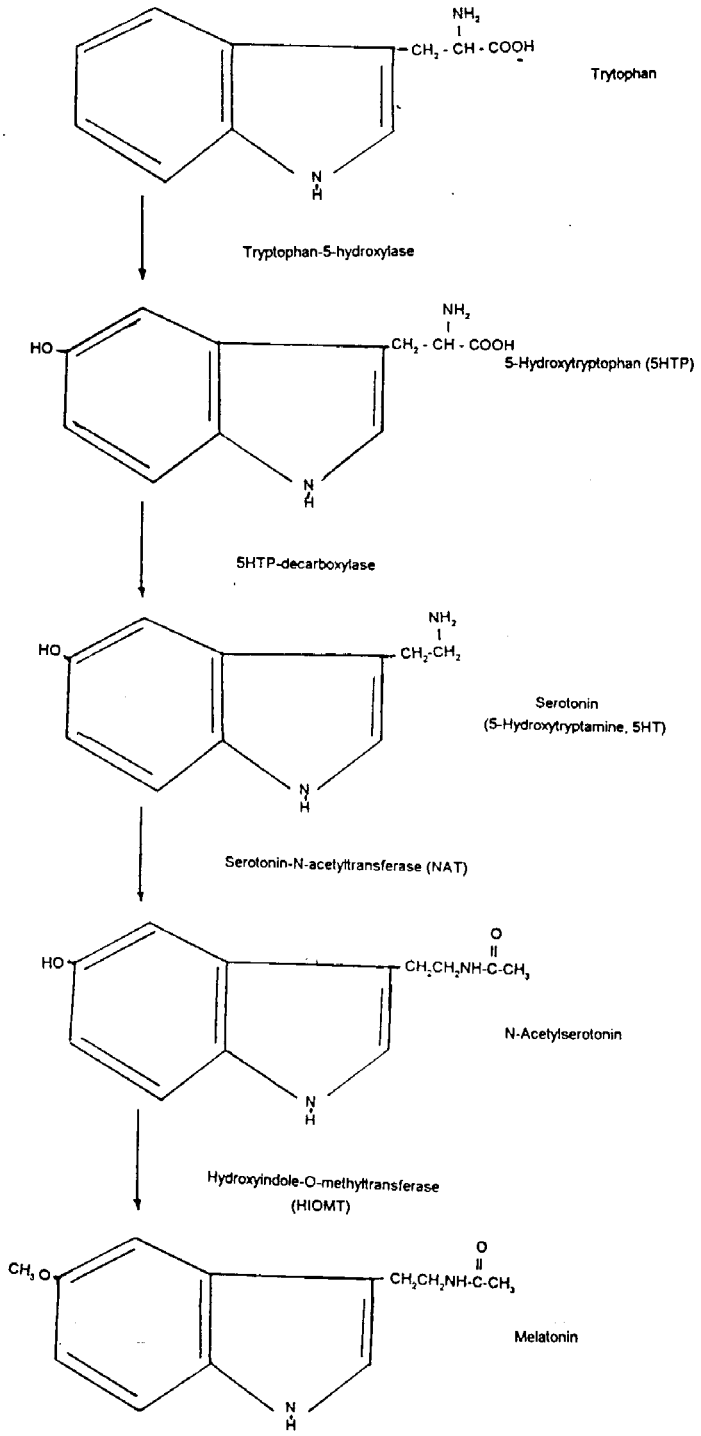


Fig 4 : Melatonin's synthetic pathway

melatonin synthesis. The final step is the transfer of a methyl group to N-acetylserotonin by the enzyme Hydroxyindole - O - methyltransferase (H I O M T).(21) It has been hypothesized that decreased melatonin biosynthesis might have been due to the impairment of calcuim dependent steps (for example binding of calcium to N - acetyltransferase activates the enzyme) in the melatonin pathway.(22)

3.3 METABOLISM / PHARMACOKINETICS

Melatonin undergoes metabolism primarily within the liver and secondarily in the kidney. It follows a microsomal phase 1 / phase 2 reaction - hydroxylation followed by sulfate or glucoronide conjugation. Exogenous melatonin is demethylated to N-acetylserotonin which is then metabolized like melatonin. Exogenous (oral or intravenous) melatonin undergoes a large hepatic first pass effect and has a short metabolic half-life.(19)(23)

Oral melatonin shows a peak level after 60 minutes. Plasma concentrations remain elevated for between 3- 7 hours (oral dose 2-5mg).

Oral melatonin has a low bioavailability (0,03- 0,76).(24)

See Table1, 2 and Fig 6 for pharmacokinetic data of melatonin after bolus intravenous (I.V.) injection, infusion over 5 hours and oral intake. The difference between the values of Iguchi and

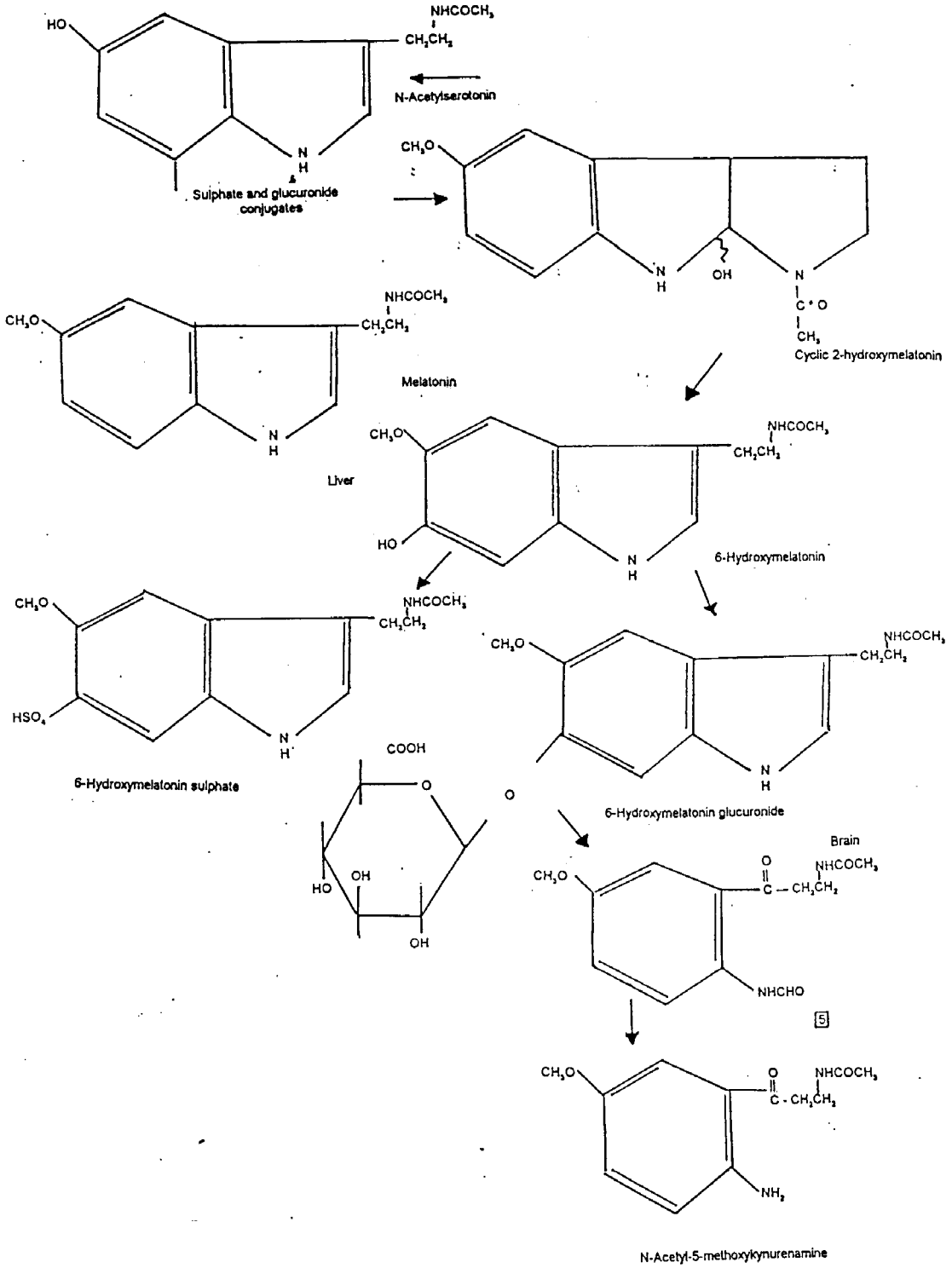


Fig 5 : Melatonin's metabolism.

those reported by Mallo et al (Table 1) are thought to reflect a difference between Asian and European people in their ability to metabolize melatonin.(23)(25)

3.4 CIRCADIAN RHYTHMS AND MELATONIN

Circadian rhythms are biological rhythms that are ± 24 hours long. (e.g. hypothalamus - hypophysis controlled cycles)
Other biological rhythms are:

* infradian (longer than 24 hours) e.g. menses, hibernation, fertility)

* ultradian (shorter than 24 hours)

* diurnal / nocturnal - events during day/night.(26)

Biological rhythms are frequently determined by a combination of external and internal factors. In the absence of any time cues, the endogenous period (tau(t)) of a rhythm is said to be free-running. (for example t in humans is 22-26 hrs)
External factors which synchronize a rhythm are known as zeitgebers.
The suprachiasmatic nucleus serves as the pacemaker for endogenous rhythms.(27)

TABLE 1 Individual and mean pharmacokinetic parameters after intravenous bolus injection of melatonin (25)

Subjects (weight, kg)	CM 66	LM 68	MD 60	RZ 63	Mean Value (SD) 64.2 (3.5)	Iguchi[a] et al.
disappearance rate constant α (min^{-1})	0.480	0.359	0.608	0.305	0.438 (0.116)	0.224
r	0.978	0.997	0.941	0.984	–	–
half-life $t_{1/2}\,\alpha$ (min, s)	1.26	1.56	1.08	2.12	1.35	3.05
disappearance rate constant β (min^{-1})	0.0232	0.0221	0.0228	0.0286	0.024 (0.002)	0.15
r	0.88	0.976	0.95	0.98	–	–
half-life $t_{1/2}\,\beta$ (min, s)	29.51	31.31	30.18	24.15	28.40	46.15
volume of central compartment (Vc, l)	11.2	19.8	11.2	10	13.0 (4.5)	13
apparent volume of distribution at steady-state V_{ss} (l)	35.7	39.7	36.2	29	35.1 (4.4)	35.2
[b] elimination rate constant k_{el} (min^{-1})	0.0854	0.0433	0.0815	0.1181	0.0821 (0.0306)	0.0453
area under curve AUC ($\mu g \cdot min \cdot ml^{-1}$)	5.2×10^{-3}	5.8×10^{-3}	5.2×10^{-3}	9.2×10^{-3}	–	15.8×10^{-3}
systemic clearance CL_s ($ml \cdot min^{-1}$)	958	856	963	1087	966 (94.5)	630

[a] recalculated from the data of Iguchi et al. 1982
[b] elimination constant rate, k_{el}, was calculated from the hybrid rate constants B_1 and B_2 and the rate constant k_{21} for transfer between compartments 2 and 1: $k_{el} = B_1 B_2 / k_{21}$

CM, LM, MD received 5 µg I.V. bolus.
RZ received 10 µg I.V. bolus.
r: goodness of fit parameter

TABLE 2 Individual and mean pharmacokinetic parameters from the falling part of the plasma melatonin profile in healthy controls after the end of the infusion (25)

Subjects (weight, kg)	BPH 87	CM 66	MD 60	MP 66	RJF 84	RZ 63	mean (SD) 71 (11.5)
disappearance rate constant α (min^{-1})	0.256	0.101	0.185	0.558	0.472	0.144	0.286[a] (0.170)
half-life $t_{1/2}\,\alpha$ (min, s)	2.42	6.51	3.44	1.14	1.28	4.48	3.27 (1.57)
disappearance rate constant β (min^{-1})	0.0126	0.0149	0.0238	0.0102	0.0184	0.0166	0.0160[b] (0.0043)
half-life $t_{1/2}\,\beta$ (min, s)	55	46.30	29.07	67.56	37.39	41.44	45.39 (12.48)
elimination rate constant k_{el} (min^{-1})	0.0231	0.0164	0.0299	0.0182	0.0271	0.0272	0.0236[c] (0.0054)
mean plasma melatonin concentration at the plateau ($pg \cdot ml^{-1}$)	100	84	76	58	53	62	72.1 (17.8)
$V_{ss}\,(l) = (1 + \frac{k_{1-2}}{k_{2-1}})\,V_1$	50.9	52.9	35.7	111.0	67.1	60.6	63.0

[a] no significant difference when compared to α given in Table 1
[b] a significant difference ($P < 0.02$) was observed using the t-test when compared to β given in Table 1
[c] a significant difference ($P < 0.001$) was observed using the t-test when compared to k_{el} given in Table 1

Subjects received Melatonin (0,4 µm / ml) as a constant infusion of 10 ml per hour for three hours starting at 11h00

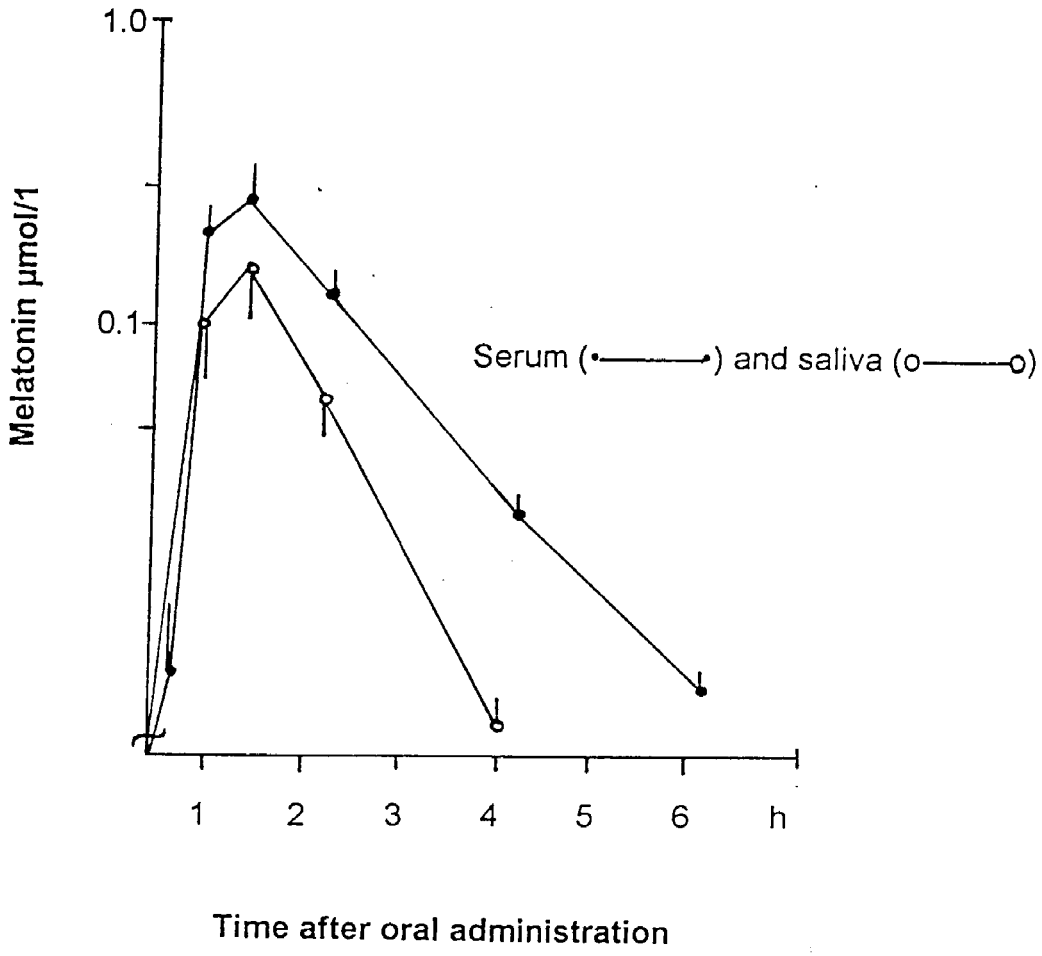


Fig 6 : Serum and saliva melatonin levels in healthy male subjects after oral administration of 100 mg melatonin.

Zeitgebers (time, light/dark, social cues) with a period T, change endogenous periods to run along their own period T. Depending on whether t is less than 24 hours, or t is more than 24 hours, there will be a phase delay/advance in the pacemaker. (28).

Melatonin is secreted during the night. Peak concentration occurs between 02-04 hrs. In absence of light, human melatonin rhythm is free-running with t greater than 24 hours. Optimal sampling times for assessment of melatonin peaks on consecutive days appears to between 23hrs-01hrs.(29)
The stability of normal melatonin rhythm in individual humans (resistance to nutritional / metabolic change) causes small changes in the phase of melatonin secretion to be of clinical significance. During winter there is a melatonin phase delay of approximately 1-2 hrs.(30)

3.5 ASSOCIATION WITH PSYCHIATRIC DISORDERS

3.5.1 Anxiety

Anxiety is a normal emotion as a response to a threat or stress.

If an individual's performance (behaviour) however deteriorates as a result of anxiety, this can be regarded as a pathological condition. (See fig 7(a): Yerkes Dodson Law).(31)

Functional psychiatric condiction	Medically induced anxiety	Substance induced anxiety
1) Anxiety disorders • Panic disorder • Social Phobia • Obsessive compulsive disorder • Acute and Post traumatic stress disorder • Generalized anxiety disorder	1) Cardiovascular • Anaemia • Angina • Heart failure • Mitral valve prolapse	1) Intoxication • Cocaine • Theophyllin • Nicotine • Caffeine
2) Depression	2) Pulmonary • Asthma • Hyperventilation	2) Withdrawal • Alcohol • Opiates • Benzodiazepines etc.
3) Psychotic disorders	3) Neurological • Stroke • Epilepsy • Migraine etc.	
4) Personality disorders etc.		

Table 3 Causes of anxiety - examples

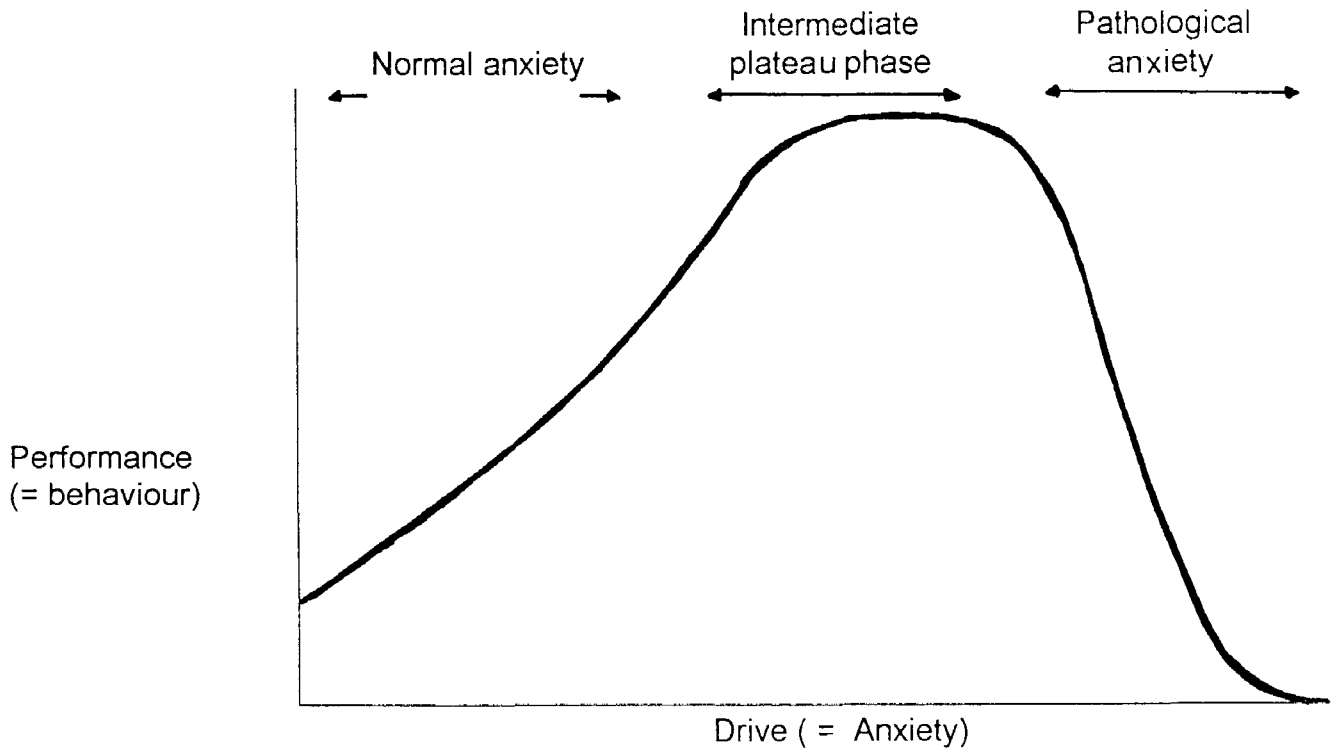


Fig 7(a) : Yerkes - Dodson Law

Anxiety can be regarded as a symptom (what patient complains of), a clinical sign (as observed by clinician, e.g. facial expression, a tremor) or a syndrome (i.e. one of the anxiety disorders).

Before the diagnosis of a functional anxiety disorder can be made the patient needs to be carefully evaluated for medical causes (mitral valve prolapse, epilepsy, Cushings etc.), substance induced anxiety (cocaine, marijuana, caffeine, theophyllin) or other psychiatric disorders in which anxiety can be prominent (depression, personality disorder, schizophrenia etc). See table 3.

More than one neurotransmitter is implicated in anxiety - gamma - aminobutyric acid (GABA), noradrenaline and serotonin may all be involved as suggested by the therapeutic response to tricyclic antidepressants, bensodiazepines, selective serotonin re-uptake inhibitors and the azapirones. A detailed discussion of these mechanisums is not attempted for the purpose of this study.

It is well known that the benzodiazepines act as a GABA - neuromodulators (increases GABA binding) - GABA being an inhibitory neurotransmitter.
Other ligands of the benzodiazepine receptor - inverse agonists - are anxiogenic.

The benzodiazepine antagonist flumazenil provokes panic when given intravenously to patients suffering from a panic disorder.(32)
From clinical experience we know that the tricyclic anti-depressant imipramine (a noradrenaline re-uptake inhibitor amongst many other things) is effective in panic disorder.

Drugs affecting the serotonergic system (for example the selective serotonin re-uptake inhibitors and the azapirones) are currently used for long term treatment of anxiety disorders and evidence is mounting that the azapirones also have antidepressant properties.(33)(34)(35)

The use of melatonin in rats has been shown to promote sleep, cause sedation and analgesia and has anticonvulsant activity. Indications are that the anxiolytic properties of melatonin in rats may be modulated through the GABA-ergic system. Flumazenil also blunts melatonin's effect.(3)(36)(37)

Melatonin treatment increases GABA - turnover in the hypothalamus and the pineal gland.(38) Melatonin was also shown to enhance GABA - binding in rat brain. There are also indications that the central (central nervous system) effect of melatonin may involve the adrenergic and serotonergic systems.(39)

Amaroli (early morning auto-urine drinking) is believed to promote meditation by restoring plasma melatonin levels to that of night-time levels.(28)

Melatonin was shown to play a role in the regulation of the serotonergic system. The effectiveness of 5-HT reuptake inhibitors in the treatment of obsessive compulsive disorder (OCD) may be related to this relationship. Fluvoxamine administration significantly increases melatonin levels after 24 hours, while tranylcypramine, pirlindole (a mono amine oxidaze type A inhibitor) and mianserin had no such effect.(40)(41)

Naloxone, which exacerbates OCD, also antagonises the analgesic and sedative effects of melatonin.(40)

There are indications from clinical work that patients with panic disorder may have a phase delay of their melatonin circadian rhythms. The fact that alprazolam and light therapy suppress nocturnal melatonin secretion suggests that seasonal affective disorder (SAD) and panic disorder, which are both seasonal, may be related by both causing phase instability of the circadian rhythm.(42)

It has been shown that the effect of stress on gastric ulceration in rats could be countered by the administration of exogenous melatonin.(43)

3.5.2 Depression

Seasonal affective disorder which occurs in autumn or winter is characterized by depression, psychomotor slowing, hypersomnia and hyperphagia. Patients respond to bright light exposure (2500 Lux) of 2 hours per day - either in the morning (gives melatonin phase response advance) or in the evening (gives melatonin phase response delay).(44)

It was noted in clinical research that 2 hours bright light exposure was more effective than 30 minutes exposure.(45)

Investigation also showed that patients with major depression and an abnormal dexamethasone suppression test (DST) had a significantly higher cortisol/melatonin (c/m) ratio than did patients with a normal DST. Low melatonin in these patients may indicate pineal gland dysfunction.(46)

Manic-depressive patients' exposed to light of 500 Lux (between 02h00 - 04h00) had a 50 per cent suppression of melatonin levels, but similar exposure had no effect on control patients' nocturnal melatonin secretion.(47)

Because acute tryptophan depletion can decrease melatonin secretion significantly, it was suggested that abnormal serotonergic function (tryptophan is the precursor of serotonin)

in some depressed patients, might be reflected as decreased melatonin levels in these patients, as serotonin is an intermediary in the production of melatonin.(48)

There are, however, no consistent findings to confirm the low melatonin - hypothesis in depression(49)(50) and there is evidence that nocturnal melatonin secretion is not reduced in depression.(51)

Currently positron emission tomography (PET) is utilised to investigate metabolic patterns in the brain caused by light therapy.(52)

Studies using 24-hour urinary output of 6-sulphatoxy - melatonin as an index of pineal activity (instead of periodic plasma/serum melatonin estimations) indicated a significantly higher output of 6-sulphatoxymelatonin when patients were on treatment with desipramine (a noradrenegic antidepressant). The fact that melatonin secretion from the pineal gland is under noradrenegic control suggests that 6-sulphatoxymelatonin reflects increased ß-adrenergic activity in the pineal gland and it could thus be useful to evaluate down regulation of ß-receptors in patients receiving antidepressant treatment.(53)

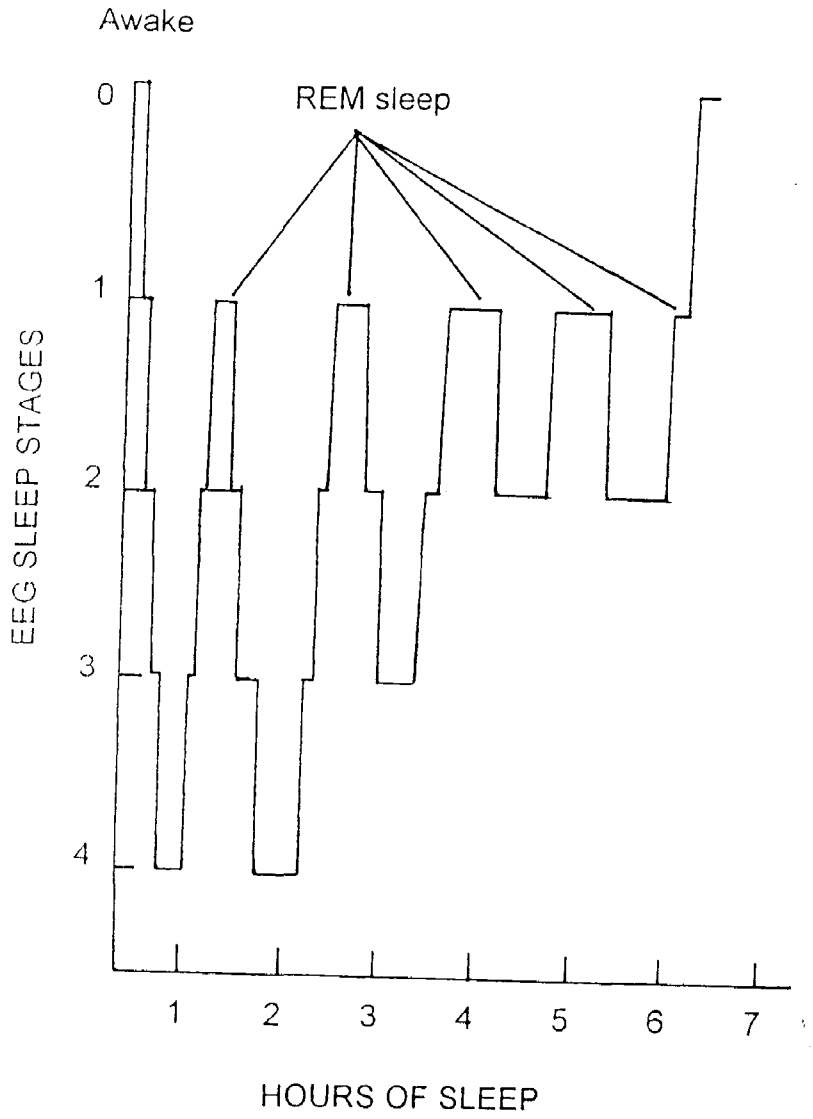


Fig 7(b) : Stages of sleep

3.5.3 Sleep

Sleep can be regarded as an active process which can be characterized by means of electroencophalogram, electro-oculogram and electromyogram recordings. (54)
This complex physiological state has been shown to consist of alternating patterns of rapid eye movement (REM) sleep and non rapid eye movement sleep.(NREM)
See fig.7(b)

The sedative effect of melatonin reported in earlier studies, which were thought to be a side-effect, is now regarded as part of the physiological role of melatonin. This is thought to occur through the regulation of the circadian rhythm rather than the hypnotic effect of melatonin as such.(55)

Light is a zeitgeber affecting circadian rhythm - in totally blind people up to 50 per cent have free running circadian rhythms greater than 24 hours.(55) This is associated with sleep disturbances.
Clinical studies suggested that exogenous melatonin may modulate the circadian rhythm in blind and non-blind people. (56)(57)

On the other hand normal melatonin secretion in a patient with Kleine-Levin Syndrome (Periodic somnolence and morbid

hunger) suggests that melatonin secretion is not directly linked to sleep.(58)

The influence of melatonin on the suprachiasmatic nucleus, which has been found to be a major sleep regulator, has recently come to light.(59)

The abundance of REM sleep during the first three months of infancy is associated with low melatonin levels - this has given rise to the theory that increased melatonin levels decrease REM sleep.(60)
It was shown that exposure to bright light increases REM-sleep latency and the duration of NREM sleep. Delayed REM sleep after bright light exposure is speculated to represent a circadian phase shift.(61)

Melatonin's sedative hypnotic effect (62) was confirmed in rodents and it was speculated that interaction with brain benzodiazepine receptors was a possibility (63) - it was for instance shown that flumazenil (a benzodiazepine antagonist) blocks the effect of melatonin on GABA binding in the rat cerebellum.(37)

Chronic insomniacs may benefit from oral intake of melatonin. A two to three day delay in response was shown.(64) The time of melatonin administration may also be important when

treating sleep disturbances - there may be a "dead zone" of the phase response curve.(65)

3.6 ASSOCIATION WITH OTHER DISORDERS

3.6.1 Cancer

Reports that cancer patients' pineal glands are larger than non-cancer patients, have led to the idea that the pineal gland may play a role in inhibiting tumor growth - possibly by inhibiting endocrine systems that stimulate tumor growth.(66)

3.6.2 Puberty

Plasma melatonin declines during development. Administration of melatonin to male and female rats delays puberty. It was shown that after induction of secondary sexual charactaristics in a patient with infantilism due to a craniopharyngioma that there was a decline in U-6-sulphatoxymelatonin (index of melatonin production). Hypothalamic amenorrhoea is also accompanied by high melatonin levels.(67)

There is a report of a patient with delayed puberty and hypogonadotrophic hypogonadism where his high plasma melatonin level (and a enlarged calcified pineal of 18mm) was suspected to be the cause of his hypogonadism. Why the

patient's melatonin secretion normalised over a seven-year period is unknown but this was accompanied by complete maturation of his pituitary-gonadal function.(68)

Growth hormone (GH) secretion is stimulated by growth hormone releasing factor (GRF) and inhibited by somatostatin. It has been established that the pineal gland plays a role in regulation of growth and GH secretion in rodents. Oral administration of melatonin causes a small increase in basal GH-levels in normal human subjects and GH respons to GRF. However, melatonin reduces GH response to insulin - induced hypoglycaemia. It was proposed that the latter stimulates GH release from the hypothalamus.(69)
It is important to remember that although nocturnal melatonin levels progressively drop by 80 per cent from childhood until adult levels are reached, this appears to be due to an increased volume of distribution (Vd) of melatonin during development. Melatonin production is constant during childhood.(49)
Nighttime melatonin plasma levels are approximately:

* 250pg/ml at age 1-3 years
* 120pg/ml at age 8-15 years
* 20pg/ml at age 50-70 years

Daytime melatonin plasma levels are $\pm$ 4-10pg/ml regardless of age.

3.6.3 Ageing

Speculation is that melatonin may exhibit anti-ageing properties.(66) It has even been suggested that ageing is a pineal failure and that a decrease in melatonin/serotonin ratio leads to arteriosclerosis and increased cell membrane thickness. This can lead to diminished diffussion of nutrients into and waste products out of cells.(70)

3.6.4 Circadian Rhythm disorder

Melatonin may play a role in development (example onset of puberty) and ageing (lifespan). It also is important in circadian rhythm (locomotor activity, body temperature) and in seasonal cycles (example reproduction).(71)(72)(73)

Evidence points to the SCN (of the hypothalamus) as the site that generates circadian rhythms in mammals.(27)

Nightwork causes a disruption in circadian rhythm that not only leads to sleep disturbances but also to an increased risk of other disorders. (Gastrointestinal, cardiovascular).(74)

Exposure to bright light (7000 - 12000 Lux) at night and complete darkness during the day contribute to circadian adaptation to daytime sleep / nighttime work.

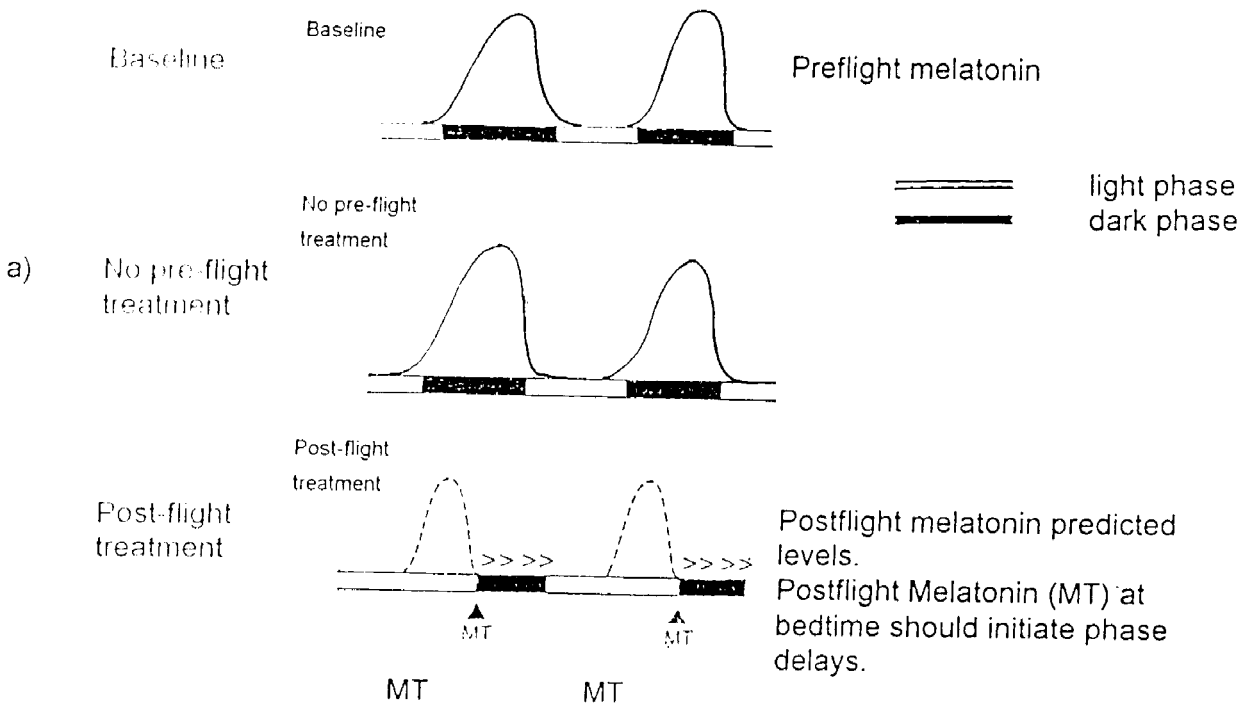
It has been proposed that changes in circadian rhythms are indicative of ageing and accompanying cognitive decline.(75) Bright light was shown to be able to reset the human circadian pacemaker - which can be important in the treatment of delayed sleep phase insommia, jet lag and shift work insommia. (76)(77)(78)

It has been shown that a 60 per cent reduction in eastward jet lag and 40 per cent reduction in westward jet lag is possible by taking 5mg melatonin, according to instructions (to induce a phase advance when travelling eastward and a phase delay when travelling westward).(79) See fig 8. It seems that eastward flights are associated with bigger acclimatisation problems.(80)

3.6.5 Pineal Tumor

Patients with pineal tumors are rare. It appears that estimation of melatonin plasma levels is a reliable way to assess the success of pinealectomy.(81) It is reported that low day time levels of melatonin are unaffected after pinealectomy. The dynamics of the decline of melatonin level after pinealectomy have not been investigated.(81)

Westwards



Eastwards

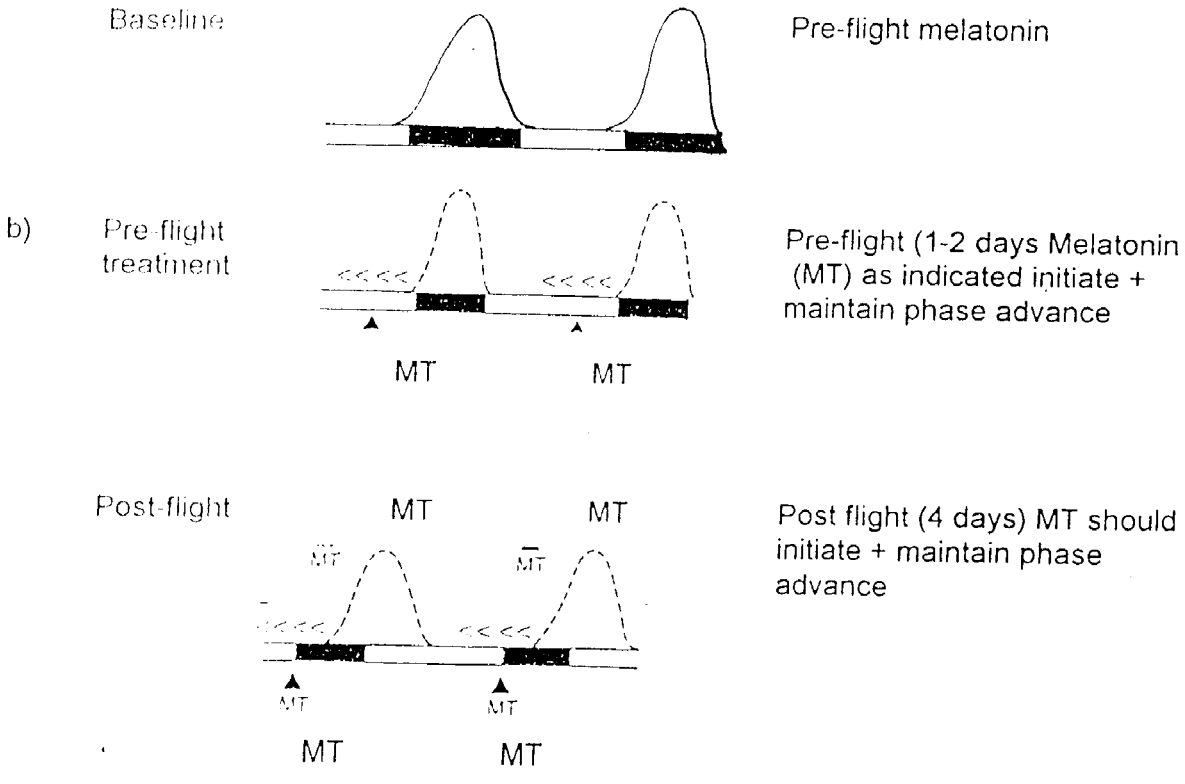


Fig 8 : Example of how melatonin can assist in treatment of jet lag.

3.6.6 Immune system

Melatonin seems to stimulate the immune response in some conditions - the opiatergic system possibly plays a role. No melatonin specific receptors have so far been found on cells of the immune system.(49) It was noted that the thymus might be one of the main targets of melatonin (81) and that melatonin is involved in the production of Interleukin-2(70).

3.6.7 Other

Patients with primary hypothyroidism were shown to secrete more melatonin during the night when compared to normal patients. Thyrotoxic and obese patients secreted normal amounts of melatonin but thyrotoxic patients showed a phase advance.(82)

3.7 MELATONIN AND SEROTONIN

3.7.1 Introduction

Serotonin is transformed to melatonin in the pineal gland. Due to this "relationship" there seems to be a very special protection mechanism to maintain a stable serotonin status in the pineal gland. Although the concentration of serotonin in the pineal gland is hundred-fold that of the brain concentration, the brain

serotonin levels in experimental animals drop before any decrease in pineal serotonin levels occurs.(83)
The interaction of melatonin with brain serotonin (and possibly other brain monoamines as well) which leads to catecholamine synthesis may be mediated through melatonin's effect on brain pyridoxal kinase activity - the latter being essential for the synthesis of brain gamma aminobutyric acid, serotonin, dopamine and norepinephrine. Both melatonin and pyridoxal kinase activity peak at midnight.

There is evidence that melatonin acts as an inhibitor on the pituitary-adrenal axis by activating the central adrenergic system that is responsible for controlling the secretion of adrenocorticotrophic hormone (ACTH).

A rise in brain catecholamines after the administration of melatonin may lead to a reduction in the stress response in animals.

It is suggested that central catecholamines and 5-HT are capable of working together at least as far as melatonin - induced behavioural modulation - (which is inhibitory in nature) is concerned.

The interactions between melatonin and other neuropeptides on behaviour cannot be ignored.(84)

3.7.2 Serotonin

3.7.2.1 General

Serotonin was first isolated from blood serum and called enteramine. It was recognised to be a vasoconstrictor and that blood platelets could concentrate serotonin.

In the central nervous system (CNS) the brainstem contains most of the sumtotal of serotonin (although the pineal serotonin concentration is the highest).

Serotonin crosses the blood-brain carrier with difficulty. It was once postulated that melatonin may be a lipid soluble derivative of serotonin, thereby enabling it to gain easy entry into nerve cells and then to be converted back to serotonin which is then the active substance.(85)

Serotonin can function as a neurotransmitter, and serotonergic transmission is important in anxiety and sleep. Benzodiazepines can depress serotonergic metabolism in the brain of the mouse - it was proposed that the availability of tryptophan as a precursor was changed by the benzodiazepines.(86)

When argentaffin cells become cancerous (carcinoid), over-production of serotonin results. These patients present with vasodilatation (flushing of skin) and severe diarrhoea. Some patients have high blood pressure.(85)

3.7.2.2 Serotonin receptors

Different 5-HT-receptors seem to be linked to different second messenger systems and as well as to ligand-gated ion channels.

Up to date 7 classes of serotonin receptors have been described (5-HT_1 - 5-HT_7). Most of these classes consist of more than one member.

With the exception of the 5-HT_3 - receptors, which are ligand-gated ion channel receptors, the 5-HT receptors interact with G-proteins. Based on their interaction with second messengers and their amino acid sequence homology the latter were seperated into distinct families e.g.:

(i) 5 HT_1 family - Negatively coupled to adenylate cyclase
(ii) 5 HT_2 family - activation of phospholipase C.
(iii) A heterogenous group (5-HT_4, 5HT_6, 5HT_7) - positively coupled to adenylate cyclase.(87)(88)

The Serotonin Receptor Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) however reclassified the 5-HT receptors in 1994 and recognised the following:

* 5-HT_1 (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1B}, 5-ht_{1E} and 5-HT_1-like)

* 5-HT_2 (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C})

* 5-HT_3

* 5-HT_4

* Recombinant (5-ht_{5A}, 5-HT_{5B}, 5-ht_6 and 5-ht_7)

* 'Orphan' receptors

It is now suggested that the terminology '5-HT_1 - like receptors' is outdated, because it does not take into account the sumatriptan-insensitive (5-HT_{1Y}) receptor which is positively coupled to adenylate cyclase (prodilator receptor).

Investigations of the 5-HT_1-like receptors thus yielded 3 distinct receptors namely sumatriptan-sensitive (5-HT_{1B}, 5-HT_{1D}) and sumatriptan-insensitive (5-HT_7).(89)

3.7.2.3 Clinical implications: Serotonin Receptors and their relation to behaviour

Speculation on the role of serotonin in psychiatric disorders, especially depression, is not new.(90)

It was shown that the serotonergic agonist (91) metachloro-phenylpiperazine (mCPP) exacerbates obsessive-compulsive symptoms in patients with obsessive-compulsive disorder. Furthermore the SSRI's are useful in the treatment of OCD. This points to a serotonergic role in the pathology of OCD.(92)

There is evidence that serotonergic neurotransmission is decreased in the presence of benzodiazepines. The 5-HT_{1A} receptor is also implicated as presynaptic 5-HT_{1A}- partial agonists e.g. buspirone, are effective anxiolytics(93) - especially for the treatment of generalized anxiety disorder.

Activation of post-synaptic 5-HT_{1A} receptor in rats produces the 5-HT-behavioral syndrome (fore-paw treading, hind limb abduction, low body posture, tremor, head weaving and straub tail).
Antidepressants were also shown to cause a diminished 5-HT_2 receptor density in the frontal cortex of rats. This was

accompanied by a reduction in the magnitude of behavioural responses elicited by 5-HT_2 - agonists.(94)

Studies of abnormal motor behaviours induced by chemical manipulation in rats implicate 5-HT receptors in a myoclonic-serotonergic syndrome.(95) Serotonin - like behaviour induced by high doses of clonidine however does not seem to be related to either serotonin or catecholamines.(96)

Indications are that serotonergic neurons may regulate the activity of the dopamine system in an inhibitory manner.

Serotonin has been implicated in a wide variety of important physiological processes : sleep, appetite, sexual behaviour and control of movement to name a few.(97)

The $5HT_2$-receptors are implicated in psychiatric disorders such as schizophrenia, anxiety and depression.
Head twitches, as seen in 5HT-behaviour syndrome, were responsible for the original definition of the $5HT_2$-receptor. Ritansenin, a 5-HT_2 antagonist has been reported to exhibit anxiolytic effects in humans.(98)

The $5HT_{2C}$ receptors are associated with a variety of pathological states, e.g. anxiety, migraine, anorexia nervosa. The atypical antipsychotic, clozapine, was shown

to have a high affinity for 5-HT_{2c} (greater than that for $5HT_2$ or Dopamine (D_1/D_2) receptors.)(98)
Ondansetron a 5-HT_3 antagonist has also been identified as a potential anxiolytic.(71)

The 5-HT_2 receptor has been proposed to be a major site of action for atypical antipsychotics. Newer atypical antipsychotics were also shown to have an affinity for 5-HT_6 receptors.(99)

Serotonin is also implicated in the control of circadian rhythms. It is speculated that serotonergic innervation of the suprachiasmatic nucleus might be involved in the control of circadian rhythms. There is however uncertainty whether rhythmic activity of the SCN, or the rhythm of 5-HT neuronal activity comes first.(100)

Studies indicate that L-5HTP (L-5-hydroxytryptophan) exerts its effect on 5-HT levels via a receptor which is distinct from those reported previously.(101)

$5HT_{1A}$ receptors have been shown to be located on cell bodies of serotonin containing neurons and that activation of these receptors reduces not only firing rates of 5-HT neurons but also inhibits the release of 5-HT.(102)

$5HT_{1B}$ and $5HT_{1D}$ are both involved in feedback control of 5-HT-release.(97)

3.7.2.4 Miscellaneous

Studies investigating the correlation between 5-hydroxyindoleacetic acid(5-HIAA) and homovanillic acid (HVA) in animals indicate that serotonin has a facilatory effect on indices of dopamine turnover in especially the brain stem and the hypothalamus. The effect of morphine on dopamine neurons in the nucleus accumbens was also inhibited by stimulation of serotonin receptors by fenfluramine (a serotonin releaser) and m-chlorophenylpiperazine (an agonist).(103)

Fenfluramine releases endogenous serotonin - its effect is blocked by p-chlorophenylalanine (pCPA).(104) It was also shown that fenfluramine could stimulate the pineal N-acetylserotonin and melatonin synthesis.(105)

3.7.3 Melatonin

3.7.3.1 Melatonin receptors

Melatonin has been discribed as an omnipresent hormone - widely distributed and affecting many organs. It has been

found that melatonin is not only found in cytosol but also located in the nuclei of cells. There is also speculation that melatonin acts as a free radical scavenger that protects DNA from damage. The fact that a pinealectomy does not abolish melatonin totally, suggests that peripheral tissue (retina, gut) may produce basal melatonin, whereas the pineal gland produces circadian rhythmic melatonin.(106)

Like all hormones, melatonin acts through specific receptors. Autoradiographic analysis with ^{125}I-melatonin showed that the density of melatonin receptors varies with species and location. Melatonin receptors on membranes interact with intracellular second messengers to affect cell functions. Melatonin inhibits cAMP in various tissues. It also inhibits cAMP, cGMP, diacylglycerol accummulation in the anterior pituitary and arachidonic acid re ease from the pineal gland.(107)

Melatonin binding sites are high affinity sites with low picomolar range Kd-values. Indications are that they are G-protein coupled receptors. Inhibition of calcium influx and membrane potentials (in the Pars distalis) also seem to be of importance in mediating melatonin's effect.(108)

The Pars tuberalis of sheep was shown to be an acceptable model for studying melatonin receptors. Tryptophan, and

other indoles also exhibits weak affinity for the melatonin receptor. Treatment with GTPyS (guanine nucleotide) did not change the Kd for the melatonin receptor and studies with saturation curves showed that an apparent decrease in receptor numbers occurs as a result of a shift of the total receptor population from a high to a low affinity form. From this it has been concluded that the melatonin receptor is a member of membrane bound receptors linked through a G-protein.(109)

Melatonin receptors may exist in two states both with pM-affinities.

* High affinity: 10 - 100pM
* Low affinity: 300 - 800pM

Binding to both the above is reversible and saturable.

In hamsters whole brain membranes, a melatonin binding site with nM affinity and different pharmacological and binding traits than those of the pM-affinity site, were identified.

ML-23 [N - (3,5 dinitrophenyl) - 5 methoxytryptamine] is a potent antagonist of melatonin mediated inhibition of the release of dopamine from the rat hypothalamus.(110)(111)

Melatonin and melatonin agonists inhibit dopamine release from the retina through activation of a different receptor than the serotonin receptor. These effects are antagonized by the melatonin receptor antagonist Luzindole (N-0779).(112)(113)

^{125}I-melatonin binding sites are abundant. Only those in the hypothalamus, medulla, pons and hipocampus exhibit diurnal variation. Timed coincidence of hormone and receptor may determine melatonin response.(110)

In defining a specific hormone receptor it is important to be able to measure a specific physiological response triggered by receptor activation.(114)

See tables 5(124) and 6(114) Fig. 10(114)

Such responses are melatonin's ability to:

* Condense pigment granules in skin melanophores
* Inhibit dopamine release in the retina
* Affect production of cAMP

These responses were used to develop in vitro bioassays for melatonin.(114)

TABLE 4 Pharmacology and distribution of melatonin binding sites (124)

	ML-Receptor (picomolar affinity)		ML-2 Site (nanomolar affinity)
2-[^{125}I] - IMEL binding			
Affinity	High	Low	
K_d	10-100 pM	300-800 pM	1 -5 nM
Sodium	Inhibited		No effect
Magnesium	Increased or no effect		Slight inhibition
Calcium	Increased or no effect		No effect
Guanine Nucleotides	Inhibited		No effect
Pharmacology	2IMEL - MEL - 6CIMEL> >60CH_3MEL >NAS ≥LUZ>>>5HT		2IMEL - 6CIMEL - 60CH_3MEL ≥MEL - NAS >> LUZ > 5HT
Localization	IPL rabbit and chicken retina SCN; PVNT; PT of rodents chicken brain areas		Hamster brain
Cellular Function	Inhibition DA release in retina Regulation of circadian rhthyms		Not known

Abbreviations:

2 IMEL	:	2-iodomelatonin
MEL	:	Melatonin
6 CLMEL	:	6 Cloromelatonin
60 CH_3 MEL	:	6 Methoxymelatonin
NAS	:	N-Acetylserotonin
5HT	:	Serotonin
IPL	:	Inner plexiform layer
SCN	:	Suprachiasmatic nuclei
PVNT	:	Paraventricular nucleus
PT	:	Pars tuberalis
DA	:	Dopamine
LUZ	:	Luzindole

Table 5 Pharmacology of functional melatonin receptors (114)

	Inhibition of DA release		Pigment condensation	
	Rabbit retina	Chicken retina	Frog skin	Isolated frog melatonin
AGONISTS	IC_{50}(nM)	IC_{50}(nM)	MEC[1](nM)	IC_{50}(nM)
2-Chloromelatonin	--	--	--	0.0003[a]
2-Iodomelatonin	0.005	0.01	--	0.0006[a]
2-Methyl-6,7-dichloro-melatonin	0.01	--	--	--
6-Choromelatonin	0.04	0.5	--	--
Melatonin	0.04	1.0	0.05	0.003 0.000009
5-Methoxyluzindole	1.3[2]	--	--	--
6-Mehtoxymelatonin	1.6	100	--	--
6-Hydroxymelatonin	2.0	30	0.1	43
N-acetyltryptamine	5.0[2]	>1000	N.E.[3]	--
N-acetyl-5-methoxy-kynurenamine	10.0	--	--	860
N-acetylserotonin	54.0	300	50,000[2]	860
5-Methoxytryptamine	63.0	200	700	86
5-Methoxytryptophol	4000	>10,000	--	430
Serotonin	>1000	>10,000	N.E.	860
Luzindole	>10,000[3]	--		--
ANTAGONISTS	K_B (nM)	K_B (nM)		
5-Methoxyluzindole	0.063	--	--	--
N-acetyltryptamine	9.0	33	+[5]	--
Luzindole	20.0	--	--	--
N-acetylserotonin	N.E[4]	--	+	N.E.

(1) Minimal effective concentrations

(2) Partial agonist

(3) Antagonist

(4) Not effective

(5) Antagonized the effect of melatonin but K_B not determined

3.7.3.2 Melatonin: Miscellaneous

5-Hydroxytryptophan administered to sheep during daytime caused elevated serum melatonin levels (sevenfold) within 2 hours. This may form the basis for a nonneural pineal function test in humans.

As 5-Hydroxytryptophan is used for depression and myoclonus, elevation of melatonin levels induced by 5-HT may also be useful in predicting which patients will respond to 5-Hydroxytryptophan; it could indicate the ability of the pineal gland to convert 5-Hydroxytrytophan to melatonin.(115)

It was shown that the amplitude of the melatonin rhythm in humans was diminished while exposed to light and deprived of food during the night. This environmental influence needs further investigation in humans.(116) High doses of propranolol (120-1280mg /day) decreased the output of urinary melatonin which may reflect a decrease in melatonin production.(116) The synthesis of melatonin is stimulated by drugs (eg. dopamine; tyramine) that are structually related to noradrenaline.(117) (118)

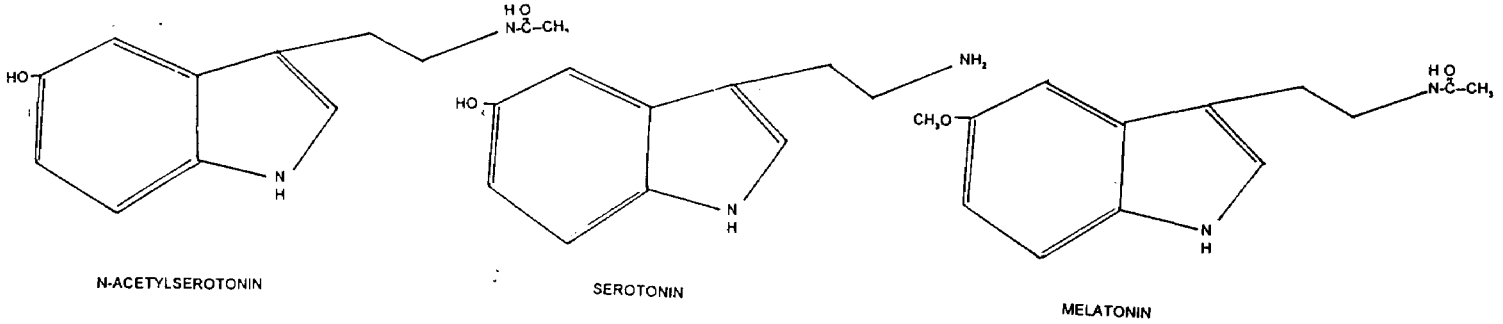
Electroconvulsive therapy (ECT) did not seem to induce melatonin production. (116)

It has also been shown that intraperitoneal injection of melatonin to rats is followed by a rise of brain serotonin. The mechanism of this response is not clear.(119)

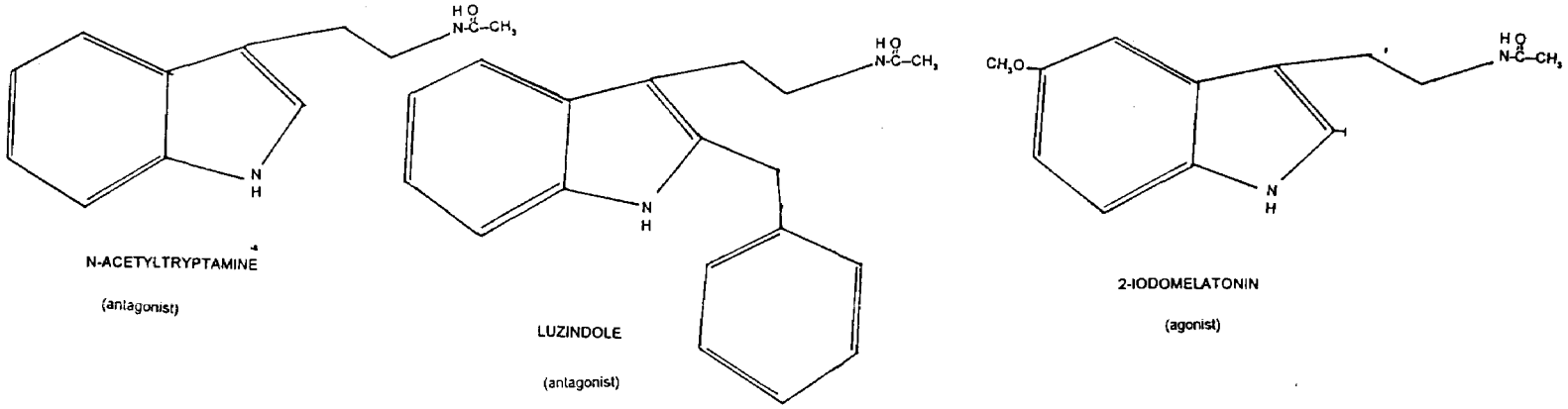
Ethanol, as ingested socially, inhibits natural melatonin secretion and increases plasma noradrenaline levels - thus interfering with endogenous rhythms.(123)
Melatonin's possible influence on striatal dopaminergic function was also demonstrated insofar that melatonin inhibits turning behaviour induced by apomorphine in rats. (66)(120)
Mounting evidence supports the theory that melatonin might be involved in all the major neuroendocrine functions of the pineal gland for example, similarities between the antigonadal and antithyroid effects of melatonin are also emerging.(121)

3.7.3.3 Exogenous melatonin in humans

It is speculated that the increase in melatonin secretion due to neuroleptic medications may protect the patient against drug-induced parkinsonism by indirectly augmenting dopamine activity. Indirect evidence suggests that



A. Chemical structures of melatonin and its Precursors



B. The melatonin receptor agonist and antagonists

Fig 9 : Structural relationships of melatonin.

melatonin acts as a 5-HT_2-receptor agonist in physiological concentrations - at higher concentrations melatonin appears to act as a 5-HT_2-receptor antagonist.
Circadian dopamine activity and the circadian response of schizophrenic patients to neuroleptics may be explained by the modulation of dopamine activity via the $5HT_2$-receptor by melatonin.(122)

3.7.4 Discussion

The literature search supports melatonin's possible involvement in the pathophysiology of anxiety. There seems to be a complex interaction between melatonin and various neurotransmitters.

Evidence points towards a special relationship between serotonin and melatonin. Not only is serotonin a precursor in melatonin's production, but the constant pineal content of serotonin is secured and protected.

Serotonin may also play a role in the regulation of the circadian rhythm via control of the SCN. See fig 10.

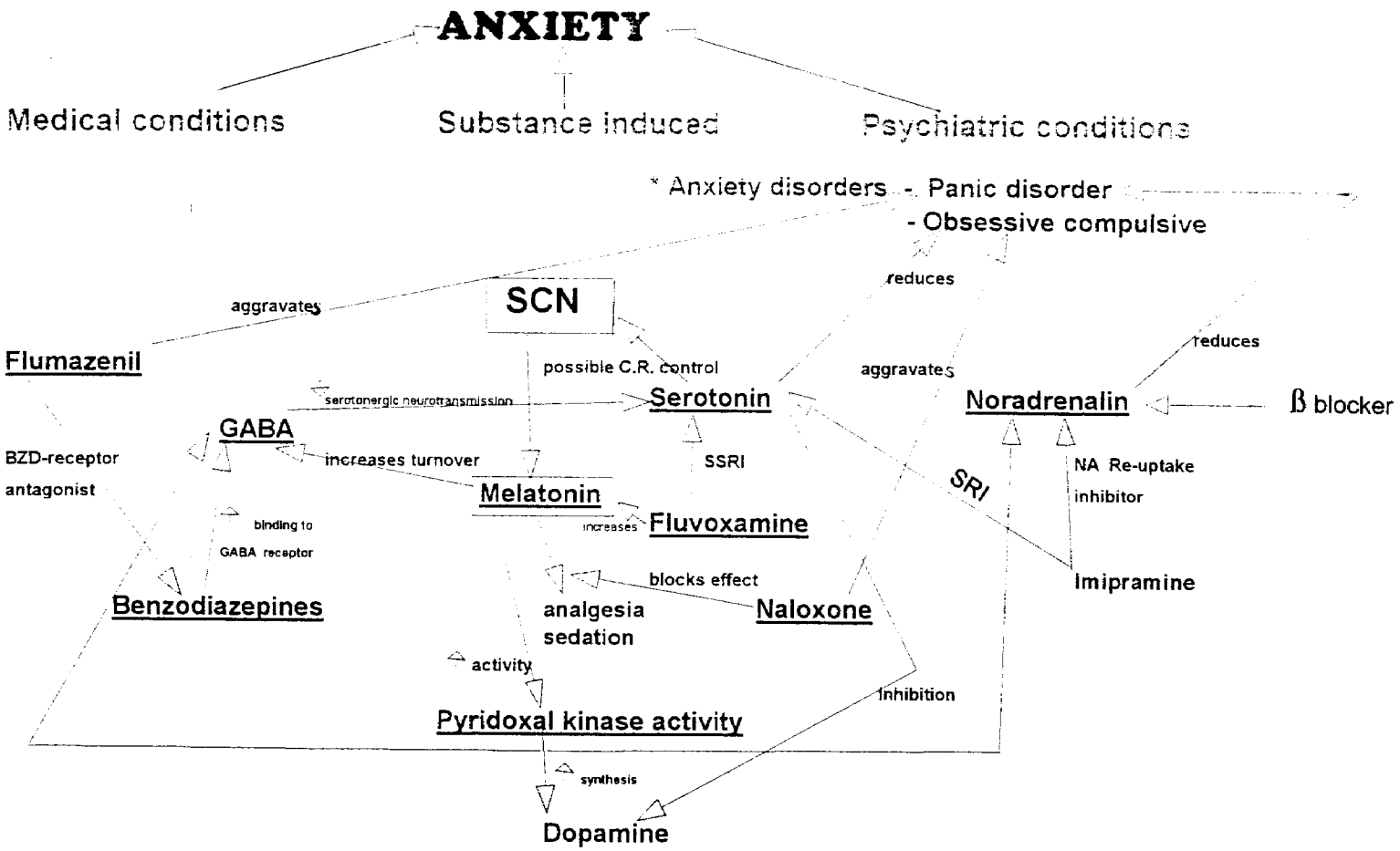


Fig10 : MELATONIN'S INTERACTIONS

CHAPTER 4: EVALUATION OF MELATONIN AS AN ANXIOLYTIC

4.1 Introduction

Many definitions of anxiety exist. Anxiety is differentiated from fear - anxiety referring to an anticipated subjective danger, fear to an immediate objective threat.(126)
A variety of rating scales for anxiety exist.(127)(128)(129) Although these scales are not for diagnostic purposes it is important to note whether pathological anxiety in patients not necessarily suffering from anxiety disorders, changes over time. In clinical practice the separation of anxiety from other emotions may be difficult. The Hamilton A anxiety rating scale was chosen for this trial because of the trialist's clinical experience with this particular rating scale.

It is clear from the literature that anxiety has a complex pathophysiology. Multiple neurotransmitters and receptors are involved. Anxiety may bear a relationship to central catecholamine hyperactivity. The combination of central and peripheral catecholamine turnover may reflect a coping response to various endogenous and exogenous stimuli.(125)

Postulations that anxiety disorders and depression may be due to dysfunctional $5HT_{1A}$ autoreceptors in different areas of the brain have been made. Others are of the opinion that anxiety and depression may be different expressions of the same underlying disorder.(5) Medications with different modes of action are used to alleviate anxiety.

Different augmentation strategies in the treatment of depression are known. This underscores the complex nature of psychiatric disorders. It would thus be reasonable to investigate the anxiolytic effect of melatonin in humans as melatonin is tolerated well by humans and there is no known toxicity.(130) Anxiolytic properties of melatonin were demonstrated in mice, but research regarding the anxiolytic properties of melatonin in humans is lacking.(3)

This could serve as a pilot study for further investigations regarding the use of melatonin in clinical psychiatry.

4.2 Protocol

Each patient completed an "N of 1" randomized double blind placebo controled trial.(131) A 3mg melatonin tablet (Melapure™, Genzyme Pharmacenticals, USA) or a placebo (Gelatine capsule fillled with starch) were administered at 21h00 during Pair 1 and Pair 2 of the trial as indicated in Table 6.

A sample of 30 adult patients suffering from anxiety was selected to participate in the trial. See Table 7 for patient profiles. The trialist obtained written informed consent from subjects before the trial commenced. (According to guidelines from the Medical Association of South Africa Research Ethics Committee.)

The complete protocol, which was approved by Rhodes University ethical committee, is attached as part of the appendix. The protocol complies with the principles expressed in the Declaration of Helsinki.

A sample of the melatonin tablets (Melapure™) which were used in this study was analysed by means of TLC to ensure that they contain melatonin and that there were no other metabolites or impurities in them. Another "over the counter" product (Melatonin, Schiff Products, Salt Lake City) was analysed by means of UV absorbance readings and HPLC. (See appendix). This was done to evaluate melatonin content of "over the counter" melatonin products. It was revealed after conclusion of the experiment that M_1 was the melatonin and M_2 the placebo.

4.3 Statistical data analysis: Discussion

The data in this analysis have been obtained from 30 patients who completed a baseline Hamilton A anxiety scale, and at the end of each of four treatment sessions (Table 8). Patients 5, 11 and 13 did not complete the experiment as indicated in Table 8. The questionnaire was the same for each session and consisted of 14 questions each on a five point scale each starting with 0 (anxiety absent) up to 4 (very severe anxiety).

Table 6 An "N of 1" Randomized double blind, placebo controlled trial of melatonin as an anxiolytic.

	<---------- Pair 1. ---------->												<---------- Pair 2.---------->												
Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Patient 1	P_1 P_1					WOP.		P_1 P_2					WOP.		P_2 P_1					WOP.		P_2 P_2					= N of 1
Patient 2	<-----M_1/M_2----- >							<-------M_1/M_2------->							<------M_1/M_2------>							<------M_1/M_2----->					= N of 1
| |	| |					| |					| |					| |					| |					| |
Patient 30																											= N of 1

* Melatonin administered in double blind, placebo controlled experiment during Pair 1 & Pair 2. (M_1/M_2)

* P_1P_1 = Pair 1 (Period 1) / P_1P_2 = Pair 1 (Period 2)

* WOP = Wash-Out Period

* P_2P_1 = Pair 2 (Period 1) / P_2P_2 = Pair 2 (Period 2)

TABLE 7 Patients Profiles

Subject	Age (Years)	Sex	Diagnosis	Medication	Duration of Illness (years)	Severity of Social stressors (0-3)
1	48	Female	Major Depression	Citalopram 20mg daily	27	3
2	38	Female	Borderline Personality disorder / Substance abuse	Fluvoxamine 100mg daily	20	2
3	41	Male	Major Depression	Paroxetine 20 mg daily	9	3
4	56	Female	Generalized anxiety disorder / Major Depression	Buspirone 5mg twice daily / Amitriptyline 100mg daily	46	3
5	36	Female	Major Depression	Fluoxetine 20mg daily	5	3
6	55	Male	Generalized anxiety disorder	Buspirone 5mg twice daily	15	1
7	47	Female	Major Depression	Moclobemide 150mg twice daily / Flupenthixol 0,5mg twice daily	20	1
8	39	Female	Major Depression	Fluoxetine 20mg daily	9	3
9	37	Male	Histrionic personality disorder	Perphenazine 4mg twice daily / Amitriptyline 75mg twice daily	20	2
10	44	Male	Schizophrenia	Clozapine 150mg daily	27	2

Subject	Age (Years)	Sex	Diagnosis	Medication	Duration of Illness (years)	Severity of Social stressors (0-3)
11	48	Female	Post - Traumatic Stress disorder	Amitriptyline 50mg daily / Perphenazine 4mg twice daily	9 months	1
12	53	Female	Panic Disorder	Sertraline 50mg daily	33	2
13	61	Female	Major Depression	Amitriptyline 25mg daily	2	1
14	40	Female	Obsessive compulsive disorder	Citalopram 40mg daily	28	3
15	45	Male	Major Depression	Trazodone 300mg daily	10	1
16	25	Female	Major Depression	Sertraline 50mg daily	2	1
17	60	Female	Major Depression	Sertraline 50mg daily	3	2
18	54	Female	Dysthymia	Clomipramine 75mg daily	1	3
19	33	Male	Social Phobia	Moclobemide 300mg twice daily	15	2
20	29	Female	Major Depression	Fluvoxamine 100mg daily	1	2

Subject	Age (Years)	Sex	Diagnosis	Medication	Duration of Illness (years)	Severity of Social stressors (0-3)
21	49	Male	Panic Disorder	Fluoxetine 40mg daily / Amitriptyline 75mg twice daily	40	1
22	43	Male	Adjustment disorder	Nil	5 months	3
23	54	Female	Major depression in Partial Remission	Nil	5	1
24	42	Female	Major Depression	Fluoxetine 60mg daily	8	2
25	47	Female	Major Depression	Nil	1 month	3
26	45	Male	Major Depression	Lithium 250mg 3 times daily / Imipramine 200mg daily / Thioridazine 150mg daily	3	3
27	43	Female	Major Depression	Mianserin 90mg daily / Thioridazine 75mg daily	30	3
28	64	Male	Schizo affective disorder	Mianserin 120mg daily / Chlorpromazine 75mg daily	50	1
29	42	Male	Borderline personality disorder	Imipramine 200mg daily	22	2
30	45	Male	Bipolar mood disorder	Lithium 1350mg daily	25	2

Table 8 Data Sheet: Hamilton A anxiety scores

Patient	Base line Day 0	P_1P_1 Day 6	P_1P_2 Day 13	P_2P_1 Day 20	P_2P_2 Day 27
1	23	26	20	31	22
2	17	11	16	16	11
3	22	6	10	2	0
4	34	32	20	14	4
5	27	16	28	withdraw re' pneumonia	
6	16	10	6	6	7
7	18	19	10	11	10
8	34	37	29	28	28
9	44	44	40	33	31
10	19	16	14	3	0

Table 8 Data Sheet: Hamilton A anxiety scores

Patient	Base line	P_1P_1	P_1P_2	P_2P_1	P_2P_2
11	32	32	34	34	withdraw re' depression
12	14	8	17	10	9
13	23	20	20	withdraw re' flu	
14	25	26	39	32	22
15	34	35	28	22	15
16	15	15	17	15	22
17	44	29	32	26	24
18	21	16	14	11	12
19	30	24	22	19	12
20	22	17	19	14	12

Table 8 Data Sheet: Hamilton A anxiety scores

Patient	Base line	P_1P_1	P_1P_2	P_2P_1	P_2P_2
21	33	22	14	8	5
22	27	27	30	29	30
23	10	17	13	4	4
24	46	50	41	41	41
25	24	16	9	14	8
26	56	56	56	53	49
27	43	46	34	29	23
28	28	23	11	5	4
29	23	20	23	16	24
30	10	6	3	6	6

Once the code was broken it transpired that the initial treatment (M1) consisted of melatonin administration and that the decrease in the mean score for this initial period was significant. However, the decrease in the mean score was also significant during the last period when treatment M2 was administered. It is likely that some other factor caused the continued decrease in the mean scores.

A graphical representation of the median, quartiles and variation of each response is given in the graph below. (Fig 12)

t-Tests for dependent samples

A cross comparison of all responses by using a t-test will reveal the statistically significant differences between each possible pair of mean scores. The data satisfies the requirements to make such a t-test valid. (Table 9(b))

TABLE 9(a) Descriptive statistics

Score	N	Mean	Median	minimum	maximum	Lower Quartile	Upper Quartile	Std.Dev	Skewness
Base	30	27.1	24.5	10	56	18	33.5	11.23	0.707
P1P1	30	24.1	21	6	56	16	30.5	12.88	0.836
P1P2	30	22.3	20	3	56	13	29.5	12.14	0.786
P2P1	28	19	15.5	2	53	9	29	12.85	0.752
P2P2	27	16.1	12	0	49	6	24	12.45	0.909

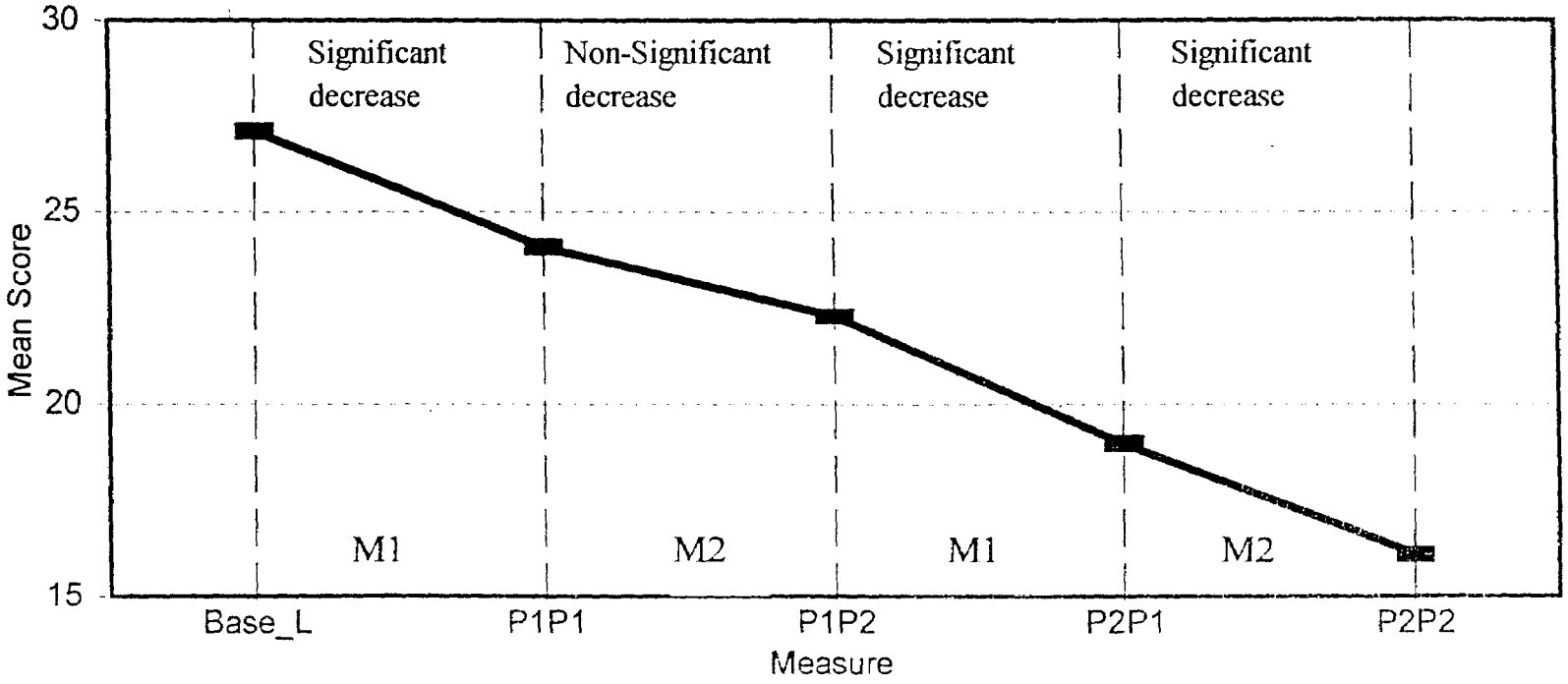


Fig 11 A graphical comparison of the mean scores at each evaluation.

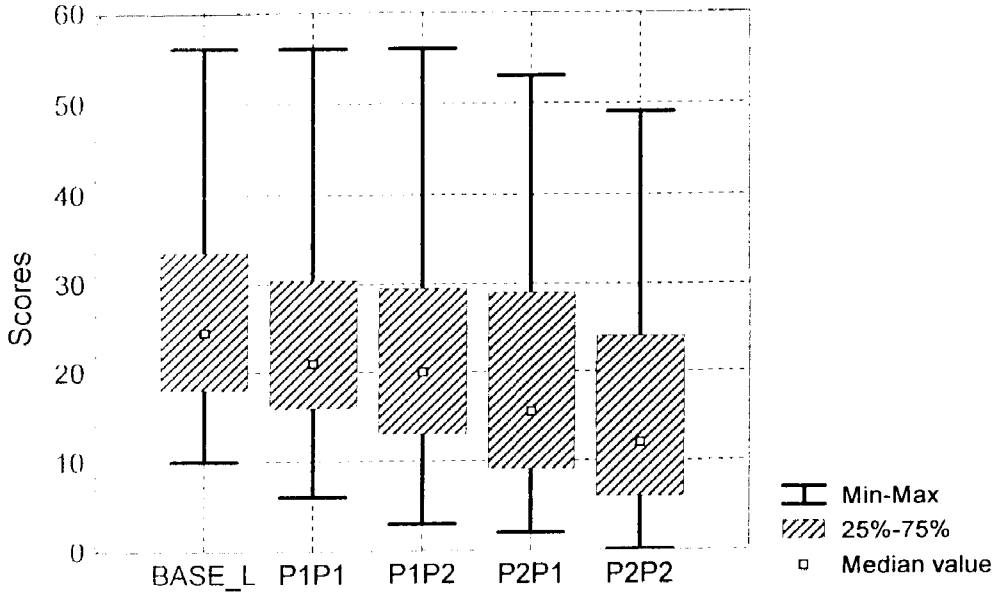


Fig 12 : Box and Whisker plot of evaluations.

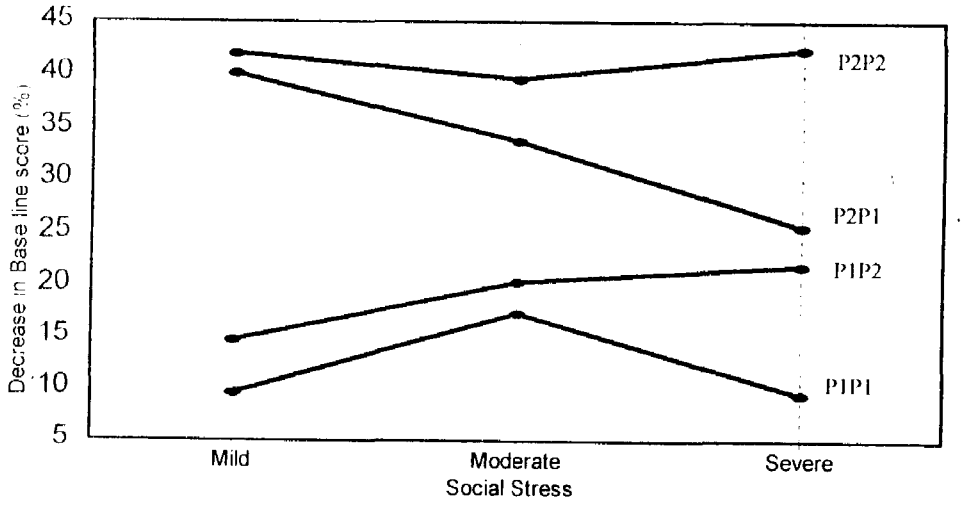


Fig 13 : Decrease in baseline score as a function of social stress.

"N of One" results

If a single patient obtains a Hamilton A score lower than his/her base line score in both periods when melatonin was administered, then we accept that melatonin was anxiolytic for that patient. Of the 27 patients who completed the full trial, 15 patients had lower scores than their baseline scores in both period 1 and period 2 for the M1 treatment. Similarly, 20 patients had lower scores in both periods for the M2 treatment. A contingency table summarizing these scores is given in Table 10.

To investigate whether sub-groups of patients can be identified who had higher rates of success, the base line scores can be categorized in four groups where each group contains approximately the same number of patients. The number of patients who improved twice in succession for a given treatment can then be identified and a possible trend can be investigated.

The observed success rate for the patients in the two lower categories of the baseline score is somewhat higher than for

TABLE 9(b) t-scores with associated p-values

	Base line X = 27.13	P1P1 (M1) X = 24.07	P1P2 (M2) X = 22.18	P2P1 (M1) X = 19.00	P2P2 (M2) X = 16.11
Base line		3.07 (0.0046)*	3.74 (0.0008)	5.21 (0.00002)	5.99 (0.000003)
P1P1 (M1)			1.44 (0.160)	4.09 (0.00035)	4.93 (0.00004)
P1P2 (M2)				3.57 (0.001)	4.80 (0.000056)
P2P1 (M1)					2.77 (0.010)

* p-values are given in brackets. P-values below 0.01 are an indication of a significant difference between the mean values of the relevant responses.

the other two groups. For this sample size, it cannot be proved that this effect is statistically significant (p- value=0.156)

A similar comparison on treatment M2 reveals that the observed success rate for treatment M2 is higher than for the treatment M1 for the patients in the two higher categories of the baseline score. Once again, this trend cannot be proved to be statistically significant (p-value=0.457). See tables 11/12.

Correlation between social stress and Hamilton score

To test whether the average decrease in the Hamilton score was related to the level of social stress the individual patients experience, the patients were categorized in three categories of social stress: (1) none to mild, (2) moderate and (3) severe. An analysis of variance (ANOVA) with social stress as an explanatory variable and each Hamilton score as response variable was carried out. There is no evidence in this sample that the reduction in the Hamilton score was correlated to social stress.

It appears that each catagory of social stress benefitted equally from the treatment. Fig 13.

To investigate possible correlations between the level of social stress and the patient's response on each item, Chi- squared tests on the relevant contingency tables were carried out. None of these correlations proved to be statistically significant. For example, see the contingency table relating level of social stress to patient's response on item 4 (ability to sleep). (Table 13)

TABLE 10 Contingency table comparing treatment with the success rate

	Success rate		
Treatment	Not successful	Successful	Total
M1	12	15	27
M2	7	20	27
Total	19	35	54

TABLE 11 Contingency table comparing baseline categories with the success rate of treatment M1

	M1		
Base line Categories	Not successful	Successful	Total
0 - 18	3	4	7
19 - 24	1	6	7
25 - 33	3	3	6
34 - 56	6	2	8
Total	13	15	28

TABLE 12 Contingency table comparing baseline categories with the success rate of treatment M2

	M2		
Base line Categories	Not successful	Successful	Total
0 - 18	3	4	7
19 - 24	1	6	7
25 - 33	2	3	5
34 - 56	1	7	8
Total	7	20	27

TABLE 13 Contingency table comparing social stress with improvement of sleep

	Ability to sleep			
Level of social stress	Improved	Did not change	Worsened	Total
Mild	6	3	0	9
Moderate	5	2	1	8
Severe	6	4	1	11
Total	17	6	5	28

* Fisher's exact test gives a p-value of 0,9685.

Conclusion

Although the treatment of the patients was successful, it could not be statistically shown whether this was due to the melatonin or the placebo. It is possible that apart from a placebo effect, the initial melatonin administration (M1) introduced a phase shift in the patient's melatonin phase response curve that could explain the sustained improvement in the patients' anxiety levels during period two of pair two (placebo, M2).

Other factors that may be responsible for the sustained improvement are a placebo effect or a therapeutic response due to prolonged contact with a clinician. Further studies are needed to elucidate melatonin's role in anxiety. From this study it may be concluded that:

a) Anxiety levels and social stressors do not correlate with the improvement of anxiety associated with the use of melatonin.

b) That a "N of 1" study is not an appropriate study design for investigating melatonin's anxiolytic effects as it does not take a possible melatonin phase shift into account.

c) Melatonin phase response curves should be estimated in each patient before and after initiating melatonin treatment to correlate anxiolytic effect with shifts in melatonin's phase response curves.

d) That non-invasive measures of melatonin levels (saliva / urine) need to be evaluated for clinical research.

e) Over the counter melatonin products content correlates well with pure melatonin and is relatively easy to verify.

APPENDIX

1. Protocol
2. Informed consent
3. Format of physical examination
4. Hamilton A - anxiety scale
5. Analysis of melatonin tablets

PROTOCOL

TRIALIST: JOHANNES JACOBUS McCALLAGHAN

PROJECT: MASTER OF SCIENCE (PHARMACY) IN THE SCHOOL OF PHARMACEUTICAL SCIENCES.

UNIVERSITY: RHODES UNIVERSITY

PROMOTOR: PROF B POTGIETER

TITLE: MELATONIN AS AN ANXIOLYTIC: AN INVESTIGATION INTO THE INTERACTION OF MELATONIN WITH SEROTONIN RECEPTORS.

PROTOCOL NO: RHODES/PHARM:1

TRIAL DESIGN: DOUBLE BLIND, PLACEBO CONTROLLED, EACH PERSON COMPLETING A " N OF 1 " STUDY.

RHODES UNIVERSITY

SCHOOL OF PHARMACEUTICAL SCIENCES

PROTOCOL FOR CLINICAL WORK

1. SPECIFIC AIMS AND OBJECTIVES

a) To conduct a literature search on the anxiolytic properties of melatonin with emphasis on the use of melatonin in humans.

To seek evidence from the literature that melatonin interacts with serotonergic receptors and to propose further investigation in this regard.

b) Conduct a pilot study evaluating the anxiolytic properties of melatonin in humans.

c) Generate ideas for further studies on the posssible role of melatonin in psychiatric disorders.

d) Publish the results in a scientific journal.

SIGNIFICANCE AND BACKGROUND INFORMATION

Melatonin is secreted by the pineal gland in a rhythmic fashion with high levels during night time.The pattern of melatonin secretion in an individual is known as its Phase Response Curve. Evidence suggests that shifts backwards or forwards of this Phase Response Curve might be the basis of melatonin's involvement in psychiatric disorders.

Melatonin is safe to administer to humans. A person receiving 200mg melatonin intravenously daily for 5 days showed no evidence of delayed toxicity 18 years later.

Researchers were unable to find an oral LD-50 for melatonin for rats.

It was also found that melatonin was not mutagenic in standard in vitro tests. Clinical trial certificates have been issued to cover long term administration of up to 300mg of melatonin per day.

Drowsiness has frequently been reported with low doses of melatonin, but doses in the range of 50mg - 6.6g melatonin per day have also given rise to complaints of headaches and abdominal cramps.

The use of melatonin in preventing jet lag is well known. Melatonin, however, shares pharmacological properties with the benzodiazepines - anticonvulsant, muscle relaxant, sedative-hypnotic and anxiolytic.

The anxiolytic properties of melatonin have been poorly documented other than in studies on the rat.

Melatonin is currently available in South Africa as a health product at pharmacies and health shops.

EXPERIMENTAL DESIGN AND METHOD

* Each subject will undergo an " N OF 1 " study in a double blind placebo controlled study.

* Each subject will be examined for exclusion criteria and informed consent will be obtained.

* Information will be gathered by means of the Hamilton A evaluation forms.

* Subjects will not be hospitalized and visits will be arranged according to the research schedule.

* The experimental population will consist of adults complaining of anxiety.They would be referred by doctors, psychologists,pharmacists and clinics for participation in the trial.

* INCLUSION CRITERIA

  - older than 21 years of age.

  - complaining of anxiety.

  - no history/ physical evidence of liver or kidney disease.

- must be able to give informed consent.

- participate in trial out of own free will.

* EXCLUSION CRITERIA

- younger than 21 years of age.

- not mentally competent to give informed consent(mentally retarded).

- if physical examination reveals evidence of liver or kidney disease.

- pregnancy/non use of contraceptives in females.

- not willing to participate in trial or if subject should wish to end his/her participation.

Subjects will undergo a physical examination and urine dipstick evaluation by a medical doctor (the trialist) before commencement of the trial. Female subjects will also undergo a pregnancy test.

No invasive investigations will be done during the trial and there will be no restrictions on diet.

Patient files will not be consulted. Evaluation will consist of history and physical examination.

Evaluation will be by means of the Hamilton A Rating Scales according to the research schedule.

A pharmacist will dispense melatonin and placebo for the purpose of the double blind study.

DATA MANAGEMENT

Hamilton A rating scales will be tabulated - see data sheet.

DATA ANALYSIS AND PRESENTATION

This will be a double blind placebo controlled experiment. Each patient will complete an " N of 1 " study. This will enable the researcher to evaluate the efficacy of melatonin as an anxiolytic in individual patients.

The Fischer exact test will be used to evaluate melatonin's effect as an anxiolytic on the whole research population (i.e. is there a statistically significant decrease in the anxiety levels experienced by patients with the use of melatonin).

SECTION 2

HUMAN SUBJECTS.

1. CHARACTERISTICS OF SUBJECT POPULATION

30 adult subjects between 21-60 years of age. No clinical evidence of renal or liver ailments. No exclusion criteria with regards to gender or race. Estimate female/male ratio 60:40 (on grounds of ratio seen in clinical practice).

2. SOURCE OF RESEARCH MATERIAL

Patients are referred by doctors, pharmacists, psychologists or clinics. No medical /personal files will be consulted.Information will be obtained by means of an interview (history), physical examination (appropriate urine analysis) and the Hamilton A anxiety scale.

3. RECRUITMENT OF SUBJECTS

Each subject will be personally informed of the research proposal, its aims and risks and the involvement of the trialist. Please see patient information leaflet/consent form.

4. POTENTIAL RISKS

As a low dose (3mg) of melatonin is to be used sedation should be expected. Patients need to be warned against driving, working with dangerous machinery and interaction with other sedating substances. If a patient suffers from depression he may experience an increase in depression. Disappointment due to no improvement in anxiety levels may be seen as a psychological risk and needs to be addressed during informed consent. Social and legal risks can be minimized by confidentiality and by obtaining informed consent.

5. PROTECTION AGAINST AND MINIMIZING POTENTIAL RISK

A thorough evaluation (physical/history) before the clinical trial will be conducted. The trialist (a medical doctor) will be available to attend to any complaints. The trialist will be the only person physically involved in conducting the trial - thus minimizing risk of breach of confidentiality.

The data sheet will contain no identification information to ensure anonymity.

The trialist is insured by the Medical Defence Union and legal advice can be obtained if needed.

6. RISKS TO SUBJECTS

Melatonin is used world wide in clinical trials. It was found to be safe without serious side effects - although depression might exacerbate. Subjects will be informed that no benefit during the pilot study must be expected as this serves only to develop a research strategy for future studies on the clinical use of melatonin.

SECTION 3

This will serve as a pilot study in which a research strategy will be developed. This will serve as a basis for studies towards a Ph.D.

PATIENT INFORMATION LEAFLET

TRIAL TITLE: Melatonin as an anxiolytic: An Investigation into the interaction of melatonin with serotonin receptors.

Clinical Trial Number: Rhodes / Pharm: 1

INTRODUCTION

You are invited to volunteer for a research study. You have been asked if you would be willing to take part in this trial. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study.

WHAT IS THE PURPOSE OF THIS TRIAL?

You are complaining of anxiety and the investigator will ask you to consider taking part in research to investigate the anxiolytic effects of melatonin.

This is a research project to develop further plans on how to use melatonin in patients suffering from anxiety - do not see participation as treatment for your anxiety. During the study you will receive 3mg melatonin at night (9pm) and an inactive substance (called a placebo) in such a way that neither yourself nor the investigator will know which one you are receiving. This is done to evaluate the true effect of melatonin.

WHAT IS THE DURATION OF THIS TRIAL?

If you decide to take part you will be one of 30 patients. The study will last for up to one month. You will be asked to visit the investigator once per week.

On the first visit you will undergo the following:

1. Hamilton A evaluation

2. General physical examination, urine dipstick and a pregnancy test if applicable.

3. Interview (including detailed medical / psychiatric history). No personal / medical files will be consulted.
   On each follow-up visit a Hamilton A evaluation will be conducted.
   (Please see appendix).

It is important that you let the investigator know of any medicines (both prescriptions or over-the-counter medicines), alcohol or other substances that you are currently taking or if you are a blood donor.

HAS THE TRIAL RECEIVED ETHICAL APPROVAL?

This clinical trial protocol was submitted to the Rhodes University ethics committee and written approval has been granted by the committee.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THIS TRIAL?

Your participation in this trial is entirely voluntary and you can refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your access to other medical care. The investigator retains the right to withdraw you from the study if he considers it to be in your best interest. If it is detected that you did not give an accurate history or did not follow the guidelines of the trial and the regulations of the trial facility, you may be withdrawn from the trial at any time.

IS ALTERNATIVE TREATMENT AVAILABLE?

It is important to note that melatonin is not a registered treatment for anxiety. Recognized effective medication is available for the treatment of anxiety.

MAY ANY OF THESE TRIAL PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

No procedures will be done. Only urine dipstick analysis.

WHAT ARE THE RISKS INVOLVED IN THIS TRIAL?

All medicines carry some risk, however small. In previous studies some patients have reported experiencing side effects which included drowsiness, headaches, and abdominal cramps.

ARE THERE ANY WARNINGS OR RESTRICTIONS FOR MY PARTICIPATION IN THIS TRIAL?

If you are a female who can become pregnant you must use suitable contraceptive measures during the trial as the safety of melatonin during pregnancy has not been established. Melatonin is not implicated in reducing the efficiency of oral or parenteral contraceptives.

DISCONTINUATION OF TRIAL TREATMENT

Uncontrolled discontinuation of trial medication is inadvisable. Special care needs to be taken for the discontinuation of this trial medication. The investigator will supervise any discontinuation with your health as first priority.

INSURANCE AND FINANCIAL ARRANGEMENTS

The investigator will be available to deal with your complaints relating to the trial. Neither you nor your medical aid will be expected to pay for these consultations, the melatonin or visits scheduled during the trial. As no serious life threatening side-effects were reported with low dose melatonin (3mg) the patient would not be entitled to any other free medical threatment during participation in this trial. (e.g. hospitalization, blood test or medication).

You must notify the investigator immediately of any complications or side effects experienced during the trial.

You have not waived any of the legal rights that you otherwise would enjoy as a participant in this trial by signing this form.

You will not be paid to participate in the trial.

Please note that if you have a life insurance policy you should notify the appropriate insurance company concerned of your intention to participate in a clinical trial. Our information to date is that it should not affect any life insurance policy taken out. Nevertheless you are strongly advised to notify the company concerned.

Any information uncovered regarding your test results or state of health as a result of your participation in this trial will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this trial but this information will not be disclosed to any third party. The only exception to this rule will be cases where a law exists compelling us to report individuals infected with communicable diseases. In this case, you will be informed of our intent to disclose such information to the authorised state agency.

INFORMED CONSENT FORM

I hereby confirm that I have been informed by the investigator, Dr J J McCallaghan, about the nature, conduct, benefits and risks of clinical trial Rhodes / Pharm: 1. I have also received, read and understood the written information (Patient Information Leaflet and Consent Form) regarding the clinical trial.

I am aware that the results of the trial, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a trial report.
I may, at any stage, without prejudice, withdraw my consent and participation in the trial.

I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the trial.

Patient's
Name .....
(Please print)

Patient's
Signature ..... Date .....

Investigator's
Name .....

(Please print)

Investigator's

Signature ..... Date .....

I, Dr ..... herewith confirm that the above patient has been fully informed about the nature, conduct and risks of the above trial.

Witness's

Name* .....

(Please print) *Consent procedure should be witnessed whenever possible.

Witness's

Signature ..... Date .....

Patient: ..... Date : .....

Age: ..... Sex: .....

Medication: ..... Allergies: .....

History:

Medical:.....

.....

.....

Surgical:.....

.....

.....

Psychiatric:.....

.....

.....

Physical examination:

Height: ..... Weight: .....BP: ..... P:.....

General: .....

Cardiovascular:. .....

Respiratory:.....

Alimentary + Genito urinary systems: .....

Urine dipstick: .....

Pregnancy Test (female): .....

Nervous system: .....

Accepted for trial:	YES		NO	

Patient No.:

Age:

Medication:

Hamilton A - anxiety scale

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27

		DAY 0	6	13	20	27
1. ANXIOUS MOOD	Worries, anticipation of the worst, fearful anticipation, irritability					
2. TENSION	Feelings of tension, fatigability, startle response, moved to tears easily, trembling, feelings of restlessness, inability to relax					
3. FEARS	Of dark, of strangers, of being left alone, of animals, of traffic of crowds					
4. INSOMNIA	Difficulty in falling asleep, broken sleep, unsatisfying sleep and fatigue on waking, dreams, nightmares, nights terrors					
5. INTELLECTUAL (Cognitive)	Difficulty in concentration, poor memory					
6. DEPRESSED MOOD	Loss of interest, lack of pleasure in hobbies, depression, early waking, diurnal swing					
7. SOMATIC (Muscular)	Pains and aches, twitching, stiffness, myoclonic jerks, grinding of teeth, unsteady voice, increased muscular tone					
8. SOMATIC (Sensory)	Tinnitus, blurring of vision, hot and cold flushes, feelings of weakness, prickling sensation					
9. CARDIOVASCULAR SYMPTOMS	Tachycardia, palpitations, pain in chest, throbbing of vessels, fainting feelings, missing beat					
10. RESPIRATORY SYMPTOMS	Pressure of constriction in chest, choking feelings, sighing, dyspnoea					
11. GASTRO INTESTINAL SYMPTOMS	Difficulty in swallowing, wind, abdominal pain, burning sensations, abdominal fullness, nausea, vomiting, borborygmi, looseness of bowels, loss of weight, constipation					
12. GENITOURINARY SYMPTOMS	Frequency of micturition, urgency of micturition, amenorrhea, menorrhagia, development of fragility, premature ejaculation, loss of libido, impotence					
13. AUTONOMIC SYMPTOMS	Dry mouth, flushing, pallor, tendency to sweat, giddiness, tension headache, raising of hair					
14. 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, exopthalmos					
	Total					

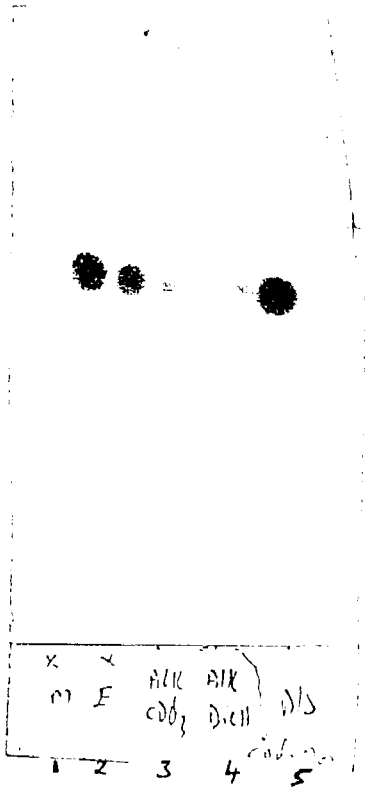
GRADING SCORES (0-4)

0 :Absent 1 :Mild 2 :Moderate 3 : Severe 4 :Very servere

A: Thin layer chromatography of pure melatonin (Sigma)and Melapure™)

Mobile phase - Chloroform : Methanol (9 : 1)

$R_f = {}^{39}/_{80} = 0,487$



1. Pure melatonin dissolved in methanol.
2. Pure melatonin dissolved in ethanol.
3. Melapure™ - Tablets dissolved in chloroform.
4. Melapure™ - Tablets dissolved in dichloromethane.
5. Double Spot.

B: Ultraviolet Analysis - Absorbance readings at 278nm

Aim: To determine the melatonin content of Melatonin™ tablets.

Experimental procedure:

a. Made stock solution of melatonin = 9mg / 100ml methanol

b. Made 5 dilutions from the stock melatonin solution with methanol:
0.9mg/100ml, 1.8mg/100ml, 3.6mg/100ml, 5.4mg/100ml and 7.2mg/100ml

c. Took absorbance readings of stock solution and 5 dilutions of melatonin (against methanol as a blank).

d. Plotted a calibration curve of concentration of melatonin vs absorbance.

e. Extracted three melatonin tablets (9mg), into chloroform.

f. Took absorbance reading of extraction (against chloroform as a blank) and determined graphically the concentration of melatonin extracted from tablets.

ABSORBANCE READING OF MELATONIN	
Concentration (mg/100ml)	Absorbance
0.9	0.235
1.8	0.43
3.6	0.863
5.4	1.271
7.2	1.675
9	2.226
Extraction sample	2.194

Melatonin concentration extracted from tablets determined from the calibration curve = 8.89 mg/100ml.

C: HPLC - Analysis

Aim: To confirm melatonin content of Melatonin™ tablets.

Experimental procedure:

UV - detector at 278nm.

Mobile phase - Chloroform : Methanol (9 : 1)

a. Made stock solution of pure melatonin (Sigma) = 9mg / 100ml methanol.

b. Three melatonin tablets extracted in chloroform.

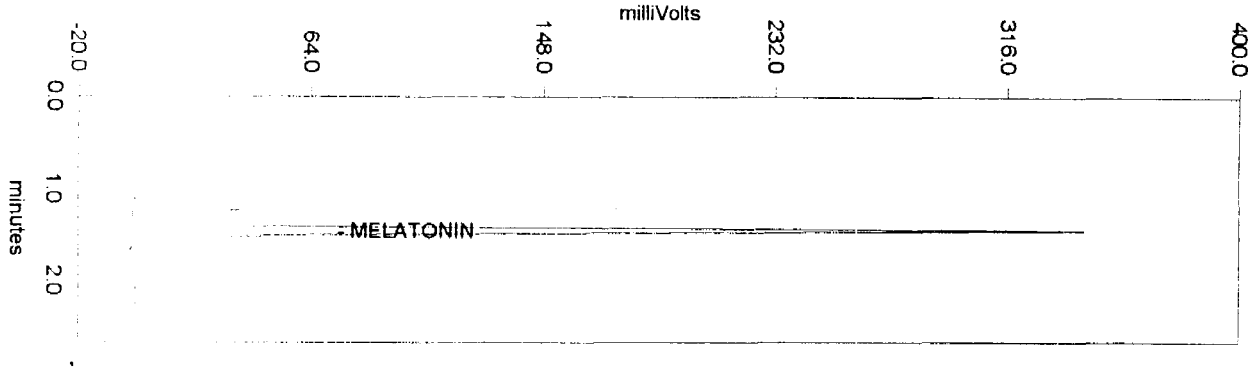
c. Injected stock solution and extracted sample to identify compound in extraction, using retention times and peak shapes for the identification.

Pure Melatonin in methanol.

```
**********************************************************************
*                                                                    *
*  TIME : 10:31 am     DELTA CHROMATOGRAPHY DATA SYSTEM    DATE :  8/10/98  *
*                            AREA PERCENT REPORT                     *
**********************************************************************

 SAMPLE NAME :  SAMPLE                    CHROMATOGRAM FILE :   A
 SAMPLE TYPE :  SAMP                      CHROMATOGRAM SOURCE : FILE
 SAMPLE AMOUNT        1.00                METHOD NAME         : APM
 DILUTE       :       1.00                INJ                 :  1 OF  1

 Chromatogram A recorded at 10:16 am on 08/10/98
 Captured using method : INTEGRAT
```



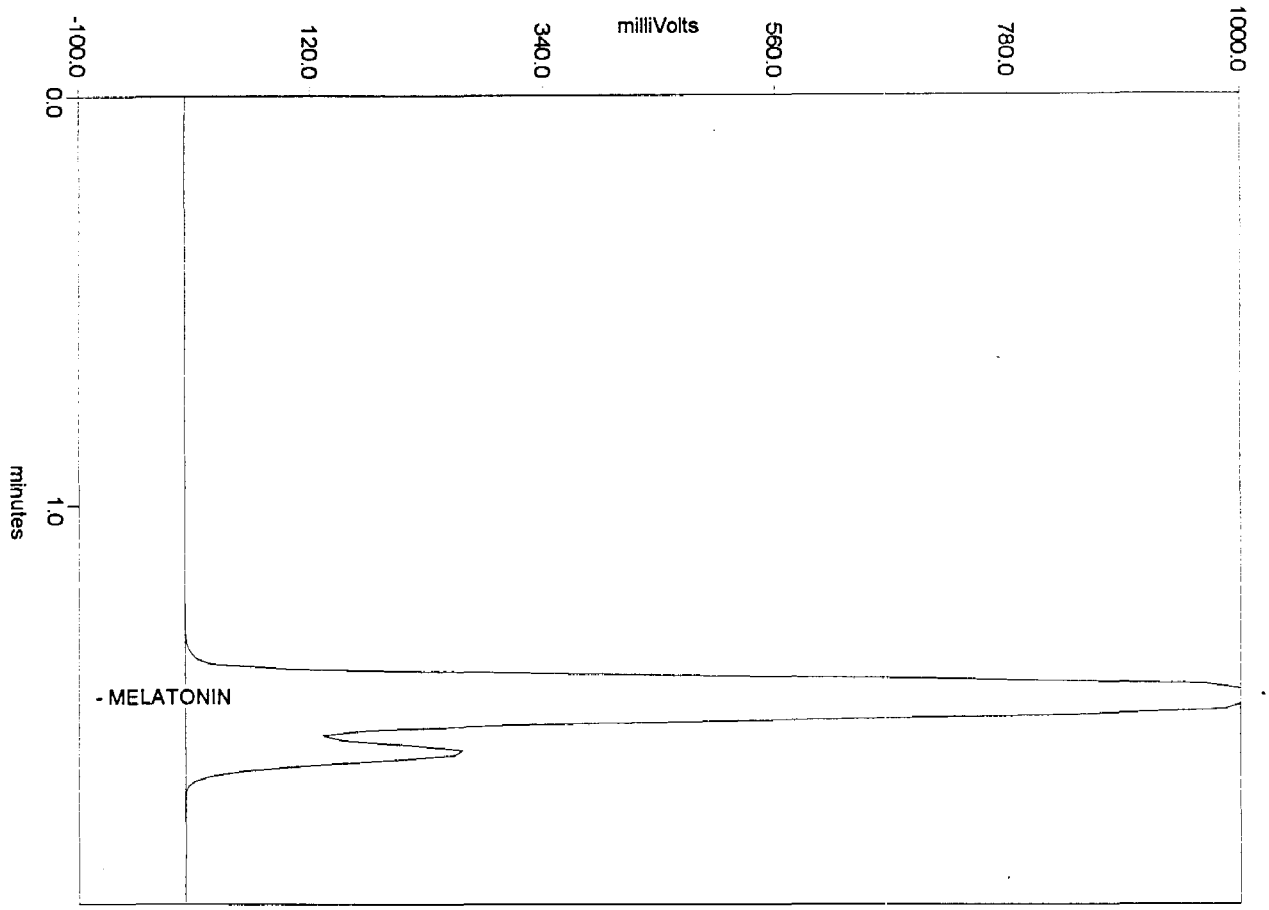
PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
1	1	PB	MELATONIN	1.46	3.10	1352.9	100.0
TOTALS :						1352.9	100.0

Melatonin™ extraction in chloroform.

```
*************************************************************************
*                                                                       *
*  TIME : 11:09 am     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :  8/10/98  *
*                              AREA PERCENT REPORT                      *
*                                                                       *
*************************************************************************

 SAMPLE NAME :  MELATONIN                CHROMATOGRAM FILE :   B3
 SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : ACQUIRE
 SAMPLE AMOUNT        1.00               METHOD NAME         : APM
 DILUTE       :       1.00               INJ                 :  1 OF  1

 Chromatogram B3 recorded at 11:05 am on 08/10/98
 Captured using method : APM
```

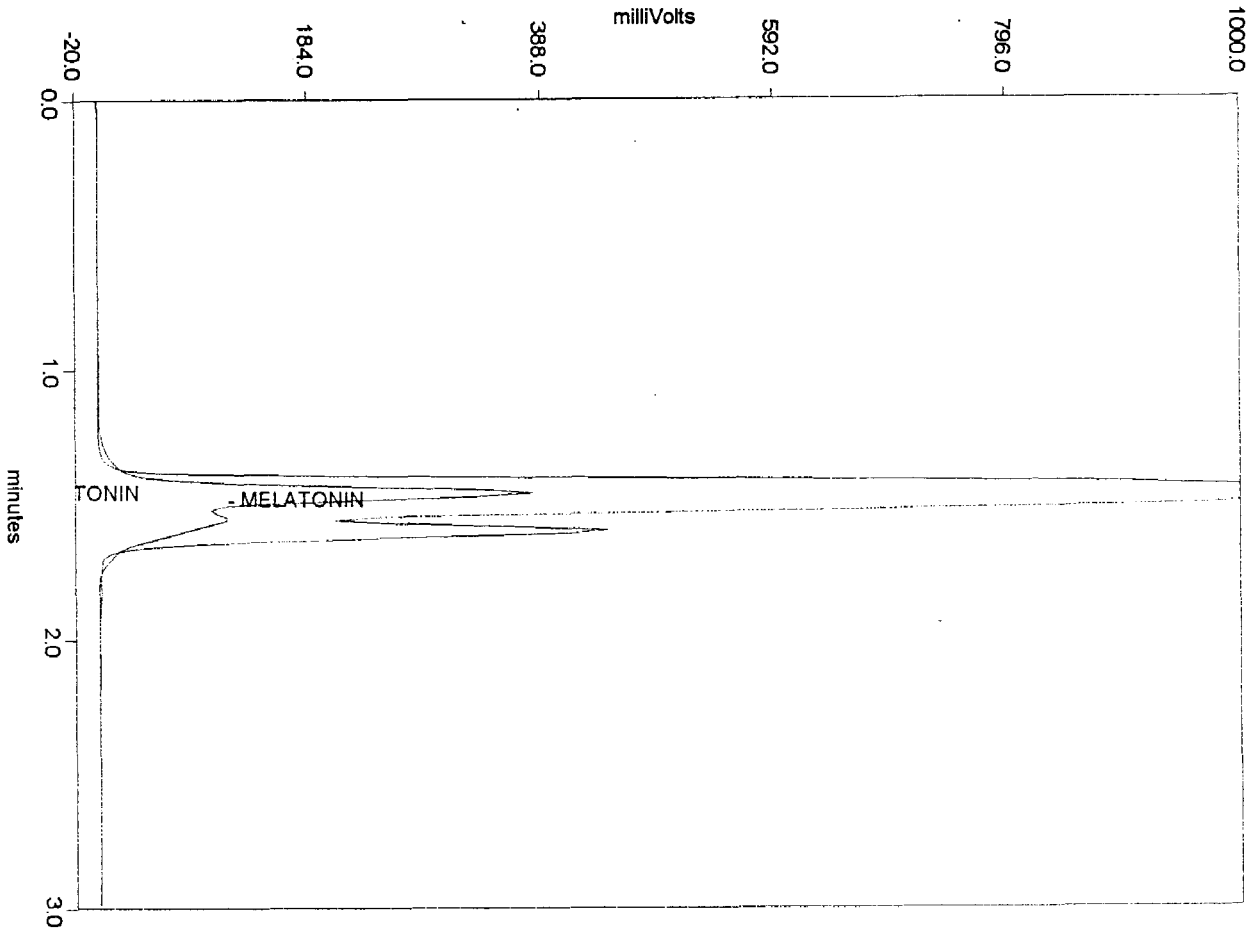


PK#	CPT#	TYPE	COMPONENT NAME	RET. TIME	WIDTH	AREA	RESULT
1	1	BE	MELATONIN	1.46	6.70	7887.0	100.0
TOTALS :						7887.0	100.0

Stripchart plot for overlay.

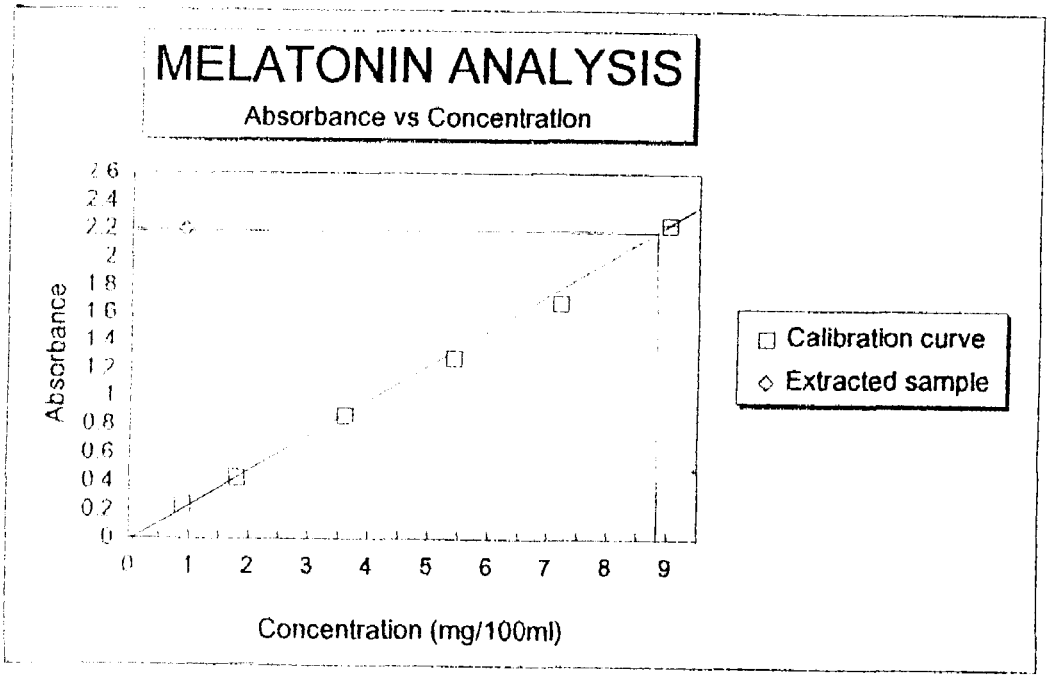
DELTA CHROMATOGRAPHY DATA SYSTEM TIME - 11:15 am DATE - Thu Oct 08 1998

STRIPCHART PLOT FOR OVERLAY



Results:

Chromatograms indicate that the pure Melatonin injection and the injection of the extracted sample are both melatonin - same retention times and same peak shapes. The second peak on the stripchart plot is propably vitamin B_6 which is also found in the Schiff melatonin product.


MELATONIN ANALYSIS
Absorbance vs Concentration
Absorbance
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9
Concentration (mg/100ml)
Calibration curve
Extracted sample

References

1. Erlich SS, Apuzzo MLJ. The pineal gland: anatomy, physiology, and clinical significance. J Neurosurg. 1985; **63**: 321-341.
2. Daya S. Melatonin: a neuropsychiatric profile. Specialist Medicine. 1994(September): 58-64.
3. Pierrefiche G, Zerbid R, Laborit H. Anxiolytic activity of melatonin in mice;involvement of benzodiazepine receptors. Research Communications in Chemical Pathology and Pharmacology. 1993; **82**: 131-141.
4. Arendt J. In: Melatonin and the Mammalian Pineal Gland. 1st ed. London. Chapman & Hall, 1995: 249-250.
5. Serotonin Specifics. Symposium: Depression and Anxiety. Montreux, Switzerland, 1995: **95/1**: 4-10.
6. Hamilton WJ, Mossman HW. In: Human Embryology. 4th ed. Cambridge: W Heffer & Sons LTD.Baltimore: The Williams & Wilkins Company; 1972: 437-460.
7. Mitchell GAG , Mayor D. In: The essentials of neuroanatomy. 4th ed. Edinburgh:Churchill Livingstone,1983: 69-76.
8. Arendt J. In: Melatonin and the Mammalian Pineal Gland. 1st ed. London. Chapman & Hall, 1995: 16-25.
9. Reiter RJ. The mammalian pineal gland: structure and function. Am J Anat. 1981; **162**: 287-313.

10. Carpenter MB. In: Core text of neuroanatomy. 2nd ed.Baltimor: The Williams & Wilkins Company, 1978: 434-461.
11. Ganong W.F. In: Review of medical physiology. 8th ed. California. Lange Medical Publications, 1977: 457.
12. Quay WB. Retrograde perfusions of the pineal region and the question of pineal vascular routes to brain and choriod plexuses. AM J Anat. 1973; **137**: 387-402.
13. Arendt J. In: Melatonin and the Mammalian Pineal Gland. 1st ed. London. Chapman & Hall, 1995: 7-15.
14. Leeson TS, Leeson CR. In: Histology. 4th ed. London. WB Saunders & Company, 1981: 480-483.
15. Tapp E, Huxley M. The histological appearance of the human pineal gland from puberty to old age. J Path. 1972; **108**: 137-144.
16. Lukaszyk A, Reiter RJ. Histophysiological evidence for the secretion of polypeptides by the pineal gland. Am J Anat. 1975; **143**: 451-464.
17. Wurtman RJ, Moskowitz MA. The pineal organ.(2). The New England Journal of Medicine.1977; **296(24)**: 1383-1386.
18. Arendt J. In: Melatonin and the Mammalian Pineal Gland. 1st ed. London. Chapman & Hall, 1995: 29.
19. Arendt J. In: Melatonin and the Mammalian Pineal Gland. 1st ed. London. Chapman & Hall, 1995: 31-42.
20. Wurtman RJ, Axelrod J, Phillips LS. Melatonin synthesis in the pineal gland: Control by light. Science. 1963; **142**: 1071-1073.

21. Wurtman RJ, Moskowitz MA. The pineal organ.(1) The New England Journal of Medicine. 1977; **296**: 1329-1333.

22. Schmid HA. Decreased melatonin biosynthesis, calcium flux, pineal gland calcification and aging: A hypothetical framework. Gerontology. 1993; **39**: 189-199.

23. Vakkuri O, Leppäluoto J, Kauppila A. Oral administration and distribution of melatonin in human serum , saliva and urine. Life Sciences. 1985; **37**: 489-495.

24. Aldhous M, et al. Plasma concentrations of melatonin in man following oral absorption of different preparations. B J Clin Pharmac. 1985; **19**: 517-521.

25. Mallo C, et al. Pharmacokinetics of melatonin in man after intravenous infusion and bolus injection. Eur J Clin Pharmacol. 1990; **38**: 297-301.

26. Lochner J de V. Biorhythms-mechanisms and clinical implications. Continuing Medical Education Journal. 1994; **12**: 1433-1443.

27. Ralph MR, et al. Transplanted suprachiasmatic nucleus determines circadian period. Science.1990; **247**: 975-978.

28. Mills MH, Faunce TA. Melatonin supplementation from early morning auto-urine drinking. Medical Hypotheses. 1991; **36**: 195-199.

29. Coetzee JA, Theron JJ, Van Der Merwe CA. Consecutive melatonin circadian rhythms in normal volunteers. SAMJ. 1989; **75**: 163-165. 90.

30. Arendt J. In: Melatonin and the Mammalian Pineal Gland. 1st ed. London. Chapman & Hall, 1995: 207-210.

31. Casey PR. In: A Guide to psychiatry in primary care. Petersfield, Wrightson Biomedical Publishing Ltd. 1993: 84.

32. Nutt D. The role of benzodiazepine receptors in anxiety. Psychiatry in practice. 1994: 41-43.

33. Blackburn TP. 5-HT receptors and anxiolytic drugs. In: Marsden CA, Heal DJ, eds. Central Serotonin Receptors and psychotropic Drugs. London,UK: Blackwell Scientific Publications, 1992: 175-197.

34. Eison AS, et al. Serotonergic mechanisms in the behavioral effects of Buspirone and Gepirone.
Pharmacology Biochemistry & Behavior.1986; **24**: 701-707.

35. Robinson DS, et al. Serotonergic anxiolytics and treatment of depression. Psychopathology. 1989; **22**(suppl 1): 27-36.

36. Golombek DA, Martini M, Cardinali DP. Melatonin as an anxiolitic in rats: time dependence and interaction with the central GABAergic system. European Journal of Pharmacology. 1993; **237**: 231-236.

37. Niles L. Melatonin interaction with the benzodiazepine-GABA receptor complex in the CNS. Adv Exp Med Biol. 1991; **294P**: 267-277.

38. Rosenstein RE, Cardinali DP. Melatonin increases in vivo GABA accumulation in rat hypothalamus, cerebellum, cerebral cortex and pineal gland. Brain Research. 1986; **398**: 403-406.

39. Niles LP, Pickering DS, Arciszewski MA. Effects of chronic melatonin administration on GABA and diazepam binding in rat brain. J Neural Transm. 1987; **70**: 117-124.

40. Sandyk R. Does melatonin mediate the therapeutic effects of 5-HT reuptake inhibitors in obsessive compulsive disorder? Intern J Neuroscience. 1992; **64**: 221-223.

41. Zohar J, et al. Serotonergic Responsivity in Obsessive-Compulsive Disorder. Arch Gen Psychiatry. 1987; **44**: 946-951.

42. Sandyk R, Dann LC. Seasonal panic disorder: A possible variant of seasonal affective disorder. Intern J Neuroscience. 1992; **62**: 263-267.

43. Khan R, Daya S, Potgieter B. Evidence for a modulation of the stress response by the pineal gland. Experientia. 1990; **46**: 860-862.

44. Wirz-Justice A, et al. Light therapy in seasonal affective disorder is independant of time of day or circadian phase. Arch Gen Psychiatry. 1993; **50**: 929-937.

45. Lewy AJ, et al. Bright artificial light treatment of a manic-depresive patient with a seasonal mood cycle. Am J Psychiatry. 1982; **139(11)**: 1496-1498.

46. Wetterberg L, et al. Melatonin and cortisol levels in psychiatric illness.The Lancet. 1982: 100.

47. Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Rosenthal NE. Manic-depressive patients may be supersensitive to light. The Lancet. 1981: 383-384.

48. Zimmermann RC, et al. Effects of acute tryptophan depletion on nocturnal melatonin secretion in humans. Journal of Clinical Endocrinology and Metabolism. 1993; **76**: 1160-1164.

49. Waldhauser F, Ehrhart B, Förster E. Clinical aspects of the melatonin action: impact of development, aging, and puberty,involvement of melatonin in psychiatric disease and importance of neuroimmunoendocrine interactions. Experientia. 1993; **49**: 671-681.

50. Rubin RT, et al. Neuroendocrine aspects of primary endogenous depression. Arch Gen Psychiatry. 1992; **49**: 558-567.

51. Thompson C, et al. A comparison of melatonIn secretion in depressed patients and normal subjects. British Journal of Psychiatry. 1988; **152**: 260-265.

52. Wetterberg L. Light therapy of depression; basal and clinical aspects. Pharmacology & Toxicology. Supplement. 1992; **71(1)**: 96-106.

53. Kennedy SH, Brown GM. Effect of chronic antidepressant treatment with Alprazolam and Desipramine on melatonin output. Psychiatry Research. 1992; **43**: 177-185.

54. Nash ES, Stoch MB, Harper GD. In: Human Behaviour. 1st ed. Cape Town Juta, 1990: 269-273.

55. Dawson D, Encel N. Melatonin and sleep in humans. J Pineal Res. 1993; **15**: 1-12.

56. Palm L, Blennow G, Wetterberg L. Correction of non-24-hour sleep/wake cycle by melatonin in a blind retarded boy. Ann Neurol. 1991; **29**: 336-339.

57. Dahlitz M, et al. Delayed sleep phase syndrome response to melatonin. Lancet. 1991; **337**: 1121-1124.

58. Thompson C, et al. Neuroendocrine rhythms in a patient with the Kleine-Levin syndrome. British Journal of Psychiatry. 1985; **147**: 440-443.

59. Jan JE, Espezel H, Appleton RE. The treatment of sleep disorders with melatonin. Developmental Medicine and Child Neurology. 1994; **36**: 97-107.

60. Sandyk R. Melatonin and maturation of REM sleep. Intern J Neuroscience. 1992; **63**: 105-114.

61. Bunnell DE, et al. Effects of evening bright light exposure on melatonin, body temperature and sleep. J Sleep Res. 1992; **1**: 17-23.

62. Dollins AB, et al. Effect of inducing nocturnal serum melatonin concentrations in daytime on sleep, mood, body temperature, and performance. Proc Natl Acad Sci. 1994; **91**: 1824-1828.

63. Sugden D. Psychopharmacological effects of melatonin in mouse and rat. The Journal of Pharmacology and Experimental Therapeutics. 1983; **227**: 587-591.

64. MacFarlane JG, et al. The effects of exogenous melatonin on the total sleep time and daytime alertness of chronic insomniacs: A preliminary study. Biol Psychiatry. 1991; **30**: 371-376.

65. Tzischinsky O, et al. The importance of timing in melatonin administration in a blind man. J Pineal Res. 1992; **12**: 105-108.

66. Grad BR, Rozencwaig R. The role of melatonin and serotonin in aging: update. Psychoneuroendocrinology. 1993; **18(4)**: 283-295.

67. Arendt J, et al. Rapid decrease in melatonin production during successfull treatment of delayed puberty. The Lancet. 1989: 1326.

68. Puig-Domingo M, et al. Brief report: Melatonin-related hypogonadotropic hypogonadism. The New England Journal of Medicine. 1992; **5**: 1356-1359.

69. Valcavi R, et al. Effect of oral administration of melatonin on GH responses to GRF 1-44 in normal subjects. Clinical Endocrinology.1987; **26**: 453-458.

70. Grad BR, Rozencwaig R. The role of melatonin and serotonin in aging: update. Psychoneuroendocrinology. 1993; **18(4)**: 283-295.

71. Marsden CA. The pharmacology of new anxiolytics acting on 5-HT neurones. Postgrad Med J. 1990; **66**(suppl): S2-S6.

72. Tamarkin L, Baird CJ, Almeida OFX. Melatonin: A coordinating signal for mammalian reproduction? Science. 1985; **227**: 714-720.

73. Binkley S. Structures and molecules involved in generation and regulation of biological rhythms in vertebrates and invertebrates. Experientia. 1993; **49(8)**: 648-653.

74. Czeisler CA, et al. Exposure to bright light and darkness to treat physiologic maladaption to night work. The New England Journal of Medicine. 1990; **322**: 1253-1259.

75. Sandyk R, Anninos PA, Tsagas N. Age-related disruption of circadian rhythms: possible relationship to memory impairment and implications for therapy with magnetic fields. Intern J Neuroscience. 1991; **59**: 259-262.

76. Czeisler CA, et al. Bright light resets the human circadian pacemaker independant of the timing of the sleep-wake cycle.Science. 1986; **233**: 667-671.

77. Laakso ML, et al. One - hour exposure to moderate illuminance (500 lux) shifts the human melatonin rhythm. J Pineal Res. 1993; **15**: 21-26.

78. Lewy AJ, et al. Antidepressant and circadian phase-shifting effects of light. Science. 1987; **235**: 352-353.

79. Arendt J. Clinical perspectives for melatonin and its agonists. Biol Psychiatry. 1994; **35**: 1-2 (editorial)

80. Arendt J, Marks V.Physiological changes underlying jet lag. British Medical Journal. 1982; **284**: 144-146.

81 Maestroni GJM. The immunoneuroendocrine role of melatonin. J Pineal Res. 1993; **14**: 1-10.

82. Röjdmark S, et al. Nocturnal melatonin secretion in thyroid disease and in obesity. Clinical Endocrinology. 1991; **35**: 61-65.

83. Reiter RJ. The melatonin rhythm: both a clock and a calender. Experientia. 1993; **49**: 654-664.

84. Datta PC, King MG. Melatonin: Effects on brain and behavior. Neurosience & Biobehavioral Reviews. 1980; **4**: 451-458.

85. Woolley DW. In: The Biochemical Bases of Psychoses. New York / London. John Wiley and Sons. 1962: 57-101.

86. Dominic JA. Suppression of brain serotonin synthesis and metabolism by benzodiazepine minor tranquilizers. In: Barchas J, Usdin E.(ed). Serotonin and behavior. New York / London. Academic Press. 1973: 149-155.

87. Blackburn TP. 5-HT receptors and anxiolytic drugs. In: Marsden CA, Heal DJ, eds. Central Serotonin Receptors and psychotropic Drugs. London,UK: Blackwell Scientific Publications, 1992: 175-197.

88. Cooper JR, Bloom FG, Roth RH. In: The Biochemical Basis of neuropharmacology. 9th ed. New York. Oxford University Press; 1996: 352-499.

89. Saxena PR, De Vries P, Villalón CM. 5-HT_1-like receptors: a time to bid goodbye. TIPS. 1998; **19**: 311-316.

90. Lapin IP, Oxenkrug GF. Intensification of the central serotoninergic processes as a possible determinant of the thymoleptic effect. The Lancet. 1969: 132-136.

91. Mueller EA, Murphy DL, Sunderland T. Further studies of the putative serotonin agonist, m-chlorophenylpiperazine: Evidence for a

serotonin receptor mediated mechanism of action in humans. Psychopharmacology. 1986; **89**: 388-391.

92. Zohar J, et al. Serotonergic Responsivity in Obsessive-Compulsive Disorder. Arch Gen Psychiatry.1987; **44**:946-951.
93. Traber J, Glaser T. 5-HT_{1A} receptor-related anxiolytics. TIPS. 1987; **8**: 432-437.
94. Lucki I. Behavioral studies of serotonin receptor agonists as antidepressant drugs. J Clin Psychiatry. 1991; **52(12)**: 24-31.
95. Pranzatelli MR, Snodgrass SR. Serotonin-lesion myoclonic syndromes.II. Analysis of individual syndrome elements, locomotor activity and behavioral correlations. Brain Research. 1986; **364**: 67-76.
96. Pranzatelli MR, Schultz L, Snodgrass SR. High-dose clonidine motor syndrome: relationship to serotonin syndrome. Behavioral Brain Research.1987; **24**: 221-232.
97. Waeber C, et al. The Serotonin 5-HT_{1D} Receptor: A Progress Review. Neurochemical Research. 1990; **15(6)**: 567-582.
98. Koek W, Jackson A, Colpaert FC. Behavioral Pharmacology of Antagonists at 5-HT_2/ 5-HT_{1c} Receptors. Neuroscience & Biobehavioral Reviews. 1992; **16**: 95-105.
99. Olanzapine: Product Monograph. Oct 1996: 9-10.

100. Martin KF. Rhythms im neurotransmitter turnover: focus on the serotonergic system. Pharmac Ther Vol. 1991; **51**: 421-429.

101. Friedman R, Barrett RJ, Sanders-Bush E. Additional evidence that L-5-Hydroxytryptophan discrimination models a unique serotonin receptor. Psychopharmacology. 1983; **80**: 209-213.

102. Lucki I. 5-HT, Receptors and Behavior. Neuroscience of Biobehavioral Reviews. 1992; **16(1)**: 83-93.

103. Agren H, et al. Interacting neurotransmitter systems. A non-experimental approach to the 5HIAA-HVA correlation in human CSF. J Psychiat Res. 1986; **20(3)**: 175-193.

104. Trulsan ME, Jacobs BL. Behavioural Evidence for the Rapid release of CNS Serotonin by PCA and Fenfluraumine. European Journal of Pharmacology. 1976; **136**: 149-154.

105. Lee Sun L, et al. Fenfluramine increases pineal melatonin synthesis by organ cultures of rat pineal glands. Med Sci Res. 1993; **21**: 385-386.

106. Menendez-Pelaez A, Reiter RJ. Distribution of melatonin in mammalian tissues: The relative importance of nuclear versus cytosolic localization. J Pineal Res. 1993; **15**: 59-69.

107. Vanecek J. Mechanism of melatonin action. Physiol Res. 1991; **40**: 11-23.

108. Morgan PJ, et al. Melatonin receptors: Localization, molecular pharmacology and physiological significance. Neurochem Int. 1994; **24**: 101- 146.

109. Morgan PJ. Molecular signalling via melatonin receptors. Advances in pineal research. 1991; **5**: 191-197.

110. Zisapel N. Melatonin receptors revisited. J Neural Transm. 1988; **73**: 1-5.

111. Zisapel N, Laudon M. A novel melatonin antagonist affects melatonin-mediated processes in vitro and in vivo. European Journal of Pharmacology. 1987; **136**: 259-260.

112. Dubocovich MJ. Pharmacology and function of melatonin receptors. FASEB. 1988; **2**: 2765-2773

113. Morgan PJ, Williams LM. Central melatonin receptors: Implications for a mode of action. Experientia. 1989; **45**: 955-965.

114. Krause DN, Dubocovich MJ. Melatonin receptors. Annu Rev Pharmacol Toxicol. 1991; **31**: 549-68.

115. Namboodirk MAA, Sugden D, Klein DC. 5-Hydroxytryptophan elevates serum melatonin. Science. 1983; **221**: 659-660.

116. Wetterberg L. Melatonin in Humans. Physiological and Clinical Studies. Journal of Neural Transmission, Suppl.13, 1978: 289-310.

117. Minneman KP, Wurtman RJ. The pharmacology of the pineal gland. Annual review of pharmacology and toxicology. 1976; **16**: 33-51.

118. Zetin M, Potkin S, Urbanchek M. Melatonin in depression. Psychiatric Annals.1987; **17(10)**: 676-681

119. Anton-Tay F, et al. Brain serotonin concentration: Elevation following intraperitoneal administration of melatonin.Science.1968; **162**: 277-278.

120. Burton S, Daya S, Potgieter B. Melatonin modulates apomorphine - induced rotational behaviour. Experientia. 1991; **47**: 466-469.

121. Vriend J. Evidence for pineal gland modulation of the neuroendocrine- thyroid axis. Neuroendocrinology. 1983; **36**: 68-78.

122. Sandyk R, Kay SR, Gillman MA. The role of melatonin in the antipsycotic and motor-side effects of neuroleptics: a hypothesis. Intern J Neuroscience. 1992; **64**: 203-207.

123. Ekman A-C, Leppäluoto J, et al. Ethanol inhibits melatonin secretion in healty volunteers in a dose-dependent randomized double blind cross-over study. Journal of Clinical Endocrinology and Metabolism. 1993; **77**: 780-783.

124. Dubocovich ML. Pharmacological characterization of melatonin binding sites. Advances in pineal research.1991; **5**: 167-173.

125. Maes M, et al. Sleep disorders and anxiety as symptoms profiles of sympathoadrenal system hyperactivity in major depression. J Affect. Disord. 1993; **27**: 197-207.

126. Dixon JJ, de Monchaux C, Sandler J. Pattens of anxiety: The Phobias. British Psychological Society. 1957; **30**: 34-40.

127. Hamilton M. The assesment of anxiety states by rating. British Journal of Medical Psychology. 1959; **(32)**: 50-55.

128. Tyrer P, Owen RT, Cicchetti DV. The brief scale for anxiety: a subdivision of the comprehensive psyhopathological rate scale. Journal of Neurology, Neurosurgry and Psychiatry. 1984; **47**: 970-975.

129. Kendall, E. The Validity of Taylor's Manifest Anxiety Scale. Journal of Consulting Psychology. 1954; **18(6)**: 429-432.

130. Reppert SM, et al. Putative melatonin receptors in a human biological clock. Science. 1988; **242**: 78-89.

131. Guyatt G, et al. Determining optional therapy - randomized trials in individial patients. The New England Journal of Medicine. 1986; **314**: 889-892.