6-SULPHATOXYMELATONIN AS AN INDEX OF PINEAL FUNCTION IN HUMAN PHYSIOLOGY

By

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SUMMARY

A radioimmunoassay has been developed for the major metabolite of melatonin, 6-sulphatoxymelatonin (aMT6s). The assay is specific, sensitive and is direct for both urine and plasma samples. Classical validation procedures have been employed and the urinary assay has been compared with an established gas chromatographic/mass spectrometric assay.

A number of physiological studies have been carried out. A marked diurnal rhythm in both urinary and plasma aMT6s excretion was found which correlated closely with plasma melatonin values. There were large inter-individual variations in aMT6s excretion but its production was consistent for any one volunteer over a four-day period. No evidence was obtained for the episodic secretion of melatonin or aMT6s when blood samples were taken every thirty seconds for ten minutes. Administration of a peripheral β-blocker to volunteers, resulted in the abolition of the night-time rise in aMT6s excretion.

In a seasonal study, a small but highly significant change in the acrophase of the aMT6s rhythm was found during the year with a phase advance in summer compared to winter. Changes in aMT6s excretion with age were also investigated. Total urinary aMT6s excretion was relatively constant in forty children aged 2-20 years. However, when aMT6s excretion was expressed as a function of body weight highly significant age-related changes were observed. In ninety adult volunteers aged 20-80 years there was a significant decline in total 24h aMT6s excretion with age, with significantly lower excretion in elderly subjects. No relationships were found between total 24h aMT6s excretion and body weight, height or pineal calcification.

In addition to the above physiological studies, the pharmacokinetics of melatonin and aMT6s were investigated following the oral administration of melatonin to normal volunteers.
ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Dr Josephine Arendt, for her enthusiastic help and support in all aspects of this work. I also thank Professor Vincent Marks. I am grateful to Dr John Wright for his help with all the clinical projects. Many thanks to all my colleagues, past and present, in laboratories 25AY19 and 26AY19 for their technical, clinical and moral support. I am greatly indebted to my cohort of long-suffering volunteers for their generous donation of body fluids: without them none of this work would have been possible. I thank Miss Tracey Bakall for her expertise in the typing and word-processing of this manuscript, and Jolanta Bojkowska-Walmsley and Dr Peter Walmsley for proof-reading. I wish to thank the Wellcome Trust and the MRC for funding this work.

Finally, I wish to express my special gratitude to my parents for their unfailing love and support throughout my education.
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<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>aMT6G</td>
<td>6-glucuronide melatonin</td>
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<tr>
<td>aMT6s</td>
<td>6-sulphatoxymelatonin</td>
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<tr>
<td>APS</td>
<td>Adenosine-5'-phosphosulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic 3',5'-adenine monophosphate</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen bromide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>df</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DMBA</td>
<td>Dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>EC</td>
<td>Electron capture</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GC/MS</td>
<td>Gas chromatography/Mass spectrometry</td>
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<tr>
<td>h</td>
<td>Hour(s)</td>
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<tr>
<td>HIOMT</td>
<td>Hydroxyindole-O-methyltransferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>K</td>
<td>Equilibrium constant</td>
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<tr>
<td>L-Dopa</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
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<tr>
<td>NADPH</td>
<td>β-Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NAS</td>
<td>N-acetylserotonin</td>
</tr>
<tr>
<td>PAPS</td>
<td>3'-Phosphoadenosine-5'-phosphosulphate</td>
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<tr>
<td>PET</td>
<td>Positive emission tomography</td>
</tr>
<tr>
<td>PPi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>TG</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>vol</td>
<td>Volume</td>
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<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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Hard students are commonly troubled with gowts, catarrhs, rheuma, cachexia, bradypepsia, bad eyes, stone, and collick, crudities, oppilations, vertigo, winds, consumption, and all such diseases as come by over-much sitting: they are most part lean, dry, ill-coloured ... and all through immoderate pains and extraordinary studies. If you will not believe the truth of this, look upon great Tostatus and Thomas Aquainas' works; and tell me whether these men took pains.

Burton's *Anatomy of Melancholy*, p.i.s.2
1. Introduction

Herophilos (325-280BC) an anatomist at the University of Alexandria, is credited with being the first to discover the human pineal. It was Galen who later coined the term 'soma konoeides' or 'konareion' for the pineal, because in a number of species it is shaped like a pine cone (Kappers, 1981). The function of the pineal gland has remained an enigma and it is only within the last twenty years that its role has begun to be understood.

1.1 Phylogenetic Development

The pineal gland exists in all classes of vertebrates, however, it has undergone major changes in the course of phylogenetic development. In lower vertebrates the epiphyseal complex (pineal gland) can consist of two components, the pineal organ proper and the parietal organ (or third eye). In the pineal gland of these species the outer segments of the sensory cells resemble retinal cones and are directly photosensitive. In birds and reptiles the pineal is directly responsive to light, but it also has a secretory function. In the course of phylogenetic development the pineal organ has changed from a predominantly photoreceptive organ in lower vertebrates to a gland, which, in mammalian species is dependent on photic information conducted via the lateral eyes and the sympathetic nervous system and is probably entirely secretory in nature (Vollrath, 1981; Collin and Oksche, 1981; Reiter, 1983).

1.2 Anatomy

Embryologically, the mammalian pineal gland develops from the diencephalic ependymal area, usually between the habenular and posterior commissures. There are great inter-species differences with respect to pineal shape.
and position. In man, the pineal lies near the posterior margin of the diencephalic roof and occupies the depression between the superior colliculi of the mesencephalon. Proximally the organ is intimately related to the third cerebral ventricle an evagination of which penetrates into the organ as the pineal recess. The weight of the pineal is about 100mg in man and it is attached to the brain by means of a stalk. In humans, the pineal gland appears to be located outside of the blood-brain barrier (Arendt et al., 1983a) and pineal blood flow is one of the highest in the body (Vollrath, 1981).

1.3 Histology

The major cellular components of the pineal organ are the pinealocytes, which account for 80-90% of the cells. Mammalian pinealocytes consist of cell bodies with long cytoplasmic processes and they contain various organelles including dense-core vesicles, synaptic ribbons and synaptic spherules (Vollrath, 1981; Karasek, 1983; Vollrath, 1985). In addition to pinealocytes, glial cells are also present in varying numbers and astrocytes have been identified in some species. Occasionally, nerve cells, fibroblasts, mast cells, plasma cells and pigment-containing cells have been observed in the pineal glands of some species (Vollrath, 1981).

1.4 Innervation

The mammalian pineal gland is innervated by three groups of nerve fibres: sympathetic, commissural and parasympathetic fibres. The major anatomical, and physiologically most important innervation is by the sympathetic postganglionic fibres from the paired superior cervical ganglia (Kappers, 1960). The neural pathway by which light information reaches the pineal involves the retina, the retinohypothalamic tract, the hypothalamic suprachiasmatic nuclei (SCN) and the paraventricular nuclei. Nerve impulses then traverse fibres in the median forebrain
bundle and reticular formation to the intermediolateral cell column of the spinal cord. They then pass to the preganglionic adrenergic fibres of the sympathetic nervous system which synapse in the superior cervical ganglion. The pineal is finally innervated by sympathetic postganglionic fibres arising from the superior cervical ganglion (Moore, 1978). There is good evidence that the major aspects of pineal synthetic and functional activity are dependent upon the sympathetic innervation.

In addition to sympathetic fibres the mammalian pineal is also innervated by fibres from the posterior and habenular commissures (Korf and Moller, 1984). These fibres are thought to be peptidergic and electrophysiological evidence suggests that the direct connections from the central nervous system (CNS) to the pineal may be of functional significance. A parasympathetic innervation of the pineal has been shown (Kenny, 1961), however, there is little experimental evidence to suggest that these fibres have an effect on pinealocyte function (Vollrath, 1981).

1.5 **Secretory Products**

There are two main chemical classes of presumptive secretory substances in the pineal. These are the indoleamines and the peptides. The most intensely studied pineal product is the indoleamine, N-acetyl-5-methoxytryptamine (melatonin). There are several other indoles which have been identified in the pineal and may have a possible hormonal role; these include 5-methoxytryptophol, 5-methoxytryptamine, N-acetylserotonin (NAS) and β-carbolines. Many peptides are also present within the pineal and may have a physiological function (Pevet, 1985).

1.6 **Synthesis of Melatonin**

A major breakthrough in pineal research occurred when Lerner and colleagues (1958) isolated and identified the active principle which caused blanching of amphibian skin. This active principle was melatonin. Melatonin is synthesized
within the pineal gland from the amino acid, tryptophan. Tryptophan is taken up by
the pinealocytes and is hydroxylated to form 5-hydroxytryptamine (serotonin). The
enzyme, serotonin N-acetyltransferase, converts 5-hydroxytryptamine to NAS and
the specific enzyme, hydroxyindole-O-methyltransferase (HIOMT), converts NAS to
melatonin (Klein et al., 1981). Most synthesis and secretion takes place at night in a
normal environment. This synthetic pathway, together with that of other indoles, is
shown in Figure 1.1. The metabolism of melatonin is outlined in Section 1.17.

The pineal is not the only site of melatonin biosynthesis. Other reported
sites of synthesis are the retina, the Harderian gland (Pang et al., 1977) and the gut
(Raikhlin et al., 1975). In most mammals, pinealectomy leads to undetectable levels
of melatonin in the plasma (Arendt et al., 1980; Markey and Buell, 1982; Tetsuo et
al., 1982a; Neuwelt and Lewy, 1983) suggesting that any effects of melatonin
derived from non-pineal sites are local.

1.7 Control of Melatonin Synthesis

As suggested previously, light plays an important role in the function of
the pineal gland. The sequence of events which controls the synthesis of melatonin
is as follows. After the onset of darkness the sympathetic nerves fire with the
subsequent release of the neurotransmitter, noradrenaline. Noradrenaline interacts
with a post-synaptic β-receptor on the pinealocyte cell membrane. The adenyl
cyclase system is activated, an increase in cyclic 3',5'-adenosine monophosphate
(cAMP) follows which, via a cAMP dependent protein kinase, brings about
phosphorylation and increased protein transcription and translation. At night there
is a 70-100 fold increase in N-acetyltransferase activity (in the rat) (Klein and
Weller, 1970) and a small increase in HIOMT activity. These mechanisms result in
the marked diurnal variation in melatonin synthesis; see Figure 1.2 (Klein et al.,
Biosynthetic Pathway for Melatonin and Other Pineal Indoles

N-Acetylserotonin

Serotonin

5-Hydroxytryptophan

Melatonin

5-Hydroxyindole acetic acid

Tryptophan
Figure 1.2  Circadian Rhythms in Indole Metabolism in the Rat Pineal Gland

The shaded area represents the dark period of the lighting cycle (Klein et al., 1981)
Recent evidence has shown that the control of melatonin synthesis is more complicated than outlined above. Noradrenaline has been shown to act on \( \alpha_1 \) receptors to potentiate the effect of \( \beta \)-adrenergic stimulation on cAMP levels (Vanecek et al., 1985) and diacylglycerol has been suggested as a possible secondary messenger of this mechanism (Sugden et al., 1985). In addition to the adrenergic receptors, pinealocytes also contain receptor sites for glutamate, \( \gamma \)-amino-n-butyric acid (GABA), dopamine, benzodiazepines and serotonin. Various putative neurotransmitters, including GABA, vasoactive intestinal peptide (VIP) and substance P, have also been identified within the pineal gland. The possible function of these receptors and neurotransmitters within the pineal remains a mystery (Ebadi and Govitrapong, 1986).

1.8 Rhythmic Melatonin Production

Melatonin is produced with an endogenous rhythm which persists in complete darkness and has a free-running period in man slightly longer than 24h (Wever, 1986). The melatonin rhythm (at least in the rat and monkey) is generated by the SCN of the hypothalamus (Klein and Moore, 1979; Reppert et al., 1981; Klein et al., 1983), the central rhythm-generating centre of the brain (Moore and Klein, 1974). Light has a dual role in controlling the melatonin rhythm. Firstly, light entrains the rhythm to 24h (Lynch et al., 1978) and secondly, light of a suitable wavelength and intensity will suppress the production of melatonin (Lewy et al., 1980; Reiter, 1985; Bojkowski et al., 1987). Thus, in most species the duration of melatonin secretion correlates closely with the length of the night (Arendt, 1985).

1.9 Secretion of Melatonin

Melatonin has not been found in granules in the pineal gland and appears to exist in the cytosol (Klein et al., 1981). Whether melatonin is directly secreted into the cerebrospinal fluid (CSF) remains uncertain and evidence suggests that
under normal circumstances melatonin is secreted primarily into the systemic circulation (Arendt et al., 1977a; Rollag et al., 1978a; Brown et al., 1979; Withyachumnarnkul and Knigge, 1980).

1.10 Exogenous Factors Influencing Pineal Function

The function of the pineal has been described as that of a 'neuroendocrine transducer' (Wurtman and Axelrod, 1965). Unlike other endocrine organs the pineal is more or less directly influenced by environmental factors. The light-dark cycle is of primary importance in the control of pineal function (Sections 1.7 and 1.8). However, other environmental factors can also influence pineal function, particularly in lower vertebrates. These include temperature, humidity and nutritional factors (Vivien-Roels and Pevet, 1983; Vivien-Roels, 1985). Magnetic fields have also been shown to influence the pineal (Semm et al., 1980; Welker et al., 1983).

1.11 Pineal Gland and Mammalian Photoperiodism

The mammalian pineal gland is a major endocrine component in the regulation of photoperiodic responses. Many mammalian species exhibit seasonal changes in, for example, body weight, pelage and reproductive behaviour. In most species the environmental cue which controls these changes is the annual change in day-length or photoperiod. The regulation of the reproductive cycle has been studied in most detail and will be discussed further.

A particular photoperiod can have different effects in different species, for example, a short-day photoperiod is stimulatory in sheep (short-day breeder), whereas it is inhibitory in hamsters (long-day breeder). Pinealectomy or superior cervical ganglionectomy have been shown to disrupt breeding cycles; animals either continue permanently in one breeding condition or continue to exhibit cycles in
fertility, however, these changes are unrelated to photoperiod. These experiments demonstrate that the pineal gland is necessary for co-ordinating the reproductive system with photoperiod. Disruption of the circadian system by lesioning the SCN has also been shown to produce a similar effect (Rusak and Morin, 1976; Stetson and Watson-Whitmyre, 1976; Tamarkin et al., 1985a; Arendt, 1986a).

It has now been shown that the photoperiodic message is translated into an endocrine signal by the secretion of melatonin. Much early work on the role of melatonin made use of continuous release implants (Tamarkin et al., 1985a). It was not until it was realised that the timing of melatonin secretion was of crucial importance to its biological effects that the functions of melatonin were understood (Tamarkin et al., 1976). Various workers have demonstrated how the duration of the melatonin signal can vary with the prevailing photoperiod (Rollag et al., 1978b; Arendt, 1979; Arendt et al., 1981a; Skene et al., 1987). It is possible to administer melatonin in a variety of ways in order to mimic long or short photoperiods. Most recently the role of the melatonin signal in translating photoperiodic information has been elegantly demonstrated in experiments involving timed subcutaneous or intravenous infusions to pinealectomized hamsters or ewes (Carter and Goldman, 1983; Bittman et al., 1983). These experiments have suggested that it is the duration of the melatonin signal which is the critical parameter in bringing about reproductive changes related to photoperiod. The daily feeding of melatonin to intact ewes in the late afternoon from mid-June, in such a way as to mimic a winter night, will advance the onset of oestrus (Kennaway et al., 1982; Arendt et al., 1983). A similar effect is observed with constant release implants (Nowak and Rodway, 1985; English et al., 1986; Arendt, 1986a).
1.12 **Mechanism of Action of Melatonin**

Two theories are used to explain photoperiodic time measurement. One theory is the 'duration' hypothesis, whereby the day-length is used as an interval timer. Alternatively, there is evidence that circadian rhythms are involved in day-length perception. In the 'external coincidence' model, a circadian rhythm in photosensitivity has been proposed whilst in the 'internal coincidence' model it is suggested that the phase-relationships between different circadian rhythms are critical (Pittendrigh, 1972; Pittendrigh, 1976).

Most evidence suggests that the duration of the nocturnal melatonin pulse indicates photoperiod length (Bittman et al., 1983; Goldman, 1983; Goldman and Darrow, 1983; Hastings et al., 1985a). No conclusive proof is available as yet and it is important to bear in mind that other properties of the melatonin signal may be of importance in the action of the hormone. These properties may include: (i) the amplitude of the nocturnal peak, (ii) the phase relationship of the peak to the light-dark cycle or to other physiological rhythms, (iii) the duration of nocturnal secretion, (iv) the total amount secreted (Goldman, 1983).

1.13 **Sites of Action of Melatonin**

The means by which melatonin exerts its effects have yet to be identified. Most workers have concentrated on possible sites of action in the brain. A specific area of the anterior hypothalamus involved with photoperiodic responses has been identified (Hastings et al., 1985b). Studies using putative melatonin binding sites have in the past yielded equivocal results. Recently, I$_{125}^i$iodomelatonin has become available and binding sites have been identified in the brain (Laudon and Zisapel, 1986; Laudon and Zisapel, 1987) including sites in the SCN and the median eminence (Vanecek et al., 1987). A specific melatonin antagonist which appears to be active in vitro and in vivo has been developed and this may prove to be a useful tool for elucidating this problem (Zisapel and Laudon, 1987).
In addition to a possible action on the brain, melatonin may also have a direct action on peripheral sites such as the ovaries. It has also been suggested that it may have a feedback effect on the pineal to modify the release of other compounds.

1.14 Other Target Organs of Pineal Substances

Many workers have studied the effect of the pineal gland on other target sites and endocrine glands, however, the results are largely contradictory and no convincing evidence has emerged. An interrelationship between the pineal and the thyroid gland has been suggested (Vriend, 1983). The pineal exerts a largely inhibitory influence on thyroid function though some studies have suggested a stimulatory effect (Vriend, 1983).

Interrelationships with other glands, including the parathyroids, the adrenals and the pancreas have been reported (Vollrath, 1981), however, no clear picture has emerged and a detailed discussion of this is beyond the scope of this thesis.

1.15 Circadian Rhythms and the Pineal

Many body rhythms are capable of self-sustaining oscillations under constant environmental conditions, ie, they are endogenous rhythms. Under these constant conditions many circadian rhythms exhibit a periodicity which does not correspond exactly to 24h. The main environmental signal that 'entrains' the endogenous oscillations to the 24h day is light. The anatomical pathway which is involved with the entrainment of circadian rhythms involves the eyes, the retinohypothalamic tract and the SCN. The dominant central circadian pacemaker is located in the SCN of the hypothalamus (Moore and Klein, 1974; Rusak and Zucker, 1979). An island of SCN isolated from its neural surroundings in the rat still shows a persistent circadian rhythm of neural activity while similar rhythmicity no longer occurs elsewhere in the brain (Inouye and Kawamura, 1979).
The exact role of the pineal gland in the mammalian circadian system is not resolved at present. In some birds there are at least two clock systems, one lies in the SCN and regulates the pineal via the usual multi-synaptic pathway through the superior cervical ganglion; the other in, for example, house sparrows, lies in the pineal itself (Zimmerman and Menaker, 1979). This 'clock' localized in the pineal is present in other vertebrates, for example, certain species of iguanid lizards (Underwood, 1977; Menaker and Wisner, 1983); however, it is absent in mammals (Follet et al., 1985).

There is nevertheless some evidence suggesting a role of the pineal gland in mammalian circadian rhythms. Pinealectomy leads to a more rapid re-entrainment of certain circadian rhythms in rats following an acute phase shift (Quay, 1970). In rodents the pharmacological administration of melatonin has been shown to have entraining or 'zeitgeber' properties (Redman et al., 1983; Murakami et al., 1983). These studies have been extended to humans and preliminary results suggest that melatonin can help alleviate the symptoms of jet-lag, possibly via its entraining properties (Arendt et al., 1987).

1.16 Melatonin in Humans

Although a role for the pineal and melatonin has been established in seasonal breeders there is as yet little evidence for a major physiological role of the human pineal gland. To investigate the function of the human pineal gland most workers have concentrated on measuring the physiological and pathological variations in the concentrations of melatonin and its principal metabolites.

1.16.1 Variations in Circulating Melatonin Levels

Circulating plasma melatonin has been shown to be a good index of pineal function. Following pinealectomy, plasma levels are undetectable (Neuwelt and Lewy, 1983). During the day circulating levels are low and increase at night to reach a maximum around 0300h (Arendt et al., 1977a).
### 1.16.1.1 Light

Experiments on subjects in environmental isolation have shown that bright light (>2500 lux) entrains human circadian rhythms (Wever et al., 1983). Light also has a suppressive effect on the nocturnal production of melatonin. Lewy and colleagues (1980) demonstrated that light of an intensity of 2500 lux would suppress melatonin production in humans, whereas light of an intensity of 500 lux was ineffective. Recent work has shown that when administered under the right conditions, light of an intensity as low as 300 lux will suppress melatonin production in man (Bojkowski et al., 1987).

### 1.16.1.2 Menstrual Cycles

When serum samples were taken at 0900 h from five women through the menstrual cycle, melatonin was found to fall during the follicular phase and rise to a peak during the luteal phase (Wetterberg et al., 1976). Whether these changes represent differences in the phase or the amplitude of the melatonin rhythm is uncertain. Measuring 6-sulphatoxymelatonin (aMT6s) Fellenberg and colleagues reported no variation in excretion throughout the menstrual cycle (Fellenberg et al., 1980; Fellenberg et al., 1982). Recently, possible phase-shifts and/or an increase in the amplitude of the rhythm during the luteal phase have been reported (Hariharasubramanian and Nair, 1985; Webley and Leidenberger, 1986). Clearly, more evidence is required before the possible variations in melatonin secretion during the menstrual cycle are established.

### 1.16.1.3 Drug Effects

A number of drugs have been shown to affect plasma melatonin concentrations. β-Blocking drugs decrease melatonin concentrations (Section 3.5). Drugs which increase noradrenaline and/or serotonin levels, such as the anti-depressant, desipramine, and the monoamine oxidase (MAO) inhibitor,
tranylcypromine, increase plasma melatonin levels (Wirz-Justice et al., 1980; Franey et al., 1986; Oxenkrug et al., 1986). The effect of desipramine was shown not to be due to the inhibition of the hepatic metabolism of melatonin. Similarly, the phosphodiesterase inhibitor, rolipram, which increases cAMP levels, increased urinary aMT6s excretion from 2400-0600h (Checkley et al., 1987). Not all MAO inhibitors have been found to increase plasma melatonin. Two- to three-fold elevations in daytime plasma melatonin occurred in depressed patients treated with clorgyline or tranylcypromine but not those treated with the MAO-B inhibitor, deprenyl (Murphy et al., 1986). Conflicting results have been obtained with the $\alpha_2$ agonist, clonidine. Lewy et al., (1986) reported a decrease in plasma melatonin levels, however, this has not been confirmed by Grasby and Cowen (1987a). The neuroleptic drug, chlorpromazine, was shown to increase serum melatonin concentrations, however, this was not due to a stimulation of the pineal but due to inhibition of hepatic metabolism (Smith et al., 1979). The peptide, destyrosine gamma endorphin, increased the urinary excretion of melatonin (Claustrat et al., 1981). The effects of a variety of other drugs and hormones including L-3,4-dihydroxyphenylalanine (L-dopa), luteinizing hormone, thyroid-stimulating hormone and 5-methoxypsoralen on melatonin levels in man have been studied (for a review see Vaughan, 1984; Souetre et al., 1987), but no consensus of opinion has emerged as to their effects as yet.

1.16.1.4 Depression

Various workers have suggested that melatonin levels are decreased in patients with depressive disorders (for a review see Grasby and Cowen, 1987b). Many of these studies have used a limited number of subjects, and control subjects and patients have not been well-matched with respect to a number of variables. A recent study has reported no differences in the plasma melatonin concentrations between patients with depression and well-matched control subjects (Thompson et al., 1988). The exact role of the pineal gland in depression needs to be resolved with further studies.
A great deal of interest was generated by the possible connection of the pineal with the psychiatric condition known as 'seasonal affective disorder'. This small group of patients suffer depressive episodes during the winter months. Lewy and Rosenthal considered that 'seasonal affective disorder' was a photoperiod related phenomenon, because the behavioural changes observed were so dependent on the season, and treated the symptoms with bright light given as a summer skeleton photoperiod (Lewy et al., 1982; Rosenthal et al., 1984). The intensity of light used was that required for suppression of melatonin secretion (>2500lux). As melatonin conveys photoperiodic information in animals via its duration of secretion, it was logical to presume that bright light compression of the melatonin profile to a summer pattern would be an effective treatment. In fact, recent evidence suggests that melatonin is not of major importance in seasonal affective disorder as light treatment given in the middle of the day is an effective treatment and the suppression of melatonin secretion by atenolol is not always an effective treatment (Wehr et al., 1986; Rosenthal et al., 1986). Recently, Wirz-Justice and colleagues (1986) have reported that both dim yellow light (250lux) and bright white light (2500lux) treatments were effective. This whole area is currently very controversial with the use of evening bright light or morning bright light being variously reported as more effective. Notably, Lewy et al., (1987) have proposed that morning bright light treatment corrects the timing of the melatonin rhythm, delayed in their patients, via its phase-advancing properties.

1.16.1.5 Cancer

For many years a relationship between the pineal gland and malignant growth has been suggested, however, the results of early experiments were contradictory (Starr, 1970; Lapin, 1979). Results of more recent experiments have shown that tumour growth can be influenced by both photoperiod and time of day of melatonin administration (Bartsch and Bartsch, 1981; Stanberry et al., 1983). In
1981, Tamarkin et al., provided compelling evidence that pinealectomy increased and melatonin dramatically decreased the incidence of dimethylbenz(a)anthracene (DMBA)-induced mammary tumours in the rat. Recently, both melatonin and the conjugated metabolites of melatonin have been shown to inhibit the growth of cancer cells in vitro (Tamarkin et al., 1985b; Leone et al., 1987a).

Clinical evidence has also suggested a relationship between pineal function and breast cancer and possibly other malignancies. The best evidence demonstrated low plasma melatonin levels in hormone-dependent breast cancer, with a correlation between plasma melatonin concentration and tumour oestrogen receptor concentration (Tamarkin et al., 1982a). However, there was no correlation between melatonin levels and other hormones studied, nor with risk status for breast cancer (Danforth et al., 1985). The exact role of the pineal in endocrine-related cancers remains to be clarified in future studies.

1.16.1.6 Seasonal Variations

Although it is well established that the pattern of melatonin secretion can vary throughout the year in many animal species, relatively few studies have examined the annual changes in human melatonin secretion. The studies which have been carried out have been largely incomplete due to methodological problems.

Some studies have only used one or two sampling points throughout the 24h period. Annual variations have nevertheless been found with peaks in summer and winter and troughs in spring and autumn (Arendt et al., 1977b; Arendt et al., 1979) and peaks in December and May with a nadir in August (Martikainen et al., 1985). Similar results to Arendt and colleagues were reported by Birau et al., (1981) when blood samples were taken at six-hourly intervals for 24h from one hundred and seventy four subjects. The same subjects, however, were only studied on two occasions.
Touitou et al., (1984) examined plasma melatonin at different times of the year, in different age groups and in patients with senile dementia. The only consistent result was the stability of the peak-time for plasma melatonin. Other workers have found no seasonal differences in total melatonin production at different times of the year (Beck-Friis et al., 1984; Griffiths et al., 1986; Sack et al., 1986).

In studies with hourly blood sampling over 24h at two different times of the year (winter and summer), the total melatonin production was the same. However, a phase-shift was observed. Illnerova et al., (1985) reported a phase advance of the summer profile by 1.5h compared to the winter profile in urbanized man in Czechoslovakia, while in the Antarctic there was a 2h phase advance of the summer profile relative to the winter profile (Arendt and Broadway, 1986). The data of Beck-Friis et al., (1984) also suggests a summer phase advance. Employing the RIA described in this thesis, Kennaway and Royles (1986) also investigated the circadian rhythm in aMT6s at two time points (winter and summer) and confirmed a phase advance of the summer profile.

1.16.1.7 Puberty

Pinealectomy hastens pubertal development in a number of species, whereas exogenous melatonin administration can delay sexual maturation in experimental animals (Lang, 1986). When lambs were implanted with melatonin there was a significant delay in the onset of puberty (Kennaway and Gilmore, 1984).

The role of the pineal and of melatonin in human sexual development remains unclear. Two lines of evidence suggest a relationship between pineal function and human puberty. Around 1900 Heubner reported a case of precocious puberty in a boy with a pinealoma and this led Marburg to suggest that the human
pineal gland was an endocrine organ which inhibited the function of the hypothalamus and thus the development of the reproductive system (Kappers, 1981). Destructive pineal tumours have been associated with precocious puberty and delayed puberty has been observed in boys with hyperactive pineal tumours (Kitay, 1954). Most of the tumours are not pinealocytes but dysgerminomas secreting human chorionic gonadotrophin (Sklar et al., 1981) and therefore it is difficult to determine the exact role of the pineal gland.

Observations on the changes in circulating melatonin levels from infancy to adulthood also indicate that there may be a relationship between the pineal and sexual maturation in humans. A great deal of controversy, however, remains in this area largely due to methodological problems. In many of the studies carried out comparing plasma melatonin levels in prepubertal and pubertal children, blood samples were collected during the daytime yielding data of questionable value (Silman et al., 1979; Lenko et al., 1982; Gupta et al., 1983).

Silman and colleagues (1979) reported a significant difference between the daytime plasma melatonin levels of prepubertal and pubertal boys. However, these findings have never been replicated by other workers. Contradictory results have also been reported in studies which have used night-time blood sampling. Workers have found no changes in the plasma melatonin profile with pubertal development (Ehrenkranz et al., 1982; Tamarkin et al., 1982b; Sizonenko et al., 1985) or a decrease with sexual maturation (Gupta et al., 1983; Waldhauser et al., 1984a; Attanasio et al., 1985).

No change in the excretion of urinary melatonin with pubertal development has been reported (Sizonenko et al., 1985), whereas Penny (1982) found an increase in melatonin excretion during puberty. An increase in 6-
hydroxymelatonin excretion in Tanner Stage II girls was reported by Tetsuo and colleagues (1982b), however, this particular group of subjects continued to show high levels throughout their development (unpublished observation, Tamarkin, 1985). No other changes in 6-hydroxymelatonin excretion during development were observed.

In this study no correction for differences in body weight was made. Studies on children with abnormal pubertal development have also yielded contradictory results (Tamarkin et al., 1982b; Lissoni et al., 1983; Attanasio et al., 1984).

To date, the most extensive studies which have been carried out with well validated methodology are those of Waldhauser and colleagues (Waldhauser and Steger, 1986). Plasma samples were taken from two hundred and eighty subjects of all ages and no variation in daytime melatonin levels with age was found, whereas night-time levels were low during the first few months of life, increasing to peak values between 1-3 years and then progressively dropping until early adulthood.

1.16.1.8 Age and Body Parameters

A number of variables including age, sex, body weight and body height, have been reported to influence melatonin production in healthy subjects. In addition to the changes in melatonin production during childhood (see Section 1.16.1.7) melatonin levels have also been demonstrated to decrease with age in adults in plasma (Touitou et al., 1981; Iguchi et al., 1982b; Beck-Friis et al., 1984; Nair et al., 1986; Waldhauser and Steger, 1986), CSF (Brown et al., 1979) and in urine (Sack et al., 1986).

The published reports on the relationship between melatonin production and body height/weight in adults have been contradictory. In a population which included both normal volunteers and subjects with major affective disorders, Beck-Friis and colleagues (1984) found a significant negative correlation between plasma melatonin and height. In contrast, Sack and co-workers (1986) with a population of
sixty normal volunteers (age range 22-94 years), reported a significant positive correlation between urinary 6-hydroxymelatonin excretion and height, which was no longer significant when height was adjusted for age.

A positive correlation between plasma melatonin and weight was reported by Arendt et al., (1982) in women and by Ferrier et al., (1982) in men. Sack et al., (1986) found no relationship between 6-hydroxymelatonin excretion and weight. These workers also found differences between the sexes, with a negative association between 6-hydroxymelatonin levels and obesity in women and an opposite association for men. Recently, in an extensive and well-controlled study Nair and colleagues (1986) reported a significant decrease in melatonin levels with age but no relationship between melatonin levels and body height, body weight or obesity index.

1.16.2 Administration of Melatonin

Melatonin was administered to normal and epileptic subjects intravenously, in doses ranging from 0.25mg/kg - 1.25mg/kg body weight, and increased sleep and a feeling of well-being and elation were found (Anton-Tay et al., 1971). When two Parkinsonians received 1.2g melatonin orally for four weeks, a marked improvement in their symptoms was detected. No undesirable effects were observed (Anton-Tay et al., 1971). However, later studies on the administration of melatonin to Parkinson's patients demonstrated that daily oral doses (starting with 100mg per day and increasing to 1g per day in divided doses) had no effect on Parkinsonian disabilities. Melatonin was well tolerated and transitory sedation was the only noticeable adverse reaction (Shaw et al., 1973).
Following the daily feeding of 1g of melatonin for 25-30 days to five human subjects with hyperpigmented skin, skin lightening was observed in one patient with untreated adrenogenital syndrome. There was no effect on patients with idiopathic hyperpigmentation (Nordlund and Lerner, 1977). Melatonin appeared to depress the levels of serum luteinizing hormone and all subjects noted increased drowsiness. No evidence of toxicity was observed (Nordlund and Lerner, 1977).

Other workers have also shown that pharmacological doses of melatonin induce sedation and sleep (Cramer et al., 1974; Vollrath et al., 1981) and affect prolactin and growth hormone secretion (Smythe and Lazarus, 1974; Waldhauser et al., 1987). When 240mg of melatonin were given orally to normal volunteers over a 2h period a decrease in self-reported alertness and increased sleepiness were observed. The effects were brief and there was no effect on sustained free motor performance, memory or visual sensitivity (Lieberman et al., 1984). Daily feeding of 2mg of melatonin in the late afternoon for one month had no effect on growth hormone, luteinizing hormone, testosterone, thyroxine and cortisol whereas the timing of prolactin secretion was slightly modified (Wright et al., 1986). The same dosing regimen significantly advanced the 'self-rated fatigue' rhythm and also phase advanced its own endogenous rhythm (Arendt et al., 1984; Arendt et al., 1985b). Recently, pharmacological doses of melatonin have been shown to alleviate the symptoms of jet-lag and it has been suggested that this may be due to the hypnotic and synchronizing properties of melatonin (Arendt et al., 1986; Arendt et al., 1987).
1.17 **General Metabolism**

The major pathway for melatonin metabolism was elucidated by Kopin and colleagues (Kopin et al., 1960; Kopin et al., 1961). Melatonin, labelled on either the methoxy group or the N-acetyl group, was injected into the tail veins of rats, and urine and faeces were collected. 60-70% of the radioactivity was recovered in the urine and 15% in the faeces. The urinary metabolites were separated and quantitated with paper chromatography. The major metabolite, accounting for 70-80% of the radioactivity, was the sulphate conjugate of 6-hydroxymelatonin (6-sulphatoxymelatonin aMT6s) whereas the glucuronide conjugate of 6-hydroxymelatonin (aMT6G) represented only 5% of the administered radioactivity. An unidentified metabolite represented 12% of the radioactivity (Figure 1.3). The administration of radioactive melatonin to mice showed that melatonin is rapidly metabolized (half-life of approximately 2 min) and a small portion is bound and retained (half-life of about 35 min). An enzyme which hydroxylated melatonin in position six was found in rat liver microsomes (Kopin et al., 1961).

![Figure 1.3 Chromatogram of the Radioactive Metabolites of $^{3}$H Melatonin](image)

Percentages of excreted radioactivity of various metabolites are shown at top of bars.
(Kopin et al., 1961)
The above results were confirmed by Kveder and McIsaac (1961). After administration of $^{14}$C$^\text{C}$ melatonin to rats, 70% and 20% of the activity was excreted in the urine and faeces, respectively. Analysis of radioactive chromatograms showed that aMT6s accounted for 55%, the aMT6G for 30%, and other minor metabolites for 15% of the activity in the urine. The major metabolic product of melatonin was identified as aMT6s by colour reactions, sulphate analysis, and by its hydrolysis to the free phenol. 5-Methoxyindoleacetic acid was found to be a metabolite of melatonin, but to account for only 2% of the dose. These authors also reported the presence of at least two metabolites which did not react with Ehrlich's reagent.

A later study confirmed that the major metabolite of melatonin in rat urine was indeed aMT6s (Taborsky et al., 1965). In addition, these workers reported that the third unidentified metabolite in the original study of Kopin et al. (1961) was free 6-hydroxymelatonin.

The first study on the metabolism of exogenous melatonin in humans was carried out by Jones et al., (1969). Radioactive melatonin was administered to five male patients. Three of the volunteers were schizophrenic, one had Huntington's Chorea and the fifth had brain damage. Analysis of the radioactivity in the urine showed that the general pattern of metabolism was similar in humans to that in rats. The major melatonin metabolites were aMT6s (59-79% of the recovered radioactivity) and aMT6G (13-29% of the recovered radioactivity). Some free 6-hydroxymelatonin was also excreted together with an unidentified acidic metabolite.

A different metabolic pathway of melatonin exists in the CNS (Hirata et al., 1974). A partially purified enzyme from rabbit brain was shown to catalyse the cleavage of the indole ring, yielding N-acetyl-N-formyl-5-methoxykynurenamine,
which was further degraded to N-acetyl-5-methoxykynurenamine by the action of formidase. When $[^{14}\text{C}]$ melatonin was injected intracisternally, the major metabolite in rat brain was shown to be N-acetyl-5-methoxykynurenamine. After intravenous administration of radioactive melatonin to rats, 15% of the total radioactivity was recovered as N-acetyl-5-methoxykynurenamine.

The deacetylation of melatonin to form 5-methoxytryptamine has been confirmed as a very minor metabolic pathway (Rogawski et al., 1979; Beck and Jonsson, 1981). The in vitro incubation of liver slices with melatonin led to a 0.3-0.8% conversion to 5-methoxytryptamine. Rapid deamination follows and the final product is 5-methoxyindoleacetic acid.

Gas chromatography/mass spectrometry (GC/MS) was used by Leone and Silman (1984) to identify a new melatonin metabolite. When deuterated melatonin was administered to rats, GC/MS analysis of the urine showed the presence of deuterated 6-hydroxymelatonin and deuterated NAS, proving that NAS was metabolized directly from melatonin. The demethylation of melatonin to form NAS was also reported to occur in man, following the intravenous injection of deuterated melatonin (Young et al., 1985). The NAS metabolite is excreted as either the sulphate or glucuronide conjugate. The importance of this demethylation pathway for the metabolism of endogenous melatonin remains to be established.

Recently Vakkuri and colleagues (1987) have identified a novel metabolite of melatonin. This metabolite was isolated by chloroform extraction and reverse phase high performance liquid chromatography (HPLC) from human and rat urine after administration of synthetic melatonin. This metabolite was shown to be 1-acetyl-1,2,3,3a,8,8a-hexahydro-8a-hydroxy-5-methoxypyrrolo 2,3-6 indole, a cyclic isomer of 2-hydroxymelatonin, by means of mass and proton magnetic resonance.
Studies with tritiated melatonin showed that this metabolite accounted for 5% of the urinary metabolites of melatonin in the rat. It remains to be determined whether this metabolite is formed from endogenously produced melatonin.

The metabolism of melatonin is summarized in Figure 1.4.

1.18 Sulphate Conjugation

Drug metabolism (and the metabolism of endogenous compounds) is normally divided into two phases: phase I (or functionalization reactions) and phase II (or conjugative reactions). Aromatic hydroxylation is a very common phase I reaction. The hydroxylation of melatonin yields a final product, 6-hydroxymelatonin, containing a chemically reactive functional group (-OH) which is, in the correct chemical state to be acted upon by the phase II or conjugative enzymes. In general, the main function of phase I metabolism is to prepare a compound for phase II metabolism.

Phase II metabolism (conjugation) involves a diverse group of enzymes (for example, UDP-glucuronyltransferases, UDP-glycosyltransferases, sulphotransferases and glutathione-S-transferase) which act on compounds to produce water-soluble products which can be excreted in urine or bile. The major conjugation pathway for melatonin is sulphation. 3'-Phosphoadenosine-5'-phosphosulphate (PAPS) is required as an 'active' donor. The production of PAPS from adenosine triphosphate (ATP) and inorganic sulphate is illustrated below:

$$\text{SO}_4^{2-} + \text{ATP} \xrightarrow{\text{ATP-sulphurylase}} \text{Adenosine-5'-phosphosulphate} + \text{PPi}$$

(APS) Inorganic phosphate

$$\text{APS} + \text{ATP} \xrightarrow{\text{APS-kinase}} \text{3'-phosphoadenosine-5'-phosphosulphate} + \text{ADP}$$

(PAPS) Adenosine diphosphate
The Metabolism of Melatonin

(a) cyclic isomer of 2-hydroxymelatonin

2-3 hexahydro-7-acetyl-5-hydroxy-5-methoxytryptophol

10 = 1-acetyl-1,2,3,7,9,9'-hexahexafluoro-7-acetyl-5-hydroxy-5-methoxytryptophol

N-acetylsomatostatin = 6

Z-methoxy-5-methoxytryptamine

acetyl-N-formyl = 7

N-acetylsomatostatin = 6

5-methoxytryptamine = 5

6 = 5-methoxy-7-indolethionic acid

6-gluconolactone melatonin

6-sulfatoxymelatonin = 3

2 = 6-hydroxymelatonin

1 = melatonin
Sulphation of 6-hydroxymelatonin occurs by its interaction with PAPS in the presence of cytosolic sulphotransferase. For many endogenous compounds there is competition between the glucuronidation pathway and the sulphation pathway. In general, sulphate conjugation predominates at low substrate concentrations and glucuronide conjugation at high substrate concentrations (Gibson and Skett, 1986).

1.19 Biological Activity of Melatonin Metabolites

Very few studies have been carried out on the biological activity of melatonin metabolites. 6-Hydroxymelatonin was reported to be the only indole besides melatonin to produce a specific effect at a low dose on amphibian melanophores (Quay and Bagnara, 1964; Quay, 1968). Blinding adult male golden hamsters led to involution of the testes and accessory sex organs and to a regression in pituitary prolactin levels within eight weeks. Subcutaneous implantations of either melatonin or 6-hydroxymelatonin were equipotent in preventing these changes, whereas NAS and 5-hydroxytryptophol were ineffective (Reiter and Vaughan, 1975). Another study demonstrated that five indoles, including 6-hydroxymelatonin and melatonin, decreased both the absolute and the relative ovarian and uterine weights of immature female mice pre-treated with human chorionic gonadotrophin (Vaughan et al., 1976a). N-Acetyl-5-methoxykynurenamine has also been shown to be biologically active. This melatonin brain metabolite was shown to be ten times more potent in inhibiting $[^3H]$ diazepam binding to rat brain synaptosomal membranes than melatonin (Marangos et al., 1981). This metabolite was also demonstrated to have a marked and time-dependent inhibiting effect on prostaglandin biosynthesis in vitro, whereas melatonin was ineffective (Kelly et al., 1984).

The influence of six pineal indoles on the sexual maturation of the male rat was investigated by Lang and colleagues (1985). In addition to melatonin, only 5-
methoxytryptamine and 6-hydroxymelatonin inhibited the neuroendocrine axis during sexual maturation. These indoles were active when injected in the late afternoon and were inactive when injected in the morning. NAS, serotonin, 5-hydroxytryptophol and 5-methoxytryptophol were all inactive. The activity of 5-methoxytryptamine was perhaps partly due to its conversion to melatonin in vivo. When injected in the late afternoon the potency of 6-hydroxymelatonin was approximately one tenth that of melatonin.

Recent evidence has emerged that the conjugated metabolites of 6-hydroxymelatonin are also biologically active. The potency of various indoles in inhibiting the growth of two human ovarian carcinoma cell lines was tested (Leone et al., 1987a). Melatonin was shown to inhibit ovarian tumour cell growth in vitro. The 6-hydroxylated melatonin metabolites were all more potent than melatonin. The most active compounds were 6-hydroxymelatonin and aMT6G. This is the first evidence to show that the conjugated metabolites of melatonin are biologically active.
ASSAY OF MELATONIN

1.20 **Bioassay**

The first methods used to measure melatonin were bioassays which relied upon the ability of melatonin to aggregate melanin granules within the dermal melanophores of amphibian skin, thereby lightening the skin (McCord and Allen, 1917). Lerner and colleagues used a bioassay to follow the isolation of melatonin from bovine pineal glands (Lerner et al., 1958; Lerner and Wright, 1960). The bioassay was sensitive and apparently specific (Quay and Bagnara, 1964). Ralph and Lynch (1970) developed another bioassay using tadpoles. The lower sensitivity limit of this assay was 100pg/ml. Pineal melatonin could be measured directly. However to measure circulating melatonin, serum had to be extracted and urine samples were concentrated by Amberlite XAD-2 column chromatography. A diurnal rhythm of a 'melatonin-like substance' in plasma and urine in normal volunteers was demonstrated (Pelham et al., 1973; Lynch et al., 1975). This assay was also used to demonstrate that the urinary melatonin rhythm was not abolished by 28h of constant artificial light (Jimerson et al., 1977).

The bioassays suffered from the disadvantages that animal breeding facilities were required, they involved tedious and lengthy extractions, and they had poor precision and specificity. Residual amounts of organic solvents used in extraction were reported to interfere with the assays (Wilson et al., 1977).

The bioassays have been largely replaced by more sensitive, specific and convenient radioimmunoassays (RIAs) (Section 1.22). Rollag and White (1986) have recently applied the tadpole bioassay to screen for high affinity melatonin receptor agonists and antagonists. These authors have also started to investigate the molecular mechanism of the action of melatonin, by testing melatonin interaction with the components of the hormone-sensitive adenylate cyclase complex in a *Xenopus* dermal melanophore bioassay (White et al., 1987).
1.21 **Spectrophotofluorometry**

All 5-hydroxy and 5-methoxyindoles have a distinctive fluorescence in strong mineral acid at 540-550nm when activated at 295nm (Udenfriend et al., 1955). A selective extraction procedure was necessary to separate 5-hydroxyindoles from 5-methoxyindoles. This approach was used by Quay (1963) to measure melatonin and a variety of different indoles, and to demonstrate a circadian rhythm in rat pineal content (Quay, 1964). Maickel and Miller (1968) enhanced the fluorescence of indole derivatives by reaction with O-phthalaldehyde. The sensitivity of the method was sufficient only to measure pineal gland melatonin or exogenously administered melatonin (Ozaki et al., 1976). Dreux (1969) reported a method for the measurement of melatonin in urine or pineal gland.

The above assays had problems with specificity, since closely-related indoles could not be separated from melatonin, and extraction solvents, including chloroform, were reported to interfere (Wilson et al., 1977). The fluorometric assays were largely replaced by RIAs which were more specific and sensitive.

1.22 **Radioimmunoassay**

1.22.1 **Principle of RIA**

In the late 1950s, patients treated with insulin were found to have developed antibodies which could bind both circulatory and iodinated insulin, in vivo and in vitro (Berson et al., 1956). Both labelled and unlabelled hormone would compete for the antibody binding sites. This competitive binding formed the theoretical basis for the principle of RIA, described by Yalow and Berson in 1960.

To perform a RIA the following are required: an antiserum binding the ligand with high specificity, standard cold ligand, labelled ligand and a method to separate bound and free ligand without disrupting the equilibrium. To assay
melatonin by RIA, an antibody specific for melatonin (Ab) and a radioactive ligand (Ag*) which can compete with melatonin (Ag) for the antibody binding sites, are required. If the number of antibody binding sites and the amount of radioactive antigen are kept constant the amount of radioactivity associated with the antibody is a quantitative function of the concentration of non-radioactive melatonin present in each tube.

<table>
<thead>
<tr>
<th>Labelled Antigen</th>
<th>Specific Antibody</th>
<th>Labelled Antigen Antibody Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag* (free)</td>
<td>+ Ab</td>
<td>Ag* - Ab (bound)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ag - Ab</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unlabelled Antigen-Antibody Complex</td>
<td></td>
</tr>
</tbody>
</table>

When equilibrium is reached, bound and free ligand are separated. Either the bound radioactivity or the free radioactivity is counted. The concentration of unknown samples is determined by comparing the radioactivity measured to that observed in a standard curve where increasing known amounts of cold ligand have been added.

1.22.2 Production of Antiserum

A molecule as small as melatonin (molecular weight 232) is not antigenic. To elicit an immune response, and to induce antibody formation, melatonin has to be conjugated to a large carrier protein. A number of different methods have been employed to couple melatonin or one of its analogues to different carrier molecules (see Table 1.1). The particular immunogen used plays an important role in determining the specificity of the antiserum produced (Brown et al., 1983).
<table>
<thead>
<tr>
<th>Method</th>
<th>Validation</th>
<th>Extraction Method</th>
<th>Label Conjugation Method</th>
<th>Protein Derivative</th>
<th>Background</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>Petrolatum Ether + Chloroform</td>
<td>Mode</td>
<td>Melamed &amp; tyramine</td>
<td>Melated BSA</td>
<td>BSA, HSA</td>
<td>1984</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Chloroform</td>
<td>Direct</td>
<td>Melated [231]</td>
<td>Melated melatonin</td>
<td>BSA, HSA</td>
<td>1987</td>
</tr>
<tr>
<td>HPLC</td>
<td>Chloroform</td>
<td>Heterogenous</td>
<td>BSA, HSA</td>
<td>Melated melatonin</td>
<td>BSA, HSA</td>
<td>1984</td>
</tr>
</tbody>
</table>

Summary of Different Melatonin RIA
1.22.3 Radiolabel

The first published assays used tritiated melatonin (Arendt et al., 1975; Levine and Riceberg, 1975). Most of the RIAs published use commercially available tritiated melatonin, 35-80Ci/mM, available from New England Nuclear, Boston (USA) and Amersham International (UK) (Kennaway et al., 1977; Wetterberg et al., 1978; Grota et al., 1981; Fraser et al., 1983a and Webley et al., 1985). Rollag and Niswender (1976) developed a RIA which had a sensitivity of 2-3pg/tube using radioiodinated melatonin analogue, iodinated N-3-(4-hydroxyphenyl)-propionyl-5-methoxytryptamine. An iodinated label has the advantage of increased sensitivity. Expensive and cumbersome liquid scintillation counting is also avoided. These advantages may be at the expense of specificity. Rollag and Niswender's assay has been improved by Claustrat et al., (1984) by using an iodinated tracer formed by reaction of Bolton Hunter's reagent (Bolton and Hunter, 1973) and 5-methoxytryptamine). Vakkuri et al., (1984) reported a technique by which melatonin was iodinated directly. The assay appears to have achieved excellent sensitivity together with the high degree of specificity required.

1.22.4 Preparation of Samples

To some extent the way samples have to be treated depends on the specificity of the antibody and the nature of the sample. Most of the melatonin RIAs published have required tedious extraction of the sample with organic solvents before assay. These extraction stages have typically included alkaline extraction with chloroform followed by petroleum ether (Arendt et al., 1977a); extraction with ethyl acetate (Jenkin et al., 1980) and extraction with water-saturated chloromethane (Wetterberg et al., 1978).

Kennaway et al., (1977) followed chloroform extraction of the samples by further purification on Lipidex 5000 columns. Similarly, following chloroform
extraction, Ozaki and colleagues (1978) purified urine and plasma samples by thin layer chromatography (TLC) before quantitation by RIA. An affinity chromatography method has been used by Thoresen (1978), where antibody has been coupled to Sepharose 4B to purify samples prior to assay. Baker reverse-phase C-18 columns were used by Sieghart and co-workers (1987) to extract melatonin from serum. With a very specific antibody it is possible to measure melatonin directly in plasma with no extraction stage. This assay has been applied to human (Fraser et al., 1983a), ovine (English et al., 1986) and rat plasma (Webley et al., 1985).

1.22.5 Separation of Samples

Samples are either incubated for a short period, for example, at 37°C for 1h (Wetterberg et al., 1978) or, more usually, they are incubated overnight at 4°C. Antibody-bound melatonin is usually separated from the free fraction by any one of three techniques; ammonium sulphate precipitation, separation with dextran-coated charcoal or precipitation using a double-antibody. A dialysis technique was used by Geffard and co-workers (1982). Following the addition of ammonium sulphate, Sieghart and colleagues (1987) replaced centrifugation with a filtration step involving Whatman GF/B glass-fibre filters.

1.22.6 Current Melatonin RIAs

RIAs are now extensively used for the measurement of melatonin in both tissues and body fluids in a variety of species. Melatonin has been assayed tissues and body fluids of many species, for examples, the pineal gland, retina, Harderian gland, brain and serum of rats and chickens (Pang et al., 1977); the plasma of man, sheep, rat, chicken, pig, donkey, cow, camel and scinzid lizard (Kennaway et al., 1977); in human serum and CSF (Arendt et al., 1977a); human urine (Lang et al., 1981), saliva (Vakkuri et al., 1985) and follicular fluid (Webley et al., 1985; Brzezinski et al., 1987).
Different RIAs have variable specificity and so it is important to validate new methods by comparison with as many other methods as possible. The first melatonin RIA was validated by comparison with the bioassay (Arendt et al., 1975). Chromatographic identity of immunoreactivity with melatonin in different solvent systems has been used to validate assays by TLC (Arendt, 1978; Ozaki et al., 1976) and HPLC (Tamarkin et al., 1982b; Vakkuri et al., 1984). The first RIA to be validated by GC/MS was that of Kennaway and co-workers (1977). Other assays have been similarly validated (Grotta et al., 1981; Fraser et al., 1983b).

A collaborative study to compare methods of measuring melatonin in human plasma was organised by Wetterberg and Eriksson (1981). Twenty laboratories were asked to participate, out of which twelve (all using RIA methodology) co-operated. Most of the laboratories using RIA methods reported comparable results. This study showed that melatonin could be accurately measured using different RIA methods.

Reliable GC/MS methods to measure melatonin have been developed (for example, Lewy and Markey, 1978). The theoretical absolute specificity of this technique has meant that the values obtained by this method are used as a 'standard', against which RIAs and other methods are compared. There is now general agreement that in normal human volunteers, daytime plasma melatonin levels are low (1-10pg/ml) with a nocturnal peak of about 30-100pg/ml. The earliest melatonin RIAs lacked complete specificity and the measured values were somewhat higher than the GC/MS values. There is now good agreement between the recently developed, very specific RIAs and GC/MS values (Fraser et al., 1983b; Claustrat et al., 1984; Waldhauser et al., 1984a; Vakkuri et al., 1984). However, in recent literature there has still been a considerable variation in the reported levels of melatonin. Daytime human plasma melatonin levels of around 100-200pg/ml have
been reported by Birkeland (1982), whereas the majority of workers using RIA technology find daytime human plasma melatonin levels to be <20pg/ml. This illustrates one of the problems with RIA, that is despite extensive validation procedures, problems with cross-reactivity can occur. Both heparin (Johansson et al., 1984) and dimethylphthalate (Wetterberg et al., 1984) have been reported to interfere with melatonin RIA, yielding anomalous melatonin values. RIAs using the same antibody have to be extensively validated for each new application, whether this involves measuring melatonin in a new species or in a new tissue/body fluid. Even after extensive validation, the results obtained may depend on the skill of the individual operator.

These problems will not be resolved until an international quality control system for melatonin measurement is adopted. Where controversial results are obtained and/or very high endogenous melatonin levels are reported, workers in the field should be encouraged to exchange samples between groups so that the methodology employed can be validated.

1.23 Assay of Urinary Melatonin

The majority of RIAs described above have been validated to measure melatonin in body fluids, such as plasma and CSF, which obviously limits the type of clinical studies which can be carried out. A small proportion of melatonin secreted by the pineal gland is excreted unmetabolized (Kopin et al., 1961). Human urinary melatonin has been measured by some workers as a non-invasive way of studying pineal function.

Lynch and colleagues (1978) applied the RIA of Levine and Riceberg (1975) to the measurement of urinary melatonin in man. Two methods were used to concentrate melatonin from urine; either Amberlite XAD-2 column chromatography
was used, or urine was directly extracted into chloroform. It was then necessary to further purify the urine samples by using the TLC method originally developed by Ozaki and co-workers (1978). The urinary melatonin levels measured using the RIA were similar to those established by bioassay (Table 1.2). The method was used to demonstrate that 5-7 days were required to re-entrain rhythmic melatonin excretion to a 12h phase shift in the lighting schedule (Lynch et al., 1978). When nine blood samples were obtained from three volunteers it was shown that urinary melatonin reflected circulating melatonin levels (r = 0.88, p<0.01).

Another RIA for urinary melatonin was developed by Wetterberg et al., (1978). Urine samples were extracted with chloromethane and washed with sodium hydroxide. Following oral administration of a melatonin solution only a small amount of the administered dose was measured in urine, indicating extensive metabolism of melatonin in man (Wetterberg et al., 1978). The correlation coefficient between serum melatonin levels at 0200h and the morning urine concentration of melatonin in twenty-four patients and healthy controls was 0.89 (Wetterberg, 1978). The correlation between total urinary melatonin output and plasma melatonin levels measured with this assay, has not been reported. The urinary assay has been used to study the re-entrainment of the melatonin rhythm following an eastward flight and a 9h time-shift (Wetterberg, 1978), to study the effect of propranolol on melatonin output (Wetterberg, 1978), to investigate melatonin production during sleep deprivation (Akerstedt et al., 1979) and to compare urinary melatonin levels found in women with breast cancer to those found in controls (Bartsch et al., 1981). Representative urinary melatonin values reported using this assay are given in Table 1.2.

A decrease in urinary melatonin excretion from birth to adulthood was demonstrated by Lemaitre and colleagues (1981). The antiserum used was reported to be specific (Lemaitre and Hartmann, 1980) and urine samples were extracted
<table>
<thead>
<tr>
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<th>Sex</th>
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<th>Day (Mean ± SD ng/h)</th>
<th>Night (Mean ± SD ng/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA Chazet et al., (1985)</td>
<td>M/F</td>
<td>10</td>
<td>1.44 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>RIA Vakker et al., (1984)</td>
<td>M</td>
<td>2</td>
<td>2.55 ± 0.35</td>
<td>0.77 ± 2.09</td>
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<tr>
<td>RIA Lang et al., (1987)</td>
<td>M/F</td>
<td>14</td>
<td>4.10 ± 1.87</td>
<td></td>
</tr>
<tr>
<td>RIA Wetterberg et al., (1978)</td>
<td>F</td>
<td>6</td>
<td>2.22 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>RIA Levine and Wetterberg, (1975)</td>
<td>M</td>
<td>4</td>
<td>1.07 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>RIA Levine, (1978)</td>
<td>M</td>
<td>6</td>
<td>0.89 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

*All values recalculated from original data. Total 24h samples.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method Used</th>
<th>Sex</th>
<th>Number of Subjects</th>
<th>Day (Mean ± SD ng/h)</th>
<th>Night (Mean ± SD ng/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chazet et al., (1985)</td>
<td>RIA</td>
<td>M/F</td>
<td>10</td>
<td>1.44 ± 0.27</td>
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<tr>
<td>Vakker et al., (1984)</td>
<td>M</td>
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<td>2.55 ± 0.35</td>
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<tr>
<td>Lang et al., (1987)</td>
<td>M/F</td>
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<tr>
<td>Wetterberg et al., (1978)</td>
<td>F</td>
<td>6</td>
<td>2.22 ± 0.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levine and Wetterberg, (1975)</td>
<td>M</td>
<td>4</td>
<td>1.07 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levine, (1978)</td>
<td>M</td>
<td>6</td>
<td>0.89 ± 0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2: Human Urinary Melatonin Levels Reported in the Literature Using Different Methods.
using the method developed by Arendt et al., (1977a) for plasma samples. The assay was validated by chromatographic identity of immunoreactivity on TLC with one solvent system.

The first RIA for the measurement of urinary melatonin which has been extensively validated was reported by Lang and colleagues (1981). The specificity of urinary melatonin measurements were confirmed by both TLC and GC/MS. These authors found that urine, unlike blood, contained many interfering substances which inhibited binding of the label to the antibody. Chloroform extraction alone did not remove all these cross-reacting derivatives from urine. In order to remove these interfering substances it proved necessary to wash the chloroform extract with 0.1M sodium hydroxide. A good correlation between plasma melatonin levels at midnight and nocturnal excretion of melatonin (2100-0700h) in thirteen adult volunteers was reported (r = 0.74, p<0.01).

The radioiodinated assay of Vakkuri et al., (1984) has also been applied to the measurement of urinary melatonin. Validation of the assay by HPLC demonstrated that the majority of urine immunoreactivity co-eluted with synthetic melatonin, but 7-23% contaminating immunoreactivity was observed. Urine samples were extracted with chloroform and, despite the incomplete specificity of the assay for urine determination, a diurnal rhythm for urinary melatonin excretion was observed.

Another radioiodinated assay, that of Rollag and Niswender (1976), has also been applied to the measurement of urinary melatonin (Claustrat et al., 1981). To attain specificity improved iodination, extraction and separation methods were used. The validation procedures employed are described by Brun et al., (1983). However, extensive validation of the urinary assay has not been reported. This assay has been used to study the effects of destyrosine gamma endorphin on urinary melatonin levels (Chazot et al., 1985).
Urinary melatonin only represents a small percentage of secreted melatonin, consequently urine concentrations of melatonin are extremely low (Kopin et al., 1961; Lang et al., 1981). A number of factors are present in urine which can cause inhibition of binding. In view of the extremely low levels and problems with cross-reactivity, it is essential that methods to measure melatonin in urine are thoroughly validated. It is not sufficient to validate a RIA for plasma measurements and then apply the techniques to urine. Very few of the methods reported above have been extensively validated and the specificity of the measurements made has to be questioned. A summary of the human urinary melatonin levels reported in the literature using some of the above mentioned methods is given in Table 1.2.

1.24 Assay of Salivary Melatonin

A useful non-invasive method to study pineal function is to measure salivary melatonin. Using an iodinated melatonin label, Vakkuri (1983) has developed a salivary melatonin assay which has been validated by HPLC. Using six-hourly sampling, a circadian variation with a nocturnal peak has been demonstrated. The same assay was used to study the distribution of melatonin in human serum, saliva and urine following the oral administration of 100mg of melatonin (Vakkuri et al., 1985). The results suggested that melatonin is passively secreted into saliva and they showed that salivary melatonin closely reflects changes in serum melatonin. The salivary melatonin concentrations corresponded to 21-56% of the serum concentrations.

Another salivary assay, modelled on the direct plasma method of Fraser and colleagues (1983a), has been developed (Miles et al., 1985). Their findings confirm the observations of Vakkuri. Paired plasma and saliva samples, taken hourly from six volunteers between 2000h and 0800h, correlated well (r = 0.971, p<0.001). The mean concentration of melatonin in saliva was 24% of the corresponding concentration in plasma.
Most recently, McIntyre and co-workers (1987) have also adapted the RIA of Fraser et al., (1983a) to the measurement of salivary melatonin. Melatonin was found to correlate in plasma and saliva, described by a linear regression equation: 

\[ y = 0.55x - 2.6 \quad (r=0.90). \]

In four subjects, the nocturnal rhythm in saliva was parallel to that observed in plasma. When one subject was challenged by exposure to 3000lux of light for 1h at 2400h a good correlation between plasma and salivary melatonin was also observed.

Further work needs to be carried out to determine the effect of changes in salivary flow-rate on melatonin levels. Extensive validation has to be carried out to ensure that artefactual results are not obtained. It has been reported that toothpaste, coffee (Laitinen and Harma, 1987) and potato crisps (McIntyre et al., 1987) can interfere with salivary melatonin RIAs.

The preliminary results with the salivary assays indicate that the method will prove to be very useful, particularly when blood samples are difficult to obtain.

1.25 Non-Isotopic Immunoassays

1.25.1 Chemiluminescence Immunoassay

Gupta and co-workers (1985) reported a chemiluminescent assay for the measurement of serum melatonin. The stable tracer is formed by reacting aminobutylethyl isoluminol with melatonin. After overnight incubation at 4°C, charcoal separation is used to separate bound and free antigens. Aliquots of supernatant are taken and luminescence initiated by oxidation of the label with a hydrogen peroxide-microperoxidase system at pH 8. The method has a similar sensitivity (standard curve range 4-500pg per tube), specificity and precision as the Rollag and Niswender RIA (1976).
The method is fairly rapid, the tracer is stable and presents none of the hazards associated with radioactivity. This method does have the disadvantage that prior extraction of the plasma sample is required.

1.25.2 Enzyme-Linked Immunoassay

A enzyme immunoassay to measure melatonin in serum, CSF and pineal gland has been developed (Ferrua and Masseyeff, 1985). Though not extensively validated, the assay is sensitive, fairly specific and values for sera were in the 'expected' range. The assay is based on the measurement of the distribution of an enzyme-labelled anti-hapten antibody (anti-melatonin - immunoglobulin G (IgG) coupled to horseradish peroxidase) between solid phase - hapten (human serum albumin-5-methoxytryptamine hemisuccinate conjugate physically absorbed onto polystyrene spheres) and liquid phase hapten. The assay has the advantages that it is simple to perform, the label is easy to prepare and chloroform extracted samples can be measured. Further evaluation of the method is necessary to confirm its specificity. The assay promises to be very useful, particularly if the sensitivity is further increased by the use of a fluorogenic substrate.

1.26 High Performance Liquid Chromatography

HPLC is a very powerful separation technique. Liquid chromatography involves the passage of a mobile liquid phase over a solid stationary phase. In HPLC, the close packed nature of the particulate stationary phase offers rapid resolution and high chromatographic efficiency but requires relatively high-pressure pumping systems to establish and maintain flow of the mobile phase through the column. For most HPLC methods 'reverse phase' chromatography is used, which involves the use of a non-polar stationary phase and a polar eluent (the mobile phase). Following separation of compounds by HPLC, detection of compounds is accomplished either by fluorometric or by electrochemical detection.
An assay using a cation exchange resin with electrochemical detection was developed by Goldman and colleagues (1980). A total of fourteen indoles were tested, including melatonin and 6-hydroxymelatonin. A reversed phase HPLC with electrochemical detection to measure tryptophan and other indole metabolites (including melatonin and 6-hydroxymelatonin) was developed by Mefford and Barchas (1980). Not all the peaks obtained from the tissue supernatants were identified and so the specificity of the method has to be questioned. The assay was later modified by using a more efficient column, a slightly higher pH, and by incorporating acetonitrile instead of methanol in the mobile phase (Mefford et al., 1983).

A fluorometric method, which was validated by using a fluorometric detector and a electrochemical detector in series, was developed by Anderson and co-workers (1982). A total of six indoles, including melatonin, could be assayed. The use of two detectors improved the specificity of the method: in order to interfere, a compound would have to have the same retention time and the same ratio of response on the two detectors as the indole of interest.

The next major advance was the incorporation of internal standards to reduce the errors during extraction procedures and to increase analytical precision. A dual detection method using 4-aminoantipyrine, an ultraviolet-absorbing substance, as an internal standard has been reported (Wakabayashi et al., 1985). The method was simplified by using veratric acid as an internal standard and a fluorometric detection system (Wakabayashi et al., 1986). Another system, which also included an internal standard, was reported by McNulty and colleagues (1985). The internal standard was 3-hydroxy-4,5-dimethoxybenzoic acid and electrochemical detection was used. In a modification of the system, using a column with 3μm reverse phase C18 particles, eleven indoles (including melatonin) could be measured in one pineal in a single run (McNulty et al., 1986).
HPLC has the major advantages that it is possible to measure a number of compounds in a single sample, the technique is specific and relatively inexpensive; sample preparation is minimal. The application of HPLC to the measurement of melatonin has, however, been restricted to measuring pineal melatonin. At the moment the detection systems are not sufficiently sensitive to measure melatonin in body fluids. An area in which HPLC has proved to be extremely valuable is in the validation of RIAs (Tamarkin et al., 1982b; Vakkuri et al., 1984).

1.27 Thin Layer Chromatography/Radiospectrometry

TLC coupled with radiospectrometry is a very useful technique to study the metabolism of indoles. A radiolabelled indole is usually incubated in vitro with pineal tissue for several hours. A TLC system is then used to separate the different metabolites, the radioactivity across the plate is subsequently measured. The technique has the advantages that it is quick, relatively cheap and, if a good separation system is used, several indoles can be measured at once. To a certain extent, the results obtained depend on where the radioactive precursor under study is labelled and few workers monitor the recovery of added radiolabel. This method has been used to study the metabolism of indoles (Wainwright, 1977) and to demonstrate metabolic changes induced by pharmacological agents (Voisin et al., 1983; Morton, 1985). The major disadvantage of the technique is that endogenous indole levels cannot be measured.

1.28 Gas Chromatography

Separation is achieved by GC because of a differential distribution between a stationary liquid phase coated on an inert column packing material and a mobile gas phase, forced through the column. Prior to injection onto the column, biological samples require a laborious extraction stage. This is followed by chemical reaction to produce a melatonin derivative with an appropriate vapour pressure for GC and to facilitate detection.
1.28.1 Gas Chromatography/Electron Capture Detection

The recombination of ions is the principle of operation of the electron capture detector. The detector has an ion chamber containing an ionizable gas, which is held at a potential sufficient for the collection of all free ions produced (the standing current of the detector). The introduction of the melatonin derivative, which has a high electron affinity, into the chamber produces a change in the standing current which is recorded.

To measure melatonin in biological material prior extraction is necessary, followed by the formation of a halogenated derivative. Melatonin has been measured in both rat and human pineal glands (Degen et al., 1972; Greiner and Chan, 1978). The specificity of the method is based on the purification of the ionizable melatonin derivative on the GC column. The preliminary extraction step is important in order to separate melatonin from other indoles which may form the same halogenated derivatives. Interfering substances in biological samples can also effect the derivatization step and the gas chromatographic separation. GC with electron capture detection has now been largely replaced by the much more specific method of GC/MS.

1.28.2 Gas Chromatography/Mass Spectrometry

This is, theoretically, the most specific method for the measurement of melatonin. Samples are separated on the GC column, concentrated and ionized. The charged fragments are accelerated through electric and magnetic fields which separate them on the basis of their charge and mass, and, theoretically, an absolute identification of the parent compound is achieved. Extensive purification of the biological samples is still necessary prior to assay. A major advance in the GC/MS measurement of melatonin was the inclusion of deuterated melatonin as an internal standard (Kennaway et al., 1977). This enabled endogenous melatonin to be
measured with great accuracy. GC/MS has been used to quantitate and identify melatonin in body fluids (Kennaway et al., 1977; Wilson et al., 1977), various tissues (Kennaway et al., 1977) and to validate RIAs (Kennaway et al., 1977; Fraser et al., 1983b).

Lewy and Markey (1978) were the first to use negative chemical-ionization for the measurement of melatonin, thereby greatly increasing the sensitivity of the method. Skene and co-workers (1983) developed a method using a double focussing magnetic mass spectrometer which increased resolution and reduced sample preparation to a minimum. Theoretically, several compounds at once can be measured by GC/MS in a single sample. Very few methods have been developed in which this has been achieved. However, Beck and colleagues (1981) reported a method for simultaneous measurement of melatonin and 5-methoxytryptophol in a single pineal extract.

The disadvantages of GC/MS are that it is expensive to run, requires trained personnel and extensive sample purification. GC/MS is not suited to the large numbers of samples associated with clinical work and research into rhythmic physiological phenomenon, because of the low sample throughout.
ASSAY OF MELATONIN METABOLITES

1.29 Assay of Melatonin Metabolites and General Considerations

Studies of human pineal function have been largely restricted by the methodology which has been available. As described above, most of the methods to measure melatonin in man rely on the measurement of melatonin in blood. In order to get meaningful results it is necessary to take blood samples over 24h, which obviously restricts the type of studies which can be carried out. Some workers have set up assays to measure melatonin in urine (Section 1.23). The disadvantage with this approach is that urinary melatonin only represents a small percentage of endogenously secreted melatonin (see Section 1.17). More recently salivary assays have been established (Section 1.24). Saliva samples are easier to obtain than plasma samples but, unless frequent sampling is undertaken they do not necessarily represent an integrated measure of endogenous melatonin production.

To circumvent the above problems workers have established assays to measure the major urinary metabolites of melatonin. These provide an integrated measure of endogenous melatonin production.

1.30 Radiospectrometry Coupled with Chromatographic Separation

In the pioneering studies on the metabolism of melatonin (Kopin et al., 1961; Kveder and McIsaac, 1961) mice and rats were injected with tritiated melatonin and urine was collected. The urinary metabolites were separated by paper chromatography and quantitative estimation of the metabolites was obtained by scanning the radioactive chromatograms (see Section 1.17). A similar approach was adopted to study melatonin metabolism in human volunteers (Jones et al., 1969). Five male patients were injected with $^{14}$C melatonin and urine was collected. The urinary metabolites were separated by extraction with ethyl acetate followed by column chromatography of the aqueous portion on Sephadex G-10. The relative
proportion of each metabolite was determined by measuring the percentage of radioactivity appearing in each peak. This technique has obvious limitations for the measurement of urinary melatonin metabolites in humans and presents considerable ethical problems.

1.31 Spectrophotofluorometry

A recent report has appeared for the fluorometric determination of the daily urinary excretion of '6-oxymelatonin' (Grinevich and Labunetz, 1986). The method used was originally reported by Zubkov and co-workers (1974) in Russian, and is based on the spectrofluorometric method for the measurement of urinary melatonin of Dreux (1969).

Exactly which of the urinary metabolites of melatonin the measurement of '6-oxymelatonin' encompasses is not made clear in the methodology. The specificity of the method has to be questioned as extensive validation procedures do not appear to have been carried out. No indication of inter- or intra-assay variations is given and the method has not been compared with any other existing assays. It is unfortunate that no circadian rhythm in excretion has been established using the assay, and only 24h urinary excretion values have been reported (Table 1.3). The values reported nevertheless, are in the range expected for the 6-hydroxylated metabolites of melatonin.

1.32 High Performance Liquid Chromatography

HPLC methods for the measurement of 6-hydroxymelatonin and NAS have been developed (Section 1.26 for example Mefford and Barchas, 1980), however, these methods have not been applied to the measurement of melatonin metabolites in urine.
No methods for the measurement of the conjugated melatonin metabolites have been reported. Recently, HPLC techniques for the measurement of the sulphate conjugates of various catecholamines and of serotonin-O-sulphate in urine samples have been developed. These techniques, a post-column hydrolysis technique and a dual-electrode electrochemical procedure (Elchisak, 1983a and b) could be applied to the measurement of the urinary conjugates of melatonin. The concentrations present in urine are relatively high and sample preparations would be minimal. With a sufficiently good separation system, all the conjugated melatonin metabolites could be measured in one run. Another important application of this technique would be to study the metabolism of melatonin in detail. The main disadvantage of the method is that it is relatively expensive to establish and maintain HPLC equipment.

1.33 Gas Chromatography

Gas chromatographic methods for the determination of melatonin and 6-hydroxymelatonin have been developed. Retention times were established on one non-polar and three polar columns with an argon ionization detection system (Greer and Williams, 1967). Attempts to detect melatonin and 6-hydroxymelatonin (free or conjugated) in normal human urine and in the urine of two patients with parenchymatous pinealoma were unsuccessful. Based on the conditions of the analysis, the authors estimated that the normal excretion of free melatonin and free 6-hydroxymelatonin was less than 10μg/day. It is likely that these authors failed to detect the metabolites in urine because of decomposition during sample preparation and derivatization.

6-Hydroxymelatonin was identified by GC/MS in normal human urine by Sisak and colleagues (1979). Urine was hydrolysed, extracted and 6-hydroxymelatonin was reacted to form the t-butyldimethylsilyl-pentafluoropropionyl derivative. The amount of 6-hydroxymelatonin detected was estimated at 20ng/ml.
The first quantitative assay for a urinary metabolite of melatonin was developed by Fellenberg and co-workers (1980), who developed a GC/MS method for the measurement of urinary aMT6s. aMT6s was chemically synthesized using the method of Baillie et al., (1973) and deuterated aMT6s, which served as an internal standard, was also prepared. The assay procedure involved isolation of aMT6s from urine using XAD-2 and Florisil columns, prior to derivatization to form a trimethylsilyl derivative. The inter- and intra-assay variations of the method were good. A marked nocturnal rise in aMT6s excretion was established and the reported values are shown in Table 1.3.

Another quantitative assay for the measurement of urinary melatonin metabolites was reported by Tetsuo and co-workers (1981a). Deuterated aMT6s was added to a urine sample as an internal standard. The urine was hydrolysed enzymatically and free 6-hydroxymelatonin extracted and reacted to form a stable t-butyldimethylsilylpentafluoropropionyl derivative. It was then separated by silica gel column chromatography, and quantitated using electron capture negative ion chemical ionization mass spectrometry. Inter- and intra-assay variations were good. This method measures the total free and conjugated 6-hydroxylated urinary metabolites of melatonin. Diurnal variations in melatonin metabolite excretion were demonstrated and the values reported are shown in Table 1.3.

An assay for the simultaneous measurement of aMT6s and aMT6G in human urine was developed by Francis and colleagues (1987). The conjugates are extracted on octadecylsilane-bonded silica cartridges and separated on silica cartridges. After hydrolysing the separated conjugates enzymatically, free 6-hydroxymelatonin is determined by GC/MS. The recoveries were low and variable, which necessitated the addition of deuterated sulphate and glucuronide conjugates to the urines before extraction. The reported inter- and intra-assay variations were
<table>
<thead>
<tr>
<th>Range</th>
<th>Sex</th>
<th>Number</th>
<th>Measured Metabolite</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3 - 41.0</td>
<td>F</td>
<td>10</td>
<td>6-oxymelatonin</td>
<td>Fluoro-</td>
<td>Chamberlain and Lander (1986)</td>
</tr>
<tr>
<td>7.6 - 36.7</td>
<td>M</td>
<td>10</td>
<td>AMTS 1</td>
<td>GC/MS</td>
<td>Felliuhbnag et al. (1980)</td>
</tr>
<tr>
<td>7.4 - 15.8</td>
<td>F</td>
<td>4</td>
<td>6-oxymelatonin</td>
<td>GC/MS</td>
<td>Tesfay et al. (1983)</td>
</tr>
<tr>
<td>6.2 - 18.8</td>
<td>M</td>
<td>10</td>
<td>6-oxymelatonin</td>
<td>GC/MS</td>
<td>Tesfay et al. (1983)</td>
</tr>
<tr>
<td>14.5 - 7.6</td>
<td>M</td>
<td>7</td>
<td>6-oxymelatonin (total conjugated)</td>
<td>GC/EC</td>
<td>Creer and Williams (1997)</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>M</td>
<td>10</td>
<td>6-oxymelatonin</td>
<td>GC/EC</td>
<td></td>
</tr>
</tbody>
</table>

Note: All values have been recalculated from original data.

**Table 1.3:** Levels of Urinary 6-Hydroxylated Melatonin Metabolites in Normal Adults Reported in the Literature.
good. No values for the endogenous levels of either compound were reported in this paper and a diurnal rhythm in excretion has not been established. This method has also been adapted for the measurement of aMT6s in hamster urine where a two-fold day/night difference in excretion has been reported (Korenman et al., 1987).

The reported GC/MS methods are sensitive and specific. However, they suffer from the disadvantages that they require laborious sample preparation and are expensive to maintain. A quantitative GC/MS assay for 5-methoxyindole-3-acetic acid, a minor melatonin metabolite, in human urine has been recently reported (Higa and Markey, 1985). The daily pattern of excretion bore no relationship to that of 6-hydroxymelatonin, indicating that the major portion of urinary 5-methoxyindole-3-acetic acid does not derive from melatonin. GC/MS has also recently been used to identify new melatonin metabolites. Young and colleagues showed that melatonin could be metabolized to form NAS as well as 6-hydroxymelatonin in man (Young et al., 1985) (Section 1.17).

1.34 Project

In view of the constraints posed by measuring melatonin in plasma samples, the aim of the project was to set up a method to measure a urinary metabolite of melatonin. We decided to develop a RIA for aMT6s the major melatonin metabolite. RIA has the advantages of being quick, relatively inexpensive and is amenable for the measurement of the large numbers of samples associated with clinical work.
CHAPTER 2

DEVELOPMENT OF 6-SULPHATOXYMELATONIN RADIOIMMUNOASSAY
Introduction

To develop a RIA which is both sensitive and specific, it is necessary to raise antibodies which have a high avidity and specificity. A number of factors can affect the quality of the antiserum produced. The conjugation method used should not alter or mask the antigenic determinants of the hapten molecule, as to be specific the antibodies produced should reflect these characteristics. A number of other factors are also important; these include the choice of carrier protein, the use of adjuvants, the choice of species and the route of administration.

The chances of eliciting a good immune response are increased by the use of an adjuvant such as mineral oil. The adjuvant is mixed with an immunogen to form a stable emulsion. Adjuvants are believed to work by acting as a depot and releasing immunogens slowly, thereby preventing rapid uptake into the circulation and degradation of the immunogen by proteolytic enzymes. The adjuvant causes local and general stimulation of the reticuloendothelial system, assists phagocytosis and attracts macrophages to the site of injection (Hurn and Landon, 1971). A modified Freund's adjuvant (Morris) was used which has the same properties as complete Freund's adjuvant without causing ulceration at the site of injection (Marks et al., 1975). The immune response of an animal to a particular compound is genetically determined and is dependent on both the species and the strain. It is best to immunize a species/strain of animal which is known to respond well to a particular immunogen or to very similar immunogens.

At the commencement of this project, no methods for the direct iodination of melatonin had been published. The approach adopted to prepare an aMT6s radiolabel was the biological transformation and subsequent purification of commercially available tritiated melatonin. Although iodinated labels have a higher specific activity, the commercially available tritiated melatonin labels are of sufficiently high specific activity to permit the development of sensitive assays.
In order to make quantitative comparisons between laboratories it is necessary to have a common standard. Unfortunately no international aMT6s standard exists. At the start of this project it was necessary to chemically synthesize aMT6s within our laboratory. Following the development of the RIA described in this thesis, several aMT6s standards from other research laboratories have been made available to us and have been compared with our own standard.

It is important to compare a newly developed method with other established methods. At the time of writing, no other RIAs for aMT6s had been published. The method has, however, been compared with a fully established GC/MS method for total 6-hydroxylated melatonin metabolites (Tetsuo et al., 1981a).

2.1 Chemicals, Reagents, Consumables and Equipment

Required Routinely in the Radioimmunoassay

Tricine Sigma Chemical Company Limited, Poole, Dorset
Sodium Chloride BDH Chemicals Limited, Speke, Liverpool
Activated Charcoal Pharmacia, Milton Keynes, Buckinghamshire
Gelatine LKB Limited, Selsdon, Surrey
Dextran T-70 Eppendorf, Supplied by BDH Chemicals Limited
Optiphase Safe Scintillant Luckham Limited, Haywards Heath, Sussex
Scintillation Vials Eppendorf, Supplied by BDH Chemicals Limited
LKB 1216 Rackbeta Liquid Labco Limited, Marlow, Buckinghamshire
Scintillation Counter Elkay Laboratory Products Limited,
LP3 Plastic Tubes Basingstoke, Hampshire
Eppendorf Multipipette 4780 Gilson, Supplied by Anachem, Luton,
Eppendorf Combitips Bedfordshire
Labco Glass Universals Gilson Adjustable Pipettes
Beckman J-6B Centrifuge

Synthesis of $[^3]H$ aMT6s
Trinitiated Melatonin NET-458
RPM I
NADPH (β-Nicotinamide adenine dinucleotide phosphate, reduced form)
PAPS
Niacinamide
Glutathione
Phenobarbitone
Sephadex LH-20
Oxygen - 5% carbon dioxide Cylinder
Nitrogen Cylinder
LKB 2112 Fraction Collector
Berthold LB2842 TLC-Plate Scanner

Synthesis of aMT6s Standard
6-Hydroxymelatonin
N-N'-dicyclohexylcarbodiimide
Dimethylformamide

Immunization
Non-Ulcerative Freund's (Morris) incomplete
Ovalbumin

Chromatography
Cellulose Plastic-Backed TLC Plates
Silica Gel Plastic-Backed TLC Plates
Whatmann No 3MM Filter Paper
Cyanogen-Bromide Activated Sepharose 4B
Amicon Disposable Columns
Ehrlich's Reagent (4-dimethylaminobenzaldehyde)
Validation of Assay

<table>
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<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
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<td>aMT6G</td>
<td>Gifts from Dr A M Leone</td>
</tr>
<tr>
<td>N-acetylsertotonin sulphate</td>
<td>St Bartholomew's Hospital, London</td>
</tr>
<tr>
<td>N-acetylsertotonin glucuronide</td>
<td>Gifts from Laboratories Plan, SA</td>
</tr>
<tr>
<td>N-acetyltrypophan</td>
<td>Geneva</td>
</tr>
<tr>
<td>5-Methoxytrypophan</td>
<td>Sigma</td>
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<tr>
<td>5-methoxy-N-acetyltrypophan</td>
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</tr>
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<td>Other indoles, steroids and</td>
<td></td>
</tr>
<tr>
<td>Catecholamine Derivative</td>
<td>Sigma</td>
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<tr>
<td>Tritiated Methoxy[^H] Melatonin</td>
<td>Amersham International, Amersham, Bucks</td>
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<tr>
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<td>Carlson Ford Limited, Ashton Under Lyme</td>
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<tr>
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<td>Creatinine Kit No. 124192</td>
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<td>Roche Products Limited, Welwyn Garden City,</td>
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<td>Bufferfly-19 Cannulae</td>
<td>Venisystems, supplied by Abbott Laboratories</td>
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<tr>
<td></td>
<td>Limited, Queensborough, Kent</td>
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<tr>
<td>Heparinised Plastic Tubes</td>
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<tr>
<td>Atenolol</td>
<td>Stuart Pharmaceuticals Limited, Cheadle</td>
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<td></td>
<td>Cheshire</td>
</tr>
</tbody>
</table>

All other chemicals were commercially available and of analytical grade.

2.2 **Synthesis of aMT6s**

aMT6s is not available commercially and was synthesized at the University of Surrey. Full details of the synthesis are given elsewhere (Franey, 1988). The method of Fellenberg and colleagues (1980) was used. Briefly, 6-
hydroxymelatonin and N,N'-dicyclohexylcarbodiimide were dissolved in anhydrous dimethylformamide. The reaction temperature was maintained at 0°C while concentrated sulphuric acid and anhydrous dimethylformamide were added. The reaction was terminated after 15min. The reaction is outlined below:

![Chemical reaction diagram]

6-hydroxymelatonin + H₂SO₄ + dicyclohexylcarbodiimide (DCC) → dimethylformamide (DMF) → 6-sulphatoxymelatonin (aMT₆s)

The Synthesis of aMT₆s

The reaction products were purified on a Sephadex LH-20 column with potassium chloride saturated methanol/chloroform (1/1 by vol; filtered until non-turbid) as the eluting solvent. aMT₆s was further purified by TLC (Silica Gel plates, n-butanol saturated with water). The final product was stored at -20°C at a concentration of 48µg/ml in methanol containing 0.1% ascorbic acid. A completely pure crystalline preparation was not obtained. Qualitative GC/MS performed by Dr G King (Queen Charlotte’s Maternity Hospital, London) gave a spectrum identical to that reported by Fellenberg and colleagues (1980).

This chemically synthesized aMT₆s has been used throughout this thesis as a RIA and chromatographic standard.
2.2.1 Crude aMT6s Preparation

In addition to the chemically synthesized standard, aMT6s has also been purified from urine. A female volunteer was given an oral dose of 500mg of melatonin, in a corn oil preparation, at 2200h and overnight urine was collected. The urine was freeze-dried and extracted with methanol. The methanol extract was purified by repeated paper chromatography (Whatman No. 3MM, propan-2-ol/5% ammonia, 4/2 by vol). The portion corresponding to the aMT6s standard was cut out, eluted with methanol and concentrated under nitrogen gas at 37°C. This fraction was further purified by TLC on cellulose plates (n-butanol/glacial acetic acid/water, 4/1/1.5 by vol). The portion corresponding to authentic aMT6s was cut out and eluted with methanol. From approximately one third of the original urine volume, 10mg of aMT6s were obtained.

2.3 Preparation of Conjugated aMT6s and Immunization of Animals

The conjugate was prepared and the animals immunized prior to the start of this project. The conjugate was prepared at the University of Surrey and full details are given elsewhere (Franey, 1988). Briefly, the Mannich reaction (Grota and Brown, 1974), a formaldehyde condensation reaction, was used to conjugate aMT6s (<4mg) to ovalbumin (55mg). No estimate of the molar ratio of hapten conjugated to protein was made. Six Suffolk cross ewes were immunized, by subcutaneous injection at six sites on the back and legs, with 500µg of conjugate emulsified in 3ml of Freund's modified adjuvant/saline (2:1 ratio) (Marks et al., 1975). The sheep were boosted seven months later with 200µg of conjugate emulsified in modified Freund's adjuvant, and were bled nine days after the booster injection.

2.4 Preparation of $\left[ ^{3}\text{H} \right]$aMT6s

aMT6s radiolabel was prepared by the biological transformation and subsequent purification of tritiated melatonin.
Wistar rats (150-200g) male/female were killed by cervical dislocation.

The liver was rapidly dissected out and cut into slices (<1mm).

600mg liver slices were incubated with approximately $1.2 \times 10^8$cpm of $[^3\text{H}]$ melatonin in 3ml of RPMI at 37°C under an atmosphere of 95% O$_2$:5% CO$_2$ for 2h with gentle shaking.

The incubate was cooled, homogenized and extracted at least five times with 3ml of methanol.

The pooled supernatants were dried down under nitrogen at 37°C.

Paper chromatography of the crude extract (Whatmann No. 3MM, propan-2-ol/5% ammonia, 4/2 by vol) revealed three peaks corresponding to melatonin ($R_f = 0.84$), aMT6s ($R_f = 0.67$) and aMT6G ($R_f = 0.45$) (Figure 2.1).

The crude extract was reconstituted in 1000μl of LH-20 column buffer (Section 2.2) and purified on a LH-20 column. 4g dry powder were used, the column dimensions were 40 x 1cm and 2ml fractions were collected.

The elution profile of the LH-20 column is shown in (Figure 2.2).

The aMT6s fractions were pooled and dried down under nitrogen at 37°C. $[^3\text{H}]$ aMT6s gave one peak chromatographically identical to authentic aMT6s (Figure 2.3). $[^3\text{H}]$ aMT6s was stored in methanol 0.1% ascorbate at -20°C and was stable for at least twelve months under these conditions.

15-25% of the starting $[^3\text{H}]$ melatonin was converted to $[^3\text{H}]$ aMT6s.
Figure 2.1  Paper Chromatogram (Whatmann No. 3MM, Propan-2-ol/5% Ammonia, 4/2 by vol) of a Methanol Extract of Rat Liver Slices After Incubation with $[^3]$H Melatonin

Figure 2.2  Elution Profile of a LH-20 Column of a Methanol Extract of Rat Liver Slices After Incubation With $[^3]$H Melatonin
Figure 2.3  

**Chromatograms of Purified {\textsuperscript{3}H} aMT6s in 4 Different Solvent Systems**

The arrows indicate the position of cold aMT6s standard.
2.4.1 Modified Label Preparation Method

Once the in vitro production of \(^{3}\text{H}\) aMT6s from melatonin under control conditions had been characterized, the procedure was modified to increase the yield of label. The following changes in the method were made:

(i) Male Wistar rats (150-200g) were treated with phenobarbitone (0.1% weight/vol) in the drinking water for seven days. On the eighth day the rats were killed.

(ii) 1200mg liver slices were incubated with approximately \(1.2 \times 10^8\) cpm \(^{3}\text{H}\) melatonin in 6ml of RPMI + 2mg of NADPH, 1mg niacinamide, 500\(\mu\)g of PAPS, 1mg of glutathione and 5mg magnesium chloride at 37°C under an atmosphere of 95% \(\text{O}_2\):5% \(\text{CO}_2\) for 3h.

(iii) The crude extract was purified on the LH-20 column (6g of dry powder). The elution profile of the LH-20 column was altered with a greater proportion of glucuronidated metabolites being produced (Figure 2.4).

Using this method approximately 25% of the starting \(^{3}\text{H}\) melatonin was converted to \(^{3}\text{H}\) aMT6s. This procedure is now routinely used in our laboratory to prepare \(^{3}\text{H}\) aMT6s.

2.4.2 Discussion of \(^{3}\text{H}\) aMT6s Preparation

The pattern of melatonin metabolism observed in the control rats was the same as that reported by Kopin et al., (1961). Paper chromatographic analysis of the incubates gave identical results to those of Kopin and colleagues, however, slightly different \(R_f\) values were obtained.
Figure 2.4

Elution Profile of a LH-20 Column of a Methanol Extract of Phenobarbitone-Induced Rat Liver Slices After Incubation with $[^3H]aMT6s$ Melatonin
A study by Wurtman and co-workers (Wurtman et al., 1968) demonstrated that treating rats with phenobarbitone increased the rate of disappearance of radioactive melatonin from plasma. This suggested that phenobarbitone induction of liver enzymes increased the rate of melatonin metabolism. Therefore to increase the yield of aMT6s label, rats were pre-treated with phenobarbitone.

Aromatic hydroxylation by microsomal mixed-function oxidases requires NADPH and molecular oxygen as co-factors. In the in vitro metabolizing system the concentration of co-factors is likely to be limiting, therefore NADPH and PAPS were included in the incubate to act as co-factors for the phase I and phase II reactions, respectively. Glutathione was included in the incubate following a report by Gregory (1962) that maximal activity of sulphate transferring enzymes in vitro only occurred in the presence of a sulphydryl compound such as glutathione. Magnesium chloride and niacinamide were added to the incubate in similar concentrations to those used in Kopin's (1961) rat liver microsomal system for the metabolism of melatonin. Phenobarbitone treatment and the inclusion of the co-factors in the incubate increased the yield of aMT6s by about 30%. Phenobarbitone treatment changed the pattern of melatonin metabolism to give a greater yield of glucuronidated metabolites than under control conditions.

The performance of a RIA depends largely on the specificity of the antibody and the purity of the label. The label gave one peak when screened in four different chromatographic systems (Figure 2.3). No evidence for the presence of the sulphate conjugate of NAS was obtained.

2.5 **Assay Buffer**

The buffer used was the tricine buffer described by Fraser and colleagues (1983a) for the direct plasma assay of melatonin.
Stock assay buffer consisted of tricine (17.9g/l, pH 5.0) containing sodium chloride (9.0g/l) and gelatine (1.0g/l). Assay buffer was made up in glass doubly-distilled water, heated at 55°C for 1h and then stored at 4°C for up to ten days.

Disposable glassware was used where possible, or else glassware was sonicated in doubly-distilled water prior to use.

2.6 **Separation of Bound and Free \(^{3}H\) aMT6s**

Bound and free \(^{3}H\) aMT6s were separated using dextran coated charcoal.

The minimum concentration of charcoal which gave non-specific binding of less than 5% was determined and found to be 100\(\mu\)l of 2% charcoal with 0.02% dextran T70, in tricine buffer. Charcoal was defined before use by mixing it with tricine for 15min. It was then centrifuged at 1000rpm for 5min before discarding the supernatant. The charcoal was resuspended in tricine buffer, dextran was added and the charcoal suspension stored at 4°C for up to a week.

Prior to use the charcoal suspension was mixed on ice for at least 30min. Bound and free aMT6s were separated by the rapid addition of charcoal. Tubes were incubated on ice for 15min. Following centrifugation at 2000g for 15min (4°C) aliquots (500\(\mu\)l) of supernatant were taken, 4.5ml of scintillant was added and the vials counted.

2.7 **Estimation of Titre**

In RIA a limited number of binding sites are incubated with an excess of radiolabel. It is essential to know how far each antiserum has to be diluted to fulfil these criteria. An antiserum dilution which binds 40-50% of the added radiolabel is usually employed, but 100% binding of radiolabel to antiserum is rarely achieved
because of various non-specific factors. The titre of an antiserum can be defined as the final dilution of antiserum which binds 50% of the maximum label bound.

2.7.1 Detection of Antibodies

Fixed amounts of radiolabel were incubated overnight at 4°C with decreasing concentrations of antiserum.

Nine days after the first booster injection $[^{3}H]$ aMT6s binding antibodies were detected in all six sheep (bleed date 11.11.82)

2.7.2 Selection of Antisera

Antisera from the three sheep (1118, 1119, 1120) which had the highest titres were selected for further characterization. Antiserum dilution curves were constructed for each antiserum, both with and without the addition of 100pg of cold aMT6s standard. The displacement obtained by adding cold aMT6s was compared.

The most sensitive antiserum was 1118. This antiserum also had the highest titre and was chosen for further characterization. The antiserum dilution curve for this antiserum, both with and without the addition of 100pg of cold aMT6s standard is shown in Figure 2.5.

2.7.3 Booster Injections of the Sheep

All six sheep have been boosted at intervals, with 200μg of conjugate and antiserum collected nine days after injection. The best antiserum remains that obtained from 1118. The titres of the antisera obtained from 1118 are presented graphically in Figure 2.6. The highest titre observed for sheep 1119 and 1120 were 1:1000 and 1:1800 (final dilutions), respectively.
Figure 2.5  Antiserum Dilution Curve for Antiserum 1118 (11.11.82) With and Without the Addition of 100 pg of aMT6s Standard

Figure 2.6  Titre of Antisera Obtained from Sheep 1118 Following Successive Booster Injections
2.8 **RIA in Buffer**

Standards (5-400µl) (see Section 2.9.5) or samples in buffer were pipetted into LP3 plastic tubes and made up to 500µl with buffer. Antiserum 1118 (11.11.82) (200µl; final dilution 1:1000) in buffer was added and the tubes mixed and incubated at room temperature for 30min. Radiolabel (100µl; 4500cpm) in buffer was added and, after mixing, tubes were either incubated at 37°C for 20min followed by 90min on ice or they were incubated at 4°C overnight. Identical results were obtained with either incubation method. Bound and free fractions were separated as described in Section 2.6. Aliquots (500µl) of the bound fraction were counted in 4.5ml of scintillant. The binding of standard samples was calculated (B/B₀), fitted to a spline function and the results of unknown samples determined by interpolation.

2.9 **Quantitation of aMT6s Standard**

No international quantitated aMT6s standard, against which our standard could be compared, exists. The method chosen to quantitate our standard was a radiolabel self-displacement technique. This technique is usually used for the estimation of the specific activity of RIA tracers (Roulston, 1979) and was adapted for the quantitation of cold aMT6s standard.

The principle is as follows: increasing amounts of labelled antigen are incubated with a constant amount of antibody under similar conditions to those employed routinely in the assay (Gocke et al., 1969). The mass of tracer present in the tubes is calculated knowing the specific activity of the radiolabel and the efficiency of the counter for the label. A radiolabel standard curve is then constructed. A standard curve with increasing concentrations of cold antigen is also constructed and the cold antigen is quantitated by comparison with the radiolabel standard curve.
The following assumptions are made. Firstly, it is assumed that labelled and unlabelled ligand are chemically identical with respect to antibody affinity. Secondly, separation of bound and free fractions is assumed to be 100% and thirdly, no loss of label is assumed to occur during the biological transformation of $[^3H]$ melatonin.

2.9.1 Experiment 1

The radiolabel used to construct the label displacement curve was methoxy-$[^3H]$ melatonin (79.8Ci/mmol). The calculation to convert a given number of counts into a mass of aMT6s is given below:

Specific activity = 79.8Ci/mmol
Efficiency of counter for $[^3H]$ = 40%
1 Ci = $2.22 \times 10^{12}$dpm
Molecular weight aMT6s = 328

\[
\text{A tube with } y \text{ cpm has } \frac{100}{40y} \times \frac{2.22 \times 10^{12} \times 79.8}{328} \text{ mg aMT6s}
\]

A standard curve with increasing concentration of $[^3H]$ aMT6s was constructed together with a standard curve with increasing concentrations of cold aMT6s. The results are presented in Table 2.1 and in Figure 2.7. The mass of tracer added to the standard curve constructed with cold aMT6s is taken into account. The potency of the cold aMT6s standard was compared to the displacement achieved by the known amounts of aMT6s in the label displacement curve and hence the aMT6s standards was quantitated.
2.9.2 Experiment 2

A standard curve was constructed using the aMT6s standard quantitated in Experiment 1. The displacement observed was compared to that achieved in another radiolabel self-displacement curve. The radiolabel used was derived from $[^3\text{H}]$ melatonin labelled on the side-chain (acetyl-5-methoxytryptamine-N-2-aminoethyl-2-$[^3\text{H}]$; 26.4Ci/mmol).

The results are given in Table 2.2. The standard curve and radiolabel displacement curve are plotted in Figure 2.8. Identical curves were obtained.

2.9.3 Comparison With Other aMT6s Standards

Once the aMT6s RIA had been established, aMT6s standards from other research groups were donated to us. aMT6s standards were kindly donated by the following: Dr D J Kennaway, Department of Obstetrics and Gynaecology, University of Adelaide, Australia, Dr A M Leone, Department of Reproductive Physiology, St Bartholomew's Hospital, London, Dr G E Webley, Institute for Hormone and Fertility Disorders, Hamburg, West Germany, Dr G M Brown, Department of Neurosciences, McMaster University, Hamilton, Ontario, Canada. These standards were synthesized by the method of Fellenberg et al., (1980) with or without modification, or by that of Leone et al., (1987b).

All standards were used to generate standard curves in order to assess their relative potency. The chromatographic properties of the standards were investigated by TLC in cellulose with eluents of n-butanol/glacial acetic acid/water (4/1/1.5 by vol) and propan-2-ol/5% ammonia (4/2 by vol).

The displacement curves obtained using the different standards are illustrated in Figure 2.9. All the standards gave displacement parallel to that of our standard, however, the relative potency of the different preparations varied approximately two-fold. All the standards were chromatographically identical.
<table>
<thead>
<tr>
<th>Mass of Unlabelled Antigen Added (pg)</th>
<th>Mass of Tracer Added (pg)</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
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<td>8.2</td>
<td>3.9</td>
</tr>
<tr>
<td>10x</td>
<td>8.2</td>
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<tr>
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<tr>
<td>0</td>
<td>9.6</td>
<td>54.5</td>
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Table 2.1 Percentage Binding with Increasing Concentrations of Cold aMT6s and for Self-Displacement Curve Using Methoxy-[^3]H Melatonin Label (79.8 Ci/mmol)
<table>
<thead>
<tr>
<th>Mass of Unlabelled Antigen Added (pg)</th>
<th>Mass of Tracer Added (pg)</th>
<th>% Bound</th>
</tr>
</thead>
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<td>0</td>
<td>35.5</td>
<td>38.5</td>
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<tr>
<td>2.3</td>
<td>35.5</td>
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<td>70.0</td>
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<td>210.2</td>
<td>10.2</td>
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Table 2.2  Percentage Binding for Cold aMT6s Standard Curve, and for Radiolabel Self-Displacement Curve Using $^{3}$H Melatonin Labelled on the Side-Chain (26.4 Ci/mmol)
Figure 2.7  Standard Curve Using Self-Displacement Technique with Methoxy-[^3]H Melatonin (79.8 Ci/mmol); the Curve with Increasing Concentrations of Cold aMT6s Standard is Superimposed

Figure 2.8  Standard Curve with Cold aMT6s and Radiolabel Self-Displacement Curve Using[^3]H Melatonin Labelled on the Side-Chain (26.4 Ci/mmol)
Figure 2.9  Standard Curves Generated by Different aMT6s Standards
2.9.4 Discussion of aMT6s Quantitation

In the absence of an international aMT6s standard or a pure crystalline preparation, a radiolabel displacement technique was used to quantitate our aMT6s standard. Using tracers labelled in different positions identical results were obtained. The relative potency of the different aMT6s standards from various research groups varied considerably. One possible explanation is differences in the efficiency of desalting in some cases. All the standards did, however, give parallel displacement indicating that they were immunoreactively identical.

When an international aMT6s standard is made available some adjustment of the absolute levels reported in this thesis may be necessary.

2.9.5 Standard Curve

Aliquots of standard, diluted with assay buffer were stored at -20°C at a concentration of 290ng/ml. For each assay an aliquot was diluted in assay buffer to provide standard solutions over the range 2.5-200pg/tube (5-400μl). Non-specific binding was always less than 10%.

2.9.6 Sensitivity

The limit of sensitivity of the assay, defined as two standard deviations from the zero standard, (Feldman and Rodbard, 1971) was 2.1pg/tube.
2.10 Cross-Reactivity

The specificity of the 1118 antiserum was assessed by comparing the ability of different compounds to displace 50% of the maximum antibody-bound $[^3]H$ aMT6s, under the conditions routinely employed in the assay. The percentage cross-reactivity is expressed as a ratio of the concentration of aMT6s to the concentration of cross-reactant (multiplied by one hundred). The other urinary melatonin metabolites; 6-glucuronide melatonin, 5-sulphatoxy-N-acetylserotonin and 5-glucuronide-N-acetylserotonin, were kindly donated by Dr A M Leone (St Bartholomew's Hospital, London) and were tested for cross-reactivity.

The percentage cross-reactivity of different compounds is given in Table 2.3. The percentage cross-reactivity was very low, maximum cross-reactivity occurring with 5-glucuronide-N-acetylserotonin (0.4%).

2.10.1 Antibody Affinity Constant

The antibody affinity constant ($K$) was calculated by the method of Muller (1980) to give a value of $K = 2 \times 10^{11}$ litres/mol (calculations not shown).

2.11 Plasma aMT6s Assay

2.11.1 Procedure to Define Charcoal

Activated charcoal was washed with doubly-distilled water, centrifuged at 1000rpm for 5min and the supernatant discarded. This was repeated three times. The charcoal was again washed with doubly-distilled water, left to settle under gravity and the supernatant discarded. This was repeated four times. The charcoal was finally washed with acetone and left to dry at 60°C in a water bath.

2.11.2 Preparation of Charcoal-Stripped Plasma

10% (weight/vol) defined charcoal was added to a pool of normal human plasma and was mixed at 4°C for 60h. The plasma was then centrifuged for 15 min
<table>
<thead>
<tr>
<th>Compound</th>
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<tbody>
<tr>
<td>aMT6s</td>
<td>100</td>
</tr>
<tr>
<td>5-glucuronide-N-acetylserotonin</td>
<td>0.4</td>
</tr>
<tr>
<td>6-glucuronide melatonin</td>
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<tr>
<td>5-sulphatoxy-N-acetylserotonin</td>
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<tr>
<td>6-hydroxymelatonin</td>
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</tr>
<tr>
<td>melatonin</td>
<td>0.001</td>
</tr>
<tr>
<td>5-methoxyindoleacetic acid</td>
<td></td>
</tr>
<tr>
<td>5-hydroxyindoleacetic acid</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-tryptamine</td>
<td></td>
</tr>
<tr>
<td>N-acetylserotonin</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-tryptophan</td>
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</tr>
<tr>
<td>tryptamine</td>
<td></td>
</tr>
<tr>
<td>tryptophan</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<tr>
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<tr>
<td>N-acetyl-5-methoxytryptophan</td>
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</tr>
<tr>
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<td>estriol 3-sulphate</td>
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</tr>
<tr>
<td>dehydroisoandrosterone sulphate</td>
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</tbody>
</table>

Table 2.3 Cross-Reactivities of Antiserum 1118 Determined at 50% Displacement of $[^3H]$ aMT6s
at 4000rpm, filtered once through a Whatmann No 1 filter and twice through a Zeiss filter.

2.11.3 Preparation of Affinity-Stripped Plasma

aMT6s-free plasma was prepared by affinity chromatography, with the antiserum (1118 23.8.84) linked to cyanogen bromide (CNBr) activated Sepharose 4B, following the manufacturer's recommended procedure (Pharmacia, 1983).

Briefly, the required amount of CNBr activated Sepharose 4B was weighed out and swollen in 1mM hydrochloric acid and washed on a sintered glass filter with the same solution. IgG obtained from 1118 (23.8.84) was dissolved in sodium hydrogen carbonate coupling buffer (0.1M, pH 8.3) containing sodium chloride (0.5M) to give 5-10mg of protein per ml of gel, when a gel/buffer ratio of 1/2 was used. This protein solution and gel suspension were mixed overnight at 4°C in an end-over-end mixer. The remaining active groups were then blocked by mixing the gel with 0.2M glycine, pH 8.0, for 2h at room temperature. Excess adsorbed protein was subsequently removed with four alternate washes with coupling buffer and acetate buffer (0.1M, pH 4.0) containing sodium chloride (0.5M). The affinity columns were stored ready for use in a bicarbonate storage buffer (0.1M), containing sodium chloride (0.5M) and 0.1% sodium azide at 4°C.

Plasma was collected from apparently healthy volunteers during the afternoon. All samples which measured <5pg/ml against a standard curve constructed in charcoal-stripped plasma were pooled. Affinity columns were prepared from 1ml of slurry, residual buffer was removed by applying slight positive pressure. To each column 2ml of plasma was added and the columns were mixed on a roller mixer for 15min at room temperature. The plasma was then collected. Up to 10ml of plasma could be processed on each column before regeneration. Columns
were regenerated by washing with 10ml of doubly-distilled water followed by 3 x 2ml washes with methanol/water (9/1 by vol) and were finally washed with storage buffer.

The measurement of samples spiked with cold aMT6s showed that 85-90% of the aMT6s was removed. When standard curves were constructed with either affinity-stripped or charcoal-stripped plasma identical results were obtained. The affinity columns retained binding activity for at least eighteen months.

2.11.4 Procedure for Plasma Assay

The RIA procedure for plasma samples was identical to that given in Section 2.8 except for the following modifications: 200-500μl of plasma sample were assayed directly and the standard curve was constructed in an equivalent volume of affinity-stripped plasma.

2.11.5 Validation of Plasma Assay

2.11.5.1 Parallelism

Parallelism was assessed when up to 500μl of plasma was assayed directly by comparing the displacement curve obtained when pooled human plasma collected at night-time was serially diluted with affinity-stripped plasma, with the standard curve.

Parallelism was achieved with unextracted plasma and is demonstrated in Figure 2.10.

2.11.5.2 Recovery

Analytical recovery studies were performed by adding known amounts of analyte and assaying the spiked samples. The results are given in Table 2.4.
2.11.5.3 Assay Performance

The sensitivity of the plasma assay varied between 5.0 pg/ml and 12.5 pg/ml depending on the volume of plasma assayed directly.

The precision of the assay was determined by intra-assay and interassay analysis. To determine interassay variation, quality control samples were measured in successive assays. The intra-assay and interassay coefficients of variation (CVs) are given in Table 2.5.

2.11.5.4 Collection of Samples

Blood samples were collected into heparinised tubes and were centrifuged immediately. Plasma was frozen and stored at -20°C.

2.11.5.5 Storage of Samples

The stability of aMT6s in plasma under various storage conditions was determined. Plasma samples were stored without preservative or with boric acid (1.0g/l) or ascorbic acid (1.0g/l) at -12°C and -20°C for up to two years. The effect of freeze-thawing was also assessed. aMT6s was measured by RIA after one day, one week, one, six, twelve, eighteen and twenty-four months.

aMT6s was found to be very stable in plasma. No differences were found between samples stored with or without preservatives, or on freeze-thawing. aMT6s was stable in plasma for up to five days when stored at 4°C and for up to two years when stored at -20°C or -12°C. Figure 2.11 compares samples stored for up to two years at -20°C, either with or without preservatives. The interassay CVs for plasma samples stored for up to two years are given in Table 2.6.
Figure 2.10  Parallelism Between aMT6s Standard Curve in Affinity-Stripped Plasma and Increasing Amounts of Unextracted, Pooled Plasma Collected at Night-Time Diluted with Affinity-Stripped Plasma.

Table 2.4  Recovery by RIA of aMT6s Added to Human Plasma Pools

<table>
<thead>
<tr>
<th>aMT6s Added (pg/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.7</td>
<td>113.9 ± 9.2</td>
</tr>
<tr>
<td>70.0</td>
<td>101.9 ± 2.9</td>
</tr>
<tr>
<td>238.2</td>
<td>84.2 ± 3.3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=5
pg/ml % CV n

Intra-assay 17.8 6.7 5
Data 77.4 5.2 5
248.8 3.1 5

Interassay 23.4 10.6 18
Data 61.0 8.8 18
130.8 6.8 18

Table 2.5  
Precision Data for the Plasma Assay

Figure 2.11 Concentrations of aMT6s in Plasma Samples Stored Without Preservative, With Boric Acid, or With Ascorbic Acid for up to 2 Years at -20°C
<table>
<thead>
<tr>
<th>Storage Condition</th>
<th>Mean ± SD (pg/ml)</th>
<th>n</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat, -12°C</td>
<td>60 ± 5.5</td>
<td>8</td>
<td>9.8</td>
</tr>
<tr>
<td>Neat, -20°C</td>
<td>59.0 ± 7.0</td>
<td>9</td>
<td>12.0</td>
</tr>
<tr>
<td>Neat, -20°C, Freeze thaw</td>
<td>57.0 ± 7.5</td>
<td>9</td>
<td>13.0</td>
</tr>
<tr>
<td>Boric Acid, -20°C</td>
<td>56.5 ± 10.0</td>
<td>9</td>
<td>18.3</td>
</tr>
<tr>
<td>Boric acid, -20°C, Freeze thaw</td>
<td>52.5 ± 6.5</td>
<td>9</td>
<td>12.3</td>
</tr>
<tr>
<td>Ascorbic acid, -20°C</td>
<td>57.5 ± 10.0</td>
<td>9</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Table 2.6  CVs for Plasma Samples Stored Under Various Conditions For Up To 2 Years
2.12 **Urine aMT6s Assay**

2.12.1 **Preparation of Charcoal-Stripped Urine**

5% (weight/vol) defined charcoal (Section 2.12.1) was added to a pool of normal human urine, collected during the afternoon, and was mixed overnight at 4°C. The urine was centrifuged at 3000rpm for 15min, then filtered once through a Zeiss filter. The charcoal-stripped urine was stored frozen at -20°C in 500µl aliquots.

2.12.2 **Procedure for Urine Assay**

The RIA procedure for urine samples was identical to that given in Section 2.8 except for the following modifications: urine samples were diluted 1:50 in assay buffer and 200µl were assayed directly. The standard curve was constructed in an equivalent volume of charcoal-stripped urine, diluted 1:50 in assay buffer.

2.12.3 **Validation of Urine Assay**

2.12.3.1 **Parallelism**

Parallelism was assessed by comparing the displacement curve obtained with a pool of human urine samples collected in the early morning serially diluted with charcoal-stripped urine, with the standard curve. The urine was diluted 1:50 with assay buffer and volumes of 1.5-200µl were made up to 200µl with charcoal-stripped urine diluted 1:50 with assay buffer. Parallelism was established by superimposing the urine dilution curve onto the standard curve and is demonstrated in Figure 2.12.

2.12.3.2 **Recovery**

Analytical recovery studies were performed by adding known amounts of analyte and assaying the spiked samples. The results are given in Table 2.7.
2.12.3.3 Assay Performance

The sensitivity of the urine assay was 0.65ng/ml.

The precision of the assay was determined by intra-assay and interassay analysis. The quality control samples used to determine the interassay variation cover a time period of fifteen months. The intra-assay and interassay CVs are given in Table 2.8. Figure 2.13 demonstrates that there was no drift of the quality control samples over a period of fifteen months.

2.12.3.4 Collection of Samples

Urine samples were collected in plastic containers and 5ml aliquots were frozen as soon as possible after collection. The volume of each collection was recorded.

2.12.3.5 Storage of Samples

The stability of aMT6s in urine under various storage conditions was determined. Urine samples were stored without preservative or with boric acid (1.0g/l) or ascorbic acid (1.0g/l) for up to five days at 4°C and room temperature (20°C) and for up to two years at -12°C and -20°C. The effect of freeze-thawing was also assessed. To determine the stability of aMT6s in urine at room temperature, aMT6s was assayed immediately after collection, and on the second, third, fourth and fifth days after collection. To determine its long-term stability, aMT6s was measured by RIA after one day, one week, one, six, twelve, eighteen and twenty-four months.

aMT6s has been found to be very stable in urine. No differences were found between samples stored with or without preservative or on freeze-thawing. aMT6s is stable for up to five days when stored at room temperature or 4°C and for up to two years when stored at -12°C or -20°C. Figure 2.14 compares the measured
amT6s concentration in three urine samples stored at room temperature (20°C) for five days without preservative. Figure 2.15 compares samples stored for up to two years at -20°C, either with or without preservatives. The interassay CVs for urine samples stored for up to two years are given in Table 2.9.

2.12.3.6 Chromatographic Identity of Immunoreactivity

The chromatographic identity of immunoreactivity was determined in three systems. Two TLC systems were used; cellulose plates with n-butanol/glacial acetic acid/water (4/1/1.5 by vol) and silica gel plates with chloroform/methanol (9/1 by vol) as the respective solvents. The paper chromatography system used was Whatmann No 3MM with propan-2-ol/5% ammonia (4/2 by vol) as the solvent. All plates were sprayed with methanol 0.1% ascorbic acid and dried before use.

The migration of standard amT6s, [3H] amT6s and the urinary immunoreactivity were compared. Urine was spotted directly onto the baseline. An equivalent volume of urine was spotted onto each track to control for the effect of urinary salts on the migration of amT6s.

To test for immunoreactivity, the plate was cut up into 1cm portions and eluted with tricine buffer. The immunoreactivity was measured by RIA. The position of the amT6s standard was detected by spraying the plate with Ehrlich's reagent (10% weight/vol in concentrated hydrochloric acid: diluted 1:5 with acetone), while the portion of the plate containing [3H] amT6s was cut up, eluted with 500μl of methanol and counted in a scintillation counter.

The results are illustrated in Figure 2.16. The migration of the urinary immunoreactivity was identical to that of amT6s standard and [3H] amT6s in each chromatographic system. Over 90% of applied immunoreactivity was recovered from all chromatograms.
Figure 2.12 Parallelism Between aMT6s Standard Curve in Charcoal-Stripped aMT6s-Free Urine Diluted 1:50 in Assay Buffer, and Increasing Amounts of Pooled Urine Collected in the Early Morning, Initially Diluted 1:50 in Assay Buffer and Further Diluted with Charcoal-Stripped Urine

<table>
<thead>
<tr>
<th>aMT6s Added (ng/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>115.1 ± 8.8</td>
</tr>
<tr>
<td>8.5</td>
<td>102.6 ± 2.1</td>
</tr>
<tr>
<td>30.6</td>
<td>83.7 ± 1.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n=5

Table 2.7 Recovery by RIA of aMT6s Added to Human Urine Pools
Figure 2.13 Concentrations of aMT6s in Quality Control Samples for the Urine Assay Measured Over a Period of 15 Months

The arrows indicate where new radiolabels were used.

<table>
<thead>
<tr>
<th>ng/ml</th>
<th>%CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay data</td>
<td>2.5</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>28.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Interassay data</td>
<td>2.3</td>
<td>16.3</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>19.5</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Table 2.8 Precision Data for the Urine Assay
Figure 2.14  The aMT6s Concentration in 3 Urine Samples Stored at Room Temperature for 5 Days Without Preservative

Figure 2.15  Concentrations of aMT6s in Urine Samples Stored Without Preservative, With Boric Acid, or With Ascorbic Acid for up to 2 Years at -20°C
<table>
<thead>
<tr>
<th>Storage Conditions</th>
<th>Mean ± SD (ng/ml)</th>
<th>n</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neat, -12°C</td>
<td>36.0 ± 3.3</td>
<td>9</td>
<td>9.0</td>
</tr>
<tr>
<td>Neat, -20°C</td>
<td>35.8 ± 4.2</td>
<td>9</td>
<td>11.7</td>
</tr>
<tr>
<td>Neat, -20°C, freeze-thaw</td>
<td>35.7 ± 3.3</td>
<td>9</td>
<td>9.2</td>
</tr>
<tr>
<td>Boric acid, -12°C</td>
<td>37.2 ± 4.3</td>
<td>8</td>
<td>11.6</td>
</tr>
<tr>
<td>Boric acid, -20°C</td>
<td>34.8 ± 2.6</td>
<td>8</td>
<td>7.6</td>
</tr>
<tr>
<td>Boric acid, -20°C, freeze-thaw</td>
<td>35.5 ± 3.6</td>
<td>9</td>
<td>10.1</td>
</tr>
<tr>
<td>Ascorbic acid, -12°C</td>
<td>36.3 ± 5.0</td>
<td>9</td>
<td>13.8</td>
</tr>
<tr>
<td>Ascorbic acid, -20°C</td>
<td>37.9 ± 6.6</td>
<td>9</td>
<td>17.4</td>
</tr>
<tr>
<td>Ascorbic acid, -20°C, freeze-thaw</td>
<td>36.2 ± 4.0</td>
<td>9</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Table 2.9

CVs for Urine Samples Stored Under Various Conditions for up to 2 Years
Figure 2.16  Migration of Urinary Immunoreactivity in 3 Chromatographic Systems

The arrows indicate the position of cold aMT6s standard.
2.12.3.7 GC/MS Validation of the Urinary Assay

One hundred urine samples from both normal volunteers and patients were assayed for both total and free 6-hydroxymelatonin by the GC/MS method of Tetsuo et al., (1981a). The assays were performed by Mrs M C Shih and Dr S P Markey of the National Institute of Mental Health, Bethesda, USA. The samples were then measured on a single-blind basis by RIA.

Least-squares regression analysis was used to calculate the slope and intercept of the best line through the data points. The standard error of regression, which gives a measure of the scatter of the points about the 'best-fit' line (Cornbleet and Gochman, 1979) was also calculated.

The correlation between the two methods was good ($r = 0.94, n = 100$) and was highly significant ($p<0.001$). Regression analysis of the data gave the equation $y = 0.47x + 0.64\text{ng/ml}$ (where $y$ is RIA) with a standard error of regression of 2.72. The results are presented graphically in Figure 2.17.

2.13 Discussion

The method described in this thesis is the first RIA developed for the measurement of aMT6s. The classical validation techniques employed for the measurement of urine and plasma samples show the method to be sensitive, specific and reproducible. The aMT6s synthesized in our laboratory is immunoreactively identical to other aMT6s standards. The availability of an internationally agreed quantitated standard is highly desirable in the interests of comparing results from different groups.

The radiolabel was prepared by the biological transformation of melatonin, labelled in two different positions, and the radiolabels produced were immunoreactively identical. No differences have been found between different
Figure 2.17 Correlation Between Total 6-Hydroxylated Melatonin Metabolites Measured by GC/MS and aMT6s Measured by RIA
batches of radiolabel and no problems with stability of the label have been encountered for periods of up to one year. The antiserum has been shown to be extremely specific with negligible cross-reactivity with other indoles. The specificity of the antiserum allows plasma and urine samples to be assayed directly, therefore lengthy extraction procedures are avoided. The cross-reactivity with the other urinary metabolites of melatonin is so negligible that it can be discounted in physiological studies.

The affinity column method employed to produce aMT6s-free plasma pools has the considerable advantage that consistent pools can be generated. There are no other reported methods for the measurement of aMT6s in plasma with which our assay can be compared. Unfortunately, the aMT6s levels in plasma are too low to investigate the chromatographic identity of the immunoreactivity.

In addition to validation using chromatographic procedures, the urine assay has been validated by comparison with an established GC/MS method. As expected, the RIA for aMT6s generated consistently lower values than the GC/MS method for total 6-hydroxylated melatonin metabolites. In the samples measured by both methods the total excreted as the sulphate conjugate was not consistent - this may be due to a variable proportion being excreted as the glucuronide conjugate. Genetic differences in the conjugating enzymes or possible drug interactions with the enzymes (Weinshilboum, 1986) may account for this difference.

aMT6s has been shown to be extremely stable. The stability of aMT6s in urine samples at room temperature is particularly important as it enables samples to be collected from volunteers in their normal social environments. The sensitivity of the assay is such that only very small volumes of urine (<1ml) need be stored for analysis.
The reported method has considerable advantages over the other methods for the measurement of urinary melatonin metabolites. It requires a minimal amount of sample with no prior extraction, it is rapid, inexpensive, requires basic equipment and no specially trained personnel. The ease with which large numbers of samples can be processed is essential for the measurement of the large number of samples associated with clinical and circadian rhythm studies. No other RIAs exist for the measurement of urinary melatonin metabolites and the reported method is considerably more rapid and robust than the reported RIAs for urinary melatonin.
Some report, a sea-maid spawn’d him. Some, that he was begot between two stockfishes. But it is certain that when he makes water, his urine is congealed ice; that I know to be true.

W. Shakespeare, Measure for Measure, 3.2.104

CHAPTER 3
BASIC PHYSIOLOGICAL STUDIES ON αMT6s PRODUCTION IN HUMANS
### 3.1 Diurnal Variation in aMT6s Excretion

#### 3.1.1 Introduction

Previous workers (Fellenberg et al., 1980; Tetsuo et al., 1980) have demonstrated a marked diurnal rhythm in the rate of excretion of urinary melatonin metabolites. We carried out a preliminary study to confirm these findings.

#### 3.1.2 Procedure

Four sequential six-hourly urine samples were collected from eight healthy volunteers (four men and four women; age range 23-27 years). aMT6s was measured by RIA.

#### 3.1.3 Results

The values for aMT6s excretion are summarized below and in Figure 3.1.

<table>
<thead>
<tr>
<th>Time</th>
<th>aMT6s Excretion, µg/Collection Mean ± SD (n = 8)</th>
<th>Range, µg/Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200 - 1800h</td>
<td>0.73 ± 0.23</td>
<td>0.32 - 1.09</td>
</tr>
<tr>
<td>1800 - 2400h</td>
<td>0.85 ± 0.18</td>
<td>0.55 - 1.16</td>
</tr>
<tr>
<td>2400 - 0600h</td>
<td>4.53 ± 2.06</td>
<td>2.10 - 8.19</td>
</tr>
<tr>
<td>0600 - 1200h</td>
<td>2.58 ± 0.87</td>
<td>0.80 - 3.84</td>
</tr>
<tr>
<td>Total 24h</td>
<td>8.70 ± 2.85</td>
<td>4.40 - 12.66</td>
</tr>
</tbody>
</table>

82% of the total aMT6s excretion occurred between midnight and midday.

#### 3.1.4 Discussion

The pattern of excretion of urinary aMT6s determined with our RIA is very similar to that reported by Fellenberg and Tetsuo. Fellenberg and colleagues
Figure 3.1

Mean ± SD Urinary aMT6s Excretion During 6-Hourly Intervals for 24h in 8 Subjects
(1980) reported that over 80% of the total 24h aMT6s production was present in the first sample voided after awakening. We found 82% of total aMT6s production was present between midnight and midday.

The aMT6s excretion rates reported (4.4-12.7μg/24h) are very similar to the previously published values. Fellenberg and co-workers (1981) reported aMT6s excretion rates of 6.3-30.9μg/24h, whereas Tetsuo et al., (1981a) quoted 6-hydroxymelatonin excretion rates of 6.2-18.8μg/24h.

The highest levels were found in the 2400-0600h collection when plasma melatonin and urinary melatonin levels are highest. High levels were also found between 0600-1200h. This is likely to be due to a delay in clearance and metabolism.

3.2 Intra-Individual Variation in aMT6s Excretion
3.2.1 Introduction

The excretion of 6-hydroxymelatonin over three consecutive days in seven male volunteers has been studied by Tetsuo et al., (1980). Over the three days the CV for consistency of 6-hydroxymelatonin excretion over 24h for all seven volunteers was 18.7 ± 20.5% (mean ± SD). In a similar study Fellenberg et al., (1981) collected urine from a single volunteer for five days. The CV for consistency of aMT6s excretion over 24h was 14%.

In the above studies the total urine voided over 24h was collected and the volume recorded. The urinary excretion of a compound is often expressed as the 'substance'/creatinine ratio. This overcomes the inconvenience involved in collecting 24h urine samples. In a study by Boyce (1985), the urinary aMT6s levels of melancholic patients have been expressed as both the total excreted (μg), and also as a creatinine ratio (μg aMT6s/g creatinine).
The following experiment was designed to further investigate the intra-individual variation in aMT6s excretion. Both the consistency of total aMT6s excretion and of aMT6s excretion when expressed as a creatinine ratio were investigated.

3.2.2 Procedure

Eighteen apparently healthy volunteers (nine men and nine women; age range 17-67 years) collected urine over 12-hourly time periods, 1000-2200h and 2200-1000h for four consecutive days. Samples were collected under normal conditions of work, activity, diet and sleep. All subjects were drug free apart from five women taking oral contraceptives.

aMT6s was measured by RIA. All the samples from any one individual were measured in the same assay. The creatinine measurements were carried out on a Cobas Bio auto-analyser using a Boehringer Mannheim kit (number 124 192). The measurements depend on the colour reaction produced when creatinine reacts with picric acid in an alkaline medium. The interassay CV of this method is <5%.

Two-way analysis of variance was used to analyse the data. The CVs for day-to-day differences in the urinary excretion of total aMT6s, and of aMT6s expressed as a creatinine ratio, were calculated for each subject. The percentage of the total 24h aMT6s production excreted at night was also calculated.

3.2.3 Results

Two-way analysis of variance of the urinary excretion of aMT6s over four consecutive days showed a significant variation between the total urinary aMT6s levels of the different subjects, for the daytime ($F = 6.34, p < 0.001, df 17, 51$), night-time ($F = 23.79, p < 0.001, df 17, 51$) and 24h samples ($F = 26.62, p < 0.001, df 17, 51$).
There was no significant within-group variation showing that for any one individual the total excretion of aMT6s was consistent within the four-day period.

Two-way analysis of variance of the urinary excretion of aMT6s over the four days, when expressed as a creatinine ratio, showed a significant variation between the urinary levels of the different subjects for the daytime ($F = 1.90, p < 0.04, df 17, 51$), night-time ($F = 2.23, p < 0.02, df 17, 51$) and 24h samples ($F = 2.31, p < 0.01, df 17, 51$). There was no significant within-group variation.

The means and the ranges of the total urinary aMT6s production are shown in Table 3.1, together with the mean CVs for consistency of total aMT6s excretion over the four days and the ranges of the CVs observed. The corresponding values for urinary aMT6s excretion, when expressed as a creatinine ratio are shown in Table 3.2.

The mean $\pm$ SD aMT6s production over the four-day period for all eighteen volunteers is shown in Figure 3.2. Figure 3.3 illustrates the aMT6s excretion in four individual volunteers over the 96h period.

Of the total 24h urinary aMT6s excretion $80.7 \pm 6.1\%$ (mean $\pm$ SD, $n = 18$) was excreted between 2200-1000h. The proportion of aMT6s excreted at night by each individual subject was remarkably consistent. The mean CV for the proportion of aMT6s excreted at night by each volunteer over the four days was $7.40 \pm 4.7\%$ (mean $\pm$ SD, $n = 18$). One-way analysis of variance showed no significant differences between sexes, in the meaned total aMT6s excretion for the daytime, night-time and 24h samples.
<table>
<thead>
<tr>
<th>Time</th>
<th>Male Subjects</th>
<th>Female Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2 - 26.7</td>
<td>12.7 ± 5.3</td>
<td>2.47 - 30.11</td>
</tr>
<tr>
<td>3.5 - 36.0</td>
<td>17.9 ± 7.9</td>
<td>1.53 - 25.66</td>
</tr>
<tr>
<td>10.5 - 63.5</td>
<td>31.6 ± 15.9</td>
<td>0.83 - 4.45</td>
</tr>
</tbody>
</table>

Total 24h
2200-0100
1000-2200

CVs for AMT6s Excretion

Table 3.1
Measures and Ranges of the Total AMT6s Excretion Over a 4-Day Period and the Ranges of the CVs Observed Together with the Mean CVs for Consistency.
Corresponding mean Cvs for consistency of AMTL6 excretion over a 4-day period, together with the means and ranges of AMTL6 excretion, expressed as a creatinine ratio, and the corresponding CVs observed.

<table>
<thead>
<tr>
<th>Time</th>
<th>Male Subjects</th>
<th>Female Subjects</th>
<th>AMTL6 excretion, mg AMTL6/g creatinine</th>
<th>Range</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>(n = 18)</td>
<td>(n = 18)</td>
<td>143.10 ± 62.41</td>
<td>19.34 - 477.86</td>
<td>40.2 ± 30.0</td>
</tr>
<tr>
<td>2200-1000</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>118.89 ± 56.69</td>
<td>22.23 - 454.19</td>
<td>42.9 ± 32.3</td>
</tr>
<tr>
<td>1000-2200</td>
<td>(n = 9)</td>
<td>(n = 9)</td>
<td>23.08 ± 10.25</td>
<td>11.21 - 40.82</td>
<td>40.2 ± 18.6</td>
</tr>
<tr>
<td>Total 24h</td>
<td></td>
<td></td>
<td>205.05 ± 203.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.2  Mean ± SD Urinary aMT6s Excretion During 12h Intervals for 96 Consecutive Hours in 18 Subjects

Figure 3.3  Urinary aMT6s Excretion, Determined at 12h Intervals for 96 Consecutive Hours in 4 Subjects
3.2.4 Discussion

The marked diurnal variation in aMT6s excretion has been confirmed and the excretion profile of total aMT6s has been shown to be consistent for any individual over four days. Consistency of excretion was greatest for the night-time values. Daytime values are often at, or near, the detection limit of the assay; in this study no restriction was made on fluid intake and slight assay variations may be magnified by large urine volumes and the subsequent multiplication factors.

The mean CV for consistency of total 24h aMT6s excretion in eighteen volunteers over four consecutive days was 13.7 ± 5.3% (mean ± SD). This is very similar to the CVs for 24h urinary metabolite excretion reported by Tetsuo and Fellenberg (for details see Section 3.2.1) which were 18.7 ± 20.5% (mean ± SD, n = 7) over three days, and 14% (n = 1) over five days, respectively.

The aMT6s values obtained are again in agreement with those previously reported. Urinary levels of aMT6s excretion in both males and females were similar and ranged from 2.47-30.11 µg aMT6s/24h. There were very large inter-individual differences in aMT6s excretion which were highly significant.

When aMT6s excretion was expressed as a creatinine ratio, the variability in aMT6s excretion from day-to-day for each individual subject, became unacceptably high. The mean CV for consistency of 24h aMT6s excretion over the four days, when expressed as a creatinine ratio was 40.2 ± 30.0% (mean ± SD, n = 18), which compares with a CV of 13.7 ± 5.3% (mean ± SD, n = 18) for total 24h aMT6s excretion.

The reasons why there is a large variability in aMT6s excretion rates when expressed as a creatinine ratio are two-fold. Firstly, there can be considerable day-to-day variations in the urine creatinine content and therefore its use as an index of
completeness of 24h urine collections is misleading. Secondly, there is a small circadian rhythm in creatinine excretion. The diurnal variation in aMT6s excretion is opposite in phase to that of creatinine and therefore using the 'creatinine ratio' again gives rise to misleading results (Daly, 1978).

In conclusion, the experiment has shown that the excretion of total aMT6s for any one individual is consistent over a four-day period. A single 24h sample is sufficient to give a representative measure of the aMT6s excretion of a particular subject. There are, however, large inter-individual variations in aMT6s excretion. All the urine excreted has to be collected and the volume recorded. Expressing the aMT6s excretion as a creatinine ratio is not a viable alternative due to the high variability of the results obtained.

3.3 The Correlation Between Plasma Melatonin Levels, Plasma aMT6s Levels, and Urinary aMT6s Excretion

3.3.1 Introduction

It is now well established that there is a marked diurnal rhythm in plasma concentrations of melatonin in humans. In normal volunteers daytime plasma melatonin levels are very low, usually undetectable by RIA technology. Melatonin secretion increases at night and reaches a peak at approximately 0100-0300h (for review, see Arendt, 1986b). In order to obtain meaningful results, hourly blood samples need to be taken at night. This is obviously an extremely difficult undertaking. If the measurement of a urinary metabolite of melatonin is to be a viable alternative, the exact relationship between plasma concentrations of melatonin and urinary aMT6s has to be established.

Fellenberg and co-workers (1981) administered deuterated-melatonin (50-1000μg doses) intravenously to two male volunteers and found that 99.7% of tracer
was excreted within 24h. About 42% of the administered dose was excreted as deuterated aMT6s confirming the significance of this pathway as a major route of excretion of melatonin and confirming that the estimation of endogenous aMT6s was a good index of melatonin production.

The first study to determine the correlation between plasma melatonin and levels of urinary melatonin metabolites was carried out by Markey et al., (1985). A significant correlation (r = 0.76, p = 0.00004) was found between night-time peak plasma melatonin levels (samples taken at 3h intervals) and the 24h urinary excretion totals for conjugated 6-hydroxymelatonin for a group of twenty-two women.

The experiment described here was carried out to determine the correlation between concentrations of plasma melatonin and urinary aMT6s. No plasma assays for melatonin metabolites have been reported and this is the first study to investigate the diurnal changes in the plasma concentrations of aMT6s.

3.3.2 Procedure

Blood was sampled (July) from eighteen apparently healthy volunteers (twelve men and six women) at two-hourly intervals from 1200-2000h and at hourly intervals from 2000-1000h with one final blood sample at 1200h. Blood was sampled by venepuncture during the day, whereas an indwelling cannula inserted into the antecubital vein was used for the night-time sampling. Night-time samples were taken using a dim red torch (< 1 lux). Simultaneously with blood sampling subjects collected timed six-hourly urine samples, 1200-1800h, 1800-2400h, 2400-0600h and 0600-1200h.

Four additional male volunteers were also included in the study. From 2000-1000h blood was sampled (May) at hourly intervals, no blood samples were collected during the day. Simultaneous urine samples were collected 2000-2400h, 2400 -1200h and 1200-2000h. The samples obtained from these four male volunteers were the 'controls' in a collaborative study (Bojkowski et al.,1987).
The age range of all the volunteers was 18-47 years. During the day subjects remained in their normal working environments and were kept at the Clinical Investigation Unit, St Luke's Hospital, Guildford, overnight. Subjects were sent to bed at 2330h and were awakened at 0700h.

All plasma samples were assayed for both melatonin (Fraser et al., 1983a) and aMT6s. Urine samples were assayed for aMT6s. The area under the curve (AUC) of the 24h plasma profiles was calculated by the trapezium method (Cornish-Bowdem, 1981). For the purposes of calculation all values below the detection limit of the assay were set at the detection limit, as were the daytime samples of the four volunteers sampled in May. In normal volunteers daytime plasma melatonin levels are very low, usually undetectable by RIA technology (Arendt, 1986b). The quantitative relationships between urinary aMT6s and plasma concentrations of melatonin and aMT6s were determined by the coefficient of linear regression.

3.3.3 Results

Plasma aMT6s was found to have a marked circadian rhythm, closely related to that of melatonin. Figure 3.4a and 3.4b illustrates the individual plasma melatonin and aMT6s profiles in all the subjects. In all the volunteers except subjects B and G daytime melatonin and aMT6s values were low and peak values were obtained at night.

In the twenty-two subjects, the mean concentrations of plasma melatonin varied from 8.0pg/ml (1400-1800h) to 49.6pg/ml (0400h) while the mean plasma aMT6s levels varied from 11.0pg/ml (1600-2100h) to 85.2pg/ml (0500h) (Figure 3.5). There were large inter-individual differences in peak melatonin values (range 10.0 - 180.0pg/ml) and peak aMT6s values (range 18.6 - 233.0pg/ml). The mean values for urinary aMT6s excretion were (μg aMT6s; mean ± SD, n = 18) 0.68 ± 0.27 (1200-1800h),
1.24 ± 0.65 (1800-2400h), 4.38 ± 1.84 (2400-0600h) and 1.87 ± 1.08 (0600-1200h). The mean total 24h urinary aMT6s excretion was 8.76 ± 3.43μg aMT6s (mean ± SD, n = 22).

The AUCs and the peak heights of the respective plasma melatonin and aMT6s profiles as well as the total 24h urinary aMT6s excretion for all twenty-two volunteers are given in Table 3.3. There were significant correlations between all the parameters (Table 3.4). The most important correlation was that between plasma melatonin (AUC of 24h profile) and total 24h urinary aMT6s (r = 0.75, p = 0.0002, n = 22) (Figure 3.6). This relationship is of great relevance and enables urinary aMT6s to be used as an index of plasma concentrations of melatonin.

The AUCs of the melatonin and aMT6s profiles and the total 24h aMT6s excretion showed no correlation with age, height or weight (p>0.05 in all cases).

3.3.4 Discussion

There is a very good correlation between urinary levels of aMT6s and plasma concentrations of melatonin (r = 0.75, p = 0.0002). This validates the use of the urinary metabolite as an index of plasma concentrations of melatonin. A very similar relationship between plasma melatonin and urinary 6-hydroxymelatonin (r = 0.76, p = 0.00004, n = 22) was reported by Markey and colleagues (1985). Whether these correlations are maintained in various disease states or after drug treatment remains to be established.

In many situations the measurement of urinary aMT6s is likely to provide a better index of pineal melatonin production than the measurement of plasma samples. Studies have been published in which single time-point sampling has been used. The marked diurnal variation in plasma concentrations of melatonin brings the validity of such results into question. In situations where plasma samples are not taken
at frequent intervals over 24h, the measurement of urinary aMT6s provides an integrated measure of pineal melatonin production over several hours and is likely to yield more valuable data.

The measurement of melatonin and its major metabolites in the same subjects indicates that low melatonin levels are not the result of more rapid metabolism and clearance, but result from low pineal production. Two of the volunteers, B and G, had no apparent rhythm in melatonin. Subject B had no aMT6s rhythm while subject G had a small night-time aMT6s peak. All the values for this subject, however, were at or near the detection limit of the assay. It is interesting to observe that neither of these subjects showed any apparent clinical abnormalities. In all the other subjects the rhythmic characteristics of the night-time increases in plasma melatonin and plasma aMT6s and in urinary aMT6s excretion were very marked. Daytime plasma levels were at or near the detection limit of the assays while the onset/decline of the night-time production of melatonin/aMT6s was very consistent between subjects.

Plasma aMT6s levels were very closely related to plasma melatonin levels. Mean peak aMT6s levels were achieved at 0500h slightly later than peak melatonin levels at 0400h, and the plasma concentrations of aMT6s remained elevated for about 2h longer than those of melatonin. There were changes in the ratio of melatonin to aMT6s over 24h, possibly due to a delay in clearance of aMT6s from the circulation compared to the rate of secretion and metabolism of melatonin. Whether there is a change in the rate of metabolism of melatonin or in the clearance of aMT6s over 24h is not known. The elimination half-life for aMT6s is greater than the corresponding half-life for melatonin (see Chapter 4) and this helps to explain why these changes in the ratio of melatonin to aMT6s are observed. The inter-individual differences in plasma concentrations of melatonin which have been previously reported (Arendt, 1978) were reflected in the plasma and urinary aMT6s levels.
Figure 3.4a  Plasma Melatonin and Plasma aMT6s (pg/ml) Profiles in 12 Subjects
Figure 3.4b
Plasma Melatonin and Plasma αMT6s
Profiles in 10 Subjects
Figure 3.5  Mean (± SEM) Concentrations of Melatonin and aMT6s in Plasma from 22 Subjects (16 Men and 6 Women) Sampled for 24h

Figure 3.6  Correlation Between Individual Levels of Plasma Melatonin (AUC of 24h Profile) and Total Urinary aMT6s Excreted Over 24h (n = 22)
<table>
<thead>
<tr>
<th>Subject</th>
<th>Plasma AUC pg/ml.h</th>
<th>Plasma Peak Height pg/ml</th>
<th>aMT6s AUC pg/ml.h</th>
<th>aMT6s Peak Height pg/ml</th>
<th>Urinary aMT6s µg/24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>345</td>
<td>28</td>
<td>589</td>
<td>49</td>
<td>6.43</td>
</tr>
<tr>
<td>B</td>
<td>276</td>
<td>18</td>
<td>339</td>
<td>19</td>
<td>3.79</td>
</tr>
<tr>
<td>C</td>
<td>868</td>
<td>97</td>
<td>1383</td>
<td>138</td>
<td>9.68</td>
</tr>
<tr>
<td>D</td>
<td>334</td>
<td>32</td>
<td>632</td>
<td>60</td>
<td>8.49</td>
</tr>
<tr>
<td>E</td>
<td>273</td>
<td>20</td>
<td>582</td>
<td>58</td>
<td>9.62</td>
</tr>
<tr>
<td>G</td>
<td>240</td>
<td>8</td>
<td>348</td>
<td>25</td>
<td>2.99</td>
</tr>
<tr>
<td>H</td>
<td>551</td>
<td>62</td>
<td>701</td>
<td>78</td>
<td>7.75</td>
</tr>
<tr>
<td>J</td>
<td>440</td>
<td>42</td>
<td>858</td>
<td>73</td>
<td>9.15</td>
</tr>
<tr>
<td>L</td>
<td>398</td>
<td>40</td>
<td>800</td>
<td>63</td>
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</tr>
<tr>
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<td>939</td>
<td>130</td>
<td>1966</td>
<td>233</td>
<td>12.11</td>
</tr>
<tr>
<td>2B</td>
<td>217</td>
<td>24</td>
<td>426</td>
<td>42</td>
<td>4.40</td>
</tr>
<tr>
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<td>592</td>
<td>72</td>
<td>948</td>
<td>132</td>
<td>9.43</td>
</tr>
<tr>
<td>2D</td>
<td>406</td>
<td>37</td>
<td>855</td>
<td>87</td>
<td>7.66</td>
</tr>
<tr>
<td>2E</td>
<td>1116</td>
<td>180</td>
<td>1138</td>
<td>152</td>
<td>12.66</td>
</tr>
<tr>
<td>2F</td>
<td>206</td>
<td>24</td>
<td>541</td>
<td>66</td>
<td>5.87</td>
</tr>
<tr>
<td>2G</td>
<td>148</td>
<td>21</td>
<td>760</td>
<td>75</td>
<td>7.80</td>
</tr>
<tr>
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<td>389</td>
<td>53</td>
<td>711</td>
<td>82</td>
<td>9.65</td>
</tr>
<tr>
<td>3A</td>
<td>546</td>
<td>76</td>
<td>846</td>
<td>101</td>
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</tr>
<tr>
<td>3C</td>
<td>938</td>
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<td>1340</td>
<td>150</td>
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<tr>
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<td>33</td>
<td>718</td>
<td>132</td>
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<tr>
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<td>476</td>
<td>55</td>
<td>1240</td>
<td>183</td>
<td>9.16</td>
</tr>
</tbody>
</table>

Table 3.3 AUCs and Peak Heights of Plasma Melatonin and aMT6s Profiles and 24h Urinary aMT6s Excretion for 22 Subjects
Comparison                                             r      p  
(n=22)                                                
AUC plasma melatonin: total 24h urinary aMT6s         0.75   0.0002 
Plasma melatonin peak height: total 24h urinary       0.70   0.0005 
aMT6s                                                
AUC plasma aMT6s: total 24h urinary aMT6s             0.70   0.0005 
Plasma aMT6s peak height: total 24h urinary           0.64   0.002 
aMT6s                                                
AUC plasma melatonin: AUC plasma aMT6s                0.76   0.0001 
Plasma melatonin peak height: plasma aMT6s peak height 0.73   0.0003 

Table 3.4 The Correlation Between Individual Levels of Plasma
Melatonin (AUC of 24h Profile and Peak Height), Plasma
aMT6s (AUC of 24h Profile and Peak Height), and Total
Urinary aMT6s Excreted Over 24h
3.4 Investigation of Short-Term Fluctuations in Plasma Melatonin and aMT6s Levels

3.4.1 Introduction

Several reports have appeared in the literature on the episodic secretion of melatonin. Coarse pulsatile rises in melatonin concentration in plasma lasting an hour or more have been observed with relatively long (20-30 min) sampling intervals (Vaughan et al., 1978; Weinberg et al., 1979). Episodic secretion of melatonin was also found in daytime samples taken from pre- and postpubertal children (Penny, 1985), and in normal volunteers when assayed by GC/MS (Mullen et al., 1980). Pulsatile patterns of melatonin secretion in humans have also been reported by Birkeland (1982) and Claustrat and colleagues (1986) with a mean of 2.5 and 4.5 peaks during the night respectively. Vaughan and co-workers (1979a) found pulses of melatonin about 10 min apart and in another study in which a sampling interval of 2-5 min was used, even more rapid fluctuations in concentration were observed (Vaughan et al., 1979b).

Further evidence for the episodic secretion of melatonin has been obtained from studies in sheep. Blood samples were taken at 12 min intervals from ovariectomized ewes and rapid fluctuations of melatonin levels were observed through the course of the night (Bittman et al., 1983). Recently, English et al., (1987a) reported very rapid fluctuations in plasma melatonin levels in sheep.

The following experiment was performed to confirm the existence of very rapid fluctuations in plasma melatonin levels in humans and to see whether these changes were reflected in the plasma concentrations of aMT6s.

3.4.2 Procedure

Three volunteers (two women and one man; aged 24, 27, and 45 years respectively) had indwelling cannulae inserted into their antecubital vein at 2330h.
They then retired to bed in darkness. At 2400h and 0300h the volunteers had 5ml blood samples taken, in darkness, with a dim red torch (<1 lux), at 30s intervals for 10 min. The plasma samples were assayed for melatonin and aMT6s, all samples from any one individual being determined in the same assay. Control or noise series were created by pooling the remaining aliquots of plasma from one or more of the volunteers to form large plasma pools. Sequential samples from these pools were then assayed in an identical manner. All measurements for a series of samples were performed in the same assay.

The following method was used to identify peaks in the data. All local minima and maxima were identified. The concentration at the minimum was multiplied by three times the concentration matched intra-assay CV to obtain the corresponding increment in hormone concentration. The next local maximum was considered a peak if it exceeded the minimum by an amount equal to or greater than this increment. The CV of the twenty samples in the noise series was used to identify peaks in that particular series and in concentration matched volunteers' samples. The number of peaks in the volunteers' samples was compared by paired Student's t-test (two-tailed) to the number of peaks in concentration matched noise series (false positives).

3.4.3 Results

The melatonin and aMT6s concentrations in the volunteers' and the control samples are shown in Figure 3.7a and Figure 3.7b, respectively. No secretory episodes, with short duration pulses of an amplitude significantly greater than that observed in the control noise series, were observed in the volunteers' samples. The CV for each series of samples was calculated. The mean CVs for the volunteers' samples were 7.5 ± 2.6% (n = 6) and 6.7 ± 1.2% (n = 6) for melatonin and aMT6s respectively, whereas for the concentration matched noise series the mean CVs were 4.3 ± 0.3% (n = 6) and 7.9 ± 0.3% (n = 6) respectively. A slightly greater variability was observed in the volunteers' melatonin samples than in the noise series.
Attempts to demonstrate pulsatile secretion of melatonin. Volunteers' samples were taken at 30s intervals for 10min; all samples from 1 individual were measured in the same assay. The control or 'noise' series were plasma pools that were assayed identically.
Attempts to demonstrate pulsatile secretion of aMT6s. Volunteers' samples were taken at 30s intervals for 10min; all samples from 1 individual were measured in the same assay. The control or 'noise' series were plasma pools that were assayed identically.
Using the three CV criterion, peaks were observed in both the melatonin noise series (eleven peaks in total) and the volunteers' melatonin samples (fourteen peaks in total). In the aMT6s noise series three peaks in total were observed, whereas in the volunteers' aMT6s samples only one peak was observed. The total number of peaks observed in the volunteers' melatonin or aMT6s samples compared to the total number of peaks in concentration matched control series showed no significant difference using the paired Student's t-test (two-tailed).

3.4.4 Discussion

In the majority of cases where workers have reported the existence of episodic secretion of melatonin the same methodology has been used (the RIA of Rollag and Niswender, 1976) adapted for the assay of human plasma. Melatonin concentrations of 500pg/ml (Weinberg et al., 1979) and 3000pg/ml (Birkeland, 1982) have been reported, which brings into doubt whether these workers were measuring melatonin. Other workers have found daytime values of around 50pg/ml (Mullen et al., 1980; Penny, 1985) and this again casts doubt on the validity of the methodology employed.

Where melatonin spikes have been reported, very little data is given on the intra-assay variation of the methodology used. With frequent blood sampling the probability of detecting false positive peaks due to assay noise is increased. It is very important to include data on the variability of repeated measurements of the same samples. Ideally, a more rigorous criterion than an increment from nadir to peak of three times the intra-assay CV should be used to identify peaks, such as multiple measurement at each time point (Ross et al., 1984). Unfortunately, with the volume of plasma required in our assay this was not possible. In only one study on the episodic secretion of melatonin in humans have such criteria been applied (Claustrat et al., 1986).
Claustrat and colleagues (1986) reported episodic secretion of melatonin with a mean of 4.5 peaks per night. The amplitude of the reported peaks was low. In the data presented in this thesis there are no 'episodic' variations in melatonin levels which are of an amplitude significantly greater than that which can be accounted for by the intra-assay variation.

The best evidence for rapid fluctuations of melatonin levels throughout the night comes from experiments in sheep. These fluctuations were first observed by Bittman and colleagues (1983) and their existence was confirmed by English and colleagues (1987a). In both these studies, samples have been taken from the jugular vein. Recently, further evidence for episodic secretion of melatonin in sheep has been obtained by Cozzi and co-workers (1987). Blood samples were obtained from the rectus sinus (a blood vessel draining the pineal gland), and the jugular vein. Episodic release of melatonin was observed at both sampling sites. It is possible that the rapid changes in concentration do not result from episodic secretion of melatonin but are caused by changes in blood flow or by changes in clearance. It is likely that human melatonin secretion is episodic but that peaks seen in the jugular vein may be 'damped' in the antecubital vein. In this study we have found no evidence for short duration pulses of melatonin or aMT6s in plasma samples taken from the antecubital vein. In 1986, using an extensively validated assay, Webley and Leidenberger also reported no evidence for the episodic secretion of melatonin in humans. In their study samples were taken from the antecubital vein at 15min intervals.
3.5 Effect of Atenolol on Urinary aMT6s

3.5.1 Introduction

It is now well established that the production of melatonin by the pineal gland is dependent on the stimulation of post-synaptic pineal β-adrenoceptors (Axelrod, 1974). Studies have shown that pineal β-adrenoceptors in the rat are of the β-subtype (Zatz et al., 1976) and lie outside the blood-brain barrier (Arendt et al., 1981b).

Previous work in humans has demonstrated that propranolol reduces the nocturnal increase in plasma melatonin (Vaughan et al., 1976b; Hanssen et al., 1977). Propranolol is a non-selective β-adrenoceptor antagonist which crosses the blood-brain barrier (Cruickshank et al., 1980). Taking plasma samples at 1400h and 2400h Cowen and colleagues (1983) were able to demonstrate that atenolol, a selective β₁-adrenoceptor antagonist which crosses the blood-brain barrier poorly (Cruickshank et al., 1980), caused a significant reduction in midnight melatonin concentration. The results suggested that human β-adrenoceptors were of the β₁-subtype.

The experimental protocol of Cowen and colleagues (1983) was adapted in order to investigate the effect of atenolol on urinary aMT6s production.

3.5.2 Procedure

Six apparently healthy subjects (three men and three women; age range 24-37 years) took 100mg atenolol or placebo (ascorbic acid) at 1800h in a single blind cross-over protocol, either on consecutive days (n = 4) or with one day between doses. Subjects had been told that the order of administration was randomized, however, in all cases they received placebo on the first day. Subjects kept to the same routine on each of the test days but no other social or dietary restrictions were applied.
Six-hourly urine samples were collected for 24h from 1200h on each of the test days. Urine samples were assayed for aMT6s and statistical significance was determined by the paired Student's t-test (one-tailed). Ethical committee approval for this study was obtained from the Ethical Committee of St Luke's Hospital, Guildford.

3.5.3 Results

The aMT6s values are shown in Table 3.5 and in Figure 3.8.

When the results were compared by paired Student's t-test significant reductions were obtained in aMT6s excretion following atenolol administration for the time periods between 2400 - 0600h (p<0.005) and 0600-1200h (p<0.005) and for the total 24h aMT6s excretion (p<0.0025).

Atenolol treatment totally abolished the night-time peak in aMT6s excretion, however, there were no significant differences in the daytime values.

3.5.4 Discussion

Our results confirm the observations made by Cowen and colleagues (1983) and show for the first time the abolition of the diurnal rhythm in urinary aMT6s excretion following a single oral dose of atenolol. In previous studies where single night-time samples have been taken, any decreases in observed plasma melatonin levels, may be due to marked phase-shifts in melatonin production, induced by the administered drug. The urinary data presented represents an integrated measure of melatonin production, therefore a decrease in aMT6s excretion following atenolol administration is due to a decrease in melatonin secretion. It may be argued that atenolol affects melatonin metabolism, however, the previously published data on plasma melatonin levels negates this hypothesis.
### Table 3.5 The Effect of Atenolol on Urinary aMT6s Levels

<table>
<thead>
<tr>
<th>Time</th>
<th>Placebo aMT6s (µg/collection) Mean ± SD</th>
<th>Atenolol aMT6s (µg/collection) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200 - 1800h</td>
<td>0.51 ± 0.10</td>
<td>0.55 ± 0.11</td>
</tr>
<tr>
<td>1800 - 2400h</td>
<td>0.85 ± 0.71</td>
<td>0.59 ± 0.25</td>
</tr>
<tr>
<td>2400 - 0600h</td>
<td>6.47 ± 3.63</td>
<td>0.82 ± 0.44</td>
</tr>
<tr>
<td>0600 - 1200h</td>
<td>2.80 ± 1.35</td>
<td>0.63 ± 0.32</td>
</tr>
<tr>
<td>Total 24h</td>
<td>10.63 ± 4.45</td>
<td>2.59 ± 0.96</td>
</tr>
</tbody>
</table>

**Figure 3.8**

Mean (± SD) Urinary aMT6s Excretion During 6h Intervals Before and After Treatment with Atenolol
This experiment confirms the suggestion that pineal melatonin production in humans is under the control of β-adrenoceptors which lie outside the blood brain barrier. Chronic treatment of hypertensive patients with atenolol has been shown to lead to lowered melatonin levels at midnight, when compared to hypertensive patients treated with diuretics (Cowen et al., 1985). It is open to speculation as to whether any of the clinical effects of β-adrenoceptor blocking drugs are due to a decrease in melatonin production.

3.6 General Discussion

The experiments described have confirmed that there are marked diurnal variations in aMT6s excretion. The excretion of aMT6s for any individual has been shown to be very consistent and a single 24h urine sample is sufficient to give a representative measure of an individual's aMT6s production. The urinary aMT6s levels we report are in close agreement with previously published values and indirectly confirm the accuracy of the method which was used to quantitate our standard (Section 2.9).

In normal volunteers, urinary aMT6s has been shown to be a good index of plasma concentrations of melatonin, thus for most purposes, nocturnal blood sampling can be avoided. Plasma aMT6s levels are very closely related to those of melatonin and plasma aMT6s measurements may prove to be a very useful tool in backing-up plasma melatonin data.

aMT6s is stable in urine and samples can be collected with ease. However, the accuracy of the measurements depends largely on the compliance of the volunteers. There are very large inter-individual differences in aMT6s excretion. Thus, for statistical reasons, large populations are required in cross-sectional studies or individuals must act as their own controls.
She found a little bottle on it, ('which certainly was not here before,' said Alice,) and round its neck a paper label, with the words 'DRINK ME' beautifully printed on it in large letters.

L. Carroll, Alice's Adventures in Wonderland, Chapter 1.
There is considerable interest in a possible therapeutic role of melatonin in man. In rodents, pharmacological doses of melatonin will entrain the rest-activity cycle (Redman et al., 1983) and accelerate the re-entrainment of the circadian rhythm in corticosterone (Murakami et al., 1983). Acute hypnotic-like effects have been observed in humans after large doses of exogenous melatonin (Vollrath et al., 1981; Lieberman et al., 1984) and timed doses of melatonin given at 1700h were found to have a significant effect on self-reported drowsiness (Arendt et al., 1984) and to shift the endogenous melatonin rhythm. Recently timed pharmacological doses of melatonin have also been shown to alleviate the symptoms of jet-lag (Arendt et al., 1986; Arendt et al., 1987). In order to interpret the results of experiments where melatonin has been administered to human subjects, it is essential to know the pharmacokinetic profiles of the preparations used.

There is little information on the pharmacokinetics of melatonin in man. Preliminary data was reported by Wetterberg et al., (1978) and Matthews et al., (1981) following the oral administration of melatonin, however, no pharmacokinetic parameters were determined. Following the intravenous injection of 10μg of melatonin in normal volunteers, the distribution half-life was found to be 5.6min and the elimination half-life was 43.6min (Iguchi et al., 1982a). Waldhauser and co-workers (1984b) reported the kinetic parameters following the oral administration of 80mg melatonin in gelatine capsules. The elimination half-life was 48min and large variations were reported in the fraction of ingested melatonin that was absorbed. The plasma concentrations of melatonin after oral ingestion of different preparations were investigated by Aldhous and colleagues (1985). The elimination half-life varied from 32-40min. The plasma samples obtained in the latter study were assayed for aMT6s and the results form the basis of this Chapter.
4.1.1 Pharmacokinetic Principles

To exert an effect, exogenously administered melatonin must be present in appropriate concentrations at its site of action. This will be a function of the amount administered and also the extent and rate of absorption, distribution, binding or localization in tissue, biotransformation and excretion. Many factors will affect the absorption of a compound; these include molecular size and shape, solubility at the site of absorption and relative lipid solubility. Drugs given in aqueous solution are more rapidly absorbed than those in oily solution, suspension or solid form because they mix more readily with the aqueous phase at the site of absorption. For drugs given in solid form, for example, as a gelatine capsule, the rate of dissolution may be the limiting factor in the absorption (Mayer et al., 1980).

The route of administration of a drug has a major effect on the blood levels observed. If intravenous administration is used the total dose injected enters the circulation, however, if oral administration is used many other factors have to be considered. Drugs in the gastrointestinal tract may be metabolized by the enzymes of the mucosa, the intestinal flora or the liver before they gain access to the general circulation. When a drug is administered subcutaneously, intramuscularly or intravenously into a peripheral vein, it enters the peripheral venous circulation and is distributed into tissues and organs though less than 30% of the dose passes through the liver during the first passage through the body (Gibaldi and Perrier, 1975). When a drug is given orally, access to the peripheral venous circulation is almost exclusively via the hepatic portal system. A compound such as melatonin, which is rapidly metabolized by the liver, may undergo substantial biotransformation before reaching the peripheral circulation when administered orally. This phenomenon is known as the first-pass effect (Gibaldi and Perrier, 1975).
Once present in the circulation a drug is then distributed around the body. The pattern of distribution will depend on physiological factors and physico-chemical properties of the drug. Drugs will enter various tissues and organs and may accumulate in certain tissues. The action of a drug is usually terminated either by metabolism or by renal or biliary excretion of the unchanged drug from the body (Mayer et al., 1980).

Pharmacokinetic principles relate specifically to the variation of drug concentrations with time, particularly in the blood or plasma, as a result of absorption, distribution and elimination. Many pharmacokinetic models used to describe the time course of drugs and/or metabolites are based on the assumptions that the systems are linear with respect to drug distribution (i.e., any changes in the plasma level of a drug quantitatively reflects any changes in the tissue levels of the drug within the same compartment) and that drug elimination is governed by first-order kinetics and occurs exclusively from the compartment containing the plasma (the central compartment). It is assumed that the central compartment is directly accessible to administered drugs regardless of the route of administration (Gibaldi and Perrier, 1975; Mayer et al., 1980).

It is important to realise the limitations of pharmacokinetic models when applied to data obtained from the oral administration of drugs. The proportion of the dose absorbed is unknown, as is the amount of drug eliminated by the first-pass effect. For a compound such as melatonin, the disappearance of melatonin in plasma is the result of renal excretion, liver metabolism, metabolism in other organs and distribution between different 'compartments' of the body. In a complex system the rate constant for the disappearance from plasma is a hybrid rate constant and the half-life calculated can be referred to as the biological half-life. As long as the limitations of the data are considered, similar pharmacokinetic parameters can be
determined for metabolites. If drugs are administered intravenously considerably more pharmacokinetic parameters can be determined. However, the intravenous administration of drugs to volunteers solely for the purposes of pharmacokinetics poses ethical problems.

Ms M Aldhous, Ms C Franey, Dr J Wright and Dr J Arendt are responsible for devising the protocol for oral administration of melatonin and for the organization of the clinical trials. The assays to determine melatonin levels following oral ingestion of different preparations were performed by Ms M Aldhous and Ms C Franey.

The plasma samples obtained following the intravenous administration of \[^{11}C\] melatonin were generously supplied by Dr B Claustrat, University of Lyons. Thanks also go to Dr D Le Bars (Lyon) who synthesized the \[^{11}C\] melatonin and to the staff of the MRC Cyclotron Unit, Hammersmith Hospital.

4.2 Methods

4.2.1 Oral Administration of Melatonin

Melatonin was obtained from Sigma Chemical Company Limited and made up in two formulations. 0.04% weight/vol of melatonin was dissolved in corn oil containing 2% vol/vol ethanol (a). Five ml of this preparation thus provided 2mg of melatonin, and for ingestion was dispersed in 50ml of milk. Gelatine-coated capsules containing 2mg of melatonin dispersed in 250mg of lactose were manufactured by St Luke's Hospital pharmacy, Guildford (b). For ingestion these were taken with approximately 50ml of water.

Ten normal healthy volunteers (five men, five women) aged 21 to 39 years and weight range 50-85kg, were assigned to two groups. A group of four were given
the corn oil preparation, and a group of six were given the gelatine preparation.

Ethical approval for this study was obtained from the Guildford Ethics Committee, St Luke's Hospital.

On the morning of the study subjects ate a standard breakfast consisting of a small bowl of cereal, two slices of toast, and tea, before 0800h. A basal blood sample was taken at 0900h and the melatonin preparation was taken immediately after a second basal sample at 1000h. Blood samples were taken from an indwelling venous cannula 15, 20, 45, 60, 120, 180, 240, 300, 360 and 420min after dosing.

All plasma samples were assayed for melatonin (Fraser et al., 1983a) and for aMT6s. The interassay CVs for the melatonin assay were 8% (25pg/ml), 7.1% (108pg/ml), 12% (141pg/ml) and 4.9% (526pg/ml). The interassay CVs for the aMT6s assay were 32.2% (35pg/ml), 12% (150pg/ml) and 2.0% (673pg/ml).

4.2.2 Intravenous Administration of $[^{11}\text{C}]$Melatonin

In order to study the distribution of melatonin in vivo in man by positron emission tomography (PET), $[^{11}\text{C}]$ melatonin was synthesized by Dr D Le Bars et al., (1987). $[^{11}\text{C}]$ melatonin (41µg, ≈ 12mCi) was injected as a bolus into the antecubital vein of a male volunteer, aged 26 years. Two basal samples were taken and twenty-three blood samples were taken at frequent intervals from an indwelling venous cannula for 75min after injection. The plasma samples were assayed for melatonin and for aMT6s.

4.2.3 Analysis of Results

The logarithm of plasma indole concentration was plotted against time. Areas under the plasma concentration curve (AUC) and the biological half-life for elimination of the terminal phase were calculated using an interactive computer
programme written for the BBC microcomputer. The AUCs of each subject's plasma indole versus time curve were compared to get a measure of the total amount of administered melatonin which was absorbed and which was also metabolized to aMT6s.

4.3 Results

Following oral administration of melatonin the plasma aMT6s levels were much higher and remained elevated for much longer than the plasma melatonin levels. The increases in plasma melatonin levels and in plasma aMT6s following an oral dose of melatonin were simultaneous. The mean plasma melatonin and aMT6s concentrations (± SEM) following gelatine and corn oil preparations are shown in Figures 4.1 and 4.2, respectively. Following the absorption phase, the elimination of melatonin and of aMT6s fitted a one-compartment model. Both preparations gave melatonin levels above baseline for 4-7h after oral administration. aMT6s levels were well above baseline until the end of the experiment (7h after oral administration) in all the volunteers. Highest melatonin and aMT6s values were observed 15-120min and 30-120min, respectively, after ingestion.

There were very large inter-individual differences in the maximum plasma melatonin concentrations achieved, these being thirty seven-fold in the case of the gelatine capsules and eight-fold for the corn-oil preparation. In contrast the maximum plasma aMT6s levels achieved were remarkably consistent. The maximum difference in peak levels between subjects was a two-fold difference in the case of the gelatine capsule. Similar results were found for the AUCs of each subjects plasma indole versus time curve. These results are summarized in Table 4.1. For the AUC of the plasma melatonin data there was a thirteen-fold and nine-fold difference between subjects for the gelatine and corn-oil preparations respectively. For the AUC of the plasma aMT6s data the maximum difference between subjects
was two-fold in the case of the gelatine capsule. The ratio of AUC aMT6s/AUC melatonin varied between subjects from a two-fold difference to a fifty-fold difference.

The biological half-lives for the elimination of melatonin and aMT6s are summarized in Table 4.2. The half-life for each compound was consistent between subjects irrespective of the preparation administered. The overall calculated mean ± SD half-life for melatonin was 44.0 ± 6.6min (n=10) whereas the half-life for aMT6s was slightly longer at 58.2 ± 8.2min (n=10).

The plasma concentrations of melatonin and aMT6s following intravenous administration of [11C] melatonin are shown in Figure 4.3. Melatonin disappearance showed a biphasic pattern with a rapid distribution phase and a slower elimination phase. The half-life of the elimination phase was 48.5min. aMT6s was detectable in the plasma 3.5min after injection of melatonin and peak aMT6s concentrations were achieved after 17.5min. The elimination of aMT6s from the plasma could be described by a one-compartment model and the biological half-life of aMT6s was calculated to be 53.5min.

4.4 Discussion

The results presented demonstrate the effect of first-pass hepatic metabolism. Following the oral administration of melatonin the amount reaching the systemic circulation is substantially less than the dose administered (Waldhauser et al., 1984b). Lane and Moss (1985) applied hepatic clearance concepts to existing data, on the intravenous and oral administration of melatonin to man, and predicted a very high hepatic extraction ratio for melatonin with reduced bioavailability of an oral dose. This prediction has been substantiated by the experimental data presented in this Chapter.
Figure 4.1  Mean (±SEM, n = 6) Plasma Melatonin and aMT6s Concentrations After 2mg Melatonin Taken as a Gelatine Capsule

Figure 4.2  Mean (±SEM, n = 4) Plasma Melatonin and aMT6s Concentrations After 2mg Melatonin Taken as a Corn Oil/Milk Preparation
<table>
<thead>
<tr>
<th></th>
<th>AUC Melatonin (ng/ml.min)</th>
<th>AUC aMT6s (ng/ml.min)</th>
<th>Factor AUC Melatonin</th>
<th>Factor AUC aMT6s</th>
<th>Ratio AUC Melatonin/aMT6s</th>
</tr>
</thead>
<tbody>
<tr>
<td>JW Oil/Milk</td>
<td>133.7</td>
<td>2365.4</td>
<td>2.1</td>
<td>1.2</td>
<td>17.7</td>
</tr>
<tr>
<td>MB Oil/Milk</td>
<td>468.4</td>
<td>2753.0</td>
<td>7.4</td>
<td>1.4</td>
<td>5.9</td>
</tr>
<tr>
<td>JE Oil/Milk</td>
<td>594.8</td>
<td>2021.8</td>
<td>9.4</td>
<td>1.0</td>
<td>3.4</td>
</tr>
<tr>
<td>NB Oil/Milk</td>
<td>63.5</td>
<td>3120.9</td>
<td>1.0</td>
<td>1.5</td>
<td>49.2</td>
</tr>
<tr>
<td>Mean ± SD Oil/Milk</td>
<td>315.1 ± 256.9</td>
<td>2565.3 ± 475.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB Gelatine</td>
<td>806.5</td>
<td>2315.0</td>
<td>8.7</td>
<td>1.6</td>
<td>2.9</td>
</tr>
<tr>
<td>DC Gelatine</td>
<td>92.5</td>
<td>1453.8</td>
<td>1.0</td>
<td>1.0</td>
<td>15.7</td>
</tr>
<tr>
<td>AH Gelatine</td>
<td>1211.0</td>
<td>2238.6</td>
<td>13.1</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>CF Gelatine</td>
<td>652.0</td>
<td>2825.9</td>
<td>7.0</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td>WA Gelatine</td>
<td>230.9</td>
<td>2187.0</td>
<td>2.5</td>
<td>1.5</td>
<td>9.5</td>
</tr>
<tr>
<td>MA Gelatine</td>
<td>492.8</td>
<td>2262.8</td>
<td>5.3</td>
<td>1.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Mean ± SD Gelatine</td>
<td>580.9 ± 405.4</td>
<td>2213.8 ± 439.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The factor was determined by assigning a value of 1 to the subject with the lowest AUC for each preparation and expressing the other AUCs as a multiple of that area.

Table 4.1 The Area Under the Plasma Indole Versus Time Curves for Each Subject Following the Ingestion of Different Oral Preparations
<table>
<thead>
<tr>
<th>Subject and Preparation</th>
<th>Melatonin</th>
<th>aMT6s</th>
</tr>
</thead>
<tbody>
<tr>
<td>JW Corn Oil/Milk</td>
<td>48.0</td>
<td>60.1</td>
</tr>
<tr>
<td>MB Corn Oil/Milk</td>
<td>39.5</td>
<td>63.6</td>
</tr>
<tr>
<td>JE Corn Oil/Milk</td>
<td>44.8</td>
<td>58.2</td>
</tr>
<tr>
<td>NB Corn Oil/Milk</td>
<td>55.7</td>
<td>44.2</td>
</tr>
<tr>
<td>Mean ± SD Corn Oil/Milk</td>
<td>47.0 ± 6.8</td>
<td>56.5 ± 8.5</td>
</tr>
<tr>
<td>CB Gelatine</td>
<td>38.3</td>
<td>61.4</td>
</tr>
<tr>
<td>DC Gelatine</td>
<td>44.7</td>
<td>44.1</td>
</tr>
<tr>
<td>AH Gelatine</td>
<td>38.1</td>
<td>66.6</td>
</tr>
<tr>
<td>CF Gelatine</td>
<td>47.5</td>
<td>60.6</td>
</tr>
<tr>
<td>WA Gelatine</td>
<td>49.8</td>
<td>67.0</td>
</tr>
<tr>
<td>MA Gelatine</td>
<td>34.0</td>
<td>55.7</td>
</tr>
<tr>
<td>Mean ± SD Gelatine</td>
<td>42.1 ± 6.2</td>
<td>59.2 ± 8.5</td>
</tr>
<tr>
<td>Overall Mean ± SD (n=10)</td>
<td>44.0 ± 6.6</td>
<td>58.2 ± 8.2</td>
</tr>
</tbody>
</table>

Table 4.2 Biological Half-Lives for the Elimination of Melatonin and aMT6s Following the Ingestion of Differential Oral Preparations
Figure 4.3  Plasma Melatonin and aMT6s Concentrations After Intravenous Administration of 41μg[11C]Melatonin
In view of the very rapid increases in aMT6s levels following oral (and intravenous) administration and the very high concentrations measured, this study confirms that the liver is a major site of melatonin metabolism in man. The importance of the route of administration on the subsequent blood levels of a drug is emphasized by the different plasma profiles obtained when melatonin was administered either orally or intravenously. Oral administration leads to a large proportion of melatonin being metabolized immediately whereas following intravenous administration all the administered dose is available for distribution around the body. After intravenous injection of melatonin, aMT6s does not appear in the plasma until after 3-5 min and only about 30% of the administered dose is subject to hepatic metabolism on the first passage through the body (Gibaldi and Perrier, 1975). When investigating the pharmacological actions of melatonin it is important to consider the route of administration. In a study conducted by Vollrath and colleagues (1981) melatonin was administered intranasally. The concentrations of melatonin obtained, particularly in the brain are likely to be considerably higher than those achieved following the intravenous or oral administration of similar doses.

The peak aMT6s levels measured and the AUCs of the aMT6s concentration time curves were remarkably consistent between subjects, whereas there were very marked differences in the same parameters for the measured melatonin levels. A large dose of melatonin was administered in this study and absorption of the drug varied between subjects. It is likely that following absorption of such a large dose of melatonin the metabolizing liver enzymes are saturated and the relatively constant amount of aMT6s produced enters the circulation, whereas the amount of unmetabolized melatonin entering the circulation is very variable due to the differences in absorption. These findings support the idea that the large inter-individual differences found in plasma melatonin and in plasma aMT6s
concentrations in normal volunteers are due to differences in the secretion of melatonin from the pineal rather than differences in metabolism.

It is of interest to consider how the proportions of the conjugated metabolites may vary with different doses of melatonin. Many melatonin metabolites have only been demonstrated following the administration of exogenous melatonin (Young et al., 1985; Vakkuri et al., 1987) and the importance of many of these metabolic pathways for endogenously produced melatonin has yet to be established. The proportion of the different metabolites produced is likely to vary depending on both the dose and the route of administration. English et al., (1987b) have shown that, in the ewe, aMT6s is produced if melatonin is given orally whereas, following subcutaneous implantation of melatonin, no aMT6s is measurable in the plasma, ie, the metabolic pathway varies depending on the route of administration.

The biological half-life for the elimination of melatonin 44.0 ± 6.6min (mean ± SD, n=10) is in close agreement with that of 48min reported by Waldhauser and colleagues (1984b) and that of 43.6 ± 3.3min (mean ± SD, n=5) reported by Iguchi et al., (1982a). The distribution of intravenously administered melatonin also followed the two-phase distribution pattern described by Iguchi et al., (1982a). The calculated biological half-life for aMT6s 58.2 ± 8.2mins (mean ± SD, n=10) is longer than the corresponding figure for melatonin. This difference in the half-life helps to explain why the proportion of melatonin to that of aMT6s in the plasma varies over 24h.

The samples were originally collected to investigate the pharmacokinetics of melatonin. At the end of the experiment melatonin concentrations had reached base-line levels, whereas aMT6s concentrations were still elevated. If samples were collected over a much longer period of time, the endogenous night-time production
of aMT6s would then be superimposed on the aMT6s elimination profile. The disappearance of aMT6s fitted a one-compartment model. Since plasma sampling was not continued until aMT6s reached base-line levels however, it is possible that aMT6s elimination may be accounted for by a two-compartment model with the last phase having an elimination half-life greater than that calculated.

No evidence for the enterohepatic recirculation of melatonin was obtained as no secondary peaks were observed. It is likely that with the high dose of melatonin administered a large proportion will be excreted unmetabolized, not only in the urine but also in the bile and faeces. The enterohepatic recirculation of melatonin therefore remains a possibility.
This will last out a night in Russia
When nights are longest there. I'll take my leave,
And leave you to the hearing of the cause;

W. Shakespeare, Measure for Measure 2, 1, 133

CHAPTER 5
ANNUAL VARIATIONS IN aMT6s EXCRETION
5.1 **Introduction**

The work of Rosenthal et al., (1986) has shown that light has an important role in human physiology. Experiments on subjects in environmental isolation have demonstrated another important effect of light: that of entraining human circadian rhythms (Wever et al., 1983). Bright light can also phase-shift the human melatonin rhythm (Lewy et al., 1987; Broadway et al., 1987) and the human temperature and cortisol rhythms (Czeisler et al., 1986). Work from our laboratory has shown that man is even more sensitive to light than previously thought. When light is administered under the right conditions, light of an intensity as low as 300 lux can suppress nocturnal plasma melatonin in normal volunteers (Bojkowski et al., 1987). This recent evidence of the effects of light on human physiology, together with inconsistent reports in the literature concerning seasonal changes in melatonin (see Section 1.16.1.6), prompted this study on the annual variation in aMT6s excretion.

5.2 **Procedure**

5.2.1 **Subjects**

Sixteen apparently healthy volunteers (nine men and seven women, aged 19-29 years at the beginning of the experiment, mean ± SD, 25.5 ± 3.3 years) recruited from the laboratory staff collected six-hourly urine samples for 24h, starting at midday. During the collection period subjects remained in their normal social environments. All the volunteers had similar daytime routines and were all subjected to approximately the same domestic and natural light intensity. Natural daylength varies from 16.4h in June to 7.5h in December at 52°N (Guildford, UK). Throughout the year in the late evenings normal domestic intensity (300-500 lux) artificial lights were used. The samples were collected at local time between the 10th and 16th day of each month over a period of thirteen months, commencing in November 1985.
Subjects were asked to abstain from alcohol on sampling days and to record any medication taken on or up to a week before sampling days. Three of the female subjects were taking oral contraceptives. One of the subjects was prescribed β-blockers for two months; no samples were collected during this period and the mean of the two adjoining months was used for the purposes of calculation. Three subjects took flights across several time zones; in these cases, samples were collected three weeks after the return to England.

5.2.2 aMT6s Assay

All samples were measured by RIA at the dilution of 1:50; where necessary samples were re-assayed at a dilution of 1:100. All the samples from any one subject were measured in the same assay. The order in which the samples for each month were measured in successive assays was formally randomized for each assay, to eliminate any within-assay trends. The interassay CVs were 11.0%, 8.4% and 9.7% (n = 32 each) at concentrations of 3.5, 7.8 and 25.7ng/ml, respectively.

5.2.3 Statistical Analysis

The 24h aMT6s excretion data was analysed by a two-way analysis of variance with replication. Close examination of the raw data demonstrated large differences in the variances within one particular time period throughout the year. A Bartlett's test for homogeneity of variances was performed (Steel and Torrie, 1981a). A significant difference was found, therefore the six-hourly aMT6s excretion data and the total 24h aMT6s excretion data were analysed non-parametrically using the Friedman two-way analysis of variance (Siegel, 1956a).

In order to eliminate the effect of the considerable inter-individual differences in aMT6s excretion, the monthly aMT6s excretion values were converted to percentages of the individual annual means. This transformed data was analysed as described above.
In order to estimate the parameters of the aMT6s rhythm for each subject in each month, cosine curves were fitted by a least-squares method to each set of four x six-hourly samples (Monk and Fort, 1983). For each 24h time span the rhythmic characteristics of the excretion are described by the following parameters; the acrophase (the estimated peak of the rhythm data); the amplitude (the measurement of the peak of the rhythm above the mean level); and the mesor (the mean value of the fitted cosine curve).

The cosinor parameters for the urinary aMT6s data throughout the year were analysed by a two-way analysis of variance. Further statistical significance was determined with the Student-Newman-Keul's test (Steel and Torrie, 1981b). In mid-March, British Summer Time was introduced and the clocks were advanced by 1h until the end of October when Greenwich Mean Time resumed. All cosinor analysis was calculated on the basis of Greenwich Mean Time. All other calculations for aMT6s excretion refer to local time.

The variability in the excretion of aMT6s over 24h for each individual was calculated \((CV = SD/\text{annual mean})\). The group means were calculated and the results for the male subjects and the female subjects were compared.

5.2.4 Urinary aMT6s Measurement as an Index of the Rhythmic Characteristics of Plasma Melatonin Secretion

The data obtained from the experiment described in Section 3.3 was re-analysed. Briefly, blood samples were obtained from eighteen volunteers for 24h at two-hourly intervals during the day and at hourly intervals at night while simultaneous six-hourly urine collections were made. Melatonin was measured in the plasma samples and aMT6s was assayed in the urines. This data was analysed using the cosine-curve fitting programme (Monk and Fort, 1983). The results obtained from the cosinor analysis of the plasma melatonin data were compared to the results from the cosinor analysis of the urinary metabolite data.
5.3 Results

Two-way analysis of variance with replication, of the raw aMT6s excretion data showed a very significant time of day effect (F = 206, p << 0.0001, df 3, 780) but no variation with months (F = 0.13, p = 0.99 df 12, 780). Bartlett's test showed that the variances in the 1200-1800h time group were not homogeneous (chi-squared = 46.5, p < 0.005 12 df). The results were therefore analysed non-parametrically. The Friedman two-way analysis of variance showed a significant seasonal variation in aMT6s excretion for the 1200-1800h time period (p = 0.015). Peaks were observed in December/January and in July, and troughs were seen in spring and autumn. No other significant differences were observed in any of the other time periods, including the total 24h excretion. The mean aMT6s excretion in each of the four x six-hourly collection periods throughout the year is shown in Figure 5.1.

When the data were transformed so that each monthly aMT6s level represented the percentage of the individual annual means, identical results were obtained on statistical analysis. Expressed this way the mean total aMT6s excretion over 24h was shown to be very consistent throughout the year and ranged from 94.5 ± 3.4% to 107.3 ± 3.1% (mean ± SEM n = 16 each).

There were good correlations between the parameters obtained from the cosinor analysis of the plasma melatonin data and the cosinor parameters obtained from the simultaneous six-hourly urinary aMT6s measurements. One subject (G) had no melatonin levels above the detection limit of the assay throughout the 24h blood sampling period (Figure 3.4a) and was excluded from the analysis. In the remaining seventeen subjects the mean acrophase for the urinary aMT6s data was 3.7 ± 1.3h (SD) and the mean acrophase for the plasma melatonin data was 3.1 ± 1.0h (SD). The
correlations between the acrophases, amplitudes and mesors obtained from the four x six-hourly urine samples and the acrophases, amplitudes and mesors from the 24h plasma melatonin data were $r = 0.73 \ (p = 0.0012)$, $r = 0.76 \ (p = 0.0007)$ and $r = 0.77 \ (p = 0.0005)$, respectively ($n = 17$ each).

A similar pattern was observed in all the urinary aMT6s excretion data, with low levels during the day and a peak in excretion during the night. Two-way analysis of variance showed no significant differences in the amplitude and mesor throughout the year. In contrast, a very significant difference was found in the acrophase of the aMT6s rhythm ($F = 3.66, \ p = 0.00015, \ df 12, 180$). Student-Newman-Keul's test showed significant differences in the acrophases: December differed from April, June, July, October ($p < 0.05$), August and September ($p < 0.01$) and January differed from August and September ($p < 0.05$). The mean monthly values for the acrophase, amplitude and mesor are shown in Figure 5.2. The three-month moving averages of the aMT6s acrophases together with the annual variation in night-length are illustrated in Figure 5.3. The linear correlation coefficient between the three-month moving averages of the aMT6s acrophases and the night-time duration for each month was $r = 0.82, \ p = 0.002 \ (n = 11)$. The linear correlation coefficient between the mean monthly aMT6s acrophases and the night-time duration for each month was $r = 0.76, \ p = 0.003 \ (n = 13)$. In December/January the mean acrophase was 4.69h whereas in August/September the mean acrophase was 3.22h; a phase advance of 1h and 30min in summer.

The means ± SD of the individual subjects' CVs for consistency of aMT6s excretion over 24h for thirteen months were 18.5 ± 5.4% ($n = 9$) for male subjects, 16.0 ± 1.3% ($n = 7$) for female subjects and 17.4 ± 4.4% ($n = 17$) for the whole group.
The only statistically significant difference in excretion during the year was observed for the 1200-1800h collection period ($p = 0.015$).

The capital and the small form of the letter indicate a significant difference between these points ($p < 0.05$, **$p < 0.01$).
Figure 5.3 Three-Month Moving Averages (±SEM) of the Acrophases of the aMT6s Rhythm Together with the Night-Time Duration (From Sunset to Sunrise) Throughout the Period of Investigation.
Discussion

The parameters of the circannual aMT6s rhythm, that is acrophase, amplitude and mesor have been ascertained together with data on urinary aMT6s excretion. We have shown that aMT6s measured in six-hourly urine collections provides a good index of the rhythmic characteristics of the nocturnal increase in plasma melatonin. This study also illustrates the advantages of urine collections. Subjects acted as their own controls and collections were made in the volunteers' normal social environment with the minimum of inconvenience.

Collections were made throughout the year so that any effects due to the extremes in day-length in winter/summer might be observed, together with the effects due to rapid changes in day-length in spring/autumn, which may be of equal or greater importance. An accurate measure of seasonal trends in urbanized man was obtained and in a sense the volunteers can be considered to be a 'wild-captured' species (Reiter et al., 1983).

The circannual change in the acrophase for aMT6s excretion is in agreement with the findings of other workers (Illnerova et al., 1985; Arendt and Broadway, 1986; Kennaway and Royles, 1986) who all reported phase advances in summer. The greatest phase advance, that of 2h, occurred under the extreme lighting conditions found in the Antarctic (Arendt and Broadway, 1986). The relatively small change in the phase position found in the Antarctic is surprising and suggests that there may be entraining cues other than light, such as social factors, which influence the phase position of the melatonin secretion profile. Whether the change in acrophase observed in this study is a masking effect due to the phase advance in the rest-activity cycle caused by the introduction of British Summer Time, has to be considered. This remains unlikely since the phase advance observed (1h and 30min) was greater than the change in the rest-activity cycle due to the
change in clock-time and secondly, because similar results have been observed in the Antarctic, where there is no manipulation of clock-time (Arendt and Broadway, 1986). It is likely therefore that the phase-advance observed is the result of environmental factors.

The shift in acrophase correlates closely with the duration of darkness, suggesting that the phase-shift is a photoperiod-related phenomenon. Together with results from studies in the Antarctic, which have shown that the application of a 12.5h skeleton spring photoperiod of 2500lux in winter, produces a 'summer melatonin profile' (Broadway et al., 1987) the results presented in this Chapter demonstrate that a residual photoperiodic response, at least as far as melatonin is concerned, is retained in man.

Recent work on the suppression of nocturnal plasma melatonin by light has shown that light of an intensity as low as 300 lux can suppress melatonin secretion in humans (Bojkowski et al., 1987). Work in animals has shown that previous lighting history is likely to be an important factor in determining an individuals' sensitivity to light (Reiter et al., 1983). If this is the case, then a variation in the ability of light to suppress melatonin may occur throughout the year and the total aMT6s excretion would be expected to change with the seasons. However, the ubiquitous use of indoor lighting may prevent the observation of any of the above changes.

In agreement with other workers we have found no changes in total melatonin production throughout the year (Beck-Friis et al., 1984; Griffiths et al., 1986; Sack et al., 1986). A seasonal variation in aMT6s excretion was observed for the 1200-1800h collection period, with peaks in summer and winter. This is similar
to the reports made by other workers who used daytime sampling (Arendt et al., 1979; Martikainen et al., 1985). Why there is a variation in the daytime and not in any of the other samples remains puzzling. It may be that during the daytime when aMT6s excretion is low, other factors such as dietary constituents may contribute to the aMT6s output. Peaks in daytime plasma tryptophan levels have been reported in January and July (Wirz-Justice et al., 1977). The amplitude of the observed daytime rhythm is low and it is not reflected in the 24h aMT6s excretion rhythm.

Throughout the year a very stable excretion of aMT6s over 24h was observed. The mean CVs for consistency of aMT6s excretion over 24h were 18.5% for male subjects and 16.0% for female subjects suggesting that there were no major menstrual cycle effects on aMT6s excretion. The CV for the whole group was 17.4% (n = 16), which is very similar to the CV of 13.7% previously reported when eighteen volunteers collected urine samples over four consecutive days (Section 3.2).

Large inter-individual differences in the aMT6s excretion were again observed, emphasizing the need to use subjects as their own controls. Night-time peaks in aMT6s excretion were always observed and the acrophase for aMT6s was relatively stable between volunteers. The large inter-individual differences in aMT6s excretion, together with the marked seasonal variation in the acrophase of aMT6s excretion suggest that perhaps it is the precise phase of melatonin secretion rather than the amplitude of the rhythm or the duration of secretion, which conveys messages about the environment to the human brain.
When the voices of children are heard on the green
And whisperings are in the dale,
The days of my youth rise fresh in my mind,
My face turns green and pale.

Then come home, my children, the sun is gone down,
And the dews of night arise;
Your spring & your day are wasted in play,
And your winter and night in disguise.

William Blake, Songs of Experience, Nurse's Song.

CHAPTER 6
CHANGES IN aMT6s EXCRETION DURING CHILDHOOD AND PUBERTY
Considerable controversy still surrounds the possible influence of the pineal gland on human puberty (see Section 1.16.1.7). The preliminary study described in this Chapter was carried out to investigate whether there are changes in aMT6s excretion related to age and pubertal development. While the study described in this Chapter was in progress, Young and colleagues (1986) using a GC/MS assay for aMT6s, reported a decrease in both the daytime and night-time excretion of aMT6s with age.

6.2 Procedure

6.2.1 Subjects and Sample Collection

Children of University personnel were recruited for the study. Two consecutive 24h urine samples were collected from forty children (twenty-four males, sixteen females) aged 2-20 years. Twenty-four hour samples were divided into three fractions: early morning urine, representing nocturnal melatonin production; midday sample, representing any carry-over of the nocturnal production; rest-of-day urine including a sample before bedtime, representing daytime melatonin production. All the urine passed over a 48h interval was collected and the volume and time of each sample collection was recorded. The detailed protocol for urine collection is shown in Figure 6.1. To ensure collections were carried out correctly, parents supervised the proceedings where appropriate.

To determine the approximate pubertal stage of the subjects, questionnaires were devised on the basis of the Tanner criteria (Marshall, 1975). The questionnaires (Figure 6.2) were completed by the subjects themselves or by their parents. To ensure confidentiality all subjects were coded. Details of age, weight, height and drug status were also recorded. All urine samples collected were assayed for aMT6s.
Figure 6.1

Protocol for Urine Collection

Urine collection - It should be stressed that all urine passed during the collection period of 48h must be collected.

Day 1
Empty bladder before going to bed and discard urine. Note down bedtime.

Day 2
- Container 1 Collect any urine which may be passed overnight. Into same container collect the first urine sample passed in the morning and note down time.
- Container 2 Collect all urine passed until midday. At midday empty bladder (if possible) and collect urine into same container.
- Container 3 From midday collect all urine passed until just before going to bed. Before going to bed empty bladder and collect urine into same container. Note down bedtime.
- Container 4 Collect any urine which may be passed overnight. Into same container collect the first urine sample passed in the morning and note down time.
- Container 5 Collect all urine passed until midday. At midday empty bladder and collect urine into same container.
- Container 6 From midday collect all urine passed until just before going to bed. Before going to bed empty bladder and collect urine into same container. Note down time.

Finished.
Figure 6.2

Questions Which Were Completed by the Subjects

1. Post-pubertal (female development is obvious but not complete)
2. Pre-pubertal (no signs of female development)
3. Breast in girls
4. Pubic hair in boy

Sexual development.

Please tick which of the following stages of puberty would best describe your

Has your voice become

Has any other facial hair started to grow?

Has any non-genital hair started to grow?

Has any other non-genital hair started to grow?

Has any period started or has menstruation occurred?

Has any sexual activity before the age of consent?

Was any sexual activity before the age of consent?

If yes, please specify age at commencement.

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Yes/No.

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6.2.2 Statistics

For the purposes of statistical analysis subjects were divided into four age groups: Group (1) 2-5.9 (n = 10), Group (2) 6-9.9 (n = 9), Group (3) 10-13.9 (n = 10) and Group (4) 14-20 years (n = 11). The aMT6s excretion over five collection periods was analysed. These collection periods were: total 24h excretion, night-time excretion, a midday sample (representing any 'carry-over' of the night-time production), total night-time production (the night-time and midday samples combined) and the daytime excretion. Urine samples were collected over two consecutive days and each aMT6s measurement used for the purposes of calculation is a mean value of two separate collections.

aMT6s excretion was expressed as the total excretion per collection interval and also as a rate of aMT6s excretion over that interval. aMT6s excretion was also expressed as a function of body weight.

One-way analysis of variance was used to determine any statistically significant differences between age groups. Further statistical significance was determined using Duncan's multiple-range test (Steel and Torrie, 1981c).

6.3 Results

There were significant differences between age groups, in the duration of sample collection for the daytime and night-time samples respectively because the older subjects retired to bed considerably later than the children. This complicated the analysis of results as aMT6s excretion had to be expressed as both total aMT6s excretion (µg) over a collection period and also as a rate of excretion over that collection period (ng aMT6s/h).
The total aMT6s excretion (µg) for the daytime collection period showed a significant difference between the oldest age group (group 4) and group (2) \( (F = 2.9, p = 0.04, df 3, 36) \). This difference is because the adult volunteers (group 4) retired to bed much later than the children (group 2), consequently, the daytime collection period was longer for the adult volunteers. If allowance was made for the differences in the duration of the collections and the data was expressed as a rate of aMT6s excretion (ng aMT6s/h) over the daytime collection period, there was no difference between age groups \( (F = 0.59, p = 0.63, df 3, 36) \). When expressed as either total aMT6s excretion, or as a rate of aMT6s excretion, there were no other significant differences between age groups in any of the other collection periods. The total aMT6s excretion for each collection period is shown in Table 6.1. Figure 6.3 illustrates the total aMT6s excretion for the 24h, total night-time and for the daytime collection periods.

When aMT6s excretion was expressed as a function of body mass very significant differences were observed between age groups. Values in the younger age group were significantly greater than values in the older age groups. Total 24h aMT6s excretion dropped from 18.63 ± 5.64 (mean ± SD) ng/h/kg in youngest children (2-6 years) to 12.10 ± 4.38 ng/h/kg in children 6-10 years, and 5.99 ± 2.73 ng/h/kg in children 14-20 years. Thus, 24h aMT6s excretion dropped by 65-70% through childhood. Similar results were obtained when aMT6s excretion was expressed as either ng aMT6s/kg, or as ng aMT6s/h/kg. In the latter case when a correction for slight differences in the duration of collection intervals between subjects was made, the levels of significance attained were greater. For the sake of clarity only the results for rates of aMT6s excretion are presented. The rate of aMT6s excretion over each collection period, expressed as a function of body weight is shown in Table 6.2 and the significant differences between the age groups are summarized. Figures 6.3 and 6.4 illustrate the differences in the rate of aMT6s excretion as a function of body mass, between age groups for the 24h, total night-time and for the daytime collection periods.
### Table 6.1: Total AMTs Excretion for Each Collection Period

<table>
<thead>
<tr>
<th>Significant Differences Between Groups</th>
<th>Daytime (Midday Sample)</th>
<th>Night-Time</th>
<th>Total Night-Time</th>
<th>Total 24h Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 4 (14-20 years)</td>
<td>1.79 ± 0.16</td>
<td>8.74 ± 3.53</td>
<td>6.92 ± 3.22</td>
<td>7.97 ± 3.09</td>
</tr>
<tr>
<td>Group 3 (10-13.9 years)</td>
<td>7.29 ± 2.68</td>
<td>8.88 ± 0.75</td>
<td>7.46 ± 2.19</td>
<td>7.26 ± 2.69</td>
</tr>
<tr>
<td>Group 2 (6.9-9.9 years)</td>
<td>6.49 ± 2.49</td>
<td>6.42 ± 0.99</td>
<td>6.91 ± 2.15</td>
<td>6.59 ± 2.50</td>
</tr>
<tr>
<td>Group 1 (2.5-4.9 years)</td>
<td>6.09 ± 2.60</td>
<td>6.09 ± 0.88</td>
<td>6.91 ± 2.15</td>
<td>6.60 ± 2.60</td>
</tr>
</tbody>
</table>

All values in AMTs (mean ± SD)
<table>
<thead>
<tr>
<th>Time</th>
<th>Group 1 (1-39 Years)</th>
<th>Group 2 (4-17 Years)</th>
<th>Group 3 (18-39 Years)</th>
<th>Group 4 (30-75 Years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daytime</td>
<td>3.3 ± 0.08</td>
<td>3.7 ± 0.9</td>
<td>3.5 ± 0.15</td>
<td>3.2 ± 0.15</td>
</tr>
<tr>
<td>Midday</td>
<td>4.0 ± 0.15</td>
<td>3.9 ± 0.6</td>
<td>3.8 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Night-time</td>
<td>3.4 ± 0.2</td>
<td>3.6 ± 0.6</td>
<td>3.5 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Total Night-Time</td>
<td>2.5 ± 0.2</td>
<td>2.7 ± 0.5</td>
<td>2.6 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Total 24h</td>
<td>2.9 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Between Groups</td>
<td>Significant Differences</td>
<td></td>
<td></td>
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</tbody>
</table>

All values in grams/72 h (mean ± SD)

Table 6.2: Expressing as a function of body weight

Rate of Ammonia Excretion for Each Collection Period
Figure 6.3  Total aMT6s Excretion and Rate of aMT6s Excretion Expressed as a Function of Body Weight, for the 24h, Total Night-Time and for the Daytime Collection Periods (Means ± SEM)

Figure 6.4  Variation in the Rate of aMT6s Excretion, Expressed as a Function of Body Weight, with Age
After analysing the answers on the questionnaires, subjects were allocated to one of four groups: pre-pubertal < 6 years, pre-pubertal 6-10 years, pubertal or post-pubertal. The allocation of subjects to different groups was on the basis of the Tanner criteria for pubertal staging (Marshall, 1975). With the exception of four subjects there was an identical distribution of subjects to that previously obtained on the basis of age.

Because of the very close agreement in the distribution pattern of subjects when grouped by age or on the basis of pubertal development the data was not re-analysed in terms of 'pubertal groups'. On the basis of crude pubertal staging obtained from the questionnaires a very similar pattern of significance to that obtained with age groups would be expected. The pre-pubertal < 6 years, pre-pubertal 6-10 years, pubertal and post-pubertal groups corresponding to age Groups 1, 2, 3 and 4, respectively.

Total 24h aMT6s excretion showed no significant differences between age groups ($F = 0.17, p = 0.91, \text{df} \ 3,36$). When 24h aMT6s excretion was expressed as a function of height, greater differences between age groups were observed, however levels of significance were not attained ($F = 2.58, p = 0.07, \text{df} \ 3,36$). As stated previously, the most dramatic differences between age groups were observed when a correction for differences in body weight was made. For total 24h aMT6s excretion as a function of body mass $F = 18.45, p = 6.4 \times 10^{-6}, \text{df} \ 3,36$.

The proportion of the 24h collection excreted in the total night-time sample was compared between age groups ($F = 2.69, p = 0.06, \text{df} \ 3,34$). Although statistical significance was not attained there appeared to be a trend, with the youngest age groups excreting the greatest proportion at night. Irrespective of whether 24h aMT6s excretion was expressed as total excretion, as a rate of
excretion or as a function of body mass one-way analysis of variance showed no sex differences (p > 0.5 in all cases).

6.4 Discussion

This study again illustrates the advantages of urine collections. Samples were obtained from children aged 2-20 years relatively easily with no ethical problems. The children were in their normal social environments, were drug free and unlike many previous studies were completely healthy and were not hospitalized. Urines were collected over 48h to enable a mean of two values to be calculated, since young children in particular may not have complete control of their bladders. An important feature of the protocol was that collections were carried out under the supervision of parents to ensure that they were carried out correctly.

When the 24h and night-time aMT6s excretion was corrected for body weight changes, there was good agreement between our data and that of Waldhauser and colleagues (1984a). Highest levels were observed in the youngest age group with a progressive decline with age. The decline occurs from the early years of life throughout pre-puberty and remains relatively constant through puberty and adolescence. A decline in daytime aMT6s excretion was also found between children aged 2-6 years and children aged 6-10 years. In contrast, Waldhauser et al., (1984a) found no change in the daytime plasma melatonin levels. One possible explanation for this discrepancy, is that plasma melatonin assays are insufficiently sensitive to detect a change, as many of the daytime samples are at or below the detection limit of the assay.

In this preliminary study no physical examination of the subjects was possible and therefore only very general conclusions can be made, as regards aMT6s levels and pubertal development. It is difficult to assess where exactly in relation
to pubertal development the drop in aMT6s levels occurs. If melatonin is an
antagonadotrophic agent then a decrease in aMT6s levels might be expected to occur
before the onset of puberty. The data presented in this Chapter does not refute this
hypothesis. In order to determine the exact relationship between melatonin levels
and pubertal development it would be essential to carry out longitudinal studies in
the same individuals with regular physical examination and sample collection.

The data presented is largely in agreement with the results of Young and
colleagues (1986). These authors reported significantly higher daytime and night­
time aMT6s levels (when expressed as a function of body weight) in children than in
adolescents and adults. These workers found a significant attenuation of the diurnal
rhythm in adults, which was not confirmed by our results. The youngest age groups
tended to start their collections earlier than the older age groups, therefore it is
difficult to draw any definite conclusions about any possible changes in the diurnal
rhythm.

Tetsuo and co-workers (1982b) reported a constant excretion of 6­
hydroxymelatonin with age and we found a similar pattern for aMT6s excretion. If
excretion remains constant, whilst body mass increases, the blood concentration of
melatonin would be expected to decrease, provided that the proportion of melatonin
metabolized to aMT6s remains constant with pubertal development.

The importance of the age-related decrease in the blood concentration of
melatonin is difficult to interpret. It is important to bear in mind that the
measurement of urinary aMT6s in this study cannot demonstrate subtle changes in
the melatonin secretion profile, for example, in the duration of melatonin secretion,
which may be of equal or greater importance. In view of the considerable problems
in obtaining blood samples from young children future studies may have to concentrate on the collection of both saliva and urine samples.

More studies on cases with abnormal pubertal development should be carried out. Recently, pinealectomy has been shown not to effect pubertal development in male rhesus monkeys (Plant and Zorub, 1986). More studies on primates should be undertaken, in particular, the effects of melatonin administration on subsequent pubertal development should be investigated.
Do not go gentle into that good night,
Old age should burn and rave at close of day;
Rage, rage against the dying of the light.

Do not go gentle into that good night.

Dylan Thomas

CHAPTER 7
FACTORS INFLUENCING aMT6s EXCRETION IN NORMAL VOLUNTEERS
7.1 Influence of Age, Sex, Weight and Height on aMT6s Excretion

7.1.1 Introduction

Many conflicting reports exist concerning the relationship of melatonin secretion to age and body parameters (see Section 1.16.1.8). Using the urinary aMT6s assay it is possible now to assess melatonin secretion in large numbers of healthy people, thus increasing the reliability of the results. Investigations into the relationships between a number of variables and urinary aMT6s levels in normal apparently healthy subjects are reported in this Chapter.

7.1.2 Subjects

Data from one hundred and thirty subjects (sixty-eight males and sixty-two females) were used for this analysis. All subjects were in good general health and were drug-free, although oral contraceptives were permitted. The age, weight, height and sex of each volunteer was recorded. The age range of the subjects was 2-80 years. The data for the youngest age group has been presented in Chapter 6.

The co-operation of Ms C Franey and Mrs M Kemp (Department of Biochemistry, University of Surrey) in providing the data for twenty-six of the subjects is acknowledged. Dr Labibe (Investigation Unit, St Luke's Hospital) kindly supplied the samples from twelve of the elderly subjects.

7.1.3 Procedure

No restrictions regarding diet, sleep, activity or light exposure were imposed on the volunteers and samples were randomly collected at different times of the year. Urine was collected over a 24h period into one to four containers depending on the study. The volume of each sample was recorded and aMT6s was measured by RIA. The total aMT6s excretion over 24h has been used for the purposes of calculation.
The samples were measured over a period of two years. Two different quality control pools were used over this time period. The interassay CVs were 13.6, 11.9, 16.3, 11.2, 11.6 and 16.7% for quality control samples which measured 19.5, 7.2, 2.3 (n = 81 each) 25.1, 7.9 and 3.3ng/ml (n = 87 each) respectively.

7.1.4 **Statistical Analysis**

To assess the influence of age, body weight and height on aMT6s excretion, the linear correlation coefficients between each of these variables and total 24h aMT6s excretion were determined. Linear correlation coefficients were calculated for the complete age range (2-80 years, n = 130), for adults (20-80 years, n = 90) and for the children (2-19.9 years, n = 40). The weights and heights of three of the elderly subjects were not obtained.

The age-related differences in aMT6s excretion were further investigated by a one-way analysis of variance. Statistical significance was determined with Duncan's test (Steel and Torrie, 1981c). The subjects were divided into ten age groups, which are outlined in Table 7.2. To investigate age-related changes in aMT6s production, the data was expressed as total excretion (µg) and also as a function of height (ng aMT6s/cm) and body weight (ng aMT6s/kg).

Differences in aMT6s excretion between the sexes were determined by unpaired t-tests for the following age ranges: 2 - 19.9, 2 - 80, 20 - 80 and 60 - 80 years.

7.1.5 **Results**

The linear correlation coefficients between total 24h aMT6s excretion and age, height and weight are shown in Table 7.1. There was no relationship between aMT6s excretion and height or weight (Figure 7.1 and Table 7.1). In contrast, there
was a highly significant decline in aMT6s excretion with age in adult volunteers ($y = -0.11x + 12.8\mu g$, $r=-0.35$, $p=0.001$, $n=90$). The decline with age for the whole age group (2-80 years) approached significance ($y = -0.04x + 9.69\mu g$, $r=-0.15$, $p=0.073$, $n=130$). However, there was no relationship between aMT6s excretion and age in the children (2 - 19.9 years).

The subjects were then divided into ten age groups (Table 7.2) and a one-way analysis of variance demonstrated age-related changes when aMT6s excretion was expressed as: total excretion ($F = 2.43$, $p = 0.014$, df 9, 120) as a function of height ($F = 1.97$, $p = 0.048$, df 9, 117) and as a function of weight ($F = 14.82$, $p = 1.1 \times 10^{-8}$, df 9,117). These results are summarized in Tables 7.2, 7.3 and 7.4 respectively and in Figures 7.2 and 7.3.

When aMT6s production was expressed as the total excretion or as a function of height, the only significant differences were between the oldest age group (60-80 years) and the other age groups (Figures 7.3a and 7.3b). When expressed as a function of weight, significant differences were observed between the two youngest age groups (2-5.9, 6-9.9 years) and all other age groups and also between the oldest age group (60-80 years) and other age groups (Figure 7.3c).

No sex differences were observed in total aMT6s excretion by unpaired t-tests in any of the following age ranges: 2 - 19.9, 2 - 80, 20 - 80 and 60 - 80 years ($p > 0.05$ in all cases).

7.1.6 Discussion

The variable most closely related to urinary aMT6s production is age. There was a significant decline in total aMT6s excretion with age in adult volunteers in agreement with the work of Sack et al., (1986). Iguchi and colleagues (1982b)
<table>
<thead>
<tr>
<th>Height</th>
<th>Weight</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Children (2-19.9 years)

Adults (20-80 years)

Complete Age Range

Correlations between 24h AMTEs Excretion and Age, Weight and Height

Table 7.1
<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.9</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>3.2</td>
<td>6.9</td>
</tr>
<tr>
<td>3</td>
<td>4.2 ± 2.75</td>
<td>6.1 ± 2.75</td>
</tr>
<tr>
<td>4</td>
<td>5.76 ± 2.15</td>
<td>6.9 ± 2.75</td>
</tr>
<tr>
<td>5</td>
<td>6.0 ± 2.50</td>
<td>6.9 ± 3.15</td>
</tr>
<tr>
<td>6</td>
<td>6.0 ± 2.50</td>
<td>6.9 ± 3.15</td>
</tr>
<tr>
<td>7</td>
<td>6.9 ± 3.25</td>
<td>6.9 ± 3.15</td>
</tr>
<tr>
<td>8</td>
<td>6.9 ± 3.25</td>
<td>6.9 ± 3.15</td>
</tr>
<tr>
<td>9</td>
<td>7.5 ± 3.53</td>
<td>6.9 ± 3.15</td>
</tr>
<tr>
<td>10</td>
<td>8.0 ± 3.70</td>
<td>6.9 ± 3.15</td>
</tr>
</tbody>
</table>

Significant Differences

10:7: p > 0.001
10:5: p > 0.01
10:3,9: p > 0.05

Table 7.2

Total 24-h Ambles Excretion in Different Age Groups
<table>
<thead>
<tr>
<th>Group</th>
<th>Mean + SD (ng h/mL)</th>
<th>Significant Differences Between Groups</th>
<th>Function of Height</th>
<th>Age (years)</th>
<th>24h AMT6s Excretion as a Function of Height in Different Age Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>60-80</td>
<td>0</td>
<td>1.2</td>
<td>0.001</td>
<td>7-6.5</td>
</tr>
<tr>
<td>16</td>
<td>46.9 ± 26.1</td>
<td>0.019</td>
<td>1.2</td>
<td>0.001</td>
<td>6-5.9</td>
</tr>
<tr>
<td>33</td>
<td>46.2 ± 23.8</td>
<td>0.023</td>
<td>1.2</td>
<td>0.001</td>
<td>6-5.9</td>
</tr>
<tr>
<td>69</td>
<td>46.8 ± 23.6</td>
<td>0.023</td>
<td>1.2</td>
<td>0.001</td>
<td>6-5.9</td>
</tr>
</tbody>
</table>

Table 7.3
<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean ± SD</th>
<th>24h AMTS Excretion as a Function of Weight in Different Age Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2-10</td>
<td>60 ± 5.9</td>
<td>42.9</td>
<td>2-5.9</td>
</tr>
<tr>
<td>2:4-9.5</td>
<td>60 ± 9.5</td>
<td>10.1</td>
<td>42.9</td>
</tr>
<tr>
<td>3:1-4</td>
<td>60 ± 4.3</td>
<td>9.7</td>
<td>42.9</td>
</tr>
<tr>
<td>4:0-3.5</td>
<td>60 ± 3.5</td>
<td>9.7</td>
<td>42.9</td>
</tr>
<tr>
<td>5:0-2.9</td>
<td>60 ± 2.9</td>
<td>9.7</td>
<td>42.9</td>
</tr>
<tr>
<td>6:0-2.4</td>
<td>60 ± 2.4</td>
<td>9.7</td>
<td>42.9</td>
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<tr>
<td>7:0-2.9</td>
<td>60 ± 2.9</td>
<td>9.7</td>
<td>42.9</td>
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<tr>
<td>8:0-2.4</td>
<td>60 ± 2.4</td>
<td>9.7</td>
<td>42.9</td>
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<td>9:0-2.9</td>
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<td>9.7</td>
<td>42.9</td>
</tr>
<tr>
<td>10:0-2.4</td>
<td>60 ± 2.4</td>
<td>9.7</td>
<td>42.9</td>
</tr>
</tbody>
</table>

Table 7.4
Figure 7.1
Relationships Between aMT6s Excretion and Weight and Height for the Complete Age Range (2-80 Years)
Figure 7.2a  
Variation in aMT6s Excretion With Age, When  
aMT6s Excretion is Expressed as Total Excretion (µg)  
(Means ± SD Superimposed)

Figure 7.2b  
Variation in aMT6s Excretion with Age, When  
aMT6s Excretion is Expressed as a Function of  
Height (ng aMT6s/cm) (Means ± SD Superimposed)
Figure 7.2c Variation in aMT6s Excretion with Age, When aMT6s Excretion is Expressed as a Function of Weight (ng aMT6s/kg) (Means ± SD Superimposed)
Figure 7.3a  Statistical Analysis of the Variation in aMT6s Excretion
With Age in the 10 Age Groups (Means$\pm$SD)
When aMT6s Excretion is Expressed as Total Excretion (µg)

Figure 7.3b  Statistical Analysis of the Variation in aMT6s Excretion
With Age in the 10 Age Groups (Means$\pm$SD) When aMT6s Excretion is Expressed as a Function of Height (ng aMT6s/cm)
Figure 7.3c  Statistical Analysis of the Variation in aMT6s Excretion
With Age in the 10 Age Groups (Means ± SD) When aMT6s Excretion is Expressed as a Function of Weight (ng aMT6s/kg)

Differences between groups were assessed by analysis of variance and further statistical significance was determined by a Duncan’s test. The capital and small form of the letter signify a significant difference between these points (p<0.05, *p<0.01, **p<0.001)
found a significant decline in daytime melatonin levels in volunteers aged 1-92 years and in this study, the decline with age for the complete age range (2-80 years) approached significance. When the oldest age group was compared to the younger age groups the levels in the aged group were significantly lower, again confirming the results of previous workers, when plasma samples have been taken over 24h (Touitou et al., 1981; Iguchi et al., 1982b). This decline in the oldest age group was observed irrespective of whether aMT6s excretion was expressed as total excretion or as a function of height/weight.

As discussed previously in Chapter 6, when aMT6s excretion is expressed as a function of weight, there is a very significant decline with age for the two youngest age groups. In addition to the precipitous drop for children up to 10 years old, there is a gradual decline in excretion in the adult volunteers and, finally, the levels in the elderly volunteers are significantly lower than those of some of the other age groups.

The decline in plasma melatonin levels described by Waldhauser and Steger (1986) for subjects aged 1 - 90 years closely resembles the decline observed in the urinary aMT6s excretion expressed as a function of weight. The urinary results confirm that the decrease observed in the plasma levels is not due to an increase in metabolism.

When collecting samples from elderly subjects it is important to consider other factors which may influence the results. Elderly subjects are more likely to have impaired bladder, kidney and liver function. However, other workers have demonstrated a decline in plasma melatonin levels in elderly subjects and this is therefore unlikely.
Although the data has been presented as total 24h excretion many collections were divided into daytime and night-time samples. Good circadian rhythms were observed in all age groups. However, it was clear that in some of the elderly subjects the circadian rhythm appeared to be dampened. The timing of collections was not identical between age groups and therefore it is not possible to draw any firm conclusions about changes in the circadian amplitude with age.

These results show conclusively that there was no relationship between total aMT6s excretion and sex, height or weight. The data was not analysed in terms of other body measures such as obesity index. Most volunteers were of normal height and weight and a parameter such as the obesity index would fall within too narrow a range to give meaningful results.

Very large inter-individual variations in aMT6s excretion were again observed, underlining the importance of large sample numbers in this kind of study.

7.2 Pineal Calcification

7.2.1 Introduction

The pineal glands of many species including humans, sheep, rats, gerbils and guinea-pigs calcify with age. Skull radiography has shown that in humans 2% of the glands are calcified in the first decade of life, 12% in the second decade, 44% in the third and 61% after the age of 30 years. After 30 years of age, the amount of pineal calcium is relatively constant, with the proportion of calcification being higher in females than males (Welsh, 1985).

The functional significance of pineal calcification remains a mystery. It has been suggested that pineal concretions are an indication of degenerative changes in the pineal gland. Another hypothesis is that the formation of pineal concretions
is related to the functional activity of the gland. Recently, Commentz and co-workers (1986) measured night-time urinary melatonin levels in eighteen subjects with pineal calcification and compared the levels to the night-time melatonin excretion of an age and sex matched control group with no evidence of calcification. The night-time melatonin excretion was the same in the two groups suggesting that calcification does not affect melatonin production.

In view of the large differences in aMT6s production observed in our normal subjects, it was of great interest to investigate any possible relationship between urinary aMT6s production and the degree of pineal calcification.

7.2.2 Procedure

Twenty-six adult volunteers (age range 20-50 years, seventeen males and nine females) gave informed consent to take part in the study. All volunteers were Caucasian, apart from one male volunteer of Asian origin. A lateral skull X-ray of each subject was taken at the Royal Surrey County Hospital. All X-rays were performed by the same radiographer using the same equipment (the co-operation of Dr Broadbridge and Mrs V Weir, X-ray Department, Royal Surrey County Hospital, is gratefully acknowledged). The degree of pineal calcification was assessed on a blind basis by Dr Broadbridge, Consultant Radiologist, Royal Surrey County Hospital. Each subject was given a score of 0-4 based on the degree of calcification: 0 = none visible, 1 = minimal, 2 = moderate, 3 = heavy, and 4 = unusually extensive. The 24h urinary aMT6s excretion of each subject was known and, in addition, the 24h plasma melatonin and aMT6s profiles of sixteen of the volunteers had been obtained in previous studies. The age, sex, weight and height of the subjects was recorded.
7.2.3 Statistics

The correlations between the degree of calcification and a number of variables were calculated using Spearman's rank correlation coefficient (Siegel, 1956b). These variables were: 24h urinary aMT6s excretion (µg), AUC of the 24h plasma melatonin profile (pg/ml.h), melatonin peak height (pg/ml), AUC of the 24h plasma aMT6s profile (pg/ml.h), aMT6s peak height (pg/ml), age, weight and height. Differences in the degree of calcification between the sexes were assessed by an unpaired t-test (two-tailed).

7.2.4 Results

Seven volunteers showed no calcification, six showed minimal calcification, eleven moderate calcification and two heavy calcification. There were no subjects with unusually extensive calcification. Thus, in the population selected, a range of calcification was observed. There was also a large range in the 24h aMT6s excretion (3.79-28.03µg) and in the AUC of the plasma melatonin (148-938pg/ml.h) and plasma aMT6s (339-1338pg/ml.h) profiles. Figure 7.4 illustrates the relationships between pineal calcification and these measures of pineal function. There was no correlation between the degree of calcification and any of the variables studied (p>0.1 in all cases).

An unpaired t-test demonstrated that the female subjects had significantly greater pineal calcification (p = 0.03).

7.2.5 Discussion

Wurtman and colleagues (1964) measured the activity of HIOMT in human pineal glands obtained at autopsy. These authors were able to demonstrate that even pineal glands which were calcified were capable of synthesizing melatonin. In a study with five male volunteers, Pelham and colleagues (1973) used bioassay to
Figure 7.4

Relationships Between Pineal Calcification and 24h aMT6s Excretion, and the AUCs of the Plasma Melatonin and Plasma aMT6s 24h Profiles

The horizontal bars represent the Means ± SD.
investigate the variations in plasma melatonin over 24h. They reported that the night-time peak levels of the 'melatonin-like' substance were inversely related to pineal calcification and/or age. This work was carried out long before the large inter-individual variation in the plasma melatonin levels of normal volunteers had been reported (Arendt, 1978). It is likely therefore, that if the study had been expanded to include more than five subjects, the reported relationship between pineal calcification and peak levels would no longer be maintained.

The results suggest that melatonin production is not influenced by pineal calcification and support the findings of Commentz et al., (1986). Although the number of subjects studied was relatively small, the range of urinary aMT6s excretion values was considerable. It is likely that if a relationship between function and calcification existed, a trend would have been observed in the data.

There was a large variation in the degree of calcification between the subjects and, with the small number of subjects studied, no correlation with age or any of the other parameters was observed. There was, however, a significant difference in the degree of calcification between the sexes, as previously reported (Welsh, 1985). No sex differences in pineal melatonin production have been reported, thus providing further indirect evidence that there is no relationship between pineal function and calcification.
We could not understand because we were too far and could not remember, because we were travelling in the night of first ages, of those ages that are gone, leaving hardly a sign - and no memories.

Joseph Conrad, Heart of Darkness, Ch.2.
8.1 Critical Evaluation of Methodology

At present no hard evidence has been produced for a major function of the human pineal gland. Progress in this field has been greatly retarded by methodological constraints. In order to study pineal gland function it has, in general, been necessary to take peripheral blood samples and measure the circulating concentrations of the pineal hormone, melatonin. In view of rhythmic production of melatonin, blood samples have to be taken at frequent intervals, particularly at night, and this is obviously a difficult undertaking. To compound these practical difficulties there have often been methodological problems with melatonin assays, some of which have lacked specificity and the necessary sensitivity.

To overcome these difficulties the urinary output of melatonin has been measured (Bartsch et al., 1981; Lang et al., 1981). However, urinary concentrations of melatonin are extremely low and only represent a small percentage of melatonin secreted from the pineal gland (Kopin et al., 1961; Lang et al., 1981). Very few studies have been carried out to validate the use of urinary melatonin as an index of plasma melatonin levels. Whether or not it is a good measure of pineal function is questionable.

The major urinary metabolite of melatonin in rats and humans, representing 40-60% of the melatonin secreted from the pineal gland, is aMT6s (Kopin et al., 1961; Jones et al., 1969). GC/MS methods to measure aMT6s exist (Fellenberg et al., 1980; Tetsuo et al., 1981a; Francis et al., 1987), however, they are expensive to run and have an extremely low sample throughput. The RIA described in this thesis, to measure aMT6s in urine and plasma, is sensitive, highly specific and is amenable to the measurement of the large numbers of samples associated with clinical work. Classical validation procedures have been employed
and the method has also been validated by comparison with an established GC/MS method. Urine samples are collected with relative ease and only very small amounts of sample need to be stored.

In addition to the validation of the assay methodology, the use of urinary aMT6s measurement as an index of pineal function has also been extensively validated. A high amplitude circadian rhythm in aMT6s excretion has been documented and urinary aMT6s concentrations are very highly correlated with plasma melatonin levels. There are, however, drawbacks in measuring aMT6s which have to be considered. It is imperative to remember that a metabolite is being measured and that factors which affect liver metabolism or kidney and bladder function may give rise to misleading results. Only one metabolite is measured and it would be important to establish techniques to measure aMT6G, the second major melatonin metabolite. Measurement of both metabolites in the same subjects would reduce misleading results due to factors interfering with the phase II conjugating enzymes.

The relationship between urinary aMT6s and plasma melatonin levels has been established in normal healthy subjects. How well this relationship is maintained in pathological cases, with or without ingestion of drugs, remains to be seen. If urinary metabolites are to be effectively used as an index of pineal function, the variables which affect melatonin metabolism must be extensively studied. It would be of great practical value as well as of academic interest to determine the influence of lighting history, genetic variables and time of day on melatonin metabolism and clearance.

Very little work has been carried out in humans to investigate the proportion of melatonin secreted from the pineal gland which enters the CSF
The possible sites of action of melatonin remain at present unknown although convincing receptors have been demonstrated in the brain (Vanecek et al., 1987). Melatonin may enter CNS tissue from both CSF and from the blood. Thus, the levels of CSF melatonin may be of considerable biological significance. It would also be worthwhile to investigate whether or not the conjugated metabolites of melatonin are present in human CSF. In view of the different metabolic pathway for melatonin in the CNS (Hirata et al., 1974) the fraction of melatonin which enters the CNS may not be accounted for by the measurement of urinary aMT6s. Likewise, the proportion of melatonin which is metabolized by pathways other than 6-hydroxylation is not accounted for by urinary aMT6s assays. The importance of these pathways for the metabolism of endogenously-produced melatonin remains to be established but is likely to be relatively small.

There are problems with the use of urinary assays in general. Frequency of sample collection is limited and by their very nature, urine samples represent an integrated measure over a particular time period. Consequently, when using urinary aMT6s measurements alone, it is very difficult to determine subtle changes in the melatonin profile. There is considerable interest in determining which feature of the melatonin secretion profile is of biological significance (Goldman, 1983). With a urinary assay it will be almost impossible to determine subtle differences in the qualitative aspect of the 'melatonin signal' such as slight differences in the timing and duration of melatonin secretion. On the other hand, differences in the amplitude of the melatonin rhythm will be reflected in urinary aMT6s measurements.

With any urinary measurement, the validity of the results depends to a great extent on the compliance of the volunteers. Where the timing of urine
collections is an important factor, as in the study on the annual variation in aMT6s excretion (Chapter 5), the motivation of the volunteers is of paramount importance. In all the studies described in this thesis, the subjects were well-motivated and dependable. Where members of the general public and patients are recruited to studies, the compliance of the subjects in collecting the samples correctly is a factor which has to be considered when analysing the results.

In spite of the above drawbacks, there are nevertheless considerable advantages with an urinary assay. In particular, the ease with which samples can be collected allows far more extensive trials to be organised. In a normal healthy population, urinary aMT6s has been shown to be remarkably well correlated with plasma melatonin.

8.2. **Control of Human Pineal Function - Evaluation by aMT6s Assay**

8.2.1 **Pharmacological Manipulations**

Because of the innervation of the pineal gland by peripheral postganglionic sympathetic neurons, the secretion of melatonin from the gland has been used as an experimental model for the evaluation of adrenergic function. The synthesis of melatonin appears to be under the control of peripheral β-adrenergic receptors, which lie outside of the blood-brain barrier in humans. The rhythm in aMT6s excretion, like that of melatonin, was abolished by a β-blocker, atenolol (Section 3.5). This approach has been exploited to investigate affective disorders (Section 1.16.1.4). In studies of this nature, either the AUC of 24h plasma melatonin or overnight aMT6s production has to be estimated (Boyce, 1985). The latter approach is obviously considerably easier.

The pineal gland is a particularly useful pharmacological model to study the effects of adrenergic drugs. The abolition of the night-time rise of melatonin by
β-blockers has already been mentioned. Checkley et al., (1987) measured urinary aMT6s to investigate the effects of a phosphodiesterase inhibitor (rolipram) and demonstrated that cAMP was a likely secondary messenger through which noradrenaline activates the synthesis of melatonin. Franey and colleagues (1986) demonstrated that desipramine, a noradrenaline uptake blocker, altered melatonin production by increasing plasma melatonin during the evening and reducing melatonin levels during the morning. By assaying both melatonin and aMT6s these authors were able to show that this change was not due to an effect on hepatic metabolism.

Stimulation of human melatonin production during the day with β-agonists is difficult, probably because the doses required would cause unpleasant side-effects. A recent area of controversy has been the effect of the α₂ agonist, clonidine. Lewy and co-workers (1986) took blood samples at thirty minute intervals between 2400h and 0500h and reported that clonidine reduced melatonin levels. However, Grasby and Cowen (1987a) used a slightly different protocol and were unable to confirm these findings. In situations such as this, a urinary aMT6s assay may resolve the problem. Recent evidence has shown that α₁-adrenoceptors are involved in the physiological control of melatonin secretion in man (Franey, personal communication). The α₁ antagonist, prazosin, significantly reduced nocturnal melatonin secretion. The observed effect was slight however, compared to that of β-adrenoceptor blockade.

In any study investigating the adrenergic function of the pineal gland, it is worthwhile to remember that although the sympathetic innervation of the pineal appears to be functionally the most important, in a number of species a central innervation of the gland has been documented (Section 1.4). The function of this central innervation of the gland remains unknown.
The variable which has been shown to have most effect on melatonin secretion is the light/dark cycle. Light plays a dual role in controlling pineal function. Bright light has an entraining effect on circadian rhythms (Wever et al., 1983) and both bright and dim light can suppress plasma melatonin levels (Lewy et al., 1980; Bojkowski et al., 1987). The manipulation of melatonin production by the acute administration of bright light during the dark phase is a unique feature of the pineal gland which has been little exploited. However, this feature of the gland has been used by Lewy et al., (1985) to investigate whether depressives are supersensitive to light.

A study to investigate annual changes in aMT6s excretion was organized with the subjects acting as their own controls. Large inter-individual differences were observed in total aMT6s excretion with no annual changes. A small but highly significant difference in the time of peak aMT6s excretion throughout the year was present. These results suggest that perhaps it is the exact phase of melatonin secretion rather than the total amount secreted which is of importance in humans. The change in the aMT6s acrophase was highly correlated with the duration of darkness suggesting that this may be a photoperiod-related phenomenon.

Broadway and colleagues (1987) investigated the changes in the plasma melatonin rhythm in response to treatment with bright or dim light during the Antarctic winter. Bright, but not dim light treatment in winter induced a marked phase advance of the melatonin rhythm, similar to that found in the summer. Kennaway and co-workers (1987) have also recently shown that evening exposure to bright light will phase-delay the aMT6s excretion rhythm. If the seasonal changes in melatonin secretion are indeed photoperiod-related, this opens up the possibility that the seasonal changes observed in other endocrine parameters are also
photoperiodic. The annual variation in melatonin secretion in response to light, together with evidence that a pharmacological dose of melatonin alters the rhythm of its own secretion (Arendt et al., 1987), suggests that the secretion of melatonin in man has a role to play in the organization and control of circadian and seasonal rhythms in humans.

It should be pointed out, however, that the evidence suggesting annual changes in melatonin secretion in man are related to photoperiod is very preliminary. The study described in Chapter 5 could be improved in a number of ways. The collection of urine samples at more frequent intervals and for a period of time longer than twelve months (preferably twenty-four) would enable the annual variation to be more precisely defined. Ideally, the lighting experienced by each individual throughout the year should be recorded. In practice, however, such refinements are probably not feasible.

The evidence which has emerged recently suggests that light has a far greater role to play in the control of the secretion of melatonin from the human pineal gland than previously thought. This may help to explain why there have been so many contradictory results in the past. In most of the published studies on melatonin secretion in man, little attention has been paid to the lighting conditions experienced by the subjects.

All animal studies on pineal function are carried out under very strictly controlled lighting conditions. In future work, lighting conditions must be carefully defined, particularly if results from different groups in different parts of the world are to be compared. Once this approach is adopted, then a more detailed examination of the other factors which may affect melatonin production will be possible. Extensive studies on the relevance of lighting history, and of exposure to
light of different wavelengths and intensities at different times of the day, need to be carried out. Further experiments under the extreme lighting conditions found in the Antarctic are under way. The practical difficulties involved in the control of light exposure may prove to be insurmountable in the majority of clinical trials.

8.2.3 aMT6s as a Circadian Rhythm Marker

aMT6s excretion shows a very stable circadian rhythm and an important use of the assay is to monitor aMT6s excretion as a circadian rhythm marker. One of the major problems in circadian rhythm research is the phenomenon known as 'masking'. Changes in the rest-activity cycle, for example, will alter and 'mask' the underlying rhythm of body temperature. As long as subjects are drug-free and the lighting intensity is carefully controlled, the rhythm in melatonin production appears to be free of masking effects. The aMT6s assay has been used to investigate the control of circadian rhythms under conditions of environmental isolation with, and without, desynchronisation (Wever, 1986) and the aMT6s rhythm has been shown to behave more like a 'strong' oscillator variable (Arendt et al., 1985b). The rhythm in aMT6s excretion will be used in future studies to investigate the phase relationships between melatonin and other circadian rhythms.

aMT6s output is also very useful as a circadian rhythm marker under field conditions. The RIA has been used as a tool to investigate the re-entrainment of circadian rhythms in jet-lag (Arendt et al., 1987) and would evidently serve to monitor circadian rhythm changes under conditions such as shift-work.

The physiological role of the pineal as a photoneuroendocrine transducer in photoperiodic species, such as the hamster, is well understood. Unfortunately, there is a paucity of information about plasma melatonin levels in rodents, largely due to technical difficulties. Because plasma melatonin levels are so low, large
volumes of blood are required. An alternative approach is to measure the rhythmic variations of pineal melatonin content, however, this has obvious limitations. The development of assays to measure aMT6s in rodent urine would have considerable importance in photoperiodic studies. In particular, repeated samples could be obtained from the same animal. This approach would be particularly useful in the study of rodent circadian rhythms where urinary aMT6s could be used as a stable circadian rhythm marker. However, this approach would be of limited value in species such as the sheep, where aMT6s is not a major melatonin metabolite (English et al., 1987b).

8.3 Establishment of 'Normal' aMT6s Levels in Humans

The urinary aMT6s assay is invaluable for the study of pineal function under field conditions and in large-scale clinical trials. Many studies are now possible which would be almost impossible to carry out otherwise. Data on aMT6s excretion (and plasma melatonin measurements) have demonstrated very large inter-individual differences in absolute levels. However, a marked circadian rhythm in excretion is present in nearly all subjects. This emphasizes the need to organize studies where subjects act as their own controls. Alternatively, large number of subjects have to be used.

In two healthy volunteers no rhythms in melatonin secretion were observed (Section 3.3). These volunteers exhibited no signs of any clinical abnormalities. Although no rhythm was apparent, this may largely be due to the lack of sensitivity of the detection methods used to measure melatonin. In one of these subjects, a very slight night-time rise in plasma aMT6s was observed and both subjects had small circadian rhythms in aMT6s excretion.
Although during the day the circulating levels of plasma melatonin are below the detection limits of the current assay, this does not necessarily mean that melatonin is not exerting an effect during the day. When circulating levels are undetectable, melatonin may be present in sufficient concentration at its putative sites of action. This may be relevant to local neuromodulator effects, such as are found in the retina (Dubocovich, 1983).

The majority of subjects exhibit a good circadian rhythm in the production of melatonin and aMT6s. For any one individual, the night-time plasma melatonin secretion profile and the rhythm in aMT6s excretion are very consistent. Whether the reproducibility of the rhythm is of clinical significance remains to be ascertained. The lack of a day-to-day variation reinforces the idea that melatonin production is a very stable circadian output which is relatively unaffected by 'masking' effects. Poor reproducibility of the melatonin 'signal' may indicate instability of the circadian system. As such, aMT6s may prove to be of diagnostic importance in diseases associated with rhythm abnormalities, such as depression, (Wehr and Goodwin, 1983) and serve to define rhythm disturbances in general.

A relatively constant output of total aMT6s excretion was found in the course of pubertal development. When aMT6s excretion was expressed as a function of weight, a very significant decline in the youngest age groups was observed. In an area of research where there have been many contradictory reports, these results support the work of Waldhauser et al., (1984) who found a decline in plasma melatonin during development. Clearly, the decline in plasma melatonin observed is not the result of increased metabolism. The fall in aMT6s excretion is most striking in the youngest age groups, long before the initiation of pubertal development. In our studies, it was not possible to confirm any relationships which may exist between stage of pubertal development and aMT6s levels, as no physical examination of subjects was carried out.
In future studies it would be important to determine accurately the sexual maturation of the subjects and to investigate how this is related to aMT6s excretion. Such a study should include the measurement of gonadotrophin levels in each subject. At present, it is uncertain whether the reported inverse relationships between the activity of the pineal gland and that of the hypothalamo-pituitary-gonadal axis is of biological significance or is merely coincidental. The decrease in plasma melatonin levels observed during childhood may simply be a result of the increases in body size which occur at that time. An interesting approach would be to study cases of delayed puberty, where body size has increased but the development of secondary sexual characteristics has not occurred. In normal subjects it will only be possible to determine accurately the relationship between melatonin levels and pubertal development with longitudinal studies in the same individuals.

The variable which had most effect on aMT6s excretion was age. A small decline in total aMT6s excretion in old age was observed, whereas weight and height had no effect. In the elderly subjects, there also appeared to be a dampened circadian rhythm in aMT6s excretion. Whether these changes are of any clinical significance and whether they affect the 'well being' of the subjects is open to speculation. It would be of great interest to try to reinforce the melatonin circadian rhythm in elderly subjects by feeding them melatonin at the appropriate time of day. It is important to realise, however, that the changes in aMT6s excretion observed in elderly subjects may be the result of other factors, such as impaired kidney and bladder function.

8.4 Pharmacokinetic Studies

The pharmacokinetics of melatonin and aMT6s have been studied and the half-life of aMT6s was found to be slightly longer than that of melatonin. The very close relationship between plasma melatonin and plasma aMT6s levels is of interest
in view of the recent evidence that the conjugated metabolites of melatonin are themselves biologically active \textit{in vitro} (Leone \textit{et al.}, 1987a). Whether or not the conjugated metabolites are also biologically active \textit{in vivo} in photoperiodic species is not known. 6-Hydroxymelatonin has low potency melatonin-like effects in some species (Reiter and Vaughan, 1975) which may be due to conjugated metabolites. At present, the purified metabolites which are available, are too precious to be used in this kind of experiment. It would be possible to investigate the potency of the conjugated metabolites on \textit{in vitro} systems such as the melanophore bioassay (Section 1.18.1). Such studies would serve to define more closely the structure-activity relationships of the melatonin receptor.

Studying the function of the pineal gland by measuring the output of melatonin has the obvious limitations that only one indole is under consideration. An important role of melatonin has been established in photoperiodic species and, as a consequence, considerable emphasis has been placed in the study of human pineal function in measuring melatonin. Many other indoles and peptides are present in the pineal gland (Section 1.5) which may have an important role to play in human physiology.

8.5 \textbf{Conclusions}

The studies described in this thesis have provided essential, basic information on human melatonin production, as evidenced by variations in aMT6s, the major metabolite of melatonin in man. They suggest fruitful avenues of research into the physiological function of the human pineal.

It is the author's personal opinion that future studies may indicate that the total output of melatonin is not of great significance, whereas the exact phase of secretion is of considerable importance. More attention will have to be paid in
future work to the lighting conditions to which human subjects are exposed. If future studies concentrate on abnormal rhythms of melatonin secretion, and on the phase of the rhythm, then we may begin to understand the role of the pineal gland in human physiology.

The mind of man is capable of anything - because everything is in it, all the past as well as all the future.

Joseph Conrad, Heart of Darkness, Ch.2.
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APPENDIX I

Papers Published:
Immunoassay of 6-Hydroxymelatonin Sulfate in Human Plasma and Urine: Abolition of the Urinary 24-Hour Rhythm with Atenolol*

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ABSTRACT. An assessment of the rhythmic characteristics of melatonin secretion in man and other species requires the determination of 24-h secretion profiles. Measurement of a major excreted metabolite would allow noninvasive study of pineal function, applicable in particular to pediatric and long term circadian rhythm studies. This report describes a simple measurement of the pineal neurohormone melatonin (MT) in human body tissues and fluids is of considerable interest, notably in psychiatric and endocrine pathology (1-3). In addition, the role of the pineal in the transmission of photoperiodic information in seasonal breeders has been intensively investigated using peripheral and pineal MT assays (4-7). To investigate pineal function in this way, it is necessary to sample pineals or blood throughout the day and night in view of the dark phase secretion of MT (8-11). In clinical practice this is difficult and has retarded progress in the field. A simple and rapid assay for an excreted MT metabolite, if it can be shown to be a good index of MT secretion, would greatly extend the range of physiological and pathological conditions amenable to study in man, most particularly in pediatric medicine. 6-Hydroxymelatonin sulfate (aMT6s) is a major MT metabolite in rodents (12, 13) and probably in man (14). Fellenberg et al. (15) described a gas chromatographic-mass spectrometric (GCMS) assay for aMT6s in man, which required a large sample volume and extensive purification. Similarly, total free and conjugated 6-hydroxymelatonin can be assayed by GCMS (16).

This report describes the development of a sensitive, specific, and reproducible RIA for aMT6s capable of measuring urinary and plasma levels in small volumes, and rapid RIA for 6-hydroxymelatonin sulfate in human plasma and urine. Physiological studies revealed that both plasma and urinary levels of 6-hydroxymelatonin sulfate were closely related to plasma melatonin, and that the urinary 24-h rhythm was abolished by the β-adrenergic antagonist atenolol. (J Clin Endocrinol Metab 60: 1166, 1985)

Materials and Methods

Materials

[3H]MT (26.4, 45.3, and 79.8 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA). N-acetyltryptophan, 5-methoxytryptophan, and 5-methoxy-N-acetyl-tryptophan were gifts from Laboratories Plan, S.A. (Geneva, Switzerland). aMT6s and [3H]aMT6s were prepared as described below. Other indoles, steroids, and catecholamine derivates together with Tricine buffer and activated charcoal were obtained from Sigma Chemical Corp. (St. Louis, MO). RPMI-1640 culture medium was obtained from Gibco Europe (Uxbridge, U.K.), Sephadex LH-20 and Dextran T-70 were purchased from Pharmacia (Piscataway, NJ), ovalbumen was obtained from British Drug Houses (Poole, U.K.) and Synperonic NX was purchased from Durham Chemicals Distributors Ltd (Chester-le-Street, U.K.). All other chemicals were commercially available and of analytical grade. Thin layer and paper chromatograms (Merck, Rahway, NJ) were washed in eluting solvent, dried, and sprayed with 0.1% ascorbic acid in ethanol before use.

Methods

1) Synthesis of aMT6s. aMT6s was synthesized from 6-hydroxymelatonin (10 mg) according to the method of Fellenberg et al. (15). The reaction products were purified on a Sephadex LH-20 column (7 g dry wt; id, 40 × 1 cm). The eluting solvent was KCl-saturated methanol-chloroform (1:1), and 5-ml fractions were collected. aMT6s eluted in fractions 18–22, as as-

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sessed by reaction with Ehrlich's reagent, after thin layer chromatography [TLC; Silica gel G, chloroform-methanol (9:1)]. Pooled fractions were dried (37 C; N2). After further purification by TLC (Silica Gel G, n-butanol saturated with water), recovery, by weight, was 40%. The product was taken up in methanol containing 0.1% ascorbic acid and stored at -20 C at a concentration of 48 g/ml. Under these conditions, aMT6s was stable for at least 1 yr, as assessed by TLC. Aliquots of this solution were further diluted 1:400 with Tricine buffer, stored at -20 C, and used for RIA standards; these diluted standards were stable for at least 3 months). No detectable indolic products other than aMT6s were present in the pooled eluate on further chromatography [TLC: cellulose, butanol-acetic acid-H2O (4:1:1.5); paper (Whatman 3MM, Whatman, Inc., Clifton, NJ); isopropanol-5% ammonia (4:2)]. Qualitative GCMS of the product, according to the method of Fellenberg et al. (15) and performed by Dr. G. King, Queen Charlotte's Maternity Hospital, London, showed a spectrum identical to that described by Fellenberg et al. (15). It should be noted, however, that the derivatization procedure employed results in loss of the sulfate group. A completely pure crystalline preparation was not obtained; hence, the product was finally quantitated by comparison of its displacement activity in the RIA with [3H]aMT6s of known specific activity (section 2), assuming no loss of label during the biological transformation of melatonin. [3H]aMT6s derived from both [methoxy-3H]melatonin (79.8 Ci/mM) and [3H]MT labeled in the side chain of melatonin. [3H]aMT6s derived from both [methoxy-3H]melatonin (79.8 Ci/mM) and [3H]MT labeled in the side chain (acetyl-5-methoxytryptamine-N-[2-aminoethyl-2-3H]; 26.4 Ci/mm) were used for this procedure with identical results.

2) Preparation of [3H]aMT6s. Female Wistar rats (150-200 g) were used. After cervical dislocation, the liver was rapidly removed and cut into slices (<1 mm). Liver slices (600 mg) were incubated with 1.4 X 10^6 cpm [3H]MT (45.3 Ci/mmol) in 3 ml RPMI-1640 at 37 C under 95% O2-5% CO2, for 2 h with gentle shaking. The incubate was cooled, homogenized, and extracted three times with 3 ml methanol. The pooled supernatants were dried at 37 C under N2. Chromatography of the crude extract [Whatman no. 3MM; isopropanol-5% ammonia (4:2)] showed three radioactive peaks, corresponding to MT (RF = 0.84), aMT6s (RF = 0.67), and probably 6-hydroxymelatonin glucuronide (RF = 0.45), as originally described by Kopin et al. (12), although a slightly higher RF was found for aMT6s than previously reported (Fig. 1). Chromatography of 6-hydroxymelatonin itself in this system resulted in decomposition.

The crude extract was purified on LH-20 (column dimensions, 40 X 1 cm; 4.0 g dry weight) as described in section 1, but 2-ml fractions were collected. [3H]aMT6s-containing fractions were pooled, dried, and stored as previously described. The product was stable for at least 3 months when stored in this way. Chromatography of the purified product showed one peak, chromatographically identical to chemically synthesized aMT6s and to the major reaction product of the liver slice incubate (Fig. 1). TLC in two further solvent systems (section 1) again showed only one radioactive product.

3) Preparation of antigen and immunization procedure. aMT6s (4 mg) was conjugated to ovalbumin (55 mg) via the Mannich reaction (17). The molar ratio of hapten conjugated to protein was not directly assessed. Six Suffolk cross eves were immunized with 500 g conjugate emulsified in 3 ml Freund's adjuvant-saline (2:1) by sc injection at six sites on the back and legs. Seven months later, 9 days after boosting with 200 g conjugate, [3H]aMT6s-binding antibodies were detected in all six sheep by RIA. The most sensitive antiserum (1118) was chosen for further characterization.

RIA. The assay procedure was based on that desribed for direct plasma assay of melatonin by Fraser et al. (18), except for the modifications described below. Antibody 1118 (final dilution, 1:1000), [3H]aMT6s, standard aMT6s, compounds tested for cross-reactivity, dextran-coated charcoal (2% charcoal and 0.02% Dextran T-70 in buffer), and urine were all diluted in assay buffer 0.1 M Tricine, pH 5, containing 0.9% NaCl and 0.1% gelatine). Antibody-bound [3H]aMT6s was counted in a cocktail consisting of 12 g PPO (2,5-diphenyloxazole) and 0.225 g POPOP (1,4-bis-[2-(5-phenyloxazolyl)]benzene) in toluene to which Sypermonic was added (750 ml Sypermonic to 1500 ml toluene scintillant).

Cross-reactivity studies: The specificity of the antiserum was assessed by comparing the displacement of antibody-bound [3H]aMT6s by a number of indoles, catecholamine metabolites, and steroids.

Plasma aMT6s assay: aMT6s-free plasma was prepared by charcoal-stripping. Dextran-T coated charcoal (10%) was incubated for 60 h with pooled heparinized human plasma taken from normal subjects, and the charcoal was separated by centrifugation.

To 200 g antibody (1118; initial dilution, 1:250), 200 g plasma sample and 300 g buffer or standard were added. Standard curves were constructed using 200 g charcoal-stripped aMT6s-free plasma, and the standards were included in the buffer volume. After incubation at 4 C for 15 min, 100 g [3H]aMT6s (4500 cpm) were added to give a final volume of 800 g. The tubes were further incubated for 20 min at 37 C and 90 min at 4 C. Bound and free aMT6s were separated by the addition of 100 g dextran-coated charcoal suspension, incubation for 15 min at 4 C and centrifugation (2000 g, 4 C;
Parallellism was assessed by comparing the displacement curves obtained with pooled nighttime human plasma, serially diluted with charcoal-stripped plasma, with the standard curve. A direct chromatographic validation of the plasma assay was not possible due to the low values recorded, the nonextracted nature of the assay, and the lack of sufficient sensitivity of current TLC and HPLC techniques.

Urine assay: The same procedure as that described for plasma was used, except that 200-µl aliquots of 1:50 diluted urine were assayed. Standard curves were initially constructed using 200 µl charcoal-stripped aMT6s-free urine diluted 1:50 or 1:100. Thereafter, urine collected from normal subjects in the midafternoon was screened in the assay, and those samples with undetectable aMT6s were pooled and used for the construction of standard curves.

Parallelism was assessed by comparing the displacement curves obtained with pooled early morning urine samples, serially diluted with charcoal-stripped urine, with the standard curve.

Sufficient immunoreactivity was present in early morning urine to assess the chromatographic specificity of the assay. Pooled early morning urine (5-50 µl) was constructed using 200 µl charcoal-stripped aMT6s-free urine diluted 1:50 or 1:100. Thereafter, urine collected from normal subjects in the midafternoon was screened in the assay, and those samples with undetectable aMT6s were pooled and used for the construction of standard curves.

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**Fig. 2.** A, Parallelism between aMT6s standard curve in aMT6s-free urine diluted 1:50 in Tricine buffer, and increasing amounts of pooled early morning urine, initially diluted 1:50 in Tricine buffer and further diluted with aMT6s-free urine. B, Parallelism between aMT6s standard curve in charcoal-stripped aMT6s-free plasma and increasing amounts of unextracted pooled nighttime plasma diluted with aMT6s-free plasma.

**Precision**

The mean and SD for each point (1.2 pg to 1.2 ng/tube) of 12 standard curves were assessed; the mean overall coefficient of variation was 9.6%. Interassay coefficients of variation for urine and plasma assay are shown in Table 2. Scatchard analysis of the binding characteristics of the antiserum gave an affinity constant of $K = 6 \times 10^{10}$ liters/mol (not shown).

**Chromatographic validation**

The migration of urinary immunoreactivity was identical to that of [3H]aMT6s and aMT6s on TLC in two different solvent systems ((chloroform-methanol (9:1); n-butanol-acetic acid-H$_2$O (4:1:1.5); Fig. 3)) and on paper chromatography in one solvent system [Whatman 3MM; isopropanol-5% ammonia, (4:2)]. Over 80% of applied immunoreactivity was recovered from all chromatograms.

**Physiological studies**

In the preliminary study, aMT6s had a marked 24-h rhythm in human plasma, closely correlated to that of melatonin. Three individual profiles chosen to represent high, medium, and low values of plasma melatonin are shown in Fig. 4. All values for both melatonin and aMT6s for subject 3 in this study (Fig. 4) were at or below the limit of detection of each assay. Thus, while there was apparently a small rise of aMT6s at night without a corresponding rise in melatonin, more sensitive techniques would be required to determine in this subject the true relationship between these two compounds at such low values.

The mean plasma levels of melatonin in the whole group varied from $10 \pm 0.5$ (±SEM) pg/ml (1800 h) to 29 ± 6 pg/ml (0200 h). The mean plasma aMT6s levels in the whole group varied from less than 13 pg/ml (most time points between 1200 and 2100 h) to 52.0 ± 11.0 pg/ml (0200 h). Levels below the limit of detection were set at the limit of detection (10 pg/ml melatonin; 13 pg/ml aMT6s) for the purposes of calculation. AUCs calculated for individual profiles of melatonin and aMT6s correlated significantly ($r = 0.82; P < 0.01$).

Mean plasma aMT6s levels in 10 normal subjects sampled for 24 h in July varied from less than 13 to 55.0 ± 9.0 pg/ml (Fig. 5). There were clear changes in the
Table 2. RIA of aMT6s: assay performance data

<table>
<thead>
<tr>
<th></th>
<th>pg/tube</th>
<th>% CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraassay CV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>8</td>
<td>3.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>3.3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>3.4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.5</td>
<td>5</td>
</tr>
<tr>
<td>Urine</td>
<td>48</td>
<td>3.3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>4.1</td>
<td>5</td>
</tr>
<tr>
<td><strong>Interassay CV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>7</td>
<td>16.2</td>
<td>6</td>
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<tr>
<td></td>
<td>26</td>
<td>12.2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>97</td>
<td>11.5</td>
<td>6</td>
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<tr>
<td>Urine</td>
<td>9</td>
<td>17.7</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>7.9</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>106</td>
<td>7.8</td>
<td>12</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>2.6 pg aMT6s/tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 pg/ml plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.65 ng/ml urine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery of aMT6s

<table>
<thead>
<tr>
<th>pg/ml</th>
<th>pg/ml added</th>
<th>% Recovery</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>52</td>
<td>130</td>
<td>87.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>91</td>
<td>130</td>
<td>87.9 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>1,930</td>
<td>4,750</td>
<td>95.5 ± 4.5</td>
</tr>
<tr>
<td>Urine</td>
<td>21,550</td>
<td>28,000</td>
<td>99.1 ± 12.5</td>
</tr>
</tbody>
</table>

CV, Coefficient of variation.

Immunoreactivity:

Fig. 3. Migration of urinary immunoreactivity, [3H]aMT6s, and aMT6s on TLC [n-butanol-acetic acid-H2O (4:1:1.5)]. The migration of urinary immunoreactivity was also identical to that of [3H]aMT6s and aMT6s on TLC in chloroform-methanol (9:1), Silica gel G, and on Whatman 3MM in isopropanol-5% ammonia (4:2).

Fig. 4. Individual plasma profiles of MT (-----) and aMT6s (---) in three subjects sampled at hourly intervals for 24 h. All of the recorded values are plotted, however, the assay limits of detection were 10 pg/ml (MT) and 13 pg/ml (aMT6s). Thus, in subject 3, all MT values were undetectable, and all aMT6s values were at or below the limit of detection.

melatonin. Whether the rate of metabolism of melatonin to aMT6s or the clearance of aMT6s varies throughout 24 h is not known.

Urinary aMT6s excretion determined at 6-h intervals for 24 h in the same 10 subjects at the same time as plasma melatonin and aMT6s is shown in Fig. 6. The values were similar to those reported by GCMS (15). As expected, a marked 24-h variation in urinary levels was found, with maximum excretion occurring between 2400 and 0600 h. A comparison of individual AUCs for plasma melatonin and plasma aMT6s and total urinary excretion of aMT6s indicated that significant correlations were present among all three parameters (Table 3). The closest relationship was found between plasma levels of both compounds (P < 0.01). Plasma melatonin correlated with urinary aMT6s at P < 0.05, an important relationship if aMT6s is to be useful as an index of melatonin secretion. It was apparent from the data, however, that the relationship was more robust at very low and very high levels of melatonin secretion than for values in the middle of the range.

**Pharmacological studies**

Urinary aMT6s excretion in six subjects during the 24 h preceding atenolol administration (Fig. 7) was quanti-
IMMUNOASSAY OF aMT6s

Fig. 5. Mean ± SEM plasma levels of MT ( ▲—▲ ) and aMT6s ( ●—● ) in 10 subjects (8 men and 2 women) sampled hourly for 24 h in July. Values recorded below the assay limit of detection were set at the limit of detection (10 pg/ml melatonin; 13 pg/ml aMT6s). Urine was collected simultaneously from the same individuals and assayed for aMT6s. The results are shown in Fig. 6.

Fig. 6. Mean ± SEM urinary aMT6s excretion during 6-h intervals for 24 h in 10 subjects (8 men and 2 women). Urine was collected simultaneously with the 24-h blood sampling for plasma melatonin and aMT6s measurement shown in Fig. 5. Samples were collected in July. Values recorded below the assay limit of detection (0.65 ng/ml) were set at the limit of detection.

Table 3. The correlation between individual levels of plasma MT (AUC of 24-h profile), plasma aMT6s (AUC of 24-h profile), and total urinary aMT6s excreted over 24 h

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Coefficient of linear regression (r)</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma melatonin: plasma aMT6s</td>
<td>0.7962</td>
<td>&lt;0.01</td>
<td>10</td>
</tr>
<tr>
<td>Plasma melatonin: total urinary aMT6s</td>
<td>0.6730</td>
<td>&lt;0.05</td>
<td>10</td>
</tr>
<tr>
<td>Plasma aMT6s: total urinary aMT6s</td>
<td>0.783</td>
<td>&lt;0.01</td>
<td>10</td>
</tr>
</tbody>
</table>

Discussion

The classical RIA validation procedures applied to aMT6s assay in plasma and urine show that this assay is accurate, reproducible, and sensitive to physiological levels of this metabolite. Further chromatographic validation of the urinary assay gave strong evidence of specificity by comparison of the migratory characteristics of immunoreactivity in different solvent systems. Clearly, it would be of interest to compare this RIA with GCMS measurements in the same samples; an internationally agreed standard preparation of aMT6s is not available, and it is likely that some adjustment of the absolute levels recorded will be necessary. As yet, however, no other published method exists for the measure-
ment of aMT6s in plasma, and the urinary levels obtained are similar to those previously reported by GCMS assay of aMT6s (15) or total free and conjugated 6-hydroxymelatonin in urine (16).

While interference by known and unknown chromatographically identical compounds cannot be excluded, physiological studies suggest that RIA of aMT6s will be a useful index of melatonin secretion in man. The assay requires little plasma, does not need an extraction procedure, and has better reproducibility than most current methods for measuring melatonin. Likewise, reported procedures for GCMS assay of aMT6s or free and conjugated 6-hydroxymelatonin in urine require large sample volumes and/or extensive preliminary sample work-up. While urine is not in short supply, a nonextracted RIA method has considerable advantages over previous methods.

In the limited physiological studies described here, both plasma and urinary aMT6s were significantly correlated to plasma melatonin. Thus, aMT6s is likely to be useful as a marker of melatonin secretion. Clearly, however, the hormone-metabolite relationship in both plasma and urine must be studied in different physiological and pathological situations; the assessment of melatonin metabolism in man is an important potential application of this assay.

One previous study described a dramatic reduction of nighttime plasma melatonin in man after administration of 100 mg atenolol (20). The pharmacological studies described here show clearly, for the first time, the acute and complete suppression of the 24-h rhythm in urinary aMT6s after a single dose. Atenolol is considered to be a selective β1-adreno-receptor antagonist which does not penetrate the blood-brain barrier extensively (21). Thus, these observations reinforce current opinion (20) that melatonin production is dependent upon β1-adrenergic receptors lying outside the blood-brain barrier.

To date, only one plasma melatonin assay does not require an extraction procedure (18), and urinary melatonin assay requires extraction and further purification (22). In addition to the evident advantage of a direct assay, the measurement of aMT6s in urine is likely to be at least as useful as that of melatonin, provided that consideration is given to possible discrepancies between hormone and metabolite. Various aspects of human pineal function are simply not amenable to study using plasma melatonin measurement. Moreover, urinary melatonin levels are very low and represent only a small percentage of secreted hormone (12-14). Development of pineal function in neonates and long term circadian rhythm studies (to determine, for example, the oscillatory control of melatonin secretion) require measurement of a suitable urinary metabolite. RIA of aMT6s is likely to provide the necessary tool for such work.

References

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Melatonin Secretion in Humans Assessed by Measuring Its Metabolite, 6-Sulfatoxymelatonin

Christopher J. Bojkowski, Josephine Arendt, Ming C. Shih, and Sanford P. Markey

Comparing a direct radioimmunoassay for 6-sulfatoxymelatonin (aMT6s) with an established gas chromatographic/mass spectrometric method for 6-hydroxymelatonin, we found a good correlation \( r = 0.94 \) \( (P < 0.001, n = 100) \). aMT6s was stable, both in urine and plasma samples, without preservative, for at least two years at \(-20^\circ C\) and for five days at room temperature. Urinary excretion of aMT6s showed considerable inter-individual differences; however, the aMT6s excretion of any one individual was consistent over a four-day period, as assessed by continuous collection from 18 normal volunteers. Total 24-h urinary excretion of aMT6s was significantly correlated with the area under the curve of the respective profiles for plasma melatonin \( (r = 0.75, P = 0.0002) \) and plasma aMT6s \( (r = 0.70, P = 0.0005) \) for 22 healthy volunteers. At 24:00 h and 03:00 h, sampling plasma at 30-s intervals provided no evidence for episodic secretion (in short pulses) of either melatonin or aMT6s.

Additional Keyphrases: radioimmunoassay • urine • serotonin metabolism • 6-hydroxymelatonin • gas chromatography/mass spectrometry compared • pineal function • inter- and intra-individual variation • sample stability • circadian rhythm markers

Currently, investigations of human pineal function depend largely on measurement of the methoxyindole, melatonin, and its major metabolites in body fluids. Melatonin is metabolized in the liver to 6-hydroxymelatonin and N-acetylserotonin, both of which are excreted as the sulfate and glucuronide conjugates \((1, 2)\). As in other species, the melatonin rhythm in humans is endogenously generated \((3)\), the bulk of secretion occurring at night in normal light-dark cycles. Because both the timing and the amplitude of melatonin secretion may be important to its possible physiological and its pathological relationships, it is essential to define the characteristics of its secretion profile.

In clinical practice, nocturnal blood sampling is difficult, and the recent development of assays \((4-6)\) for melatonin metabolites in urine greatly increases the number of clinical cases amenable to study and also permits longitudinal studies in the same individual. However, the relationship of concentrations of melatonin metabolites in urine to those in plasma must be thoroughly established before urinary data can be unambiguously interpreted.

We have developed a sensitive, specific direct radioimmunoassay (RIA) for 6-sulfatoxymelatonin (aMT6s) in urine and plasma \((6)\); reportedly, aMT6s is the major melatonin metabolite in humans \((7, 8)\). We now describe the performance of the assay and present data comparing the measured concentrations of aMT6s with those of total 6-hydroxylated melatonin metabolites as determined by gas chromatography/mass spectrometry (GC/MS). We have also assessed the reliability of urinary aMT6s as an index of the endogenous concentrations of melatonin in plasma and report the characteristics of individual aMT6s production.

Materials and Methods

**Materials**

We prepared \[^{[3]H}]aMT6s \) by biologically transforming \[^{[3]H}]melatonin \((56.7, 48.2, 45.1, \text{ and } 49.4 \text{ kCi/mol; New England Nuclear Corp., Boston, MA}) \) as previously described \((6)\). A specific antiserum \((1118 \text{ II.18.82; Guildhay, Surrey, U.K.}) \) raised in sheep against aMT6s-ovalbumin was stored lyophilized at \(-20^\circ C\). Just before use, we diluted the antiserum 1000-fold with assay buffer.

Standard aMT6s was synthesized in our laboratory from 6-hydroxymelatonin according to the method of Fellenberg et al. \((4)\) and purified as previously described \((6)\). Aliquots of standard diluted with assay buffer were stored at \(-20^\circ C\), at a concentration of 290 \( \mu\text{g/L} \). Standard aMT6s was also generously supplied by each of the following: Dr. D. J. Kennaway, Department of Obstetrics and Gynaecology, University of Adelaide, Australia; Dr. A. M. Leone, Department of Reproductive Physiology, St. Bartholomew’s Hospital, London, U.K.; Dr. G. E. Webley, Institute of Hormone and Fertility Disorders, Hamburg, F.R.G.; and Dr. G. M. Brown, Department of Neurosciences, McMaster University, Hamilton, Ontario, Canada. Each standard was tested in the RIA. These standards were synthesized by the method of Fellenberg et al., with or without modifications \((4)\), or by that of Leone et al. \((9)\).

The assay buffer contained 17.9 g of “Tricine” \(N\text{-}[\text{tris(hydroxymethyl)methyl]glycine; Sigma Chemical Co., Poole, Dorset, U.K.}) \), 9.0 g of sodium chloride, and 1.0 g of gelatin per liter. The charcoal suspension consisted of 2.0 g of activated charcoal (Sigma Chemical Co.) and 0.2 g of Dextran T70 (Pharmacia Fine Chemicals, Upsala, Sweden) per liter of assay buffer. The assay buffer and charcoal suspension could be used for up to 10 days if stored at 4 \(^\circ C\). Cyanogen bromide-activated Sepharose 4B was obtained from Sigma Chemical Co.

**Procedures**

aMT6s RIA. We follow the procedure originally described by Arendt et al. \((6)\), but with several modifications. In brief, the procedure is as follows. We incubate 100 \( \mu\text{L} \) of \[^{[3]H}]aMT6s \((4500 \text{ counts/min})\) with 200 \( \mu\text{L} \) of antiserum and the sample \((200-500 \mu\text{L} \text{ of plasma or } 200 \mu\text{L} \text{ of urine diluted 50-fold with assay buffer})\) or \(5-400 \mu\text{L} \) standard \((2.5-200 \text{ pg per tube})\) for 20 min at 37 \(^\circ C\), then for 90 min at 4 \(^\circ C\). To construct the standard curves, we use either affinity-stripped plasma (in place of charcoal-stripped plasma), or charcoal-stripped aMT6s-free urine diluted 50-fold with assay buffer, in volumes equivalent to those of the test material being measured. The final volume of each sample is brought to 800 \( \mu\text{L} \) with assay buffer. To separate bound and free aMT6s, we add 100 \( \mu\text{L} \) of dextran-coated charcoal suspension, incubate at 4 \(^\circ C\) for 15 min, and centrifuge at 2000 \( \times \text{g} \) for 15 min at 4 \(^\circ C\). The radioactivity of 500-\(\mu\text{L} \text{ of}
Results were identical when standard curves were constructed with either affinity-stripped plasma or charcoal-stripped plasma. We then applied 2 mL of plasma sample to each column, placed the columns on a roller-mixer for 1 h at room temperature, and then collected the plasma. We could process up to 8 mL of plasma on each column before regenerating the columns by washing them with 10 mL of doubly distilled water, followed by three 2-mL washes with methanol/water (9/1 by vol). We prepared aMT6s-free plasma by affinity chromatography, using antiserum (no. 1118 23.8.84) linked to cyanogen bromide-activated Sepharose 4B, according to the manufacturer’s recommended procedure (10). Columns were stored, ready for use, at 4 °C in 84 g/L bicarbonate buffer containing 29.2 g of sodium chloride and 0.1 g of sodium azide per liter. We used plasma collected from normal volunteers in the mid-afternoon, pooling those samples that measured <5 ng/L in (comparison with charcoal-stripped plasma). We poured 1 mL of antibody-Sepharose slurry into disposable columns (Amicon Ltd., Stonehouse, Glos., U.K.) and removed the residual buffer by applying slight positive pressure. We then applied 2 mL of plasma sample to each column, placed the columns on a roller-mixer for 1 h at room temperature, and then collected the plasma. We could process up to 8 mL of plasma on each column before regenerating the columns by washing them with 10 mL of doubly distilled water, followed by three 2-mL washes with methanol/water (9/1 by vol). Measurement of the affinity-stripped spiked samples showed that 85–90% of unlabeled aMT6s was removed. Results were identical when standard curves were constructed with either affinity-stripped plasma or charcoal-stripped plasma. The antibody-Sepharose preparation retained its binding activity for at least a year.

To assess assay parallelism, we compared the standard curve with the displacement curves obtained for pooled human plasma collected during the night and serially diluted with affinity-stripped plasma.

Melatonin RIA. We followed the method described by Fraser et al. (11). The isotope assay CVs were 10.0, 12.6, and 20.6% (n = 14 each) at 134.4, 47.8, and 25.5 ng/L, respectively. Intra-assay CVs were 3.5 and 4.4% (n = 19 each) at 48.7 and 101.0 ng/L.

Comparison with GC/MS method for total 6-hydroxylated melatonin metabolites. We assayed 100 urine samples from healthy volunteers for total free and conjugated 6-hydroxymelatonin by the gas-chromatographic/mass-spectrometric method of Tetsuo et al. (5). We then assayed the same samples for aMT6s, on a single-blind basis, by RIA. We used least-squares regression to calculate the slope and intercept of the best-fit line through the data points, and also determined the standard error of regression (12).

Studies of Analytical Variables

Comparison of standards. We used six aMT6s standards from different sources (see Materials) to generate standard curves and assessed their relative potency. Their purity was evaluated by thin-layer chromatography on cellulose (Merck; supplied by BDH Chemicals Ltd., Eastleigh, Hampshire, U.K.), with butanol/acetic acid/water (4/1/1.5, by vol) and propan-2-ol/ammonia, 50 mL/L (4/2 by vol) as eluents.

Cross-reactivity studies. The specificity of the antiserum has been reported previously (6), but we performed additional cross-reactivity studies with 6-glucuronide melatonin, 5-sulfatoxy-N-acetylserotonin, and 5-glucuronide-N-acetylserotonin (all kindly supplied by Dr. A.M. Leone, St. Bartholomew’s Hospital).

Storage of samples. To determine the stability of aMT6s in plasma under various storage conditions, we stored plasma samples without preservative or with boric acid (final concentration 1.0 g/L) or ascorbic acid (1.0 g/L) at −12 °C and −20 °C for up to two years. We also assessed the analyte’s stability in plasma after freezing and thawing. As a routine procedure we froze plasma samples without delay and stored them at −20 °C.

The stability of aMT6s in urine was determined as for plasma; i.e., samples were stored without preservative or with boric acid (1.0 g/L) or ascorbic acid (1.0 g/L) for up to five days at 4 °C and at room temperature, and for up to two years at −12 °C or −20 °C. As a routine procedure, urine samples were frozen as soon as possible after collection and stored frozen at −20 °C. The total volume of each collection was recorded.

We measured aMT6s in the stored urine and plasma samples after one day, one week, and one, six, 12, 18, and 24 months.

Physiological Studies

Intra-individual variations in aMT6s excretion. For four consecutive days, 18 apparently healthy volunteers (nine men and nine women, ages 17–67 years) collected urine over 12-h periods: 10:00–22:00 h and 22:00–10:00 h. The only restriction placed on the subjects was the timing of their urine collection. Five women were taking oral contraceptives, but the subjects were otherwise drug free.

We used two-way analysis of variance to analyze the data. We also calculated the coefficients of variation for day-to-day differences in each subject’s urinary excretion of aMT6s. Short-term fluctuations in concentrations of melatonin and aMT6s in plasma. Previous workers have reported episodic secretion of melatonin in short pulses in humans and sheep (13–15). To confirm these observations and to see if the reported melatonin peaks were also reflected in peaks for aMT6s, we performed the following experiment.

Three volunteers (two women and one man) had indwelling cannulas inserted into their antecubital veins at 23:30 h. They then retired to bed in darkness. At 24:00 h and 08:00 h, 5-mL blood samples were taken at 30-s intervals for 10 min, in darkness with a dim red torch (<1 lux). We assayed these plasma samples for melatonin and aMT6s, determining all samples from any one individual in the same assay. Control or "noise" series were created by pooling the remaining aliquots of plasma from one or more of the volunteers to form large pooled specimens of plasma. We then assayed sequential samples from these pools in the same manner, again performing all measurements for a series of samples in the same assay.

To identify peaks in the data, we identified all local minima and maxima, then multiplied the concentration at the minimum by threefold the intra-assay CV for that concentration, to obtain the corresponding increment in hormone concentration. The next local maximum was considered a peak if it exceeded the minimum by an amount equal to or greater than this increment. We used the CV for 20 samples in the noise series to identify peaks in that particular series and in concentration-matched volunteers’ samples. We compared the number of peaks in the volunteers’ samples with the number of peaks in concentration-matched noise series (false positives) by paired Student’s t-test (two-tailed).

Correlation between plasma concentrations of melatonin and urinary aMT6s excretion. Blood was sampled (in July) from 18 apparently healthy volunteers (12 men and six women, at 2-h intervals from 12:00–20:00 h, at 1-h intervals from 20:00 to 10:00 h, with one final blood sample at 12:00 h.
During the day, blood was sampled by venipuncture, whereas at night we used an indwelling cannula inserted into the antecubital vein. At night, samples were taken with illumination from a dim red torch (<1 lux). Simultaneously with blood sampling, subjects collected timed 6-h urine samples: 12:00–18:00 h, 18:00–24:00 h, 24:00–06:00 h, and 06:00–12:00 h.

Four additional male volunteers were also included in the study. From 20:00 to 10:00 h blood was sampled (in May) at 1-h intervals; no blood samples were collected during the day. Urine specimens were collected concurrently, at 20:00–24:00 h, 24:00–12:00 h, and 12:00–20:00 h intervals. The age range of all the volunteers was 18–47 years.

We assayed all plasma samples for both melatonin and aMT6s; urine samples were assayed for aMT6s. We calculated the area under the curve (AUC) of the plasma profiles by the trapezium method (16). For the purposes of calculation we set all values below the detection limit of the assay and all the daytime-collected plasma samples from the four volunteers sampled in May at the detection limit. In normal volunteers daytime concentrations of melatonin in plasma are usually so low as to be undetectable by RIA technology (17). We used the coefficient of linear regression to establish the quantitative relationships between concentrations of aMT6s in urine and the concentrations of melatonin and of aMT6s in plasma.

Results

Assay Performance

The lowest detectable concentration of aMT6s standard, defined as the amount of aMT6s producing a displacement of 2 SD from maximum binding, was 2.1 pg per tube. The sensitivity of the urine assay was 0.65 μg/L. That of the plasma assay ranged from 5.0 ng/L to 12.5 ng/L, depending on the volume of plasma assayed directly.

Pooled human plasma collected during the night, serially diluted with affinity-stripped plasma, gave displacement parallel to that of aMT6s standard when up to 500 μL of plasma was assayed directly.

Analytical recoveries of three different concentrations of aMT6s (18.7, 70.0, and 238.2 ng/L) from endogenous human plasma pools were 113.9 ± 9.2, 101.9 ± 2.9, and 84.2 ± 3.3%, respectively. These values are mean ± SEM (n = 5 each).

Data for parallelism, recovery, and chromatographic identity of urinary immunoreactivity for the urine assay have been reported previously (6).

Precision. We determined the intra-assay and interassay precision of the assays.

The intra-assay CVs for the modified plasma assay were 6.7, 5.2, and 3.1% (n = 5 each) for samples with mean values of 17.8, 77.4, and 248.8 ng/L, respectively.

We assessed the interassay CVs for the modified plasma assay by measuring quality-control samples in successive assays. The CVs were 10.6, 8.8, and 6.8% (n = 18 each) for mean aMT6s values of 23.4, 61.0, and 130.8 ng/L, respectively.

We assessed the interassay CVs for the urine assay from quality-control samples taken during 15 months. The CVs were 16.3, 11.9, and 13.6% (n = 81 each) for mean aMT6s values of 2.3, 7.2, and 19.5 μg/L, respectively. No drift in the quality-control samples was observed after 15 months.

The intra-assay CVs for the urine assay have been reported previously (6).

Comparison of standards. All the standards tested gave displacement parallel to that of our standard and were chromatographically identical. The relative potency of the different preparations, however, varied approximately two-fold, possibly due to the efficiency of desalting in some cases.

Cross-reactivity studies. Original cross-reactivity studies with this antiserum showed it to be extremely specific but did not include the other urinary metabolites of melatonin: 6-glucuronic acid melatonin has recently become available and two new urinary metabolites of melatonin have been identified, 5-sulphatoxy-N-acetylserylserotonin and 5-glucuronic N-acetylserylserotonin (8). They cross reacted with the antiserum by 0.03%, 0.003%, and 0.4%, respectively.

Storage of Samples

aMT6s is extremely stable in plasma and urine. We found no differences for samples stored either with or without preservative, or between fresh urine samples and those stored frozen, nor were there losses on thawing and refreezing. aMT6s was stable in plasma and urine for up to five days at 4 °C or at room temperature, and for up to two years when stored at −20 °C or −12 °C. Interassay CVs for urine and plasma samples stored without preservative at −20 °C over a two-year period were 11.7% and 12.0% (n = 9 each) at concentrations of 36.0 μg/L and 59.0 ng/L, respectively.

Comparison with GC/MS Method for Total 6-Hydroxylated Melatonin Metabolites

A good correlation was obtained between the two methods (r = 0.94, n = 100), which was highly significant (P < 0.001). Regression analysis of the data gave the equation y = 0.47x + 0.64 μg/L (where y is the RIA) with a standard error of regression of 2.72 (12). The regression line is shown in Figure 1.
Biological Variation Factors

**Intra-individual variations in aMT6s excretion.** Two-way analysis of variance of the urinary excretion of aMT6s over four consecutive days showed a significant variation between the urinary aMT6s excretion by the different subjects for the daytime ($F = 6.34, P < 0.001, \text{df} = 17,51$), nighttime ($F = 23.79, P < 0.001, \text{df} = 17,51$), and 24-h samples ($F = 26.62, P < 0.001, \text{df} = 17,51$). We found no significant within-group variation, showing that for each individual the excretion of aMT6s was consistent within the four-day period. Figure 2 illustrates the intra-individual variations in aMT6s excretion for four subjects during the collection period. 

Table 1 shows the mean excretion of aMT6s, together with the mean CVs for consistency of aMT6s excretion during the four days, and the observed ranges of the CVs.

Of the mean total 24-h urinary aMT6s excretion, 80.7% (SD 6.1%; $n = 18$) was excreted between 22:00 and 10:00 h. This finding is in harmony with the study of Fellenberg et al. (4).

**Short-term fluctuations in plasma concentrations of melatonin and aMT6s.** Figure 3 compares melatonin and aMT6s concentrations in representative control samples and samples from volunteers. No secretory episodes, with short duration pulses, of an amplitude significantly greater than that observed in the control noise series were observed in the volunteers' samples. We calculated the CV for each series of samples. The mean CVs for the volunteers' samples were 7.5% (SD 6.1%; $n = 6$) and 6.7% (SD 1.2%; $n = 6$) for melatonin and aMT6s, respectively, whereas for the concentration-matched noise series the mean CVs were 4.3% (SD 0.3%; $n = 6$) and 7.9% (SD 0.3%; $n = 6$), respectively. We thus observed a slightly greater variability in the volunteer melatonin samples than in the noise series.

Using the three-CV criterion, we observed peaks in both the melatonin noise series (11 peaks in total) and the volunteer melatonin samples (14 peaks in total). In the aMT6s noise series we observed a total of three peaks, whereas in the volunteer aMT6s samples only one peak was observed. The total number of peaks observed in the volunteer melatonin or aMT6s samples compared with the total number of peaks in concentration-matched control series showed no significant difference by the paired Student's $t$-test (two-tailed).

**Correlation between concentration of melatonin in plasma**
and urinary aMT6s excretion. Plasma aMT6s, like melatonin, has a marked circadian rhythm, as previously shown by use of a small number of subjects (6). In the 22 subjects reported here, the mean concentrations of melatonin in plasma ranged from 8.0 ng/L (14:00–18:00 h) to 49.6 ng/L (04:00 h), while the mean concentrations of aMT6s in plasma ranged from 11.0 ng/L (16:00–21:00 h) to 85.2 ng/L (05:00 h) (Figure 4).

The mean excretion of aMT6s was (µg aMT6s; mean ± SD, n = 18 each) 0.68 ± 0.27 (12:00–18:00 h), 1.24 ± 0.65 (18:00–24:00 h), 4.38 ± 1.84 (24:00–06:00 h), and 1.87 ± 1.08 (06:00–12:00 h). The mean total 24-h urinary aMT6s excretion was 8.76 ± 3.43 µg aMT6s (mean ± SD, n = 22).

We used the coefficient of linear regression to establish the relationships between the AUC of the individual plasma melatonin and plasma aMT6s profiles and the respective peak height of the profiles, with the individual 24-h urinary aMT6s excretion. We observed significant correlations between all these (Table 2). The most important correlation was that between plasma melatonin (AUC of 24-h profile) and total 24-h urinary aMT6s (r = 0.75, P = 0.0002, n = 22).

### Discussion

We show that this RIA for aMT6s is sensitive, specific, relatively simple, and very robust. The assay is direct for both plasma and urine, obviating lengthy extraction procedures. There is little interference in the assay by the other urinary metabolites of melatonin, and it can be ignored in physiological studies. The use of affinity-stripped plasma to construct standard curves has decreased the concentration detectable in plasma from 13.0 ng/L to 5.0 ng/L, and enables reproducible aMT6s-free plasma pools to be generated.

The aMT6s standards supplied by other groups were all immunoreactively and chromatographically identical to our standard, although their potency varied somewhat. The commercial availability of a pure standard preparation of aMT6s would be highly desirable in the interests of comparing results from different laboratories.

The RIA shows a very good correlation with the gas-chromatographic/mass-spectrometric method. As expected, the RIA for aMT6s generated consistently lower values than the gas-chromatographic/mass-spectrometric method, which measures total (free and conjugated) 6-hydroxylated melatonin metabolites. In the samples measured by both methods the proportion of 6-hydroxylated metabolites excreted as the sulfate conjugate was not consistent. Whether this is ascribable to a variable proportion being excreted as the glucuronide conjugate remains to be established. Possible reasons for this could be genetic differences in the conjugating enzymes or possible drug interactions with the conjugating enzymes (18). When we repeatedly measured the same sample pools by both methods, we found that the proportion excreted as the sulfate conjugate remained consistent (data not shown), implying that methodological inconsistencies were not the cause of the inconsistency.

The observed stability of aMT6s in urine (for up to five days at room temperature) is particularly important when one is carrying out studies on volunteers in their normal social environments. In such volunteers the 24-h excretion of aMT6s was consistent during a four-day period, the nighttime values being most consistent. Daytime values are often at or near the detection limit of the assay; in this study we did not restrict fluid intake, and slight assay variations might be magnified by larger urine volumes and consequent multiplication factors.

The consistency of individual aMT6s excretion very probably reflects a stable rate of melatonin production in the same individuals (19). One consequence of practical importance is the reliability of both compounds as circadian rhythm markers (17).

In this preliminary study, we found no evidence for short-duration pulses of melatonin or aMT6s in human plasma taken from the antecubital vein. If blood sampling is frequent, the probability of detecting false-positive peaks due to assay noise is increased. It is important to include data on the variability in repeated measurements of the same samples, something that previous reports on short-duration pulses and episodic secretion of melatonin have omitted (13, 14). Ideally, a more rigorous criterion than an increment from nadir to peak of three times the intra-assay CV should be used to identify peaks, such as multiple measurement at

| Table 2. Correlation between Individual Values for Plasma Melatonin, Plasma aMT6s, and Total Urinary aMT6s Excreted during 24-h Excretion |
|-----------------------------|----------------|----------------|
| Comparison                  | r   | P       |
| 24-h urinary aMT6s vs:      |     |         |
| Plasma melatonin AUC        | 0.75| 0.0002  |
| Plasma melatonin PH         | 0.70| 0.0055  |
| Plasma aMT6s AUC            | 0.70| 0.0005  |
| Plasma aMT6s PH             | 0.64| 0.002   |
| AUC plasma melatonin vs AUC plasma aMT6s | 0.76| 0.0001 |
| Plasma melatonin PH vs plasma aMT6s PH | 0.73| 0.0003 |

AUC, area under the curve; PH, peak height, n = 22 each.
each time point (20). Unfortunately, with the volume of plasma required in our assays this was not possible. In the melatonin assay a slightly greater variability was observed in the volunteers' samples than in the control noise series. In view of other reports, notably studies of sheep, it is likely that human melatonin secretion is episodic but that concentration differences, for example, in the jugular vein (14, 15) may be "damped" in the antecubital vein.

The correlation between urinary aMT6s excretion and concentrations of melatonin in plasma is a very important relationship, because it validates the use of the urine assay as an index of the endogenous concentrations of melatonin in plasma. Markey et al. (21) reported a correlation coefficient of 0.76 between plasma melatonin and urinary 6-hydroxymelatonin. This is confirmed by our correlation coefficient (0.75) between plasma melatonin and urinary aMT6s.

In conclusion: measurement of urinary aMT6s evidently is a very useful tool in the study of pineal function. In normal volunteers urinary aMT6s is a good integrative measure of plasma melatonin. Whether or not this relationship is maintained in pathological and clinical situations remains to be established.

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Annual Changes in 6-Sulphatoxymelatonin Excretion in Man

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Abstract

A recently developed RIA for 6-sulphatoxymelatonin, the major urinary metabolite of melatonin, has been used to investigate the annual change in melatonin secretion in humans. Twenty plasma samples were taken from 18 volunteers throughout a 24h period and simultaneous 6-hourly urine samples were also collected. Plasma melatonin and urinary 6-sulphatoxymelatonin were measured by RIA. 6-sulphatoxymelatonin assayed in the urine samples was shown to be a good index of the rhythmic characteristics of the plasma melatonin secretion.

To study annual changes in excretion, four sequential 6-hourly urine samples were collected at monthly intervals from 16 normal volunteers for 13 months. Cosinor curves were fitted to the 6-sulphatoxymelatonin excretion data and the 24h rhythm was described by the cosinor parameters; amplitude, mesor and acrophase. Significant differences in the acrophase were found during the year. The summer acrophase was phase advanced relative to the winter acrophase by about 1.5h while intermediate phase positions were observed in spring/autumn.

The 24h excretion of urinary 6-sulphatoxymelatonin was remarkably consistent and there was no annual rhythm. In contrast, the daytime 6-sulphatoxymelatonin excretion between 1200-1800h showed a statistically significant seasonal rhythm, with peaks in December/January and in July.
LIST OF PUBLICATIONS


