Determination of melatonin in edible plants and medicinal herbs: Comparison of different methods, including UV spectroscopy and GC/MS, with reference to its application in immunodegenerative disorders.

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Summary
This research project aims to develop methods for the determination of melatonin in plant tissue. Two independent methods are to be devised one using UV spectrometry and the other GC/MS. Both methods are to be preceded by extraction of the molecules from the plant tissue using solid phase extraction (SPE) and/or liquid chromatography techniques.

Introduction
The pineal hormone melatonin has been shown to have beneficial effects in many aspects of health. Melatonin has been shown to be an important hormone in the regulation of important biological cycles. It has been shown, in human beings, to induce restfulness, and appears to play an important role in reproduction, having an influence on the onset of puberty (Cavallo, 1993; Puig-Domingo, Webb et al., 1992; Waldhauser, Boeppele et al., 1991). In addition to its hormone function melatonin is also a far more powerful antioxidant than Vitamins C, E, and K, able to protect biological tissue from the harmful effects of free radicals (Poeggeler, Thuermann et al., 2002). This is because melatonin can penetrate all cells, whereas the other vitamins are capable of only selective penetration.

In the human body, melatonin is secreted in a distinctive daily cycle which peaks significantly during the hours of darkness. It is thought that inducing sleep is one of melatonin’s primary roles (Dawson, Encel et al., 1993). A sleep research project carried out at Massachusetts Institute of Technology reported that a very small amount (0.1mg) of melatonin enhanced sleep in healthy young volunteers (Dolins, Wurtman et al., 1994).

For people who suffer from disrupted sleep or insomnia the prescribed sleeping remedies are usually medicines from the benzodiazapine group of drugs. Benzodiazapines have a tranquilizing effect on the body but with this positive effect comes a myriad of less desirable side-effects including next day drowsiness, memory loss and disruption of essential sleep ‘levels’. These drugs should only be a short term solution but in many cases are prescribed to patients for a number of years. Benzodiazapines, despite their tranquilizing effect, disrupt the ‘normal’ sleep pattern often leaving users with a deficiency of REM and deep sleep (Steinburg,1989).

Sleep induced by melatonin shows the same sleep level patterns and behavior as natural sleep (Cramer, 1974). Melatonin shows none of the side effects observed with traditional sleep medication. Studies on elderly patients, who are generally very sensitive to drug side effects, showed that even high dose (50mg) melatonin did not interfere with memory, concentration or motor control (Singer, 1994).

The requirement of only small doses of melatonin to promote sleep was highlighted in a study by Richard Wurtman. In his first study melatonin was administered at a very high dose (240mg), this amount caused fatigue and sleepiness in the human subjects (Wurtman, 1984). In a further study the amounts of melatonin were reduced to doses of 1 and 0.3mg, Wurtman found that even these minute quantities were able to decrease the amount of time taken for the subject to fall asleep (Wurtman, 1995).

There seems to be a definite link between physiological melatonin levels and cancer incidence. Suppression of the normal light/dark melatonin cycle by light during darkness hours can lead to a increase in the progression of cancerous tissue (Blask, Dauchy, 2002; Reiter, 2002; Sanchez-Barcelo, 2003). It has also been shown that cancer patients tend to have a lower secretion of melatonin when compared to healthy subjects (Kos-Kudla, 2002). Melatonin has been shown to have an inhibitory effect on the growth of endometrial (Kobayashi Y 2003), breast (Dillon, 2002; Anisimov, 2003; Bizzarri, 2003) and prostate (Shiu, 2003) cancer tumors.

Studies have shown that melatonin can be very effective in lessening the toxicity of chemotherapy drugs. A five year survival study of non-small cell lung cancer patients taking the chemotherapy drugs cisplatin and etoposide with or without the concomitant administration of melatonin has been carried out (Lissoni, 2003). Both the tumor regression and survival rates were considerably higher in the cases where melatonin had been used. No patient treated with chemotherapy alone was alive after two years, in contrast 6% of the patients treated with chemotherapy and melatonin achieved a five year survival. In a separate paper Lissoni also reports melatonin’s potential for disease control in the case of metastatic solid tumours (Lissoni, Malugani , 2003). A
similar study of concomitant administration of melatonin and irinotecan to metastatic colorectal cancer patients shows the best disease control was achieved in patients receiving both compared to chemotherapy alone (Cerea, 2003).

In humans, one early study showed a correlation between blood pressure and melatonin (Birau, Peterssen et al., 1981). This particular study involved the administration of nose drops to twenty hypertension sufferers, with some of the nose drops containing melatonin, the others a placebo. At the end of the first week the treatments were reversed. The authors reported that when the patients were taking the melatonin-containing nose drops the recipients’ blood pressure fell to within the normal range.

From the literature it can be seen that melatonin has a very important role in protecting biological tissue from damage which can cause cell disease, mutation and death. Deficiencies in melatonin can lead to increases incidence of cancers and other debilitating diseases (Kenny, 2001; Kos-Kudla, 2002). This property of melatonin is often attributed to it acting as an antioxidant (Reiter RJ, Melchiorri D 1995).

Interestingly, many medicinal plants have been shown to contain high levels of melatonin which may be a key factor in their therapeutic actions (Chen G., Huo Y. et al, 2003). Not only medicinal plants but some examples of everyday edible plants have been reported to show significant levels of melatonin (Hattori A., Hiro M. et al, 1995, Burkhardt S., Tan D.X. et al, 2001).

In view of the link with cancer prophylaxis or treatment, we have begun developing a database of melatonin levels in edible and medicinal plants. We believe that such a resource would be of value to healthcare professionals, cancer epidemiologists, cancer and diet researchers, and members of the public. In the latter category would be those who may wish to boost their natural melatonin intake without recourse to synthetic concentrates or tablets. In addition, the information could be valuable in formulating therapeutic diets as a supplement to recognised anti cancer treatments.

Materials and Methods

Melatonin (N-Acetyl-5-methoxy tryptamine M = 238) was obtained from Sigma. UV spectra were obtained using Unicam UV2 and SP6-500 machines equipped with flow cells. Column and thin layer chromatographic separations employed standard silica support materials. Melatonin (λ=280; ε=5500 in ethanol) was determined in methanol, ethanol and 0.1N HCl. 0.1N, NaOH aqueous and ethanolic solutions. Extraction from plants involved crushing the material in a commercial food juicer and employing various methods for optimising extraction of the melatonin from the solid material, including ultrasonic disintegration. Filtrates were subjected to solvent extraction. Gas chromatography/Mass spectrometry employed a Finnigan Polaris Q ion quadrupole GC/MS system.

Results/Conclusion

UV methods enabled easy analysis of the optimisation of precursor conditions for extraction of the melatonin from the crude plant material. Owing to the relatively high extinction at 280nm, the limits of detection in our systems of unconcentrated extracts were about 5 E-6 M or 1µg/ml (1mg/l) at 0.1Abs and 5cm cuvettes. Since measured concentrations of melatonin in plants are in this range and can be as high as 5mg/kg dry weight, the method is capable in principle of determining levels of the material. The absorption peak at 280nm is in a region where there are likely to be several other substances absorbing, notably the amino acid tryptophan so prior separation was developed using thin-layer and column chromatography. The material was then assayed using a UV flow cell detector on the column eluate. GC/MS procedures are in the process of initial development.

Discussion

Radio immunoassay (RIA) has been a favoured method of melatonin determination in biological fluids due to its simplicity and sensitivity. Wide usage of RIA can also be attributed to the wide availability of commercial melatonin kits. RIA reported to be sensitive to 4 or 5ng/L depending on the extraction technique employed prior to assay (Di W.L., Kadva A., et al., 1998). A miniature RIA for use with samples from invertebrates has been shown to be sensitive to 2pg melatonin (Gorbet and Steel, 2003). Levels of melatonin in plant tissue were determined by Hattori et al. by RIA (Hattori, Migitaka, et al., 1995). A significant problem with RIA is lack of specificity of the antibody causing erroneous results due to cross reaction with other closely related indolic and naphthalenic analogues and it is not at all clear whether the results quoted are accurate or specific to melatonin. Extraction of melatonin from the sample in question followed by the RIA may go some way towards alleviating this problem. However, there are specific health and safety issues to consider when using RIA. For this reason we have been exploring the other techniques outlined in this communication.
In the past, GC/MS techniques for the determination of melatonin, although in principle very sensitive have been used to a lesser extent than RIA mainly due to difficulties in developing simple, quick automated analyses in comparison to the bioassay methods.

Melatonin in human saliva has been determined using HPLC-tandem mass spectrometry with the limit of detection and quantification 1.05pg/mL and 3pg/mL respectively (Eriksson K., Ostin A., et al., 2003). Determination of the daytime concentration of melatonin in mouse pineal gland has been achieved with reversed-phase micro-HPLC with fluorescence detection. The amount reported was 74fmol/pineal gland or 20pg per pineal gland (Tomita T., Hamase K., et al., 2001). HPLC with fluorescence detection has also been used to quantify melatonin in samples of Chinese herbs, with a detection limit of 50pg (Zhang Y., Chen G., et al., 2003). In a recent publication HPLC with fluorescence detection has been used to determine melatonin in cell culture containing phytohemagglutinin –stimulated lymphocytes, in this case the detection limit was 10pg per injection (Carrillo-Vico A., Calvo J.R., et al., 2004).

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