

## Effect of a synthetic pineal tetrapeptide (Ala-Glu-Asp-Gly) on melatonin secretion by the pineal gland of young and old rats

Y. Djeridane\*, V.Kh. Khavinson\*\*, V.N. Anisimov\*\*, and Y. Touitou\*

\*Faculty of Medicine Pitié-Salpêtrière, Medical Biochemistry and Molecular Biology, Paris, France,  
\*\*Saint-Petersburg Institute of Bioregulation and Gerontology, St. Petersburg, Russia

**ABSTRACT.** The pineal gland contains many peptides known to be implicated in melatonin production. We examined the effects of a synthetic pineal tetrapeptide Ala-Glu-Asp-Gly on melatonin secretion by the pineal gland. The tetrapeptide effects on pineal gland melatonin secretion were studied in young (9 weeks) and old (27 months) male Wistar rats using a perfusion device. Pineal tetrapeptide at the concentrations used ( $10^{-4}$  to  $10^{-6}$  M) had no significant effect upon melatonin secretion whatever the

age of the animals, young or old. We also looked at the effect of the tetrapeptide on pineal melatonin stimulated by a  $\beta$ -adrenergic agonist, isoproterenol. We found that isoproterenol-induced melatonin increase was not modified by the tetrapeptide. Our results suggest that the pineal tetrapeptide Ala-Glu-Asp-Gly, does not seem to play a role, at least *in vitro*, in the control of melatonin secretion by the rat pineal gland. (J. Endocrinol. Invest. 26: 211-215, 2003)

©2003, Editrice Kurtis

### INTRODUCTION

Several studies showed that blood melatonin levels markedly decrease with age (1-5). Melatonin, which is the major secretory product of the pineal gland, plays a key role in the synchronization of the biological rhythms and modulates functions of the endocrine, nervous and immune systems (1). The hormone appears to have some anti-oxidative effects and geroprotective activity (6, 7), which led to the hypothesis that it might delay the ageing process and inhibit the growth of tumor cells. Thus, it appears that the search for new stimulators of endogenous melatonin is of considerable importance. The pineal gland contains many peptides such as vasoactive intestinal peptide, neuropeptide Y, vasopressin, oxytocin, pituitary adenylate cyclase-activating polypeptide, secretoneurin and orexin (8-13). Some of these peptides have been found to be active on melatonin secretion *in vitro* (9, 10, 13). Previous studies showed that a com-

plex of peptides extracted from the pineal gland (epithalamin), increased melatonin synthesis in old and not in young rat pineal gland resulting in higher plasma concentration of the hormone (14, 15). This complex was also found to prolong the life span of rodents and to diminish the incidence of spontaneous tumor development (14, 16, 17). A recently synthesized polypeptide on the basis of the analysis of epithalamin amino acids, epitalon (Ala-Glu-Asp-Gly) has been shown (18) to possess geroprotective and anti-tumor effects (17), and to stimulate melatonin production in old but not in young rhesus monkeys *in vivo* (19, 20). We therefore found it worth looking at the effects of this pineal tetrapeptide Ala-Glu-Asp-Gly on melatonin secretion in the pineal gland of young and old rats *in vitro* using an organ perfusion device. The release of norepinephrine from nerve endings in the pineal gland acts primarily on  $\beta$ -adrenergic receptors (1). This causes a rise of intracellular cAMP, increasing the activity of the rate-limiting enzyme serotonin-N-acetyltransferase and thus, the production of melatonin. It has been assumed that the pineal tetrapeptide Ala-Glu-Asp-Gly increases the number and/or affinity of  $\beta$ -adrenergic receptors on pinealocyte membranes for norepinephrine (19). We thus examined the effects of the tetrapeptide on basal and on adrenergically stimulated pineal melatonin secretion.

Key-words: Pineal tetrapeptide, melatonin, pineal gland, aging, rat.

Correspondence: Prof. Yvan Touitou, Faculté de Médecine Pitié-Salpêtrière, Service de Biochimie Médicale et Biologie Moléculaire, 91, Boulevard de l'Hôpital, 75013, Paris, France.

E-mail: touitou@ccr.jussieu.fr

Accepted September 25, 2002.

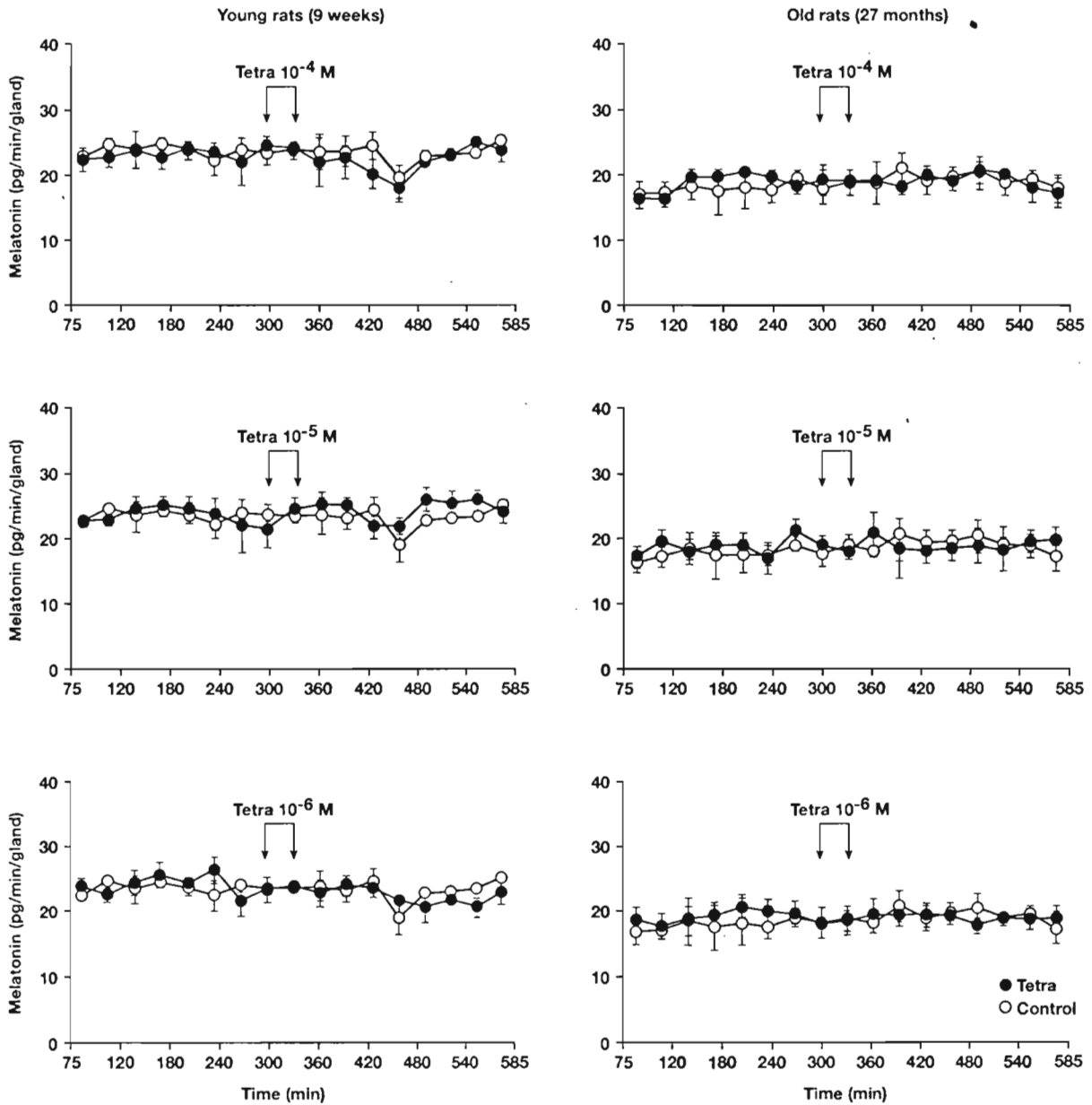


Fig. 1 - Effect of a synthetic pineal tetrapeptide (Tetra,  $10^{-4}$  to  $10^{-6}$  M) on melatonin secretion by perfused pineal glands of young (9 weeks) and old (27 months) rats obtained in the middle of the dark span. Each value is the mean  $\pm$  SE of data obtained in three perfusion chambers (one gland per chamber). The drug (arrows) was infused for 30 min, 300 min after the beginning of the perfusion.

## MATERIALS AND METHODS

### Animals

Twenty-four young (6 weeks) and twelve old (24 months) male albino Wistar rats (Iffa Credo, L'Arbresle, France), on arrival at the laboratory, were used in the experiments. Animals were housed in a chronobiologic animal facility (Enceinte Autonome

d'Animalerie, Ref. A 110-SP-6, ESI Flufrance, Arcueil, France) with food and water available *ad libitum*. The facility was equipped with sound-proof, temperature-controlled ( $21 \pm 1.0$  C) compartments, all the same size and provided with independent light-dark cycles. The rats were synchronized to a light-dark cycle regimen of 12:12 under a reverse lighting schedule (lights

on from 15:30 h to 03:30 h); this enabled us to perform the dark-span sampling during the day. The rats were synchronized to this lighting regimen for 3 weeks before the experiments. These animal experiments were performed in accordance with the principles of laboratory Animal Care (NIH) and with French laws. Rats were sacrificed by decapitation at the age of 9 weeks for young rats and 27 months for old rats, in the middle of the dark phase and, to avoid alteration of melatonin production, under dim red light.

### Experimental device and procedure

The perfusion system has been described previously (21). Briefly, it consisted of a plastic column closed with two pistons, a thermostatic bath, and a peristaltic pump. The pineal glands were quickly removed and kept in 4 C Krebs-Ringer solution at pH 7.4, gassed with 5% CO<sub>2</sub> and 95% O<sub>2</sub> until transfer into the perfusion chambers. The Krebs-Ringer medium contained (mM) NaCl 120, KCl 5, KH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2.6, MgSO<sub>4</sub> 0.67, NaHCO<sub>3</sub> 22.5, and glucose 10. The pineal glands (one gland per chamber) were perfused with oxygenated Krebs-Ringer solution at a constant flow rate of 0.1 ml/min. The pineal glands and medium were maintained at 37 C by the thermostatic bath (Techne TE-8J). The pineal glands were perfused for 9 h 30 min (570 min). Perfusates were collected into plastic tubes every 30 min with an automatic fraction collector and stored at -20 C until melatonin assays.

### Effects of the pineal tetrapeptide on melatonin secretion by perfused pineal glands

This experiment was undertaken in order to determine the effects of different doses of a synthetic pineal tetrapeptide Ala-Glu-Asp-Gly on melatonin release by the pineal gland of young and old rats. The peptide in injectable solution was prepared as 10<sup>-4</sup> M, 10<sup>-5</sup> M, and 10<sup>-6</sup> M solutions in Krebs-Ringer. Since melatonin secretion by perfused rat pineal glands has been shown to be stabilized 3-4 h after the perfusion begins, the peptide was infused for 30 min, 300 min after the beginning of the perfusion. The data were compared with control glands that underwent no tetrapeptide infusion but only Krebs-Ringer solution infusion.

### Effects of the pineal tetrapeptide on isoproterenol-stimulated melatonin secretion by perfused pineal glands

We examined how  $\beta$ -adrenergic stimulated melatonin secretion by young rat pineal glands is affected by the pineal tetrapeptide Ala-Glu-Asp-Gly, using isoproterenol, a  $\beta$ -adrenergic agonist. Isoproterenol was diluted directly in a Krebs-Ringer solution. This drug was purchased from Sigma (St. Louis, Mo., USA) and was used at a final concentration of 10<sup>-4</sup> M. This concentration was chosen for its optimal effect on pineal melatonin production *in vitro* as observed in a previous study (22). Isoproterenol was infused for 30 min (300-330 min) either alone or together with 10<sup>-4</sup> M pineal tetrapeptide. In each case the data were compared to corresponding controls.

### Melatonin radioimmunoassay

Melatonin in the perfusate fractions was assayed directly by a modification of the RIA method of Fraser et al. (23), with a spe-

cific rabbit antiserum (R 19540) provided by INRA (Nouzilly, France) and 125I labeled melatonin tracer (NEN-Dupont, Boston, MA, U.S.A.). The detection limit of the melatonin assay was 5 pg/tube. The intra-assay and inter-assay coefficients of variation were, respectively, 10 and 18% for a concentration of 50 pg/ml of melatonin, and 7 and 11% for 200 pg/ml.

### Presentation of data and statistical analysis

The melatonin production in each chamber was expressed in pg/min/gland. Results were expressed as mean  $\pm$ SE. The experimental data were analyzed for statistical significance with Student's *t*-test. Differences were defined as significant at probability levels  $p < 0.05$ .

## RESULTS

The mean value of the basal level of melatonin released from perfused rat pineal glands removed in the mid-dark-span was 23.7  $\pm$  1.6 pg melatonin/min/gland in young rats vs 18.52  $\pm$  0.3 pg melatonin/min/gland in old rats;  $p < 0.01$ .

The synthetic pineal tetrapeptide at the concentrations used (10<sup>-4</sup> M to 10<sup>-6</sup> M) did not result in any detectable effect on melatonin secretion by the rat pineal gland, neither in young nor in old animals (Fig. 1).

The  $\beta$ -adrenergic agonist isoproterenol significantly increased melatonin secretion by pineal glands by 70% compared with control glands that underwent no isoproterenol infusion ( $p < 0.05$ ) (Fig. 2).

The pineal tetrapeptide (10<sup>-4</sup> M) had no significant effect on the isoproterenol-induced increase (Fig. 3).

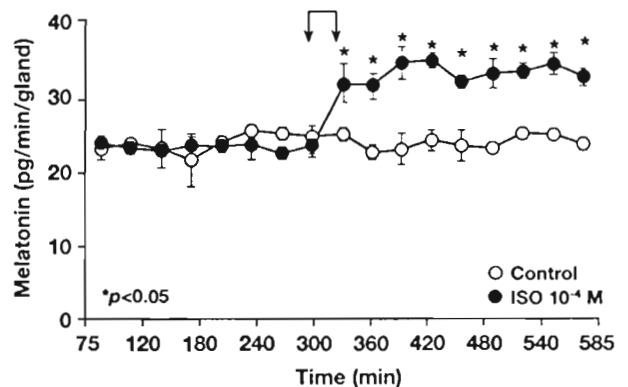


Fig. 2 - Profiles of melatonin release from perfused pineal glands of young (9 weeks) rats obtained in the middle of the dark span after infusion of isoproterenol (ISO, 10<sup>-4</sup> M). Each value is the mean  $\pm$ SE of data obtained in three perfusion chambers (one gland per chamber). The arrows show the duration (30 min) of drug infusions.

## DISCUSSION

The pineal gland contains many peptides such as vasoactive intestinal peptide, neuropeptide Y, vasopressin, oxytocin, pituitary adenylate cyclase-activating polypeptide, secretoneurin, and orexin, which all have been shown to be implicated in the regulation of the methoxyindole synthesis in the pineal gland (9, 10, 13). The complex of peptides epithalamin, and the tetrapeptide Ala-Glu-Asp-Gly are physiologically active preparations of the pineal gland (18). It has been previously shown that a subcutaneous injection of epithalamin to old rats (18-20 months) results in an increased pineal melatonin production (14, 15). Moreover, the tetrapeptide Ala-Glu-Asp-Gly has been found to increase plasma melatonin levels only in old rhesus monkeys (20-26 yr) but not in young animals (6-8 yr) (19, 20). These data prompted us to compare *in vitro* the effect of Ala-Glu-Asp-Gly on pineal melatonin secretion in both young and old rats.

We found that the pineal content of melatonin in old rats was 1.3 times lower than in young animals, which is consistent with published data on the decrease of blood melatonin concentrations during aging (2-5). We showed that whatever the age of the animals, young (9 weeks) or old (27 months), Ala-Glu-Asp-Gly had no effect on melatonin secretion in the rat pineal gland in our experimental perfusion conditions. These differences in the *in vivo* and *in vitro* effects of the pineal tetrapeptide upon pineal melatonin may be related to a difference in the physiological state of the gland or might also be related to interspecies variations.

Indeed, other papers (19, 20) deal with the stimulating effect of the peptide on monkeys *in vivo*, while in the present study we used *in vitro* perfusion on rat pineal gland.

Adrenergic innervation of the pineal gland plays a key role in the regulation of melatonin production (1). It has been assumed that Ala-Glu-Asp-Gly increases the number and/or affinity of  $\beta$ -adrenoreceptors on pinealocyte membranes for norepinephrine (19). We have therefore examined the effect of the tetrapeptide Ala-Glu-Asp-Gly on adrenergically stimulated melatonin secretion with a  $\beta$ -adrenergic agonist, isoproterenol. Our data showed that isoproterenol stimulated, as expected, the pineal melatonin secretion and that this effect was persistent, which is in good agreement with previous data (21, 22). The tetrapeptide had no additional influence on the isoproterenol-induced melatonin increase. In conclusion, we found here that the pineal tetrapeptide Ala-Glu-Asp-Gly did not alter nocturnal pineal melatonin secretion which strongly suggests that this peptide does not seem to play a role in the control of melatonin secretion in the rat pineal gland, at least *in vitro*.

## ACKNOWLEDGMENTS

The Authors are deeply indebted to Dr. J.P. Ravault (INRA, Nouzilly, France) for kindly supplying the melatonin antiserum, to H. Charbuy and F. Camus for their technical help, and to M. Soutarson for taking care of the animals.

## REFERENCES

1. Reiter R.J. Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. *Endocr. Rev.* 1991, 12: 151-180.
2. Touitou Y., Fevre M., Bogdan A. et al. Patterns of plasma melatonin with ageing and mental condition: stability of nyctohemeral rhythms and differences in seasonal variation. *Acta Endocrinol.* 1984, 106: 145-151.
3. Waldhauser F., Kovacs J., Reiter E. Age-related changes in melatonin levels in humans and its potential consequences for sleep disorders. *Exp. Gerontol.* 1998, 33: 759-772.
4. Touitou Y., Haus E. Alterations with aging of the endocrine and neuroendocrine circadian system in humans. *Chronobiol. Int.* 2000, 17: 369-390.
5. Touitou Y. Human aging and melatonin. Clinical relevance. *Exp. Gerontol.* 2001, 36: 1083-1100.
6. Reiter R.J., Tan D.X., Cabrera J. et al. The oxidant/antioxidant network: role of melatonin. *Biol. Signals Recept.* 1999, 8: 56-63.
7. Gitto E., Tan D.X., Reiter R.J. et al. Individual and synergistic antioxidative actions of melatonin: studies with vitamin E, vitamin C, glutathione and desferrioxamine (des-

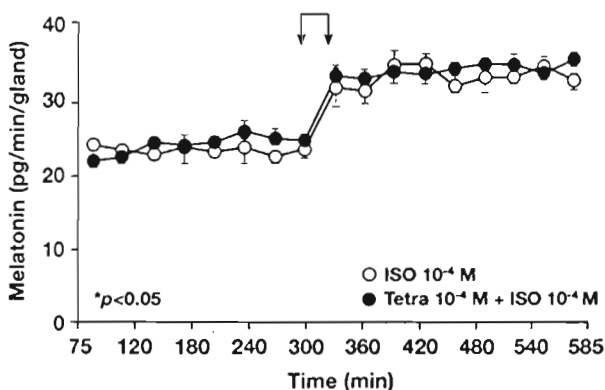


Fig. 3 - Effect of synthetic pineal tetrapeptide (Tetra,  $10^{-4}$  M) on  $10^{-4}$  M isoproterenol (ISO)-stimulated melatonin release from perfused pineal glands of young (9 weeks) rats obtained in the middle of the dark span. Each value is the mean  $\pm$  SE of data obtained in three perfusion chambers (one gland per chamber). The drugs (arrows) were infused for 30 min, 300 min after the beginning of the perfusion.

- feroxamine) in rat liver homogenates. *J. Pharm. Pharmacol.* 2001, 53: 1393-1401.
8. Buijs R.M., Pévet P. Vasopressin- and oxytocin-containing fibres in the pineal gland and subcommissural organ of the rat. *Cell Tissue Res.* 1980, 205: 11-17.
  9. Simonneaux V., Ribeleyga C., Miguez J.M., Pévet P. Physiological role of neuropeptides in the mammalian pineal gland. In: Tang P.L., Pang S.F., Reiter R.J. (Eds.), *Melatonin: A universal photoperiodic signal with diverse actions.* Front. Horm. Res. Karger, Basel, 1996, p. 24.
  10. Simonneaux V., Vuillez P., Eder U., Miguez J.M., Pévet P., Fischer-Colbrie R. Secretoneurin: a new neuropeptide in the rodent pineal gland. *Cell Tissue Res.* 1997, 288: 427-434.
  11. Yuwiler A., Brammer G.L. Neurotransmitters and peptides in the pineal gland. In: Wetterberg L. (Ed.), *light and biological rhythms in man.* Pergamon Press, Oxford, 1993, p. 133.
  12. Yuwiler A., Brammer, G.L., Bennett B.L. Interaction between adrenergic and peptide stimulation in the rat pineal: pituitary adenylate cyclase-activating peptide. *J. Neurochem.* 1995, 64: 2273-2280.
  13. Mikkelsen J.D., Hauser F., deLecea L. et al. Hypocretin (orexin) in the rat pineal gland: a central transmitter with effects on noradrenaline-induced release of melatonin. *Eur. J. Neurosci.* 2001, 14: 419-425.
  14. Anisimov V.N., Bondarenko L.A., Khavinson V.Kh. The pineal peptides: Interaction with indoles and the role in aging and cancer. In: Gupta D., Wollmann H.A., Ranke M.B. (Eds.), *Neuroendocrinology: New frontiers.* Brain Research Promotion, London, 1990, p. 317.
  15. Anisimov V.N., Bondarenko L.A., Khavinson V.Kh. Effect of pineal peptide preparation (Epithalamin) on life span and pineal and serum melatonin level in old rats. *Ann. NY. Acad. Sci.* 1992, 673: 53-57.
  16. Anisimov V.N., Khavinson V.Kh., Mikhalski A.I., Yashin I. Effect of synthetic thymic and pineal peptides on biomarkers of ageing, survival and spontaneous tumour incidence in female CBA mice. *Mech. Ageing Dev.* 2001, 122: 41-68.
  17. Anisimov V.N., Arutjunyan A.V., Khavinson V.Kh. Effects of pineal peptide preparation epithalamin on free-radical processes in human and animals. *Neuroendocrinol. Lett.* 2001, 22: 9-18.
  18. Khavinson V.Kh., Morozov V.G. Novel approaches to the prevention and treatment of age-associated pathology using peptide bioregulators - Epithalamin and thymalin. In: *aging in the americas: frontiers of care, policy and research.* Abstr. of Pan-American Congress, February 21-24, San Antonio, 1999, p. 46.
  19. Goncharova N.D., Khavinson V.Kh., Lapin B.A. Regulatory effect of epithalon on production of melatonin and cortisol in old monkeys. *Bull. Exp. Biol. Med.* 2001, 131: 394-396.
  20. Khavinson V.Kh., Goncharova N., Lapin B. Synthetic tetrapeptide epitalon restores disturbed neuroendocrine regulation in senescent monkeys. *Neuroendocrinol. Lett.* 2001, 22: 251-254.
  21. Zhao Z.Y., Touitou Y. Kinetic changes of melatonin release in rat pineal perfusion at different circadian stages. Effects of corticosteroids. *Acta Endocrinol.* 1993, 129: 81-88.
  22. Zhao Z.Y., Touitou Y. Differences in isoproterenol-stimulated melatonin production by perfused rat pineal glands: Time dependent effects and role of enantiomeric forms. *Life Sci.* 1994, 54: 497-502.
  23. Fraser S., Cowen P., Franklin M., Franey C., Arendt J. Direct radioimmunoassay for melatonin in plasma. *Clin. Chem.* 1983, 29: 396-397.