Chapter 5

Urinary Excretion of 5-Hydroxyindoleacetic Acid, Serotonin and 6-Sulphatoxymelatonin in Normoserotonemic and Hyperserotonemic Autistic Individuals

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Abstract

The presence of elevated levels of platelet serotonin (5-hydroxytryptamine, 5-HT) in a substantial proportion of individuals with autism is one of the more well replicated findings in neuropsychiatry. The issue of gut 5-HT production and platelet hyperserotonemia was studied by comparing urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) and 5-HT in 10 normoserotonemic and 10 hyperserotonemic age-matched autistic individuals. The relationship of urinary 6-sulfatoxymelatonin (6-SM) excretion to platelet 5-HT, and to urinary 5-HT and 5-HIAA excretion, was also examined. In the hyperserotonemic group, a trend-level significant increase for urinary excretion of 5-HIAA or 5-HT and a significant decrease for 6-SM were found, while the urinary 5-HIAA / 5-HT ratio was similar in the two groups. The results suggest that the catabolism of 5-HT does not differ in the groups, but that greater exposure of the platelet to 5-HT can not be ruled out as a cause of the platelet hyperserotonemia of autism. Larger studies are needed to examine the relationship between gut 5-HT production and the platelet 5-HT elevation of autism, and to characterize the relationship between melatonin production and platelet hyperserotonemia more thoroughly.

Introduction

Elevated levels of the neurotransmitter/neurohormone serotonin (5-hydroxytryptamine, 5-HT) in blood platelets of individuals with autism is one of the most well replicated findings in neuropsychiatry (Anderson, 2002). Typically, the group mean has been reported to be elevated 25-50% in groups of individuals with autism compared to normally developing individuals. Recently, we have reported data that suggest a bimodal distribution of platelet serotonin in Dutch individuals diagnosed with autism and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) (Mulder et al., 2004). It appeared that about half of the individuals with autism or PDD-NOS had platelet 5-HT values distributed in an upper mode, with a mean upper mode value nearly twice that seen for the lower mode. The lower mode in the autistic group appeared to largely overlap the distribution seen in normal control and mental retardation contrast groups.

While this further characterization of the platelet hyperserotonemia of autism does not immediately shed light on the mechanism responsible for the elevation, it may lead to improved strategies for elucidating underlying alterations and mechanisms. The mechanism has been hypothesized to be due either to increased exposure of the platelet to 5-HT or to an alteration in the platelet's handling of 5-HT (Anderson, et al., 1990; Anderson, 2002).

More than 90% of peripheral 5-HT is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut wall, stored within the enterochromaffin cells and released into the mucosal and myenteric layers of the gut, where 5-HT plays a critical role in stimulating peristalsis and other enteric processes (Gershon, 2003). Released 5-HT is metabolized locally by uptake into enterocytes and after entering the general circulation by efficient clearance by the endothelium, lung and liver. In both cases, 5-HT is predominantly metabolized by monoamine oxidase-A (MAO-A) to 5-hydroxyindoleacetic acid (5-HIAA) (Grahame-Smith, 1988).

Plasma free 5-HT can also be taken up by circulating platelets and nearly all 5-HT in the blood (>99%) is found stored within the platelet, with the typical blood concentration being approximately 150 ng/ml (~600 ng/109 platelets, ~3.5 nmol/109 platelets). Levels of free plasma 5-HT in the general circulation appear to be quite low (<300 pg/ml), and it is not clear whether most loading of platelets with 5-HT occurs in gut capillaries and the portal circulation or in the general circulation. It is clear that increased gut production of 5-HT, such as seen in carcinoid syndrome, a

cancerous proliferation of the enterochromaffin cells, leads to increased levels of platelet 5-HT and increased urinary excretion of 5-HIAA (Anderson et al., 1987; Kema et al., 2000). Urinary 5-HT is less well established to be proportional to gut serotonin synthesis; however, much of urine 5-HT can also be assumed to be derived from the enterochromaffin cell.

Several studies have measured urinary excretion of 5-HIAA in autism in order to address the issue of 5-HT production and platelet 5-HT exposure. A few studies have also reported urinary 5-HT levels and free plasma 5-HT concentrations. Most studies have not found differences in urinary 5-HIAA excretion between autistic individuals and normal or mentally retarded controls (Schain and Freedman, 1961; Partington et al., 1973; Minderaa et al., 1987; Launay et al., 1988; Martineau et al., 1992; Herault et al., 1996; Croonenberghs et al., 2000). However Hanley et al. (1977) reported elevated levels in urinary 5-HIAA and urinary serotonin in autistic subjects compared to a group of subjects with mental retardation. Minderaa et al. (1987) reported borderline significantly higher levels of 5-HIAA excretion in four hyperserotonemic autistic individuals, while finding a very similar urinary 5-HIAA excretion in the whole group of unmedicated autistic individuals compared to normal controls. Three studies have reported elevated urinary 5-HT excretion in groups of autistic subjects, but no differences in 5-HIAA excretion (Launay et al., 1988; Martineau et al., 1992; Herault et al., 1996), while Anderson et al. (1989) reported that urinary excretion of serotonin was unaltered in autism. Taken together, the prior studies tend to suggest that gut 5-HT synthesis is not altered in autism. However, in general, the group sizes have been small and only a limited number of subjects with elevated platelet 5-HT have been studied.

The neurohormone melatonin is synthesized from 5-HT, predominantly in the pineal gland, and has a major role in the regulation of circadian rhythms, sleep and mood. To date, melatonin production has not been assessed in relation to the issue of hyperserotonemia in autism. Production of pineal melatonin is regulated by photic information from the retina and during the day circulating melatonin levels are quite low. Pineal melatonin production increases swiftly after the onset of darkness and stays high until dawn (see for a review Brzezinksi, 1997). Another source of melatonin is the enterochromaffin cells of the gut wall. Several studies show that the melatonin concentration in the gastro-intestinal tract surpasses the blood concentration 10 to 100 times. Studies in animals indicate that melatonin derived

from the gastro-intestinal tract accounts for most of the meager day-time blood concentration of melatonin, whereas night time circulating levels of melatonin are determined by pineal gland production (as reviewed by Bubenik, 2002). Melatonin is metabolized in the liver and kidney to 6-sulphatoxymelatonin (6-SM) and excreted in urine. Urinary excretion of 6-SM closely follows the melatonin blood concentration (Lynch et al., 1975). The limited available data regarding melatonin levels in autism suggest that group mean melatonin production is reduced in autism (Nir et al., 1995; Hayashi et al., 2000; Kulman et al. 2000; Tordjman et al., 2005). These prior results, and the fact that 5-HT is the immediate precursor for melatonin in the pineal and the gut, suggested that it would be of interest to examine 6-SM excretion along with that of 5-HT and 5-HIAA.

We examined the issue of gut 5-HT production and platelet hyperserotonemia by comparing urinary excretion of 5-HIAA and 5-HT in groups of normoserotonemic and hyperserotonemic autistic individuals. Possible group differences in melatonin excretion and the interrelationships between the urinary measures were also examined.

Method

Subjects

Subjects from our prior study (Mulder et al., 2004), examining the distribution of platelet 5-HT values in autism spectrum disorders, were invited to participate in a follow-up study of indole metabolism. Diagnoses were established through comprehensive assessment, using the Autism Interview Schedule-Revised (ADI-R, Lord et al., 1994), Autism Diagnostic Observation Schedule-Generic (ADOS-G, Lord et al., 2000) and DSM-IV-TR diagnosis of autism, Asperger disorder or PDDNOS (APA, 2000) as described previously. All subjects (N = 20) were male, unmedicated and had an ADI-R classification of autism corroborated by an ADOS-G and DSM-IV-TR diagnosis of PDD. Autistic subjects were assigned to hyperserotonemic and normoserotonemic subgroups using a cut-off of 4.55 nmol/10^9 platelets derived from the aforementioned study (Mulder et al., 2004). Hyper- and normoserotonemic subjects were matched on age: group mean ages [mean ± SD, (median)] 15.3 ± 4.4, (15.5) and 15.3 ± 4.0, (15.5) yrs., respectively (Mann-Whitney U; Z_2 = 0.038, p = .971). Group mean IQs were 80.5 ± 36.4, (82.0) and 46.3 ± 20.5, (55.0) respectively; Mann-Whitney U; Z_2 = 1.965, p = .052. Approval for the study was given by the

Central Committee for Research in Humans in The Hague, NL. Informed consent/assent was obtained from all subjects and their parents.

Laboratory Measures

Blood samples were collected in 10-mL Vacutainer tubes (Becton-Dickinson, Meylan Cedex, France) containing 0.12 mL (0.34 mol/L) dipotassium EDTA solution. Platelet-rich plasma was prepared within 1 hour after sampling by centrifuging for 30 min at 120 × g and 4°C, and a platelet count was obtained. Platelet rich plasma 5-HT and plasma tryptophan were determined using a previously described HPLC method with fluorometric detection (Kema et al., 2001).

Twenty four-hour urine samples were collected from the subjects in brown propylene bottles (Sarstedt). Instructions concerning dietary intake (Kema et al., 2000) were issued to the parents and no consumption of prohibited foods was reported. One subject was excluded because of incompleteness of the 24 hour urine collection. Urinary 5-HIAA and 5-HT were measured using previously described methods (Kwarts et al., 1984; Mulder et al., 2005). 6-Sulphatoxymelatonin (6-SM) analysis was performed by enzyme-linked immunoabsorbent assay (ELISA) using a 6-SM ELISA Kit from Bühlmann AG (Schönenbuch, Switzerland). The urine samples were diluted prior to assay (1/200). The intra–assay and inter–assay coefficients of variation were 13.6% and 7.8% (n = 35) respectively for a 23.9 ng/mL (~100 nmol/mL) control sample value (based on approximately 1 liter per day urine volume this control sample would be equivalent to 0.10 nmol/24 hr).

Statistical Analysis

Age, IQ, platelet 5-HT, urinary 5-HIAA, urinary 5-HT, urinary 6-SM, plasma tryptophan levels, and urinary 5-HIAA / 5-HT ratios between hyper- and normoserotonemic groups were compared using the non-parametric Mann-Whitney U test. Spearman correlations were used to examine relationships between biochemical variables. For urinary 5-HIAA and urinary 5-HT the analyses are one-tailed, since there was an a priori expected direction of the difference. Despite the number of planned comparisons, α was set to .05 to reduce the potential for errors of the second type.

Results

Group means, SDs and medians observed for platelet 5-HT levels and urinary 5-HIAA, 5-HT and 6-SM excretion rates are given in Table 5.1 and the individual data plotted in Figure 5.1. Comparison of urinary 5-HIAA and of urinary 5-HT excretion in hyper- and normoserotonemic groups revealed trend-level significant differences between groups, with one-tailed p-values just above the .05 level (p = .061 and p = .071 for urinary 5-HIAA and 5-HT respectively). Excretion of urinary 6-SM appeared to be decreased in the hyperserotonemic group at a trend-level when expressed per 24 hours and was significantly lower when expressed per mol of creatinine (see Table 5.1). Plasma tryptophan concentrations were not significantly different in the two groups [mean ± SD, (median): 52.3 ± 15.2, (50.1) vs. 55.3 ± 9.89, (53.6) μM; Mann-Whitney U; Z_2 = 0.643, p = .523].

Table 5.1: Twenty four-hour urinary excretion (mean ± SD, median) of 5-HIAA, 5-HT and 6-SM in normoserotonemic (N=10) and hyperserotonemic (N=9) autistic subjects.

	Normoserotonemic *(< 4.5 nmol/10^9 plts)*	***Hyperserotonemic*** *(≥ 4.5 nmol/10^9 plts)*
Platelet Rich Plasma (PRP) 5-HT		
nmol/10^9 platelets [a]	3.14 ± 0.70, 2.95	6.02 ± 1.33, 5.60
Urinary 5-HIAA Excretion		
μmol/24 hours [b]	14.0 ± 6.27, 14.2	19.2 ± 10.3, 16.8
mmol/mol creat [c]	1.69 ± 0.86, 1.70	1.79 ± 0.75, 1.60
Urinary 5-HT Excretion		
μmol/24 hours [d]	0.52 ± 0.20, 0.55	0.68 ± 0.26, 0.65
μmol/mol creat [e]	60.1 ± 21.4, 59.0	63.2 ± 19.2, 56.0
Urinary 6-SM Excretion		
nmol/24hours [f]	77.1 ± 35.1, 84.2	53.0 ± 32.7, 57.2
μmol/mol creat [g]	9.03 ± 4.55, 9.07	4.90 ± 2.84, 4.81

NOTE: a. Mann-Whitney U; $Z_{(2)}$=3.787, p<.0001, b. Mann-Whitney U; $Z_{(2)}$=1.55, p=.061 (one tailed) c. Mann-Whitney U, $Z_{(2)}$=0.328, p=.390 (one-tailed) d. Mann-Whitney U; $Z_{(2)}$=1.47, p=.071 (one-tailed) e. Mann-Whitney U, $Z_{(2)}$=0.368, p=.360 (one-tailed) f. Mann-Whitney U, $Z_{(2)}$=1.470, p=.142 g. Mann-Whitney U; $Z_{(2)}$=2.205, p=.027

Across all subjects, urinary 5-HIAA and 5-HT excretion rates (per 24 hours) were not significantly correlated with platelet serotonin (r = 0.128, p = .60 and r = 0.149, p = .54, respectively); however, urinary 5-HIAA and urinary serotonin were highly correlated (r = 0.840, p < .0001). Urinary 6-SM was trend-level significant

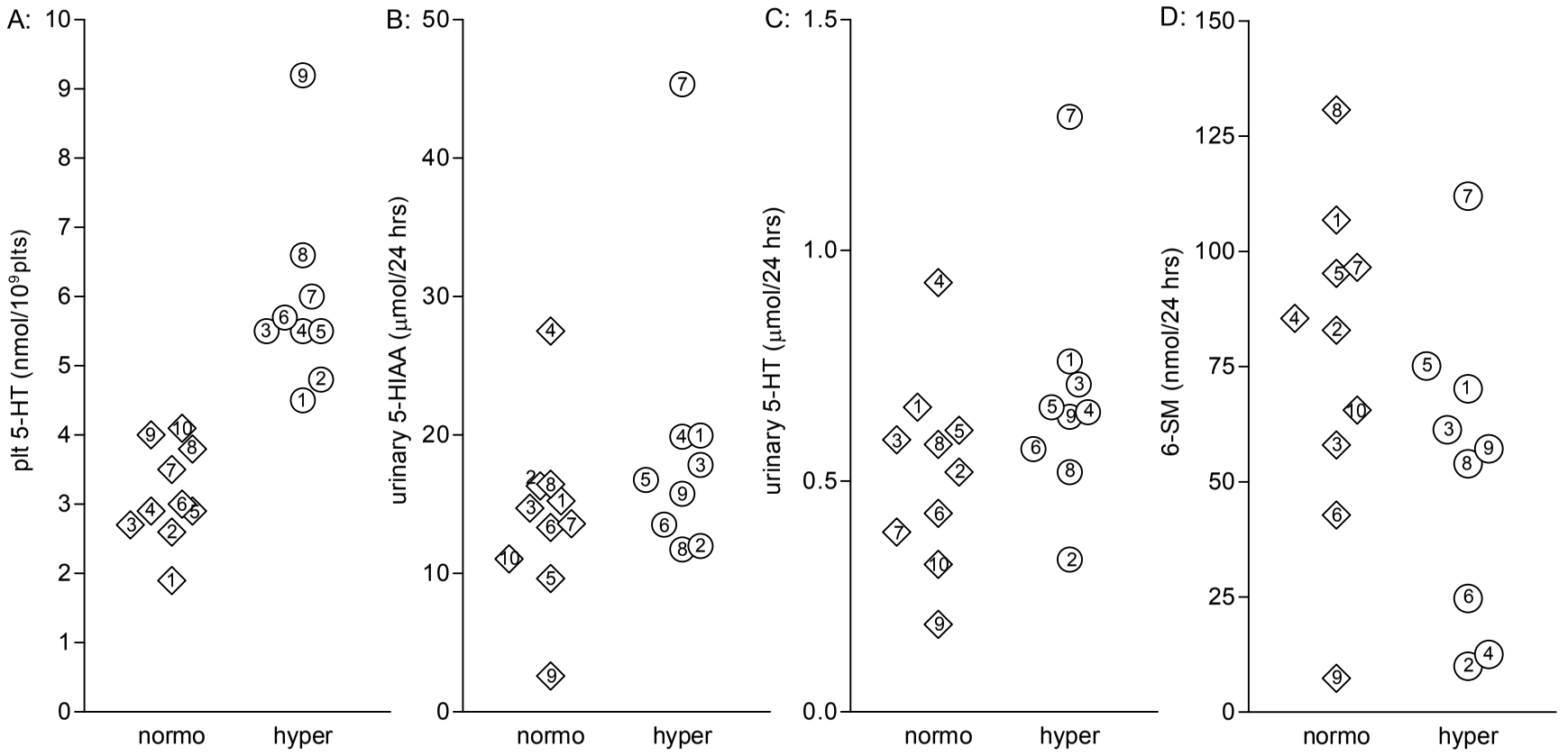

Figure 5.1. Individual platelet 5-HT concentrations (nmol/10^9 plts; A) and twenty four-hour urinary excretion of 5-HIAA (µmol/24 hrs; B), 5-HT (µmol/24 hrs; C) and 6-SM (nmol/24hrs) of the normoserotonemic (N=10) and hyperserotonemic (N=9) autistic subjects. The enclosed numbers are the subjects' ID numbers; diamonds depict the normoserotonemic and circles the hyperserotonemic subjects.

negatively correlated with platelet serotonin and positively correlated with urinary 5-HIAA and 5-HT when expressed on a 24 hour basis ($r = -0.365$, $p = .125$, $r = 0.458$, $p = .049$, $r = 0.433$, $p = .064$, respectively). No significant correlations were observed between plasma tryptophan and any of the other three biochemical measures, apart from urinary 5-HT where a positive correlation of $r = 0.633$, $p = .004$ was found. Similar correlative relationships were observed between the biochemical measures when excretion was expressed per mol of creatinine (platelet 5-HT vs. urinary 5-HIAA and 5-HT; $r = -0.034$, $p = .89$ and $r = -0.069$, $p = .78$, respectively; urinary 5-HIAA vs. 5-HT; $r = 0.927$, $p < .0001$). However when 6-SM excretion was expressed per mol of creatinine all correlations were significant ($r = -0.491$, $p = .033$, $r = 0.553$, $p = .014$, $r = 0.515$, $p = .024$, respectively). Correlations within the normoserotonemic and hyperserotonemic subgroups were similar as compared to the whole group (data not given).

When urinary product / substrate (5-HIAA / 5-HT) ratios were compared between hyper- and normoserotonemic groups, no significant difference was observed between groups (mean ± SD, median; 270 ± 70, 280 and 280 ± 50, 260, respectively; Mann-Whitney U: $Z_2 = 0.82$, $p = .935$).

The group means (± SD) for moles of creatinine/24 hr excreted in the hyper- and normoserotonemic groups were similar (11.3 ± 3.83, 12.40 versus 9.07 ± 3.48, 7.91 mmol/24hrs; Mann-Whitney U, $Z_2 = 1.470$, $p = .156$); the amount of urine collected per 24 hours was also similar in the two groups (1,327 ± 552, 1,330 versus 1,075 ± 555, 975 ml/24hrs; Mann-Whitney U, $Z_2 = 1.184$, $p = .236$, respectively).

The excretion of 5-HIAA, 5-HT and 6-SM expressed on a 24 hour basis was uncorrelated with age ($r = -0.194$, $p = .43$, $r = 0.091$, $p = .71$ and $r = -0.042$, $p = .89$, respectively). The excretion rates of 5-HIAA, 5-HT and 6-SM expressed per mole of creatinine were negatively correlated with age ($r = -0.82$, $p < .0001$. $r = -0.80$, $p < .0001$ and $r = -0.402$, $p = .087$), apparently mainly due to a positive correlation of creatinine with age ($r = 0.62$, $p = .005$).

Discussion

Urinary excretion rates of 5-HIAA, 5-HT and 6-SM were examined in subgroups of autistic patients as part of an investigation of the underlying causes of the platelet hyperserotonemia of autism. The present study revealed trend-level significant differences in urinary excretion of 5-HIAA and 5-HT between hyperserotonemic and

normoserotonemic individuals with autism. Group mean excretion of 6-SM appeared to be decreased in the hyperserotonemic individuals. In normoserotonemic as well as in hyperserotonemic individuals with autism, excretion rates of 6-SM were negatively correlated to platelet 5-HT and positively correlated to urinary excretion rates of 5-HIAA and 5-HT. Additionaly, plasma tryptophan concentration was correlated to urinary 5-HT excretion. The group mean excretion values reported here for 5-HIAA, 5-HT and 6-SM were within normal ranges established at the University Medical Center Groningen and were in excellent agreement with the prior reports (Minderaa et al., 1987; Launay et al., 1988; Anderson et al., 1989; Deacon, 1994).

This test of the relative excretion rates of 5-HIAA and 5-HT excretion in well defined hyperserotonemic and normoserotonemic subgroups suggests that greater exposure of the platelet to 5-HT due to greater gut production of 5-HT can not be ruled out as a cause of the platelet hyperserotonemia of autism. However, the similar urinary 5-HIAA / 5-HT ratios observed across normo- and hyperserotonemic groups suggests that the catabolism of 5-HT does not differ in the groups. The decreased excretion of 6-SM in hyperserotonemic individuals and the negative correlation of urinary excretion of 6-SM with platelet serotonin provide internally consistent data suggesting that there may be some link between altered (elevated) platelet 5-HT and abnormal (lower) melatonin production in autism.

In summary, larger studies in well characterized autistic and contrast groups and subgroups are needed to sort out the underlying cause(s) of, and possible relationship between, the platelet hyperserotonemia and the reduced melatonin production observed in autism.

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