Chapter 7

Application of 5-HT-SO_4 in Biomarker Research

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Abstract

A serotonin catabolite, serotonin O-sulphate (5-HT-SO_4), is hypothesised to accentuate the intensity of serotonin metabolism in the central nervous system (CNS). We hypothesised that serotonin O-sulphate could be quantified in human plasma using modern liquid chromatography-mass spectrometry. To test our hypothesis, we performed a critical literature review and a three-stage trial. First, a suitable liquid chromatography-mass spectrometry (LC-MS/MS) method for detection of 5-HT-SO_4 in human plasma samples was developed. Second, a pilot phase involving four healthy volunteers was executed. Finally, nine healthy volunteers were selected for the main study, where a basal plasma level of 5-HT-SO_4 was measured before and after serotonergic stimulation of the central nervous system. One h after stimulation, six study subjects showed a decrease in 5-HT-SO_4 levels, while three subjects showed an increase. This was the first study in which naturally occurring 5-HT-SO_4 was detected by liquid chromatography–mass spectrometry (LC-MS/MS) in the samples of human plasma obtained from healthy volunteers. The method developed was specific to the measurement of 5-HT-SO_4 and opens up new possibilities to evaluate minor pathways or serotonin metabolism by minimally invasive methods.

Keywords: serotonin, serotonin O-sulphate, biomarkers, depression

1. Introduction

For several decades, it is noted that biomarkers are playing an increasingly important role in drug discovery and development from target identification and validation to clinical application, thereby making the overall process a more rational approach.

Indisputably, serotonin (5-HT) plays a significant role in the course of depressive disorders, and majority of the drugs developed interferes with this pathway. Therefore, we decided to ascertain the metabolic processes to find perspective areas of research based on experience gathered so far. The most exploited laboratory biomarker methods investigating central nervous system

(CNS) processes use the cerebrospinal fluid (CSF) as far as it is site specific and reveals local processes. Thus, measurement of indoleamine metabolites in the cerebrospinal fluid (CSF) remains an analytical evaluation method of drug efficacy during clinical trials. Nevertheless, due to patient's safety concerns and clinical convenience, a method employing less invasive approach is highly appreciated by health care professionals.

Thus, our objective was based on critical literature review approach and practical liquid chromatography-mass spectrometry (LC-MS/MS) method implementation to investigate a possibility to use 5-HT-SO_4 as a potential CNS-specific serotonin metabolism biomarker based on a less invasive laboratory method suitable for clinical and pharmacological studies.

2. Literature evidence related to 5-HT-SO_4 appearance in animal and humans

Sulphonate conjugation was first described in 1876 and has since been shown to be a significant pathway in the biotransformation of many neurotransmitters.

During a critical literature review, our special attention was drawn to the final phase of 5-HT degradation by sulphotransferases (SULT) and the end product of biotransformation, 5-HT-SO_4. We identified 66 papers and excluded 51 papers that did not contain data related to 5-HT-SO_4. In total, 15 papers were included in the final review with 10 analysed in terms of outcomes.

We found that during the last century, a sulphation of serotonin was described by Kishimoto and a final product of such biotransformation, serotonin-O-sulphate, was found [1]. Furthermore, during later years in animal experiments, it was approved that 5-HT-SO_4 is the final product of serotonin metabolism which is rapidly excreted from the organism [2, 3]. Later, a similar compound was found in human urine, cerebrospinal liquor and platelets [4–6]. During recent years, some research was done with marine molluscs determining 5-HT-SO_4 in their nervous system [7, 8]. Findings showed that the serotonin metabolite 5-HT-SO_4 forms from 5-HT uptake and metabolism in central ganglia and other structures of nervous system but not in haemolymph itself.

Table 1 summarises the evidence related to 5-HT-SO_4 appearance in animals and humans.

As shown in **Table 1**, 5-HT-O-SO_4 was intensively investigated by G.M. Tyce during the 1980s and 1990s. Initially, a considerable amount of acid-hydrolysable conjugates of dopamine, norepinephrine (NE) and 5-HT were detected in lumbar CSF of normal individuals. The amounts of conjugated amines were small in comparison to the amounts of homovanillic acid and 5-hydroxyindoleaceticacid [9]. In a further study performed with CSF from humans and ventriculocisternal perfusion of African green monkeys found that the sulphates of NE, dopamine and 5-HT are present in the CSF of laboratory animals and humans. All amines and metabolites were quantitated by using high-performance liquid chromatography (HPLC) with electrochemical detection. The amounts of sulphated amines in human CSF always greatly exceed the amounts of the free amines [6]. This gave us a preliminary impression of 5-HT-SO_4 site

Date	Research name	Results	Analytical methods applied
2009	Analysis of intact glucuronides and sulphates of serotonin, dopamine, and their phase I metabolites in rat brain microdialysates by liquid chromatography-tandem mass spectrometry	The LC-MS/MS method was validated by determining the limits of detection and quantitation, linearity and repeatability for the quantitative analysis of 5-HT and DA and their glucuronides, as well as of 5-HIAA, DOPAC and HVA and their sulphateconjugates.	LC-MS/MS
2008	5-HT and 5-HT-SO_4 but not tryptophan or 5-HIAA levels in single feeding neurons track animal hunger state	Changes in levels of 5-HT-SO_4 in the metacerebral giant neurons of Pleurobranchaea californica related to feeding were observed	Capillary electrophoresis with laser-induced wavelength-resolved fluorescence detection (CE-LIF)
2007	Serotonin catabolism in the central and enteric nervous systems of rats upon induction of serotonin syndrome	Serotonin sulphate showed surprisingly large increases in rat intestinal tissues after induction of serotonin syndrome	*Capillary electrophoresis with laser-induced native fluorescence detection (CE-LINF)*
2004	Systemic serotonin sulphate in opisthobranch molluscs	5-HT-SO_4 forms in neural cells. Not detected in haemolymph	Capillary electrophoresis (CE) system
2003	Serotonin catabolism depends upon location of release: characterisation of sulphated and gamma-glutamylated serotonin metabolites in Aplysia californica	The pathway of serotonin inactivation with further formation of 5-HT-SO_4 depends upon the type of neuronal tissue subjected to neurotransmitter incubation	CE-LIF LC-MS
1988	Presence of phenolsulphotransferase activity in microvascular endothelial cells: formation of 5-HT-O-sulphate in intact cells	Existence of phenolsulphotransferase in the endothelial cells and formation of 5-HT-SO_4verified	Not available
1986	Amine sulphate formation in the central nervous system	Origin of the central nervous system of amine sulphates (also 5-HT-SO_4) in monkeys and humans observed. 5-HT-SO_4 was detected in CSF of monkeys and humans but not in the plasma	High performance liquid chromatography (HPLC) with electrochemical detection
1985	Free and conjugated amines in human lumbar cerebrospinal fluid	5-HT-SO_4 was detected in the CSF of healthy humans	HPLC with electrochemical detection
1983	Exploration of the role of phenolsulphotransferase in the disposition of serotonin in human platelets: implications for a novel therapeutic strategy against depression	Existence of phenolsulphotransferase in the platelets and formation of 5-HT-SO_4 verified	The assay technique-purified alveolysin toxin
1966	Isolation of serotonin-O-sulphate from human urine	5-HT-SO_4 isolated from human urine	Ion exchange resins

Table 1. Summary of the evidence found related to 5-HT-SO_4 appearance in animals and humans.

specificity. At the time of above mentioned studies, 5-HT-O-SO_4 could not be detected in the plasma of untreated monkeys and the concentration of 5-HT-O-SO_4 in brain perfusates versus plasma increased after injection of 5-HT sulphate. The ratio of amine sulphate in the brain versus amine sulphate in plasma was greater for 5-HT-O-SO_4 than for DA-O-sulphate at 60 and 100 min after injection. Finally, it was concluded that although 5-HT-O-SO_4 could not be detected in the plasma of monkeys or humans under normal conditions, the 5-HT-O-SO_4 in ventriculocisternal perfusates undoubtedly originates in the CNS [6].

This obstacle inspired us to analyse more recent studies selected during review.

Some research was conducted with marine molluscs determining 5-HT-O-SO_4 in their nervous system [7, 8].

In one such research done in 2003, incubation of neuronal tissue of Aplysia revealed three novel 5-HT catabolites. **Figure 1** summarises the metabolism of 5-HT found in Aplysia central ganglia compared to human.

As seen from **Figure 1**, there is no difference of 5-HT-O-SO_4 formation between humans and molluscs.

As shown in **Table 2**, the 5-HT-O-SO_4 can be detected in CNS and its formation though is site specific, and later animal studies confirm detection in periphery. Moreover, as far similar sulphotransfares exist in sea molluscs and mammals, an equal process should be theorised for humans [10]. Also, literature evidence exists that 5-HT-SO_4 was proposed to be measured in the animal urine or plasma which makes it to be relative simply detected by HPLC with various detectors [11].

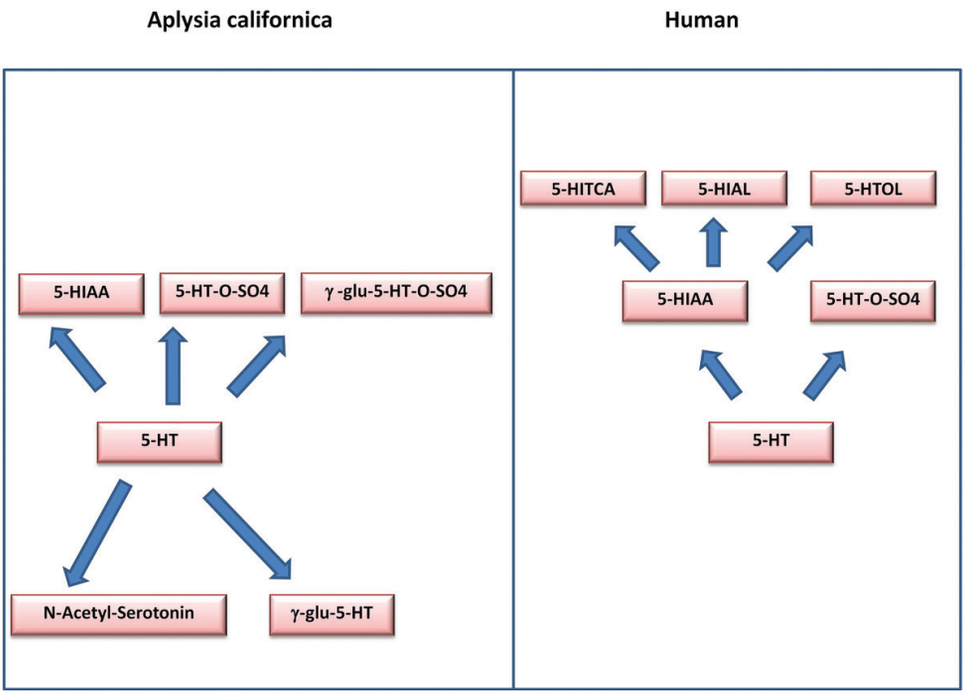

Figure 1. Metabolites of 5-HT in the marine mollusc *Aplysia californica* versus humans. The novel metabolites are shown for *Aplysia californica*. Only detailed 5-HIAA metabolism is shown for humans.

Trial	Quantity of trial subjects	Species	5-HT-O-SO_4 qualified	5-HT-O-SO_4 quantified	5-HT-O-SO_4 found in nervous system	5-HT-O-SO_4 found outside nervous system	Conclusions
Tyce et al. [9]	22	Humans	No. defined as conjugate of 5-HT	No. defined as conjugate of 5-HT	No. defined as conjugate of 5-HT	No	CSF contains 5-HT conjugate
Tyce et al. [6]	12/4	Humans/ animals	Yes	Yes	Yes	No	5-HT-O-SO_4 originates from CNS
Stuart et al. [7]	N/A	Animals	Yes	No	Yes	No [c]	5-HT-O-SO_4 formation is nervous-system specific
Stuart et al. [8]	11	Animals	Yes	Yes	Yes	Yes	5-HT-O-SO_4 forms in the nervous system but not in haemolymph itself
Uutela et al. [12]	N/A	Animals	Yes	Yes	Yes	No	5-HT-O-SO_4 in rat brain microdialysates was analysed using a direct LC-MS/MS method

Table 2. Summary of trials involving 5-HT-O-SO_4.

Summarising the literature review, there is evidence of similarities between human and animal metabolic pathways, and as far as there is literature evidence of site-specific 5-HT-SO_4 formation in animals, we can extrapolate the same to humans.

We noted that historically used methods assumed highly invasive approach of CSF sample collection from human subjects. Therefore, search for a minimally invasive method has significant clinical benefit. Moreover, the latest 5-HT-SO_4 research revealed experience with LC-MS application for such a purpose.

3. The latest findings related to 5-HT-SO_4 appearance in humans

The evidence in the scientific literature justifies the decision to employ detection of 5-HT-SO_4 in clinical practice. As far as there was no literature data particularly on 5-HT-SO_4 detection by LC-MS/MS in the human plasma, we initiated a development of the chromatography method based on literature evidence related to detection of similar compounds such as indoleamines in the human plasma. The objective concerning analytical procedure was to demonstrate that it is suitable for its intended purpose—qualitative detection of 5-HT-SO_4 in the human plasma.

Tandem mass spectrometric analysis (MS/MS) was made in a positive-ion mode (ESI+). The electrospray ionisation of 5-HT-SO_4 was weak. Thus, for the further quantitative analysis, a following ion transition was used: (257>>160) + (240>>160).

Specificity of the method was assessed visually by comparing multiple reaction monitoring (MRM) chromatograms of plasma sample spiked with serotonin O-sulphate, samples of plasma and purified water. As seen in **Figure 2**, in the plasma-based calibration standard (A) and plasma (B), some 1.79–1.80 min retention time peaks can be observed [13]. In the plasma-based calibration standard and "pure" plasma, some peaks with a retention time of 1.79–1.80 min were observed. The purified water samples treated similarly do not show such signals (C).This signal might be induced by native content of serotonin sulphate found in plasma samples. Conclusion was reached because in the analytical solution made of 5% serum albumin, such a signal was not seen [13]. For the MRM chromatograms, the test solution of 5% serum albumin (buffered to pH = 7 in a phosphate buffer) was prepared.

The results obtained lead to conclusion that the method developed is specific to the compound of interest, 5-HT-SO_4.

The results obtained lead to the conclusion that the method developed is specific to the compound of interest, 5-HT-SO_4, in samples of human plasma. The linearity of detection was evaluated three times in different days by analysing calibration standard solutions of 5-HT-SO_4 [13].

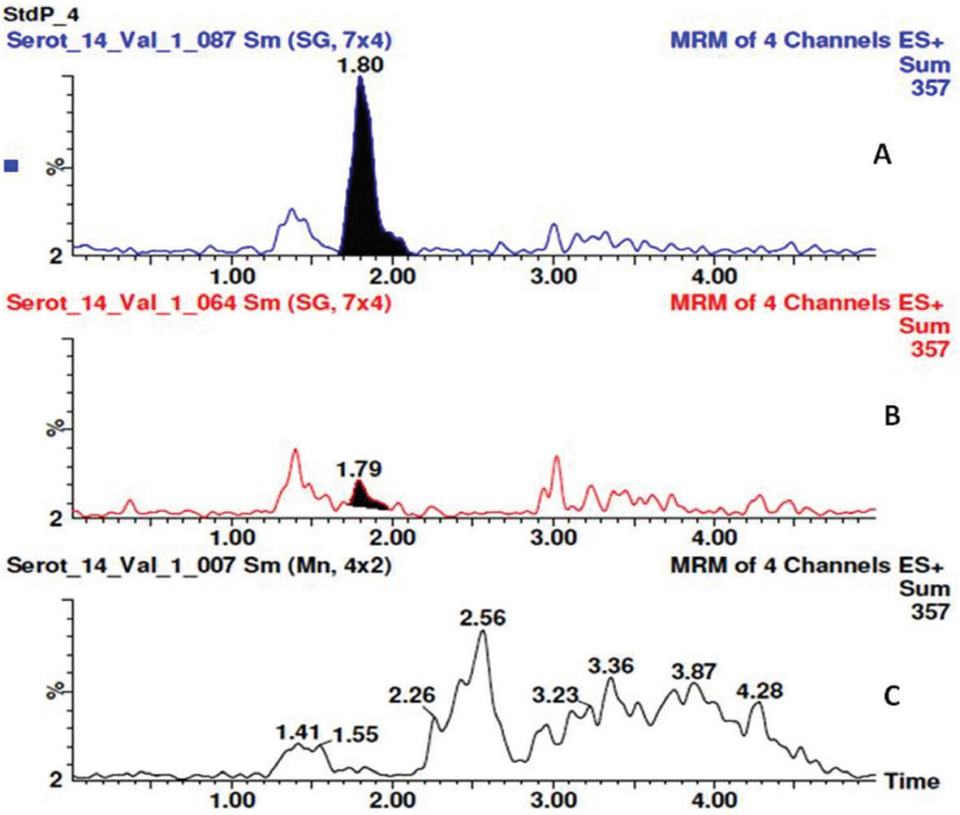

Figure 2. Chromatograms of 5-HT-SO_4 samples. (A) Plasma standard solution (containing 96 ng/mL of 5-HT-SO_4); (B) "pure" plasma sample and (C) water.

This method resulted in a linear relationship between concentration of the analyte (from 10 to 225 ng/mL) and a mass spectral signal of 5-HT-SO_4 with a calibration curve correlation coefficient of >0.98.

The optimal detection limit of 5-HT-SO_4 in the plasma sample was determined to be 26.5 ng/mL. Four different concentrations of 5-HT-SO_4 were used for a recovery testing and the method gave correct 5-HT-SO_4 detection results, which were justified by the average level of recovery of the analyte at 116 ± 8%. The relatively high interval of recovery can be explained due to the matrix effect [13].

The intra-laboratory accuracy of the method over a 3-day period was characterised by a standard deviation of ± 11.95% [13]. Taking into account above mentioned results, the method was concluded to be a suitable technique for measuring 5-HT-SO_4 in human blood samples.

The findings of method development phase led to the decision to perform the first-in-humans study in order to assess the clinical applicability of the LC-MS/MS method developed, and the studies were designed to quantify intra-individual results using a cohort of healthy subjects. The clinical study had a two-stage design: a plot study and main Study.

The pilot study confirmed that the peaks with retention time 1.79–1.80 min are detected in the samples of plasma of healthy volunteers. These peaks corresponded to the signal of 5-HT-SO_4. One study subject was exposed to oral intake of L-5-hydroxytryptophan (5-HTP) containing food supplement to observe the influence of serotonergic stimulation to 5-HT-SO_4 level. The pilot study proved that the 5-HT-SO_4 could be qualified in plasma samples obtained from healthy volunteers. The increase in 5-HT-SO_4 level after serotonergic stimulation was observed. Unfortunately, all results were below the detection limit of the method and probably due to several matrix-, method-, or analyte-specific reasons.

Our primary interest was to ascertain quantitative differences of basal 5-HT-SO_4 levels, the intra-individual sensitivity of the quantitation as well as detection limit issues obtained in the pilot study on a larger number of subjects.

Thus, after measurement of the basal 5-HT-SO_4 levels in nine subjects, all of them were exposed to serotonergic stimulation with a food supplement containing 100 mg of 5-HTP and a second blood sample was analysed. In six study subjects, a decrease in 5-HT-SO_4 levels was observed 1 h after 5-HTP ingestion. Three subjects, however, showed an increase in 5-HT-SO_4 1 h after 5-HTP ingestion. Out of nine study subjects and one pilot subject, an increase in 5-HT-SO_4 1 h after 5-HTP ingestion was observed in three women and one man, respectively. The others—five men and one woman—showed decrease. A graphical chart of study results is shown in **Figure 3**.

The outcome of our studies is that we developed a liquid chromatography method, which is specific to the measurement of 5-HT-SO_4 in the samples of human plasma. It is the first time when 5-HT-SO_4 was detected in the plasma obtained from healthy volunteers [14]. The sensibility of the LC-MS/MS method to detect intra-individual changes of the compound in the healthy volunteers undergoing supplementation with 5-HTP was observed, but the majority of results were below detection limit [13].

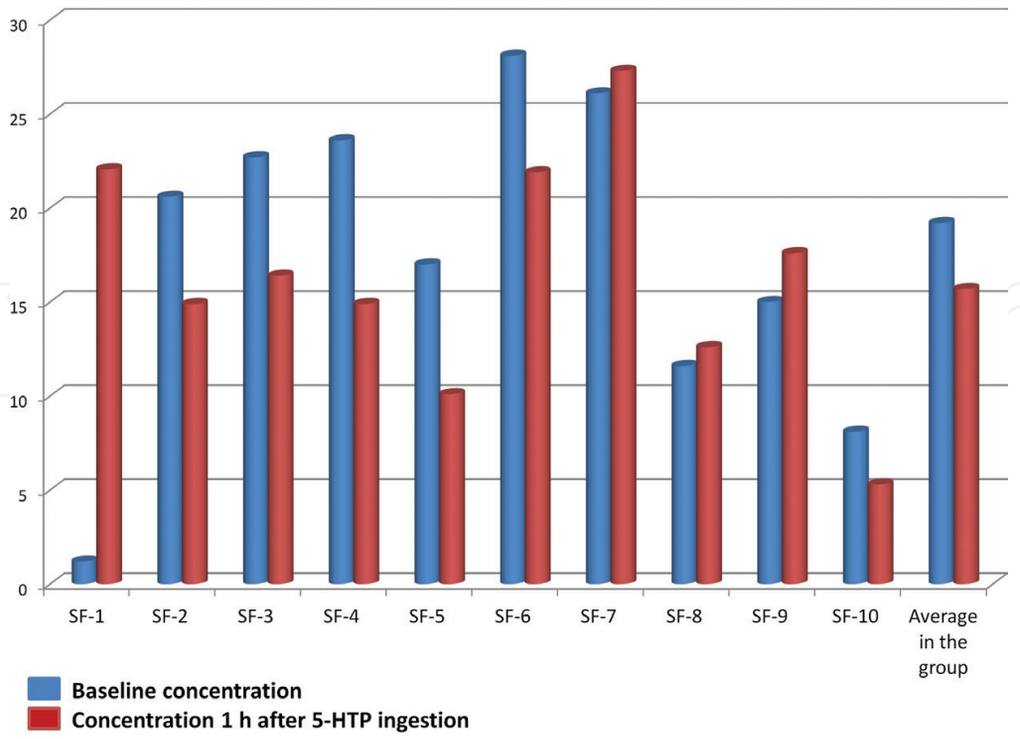

Figure 3. A graphical view of studies results.

4. Conclusions and discussion

This chapter ascertains a possibility for **5-HT-SO$_4$** to be employed as a potential serotonin metabolism biomarker based on a less invasive laboratory method. Based on critical literature review, **5-HT-SO$_4$** is identified as a potential 5-HT metabolism biomarker to be detected by a minimally invasive approach in human plasma. The novel LC-MS/MS method, which is specific to the measurement of **5-HT-SO$_4$** in the human plasma, has been developed [13]. It was the first time when **5-HT-SO$_4$** was detected in the plasma obtained from healthy volunteers [13]. However, the clinical applicability of the method was not justified as the majority of results were below detection limit of 26.5 ng/mL [13].

Concerns regarding the issue of whether 5-HT-SO$_4$ we found in the plasma has CNS origin or not should be evaluated. It is known that L-amino acid decarboxylase acts both in the periphery and in the CNS which can result with the ingested 5-HTP being converted into serotonin in the periphery of the body too [15], but plasmatic serotonin mostly derived from peripheral tissues is primarily metabolised in the liver to 5-hydroxyindole acetate and then excreted in the urine [16, 17]. Regarding 5-HT locating in the gastrointestinal tract, it is known that once serotonin reuptake transporter (SERT) has brought serotonin into the epithelial cells, it is metabolised to 5-HIAA by monoamine oxidase which is localised to all intestinal epithelial cells [18]. Alternatively, 5-HT released into the lamina propria may enter the portal vein circulation and be detected either as free serotonin or within platelets (via the actions of SERT). As the liver processes the portal circulation, enzymes rapidly degrade the

free 5-HT. The monoamine oxidase degrades about one-third to urine detectable 5-HIAAn. The remaining two-thirds of serotonin is degraded to the metabolite 5-HT-O-glucuronide. It should be noted that 5-HT taken up by platelets is protected from degradation in the liver and enters the general blood circulation [18]. Coincidentally, the only sites of 5-HT sulphation identified in humans are the CNS and enteric nervous systems [6, 19] while enteric nervous system 5-HT-SO_4 was found only after induction of serotonin syndrome [19]. Some isoforms of SULT have been shown to have sulphate serotonin [20]. Also, findings in sea molluscs indicate that metabolism of serotonin with further formation of 5-HT-SO_4 depends upon the location of release. Thus, haemolymph 5-HT- SO_4 most probably originates from the nervous system [7, 8]. There is also no doubt about the entrance of ingested 5-HTP into CNS [21]. Also, earlier studies concluded that under normal conditions, the 5-HT-O-SO_4 originates from CNS [6]. The most significant finding was that 5-HT-O-SO_4 freely crosses blood-CSF barrier, so physiological circumstances are not preventing the appearance of CNS-originated 5-HT-O-SO_4 in the venous blood circulation. Therefore, taking into account all aforementioned facts, we are more concerned that 5-HT- SO_4 detected in the study [13] mimics serotonin metabolism in CNS. Future investigations are needed to justify this assumption.

The most disputable outcome of our research is the elevation or reduction of 5-HT-SO_4. The majority of volunteers from the study phase, six out of nine, had a drop of plasma sulphate concentration [12]. Although there is no data available regarding diurnal rhythmicity of serotonin sulphate levels in the human plasma, we are not able to confirm whether these changes are due to the direct influence of serotonergic stimulation. To investigate the possible link to the health status of study subjects with 5-HT-SO_4 level changes, we performed questioning. Hamilton depression rating scale results revealed mild depression in four subjects. Unfortunately, we cannot make any clinical conclusion related to the correlation between symptoms and laboratory findings due to results below the detection limit, but the trend seems to be very intriguing.

Nevertheless, in the light of literature data, we tend to explain phenomena observed by substrate inhibition of SULT 1A3 [22, 23]. It could be concluded that under normal circumstances, quantity of serotonin synthesised and metabolised is kept under certain limits [20]. Data favouring this is evidence of paradoxical actions of the 5-HTP on the activity of identified serotonergic neurons in a simple motor circuit. It was found that more serotonin did not lead to more potent swim motoraction, implying that serotonin synthesis must be kept withincertain limits for the circuit to function properly. Also, alteration of neurotransmitter synthesis can lead to grave consequencesfor the output of neuralnetworks [24]. Described mechanisms could be taken into account explaining our results. Thus, we hypothesise that a drop of 5-HT-SO_4 in plasma would be related to the overproduction of serotonin, leading to inhibition of SULT 1A3. Elevation of 5-HT-SO_4 was probably a sign of serotonin deficiency, but such an opinion also requires further investigation. The latter would correlate with the experiment made in sea molluscs when hungry animals had significantly higher levels of serotonin and 5-HT-SO_4 than their satiated partners [25]. It remains for future investigations to determine whether serotonin sulphate found in plasma has central nervous system origin and the reason for elevated or lowered 5-HT-SO_4 levels after serotonergic stimulation [13].

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