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Enzymatic Detection and Quantification Assay of Isatin, a Putative Stress Biomarker in Blood

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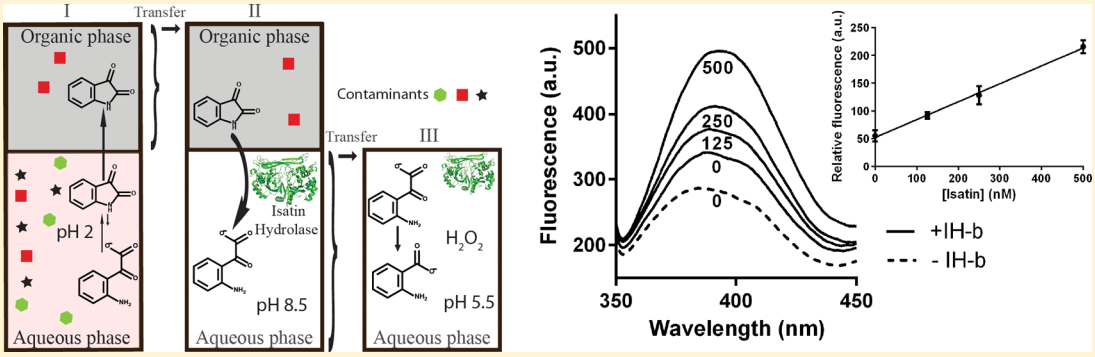
S *Supporting Information*

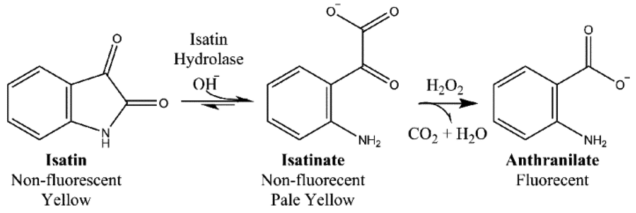


ABSTRACT: Isatin is an endogenous inhibitor of monoamine oxidase B and is found in human blood and tissue. Increased levels of isatin have been linked to stress and anxiety in rodents and humans; however, the metabolism of isatin in humans is largely unknown. We have developed a fluorescence-based enzymatic assay that can quantify isatin in blood samples. A phase extraction of isatin followed by a second phase extraction combined with an enzymatic reaction performed by an isatin hydrolase is used to extract and quantify isatin in whole blood samples. This results in a purity of more than 95% estimated from RP-HPLC. The hydrophobic molecule isatin is in equilibrium between an organic and aqueous phase; however, conversion by isatin hydrolase to the hydrophilic product isatinate traps it in the aqueous phase, making this step highly specific for isatin. The described protocol also offers a novel method for fast and efficient removal of isatin from any type of sample. The isolated isatinate is converted chemically to anthranilate that allows fluorescent detection and quantification. Pig plasma isatin levels are quantified to a mean of 458 nM ± 91 nM. Biophysical characterization of the isatin hydrolase shows enzymatic functionality between pH 6 and 9 and at temperatures up to 50 °C. Isatin hydrolase is highly selective for manganese ions with a dissociation constant determined to be 9.5 μM. We deliver proof-of-concept for the enzymatic quantification of isatin in blood and provide a straightforward method for further investigation of isatin as a biomarker in human health.

KEYWORDS: *Anthranilate, monoamine oxidase, MAO inhibitor, Parkinson's disease, enzymatic extraction, anxiety*

Isatin (indoline-2,3-dione), shown in Scheme 1, is a heterocyclic neuroactive compound found to be present endogenous in humans and detected in blood, urine, and tissue.[1]

Scheme 1



The hydrophobic nature of the molecule allows it to readily cross the blood–brain barrier.[2] Isatin is an inhibitor of monoamine oxidase B (MAO-B) and constitutes the main component of the inhibitor mix tribulin.[1] MAO-B is predominantly found in neurons, astroglia, and blood platelets. MAO-B metabolizes a number of neurotransmitters such as phenylethylamine and dopamine.[3] Inhibitors of MAO-B are used as strong antidepressants and in symptom relief treatment of Parkinson's disease, e.g., selegiline.

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Isatin has been shown to inhibit MAO-B with an IC_{50} in the range of 3−20 μM.[1,4] The levels of certain monoamines, including serotonin and dopamine, increase in the brain of rats upon administration of 20−200 mg/kg of isatin, which correlate with the inhibition of MAO-B by isatin.[5,6] Studies in rodents and primates have shown that isatin doses of 20 mg/kg have an anxiogenic effect, whereas doses of 50 mg/kg are sedative.[7,8] Anticonvulsant activity has been reported at even higher doses.[9] A single study has shown that isatin levels in pregnant women increase almost 3-fold in the third trimester when compared to those in nonpregnant women.[10] The authors also show that anxiety and psychological stress during pregnancy cause a 3-fold increase in isatin levels compared to those in normal pregnancies and that isatin levels correlate with cortisol levels.[10]

Isatin shows distinct tissue distribution in rats.[11] In the brain, the highest level of isatin per gram tissue is found in the hippocampus: ∼0.13 μg/g (∼0.7 μM assuming 80% water in tissue). The highest mean concentration outside of the brain is found in the vas deferens, however, with a variance that ranges from 0.2 to 11 μg/g (1−55 μM assuming 80% water in tissue).

Preliminary studies on patients suffering from Parkinson's disease suggest a linear correlation between the progression state of the disease, as measured by the Hoehn−Yahr scale, and the urinary output of isatin.[12,13] There is no indication that isatin is involved in the etiology of the disease.

Isatin is also an antagonist to the atrial natriuretic peptide (ANP) secreted by heart muscle cells.[14,15] In the heart, isatin levels have been measured to ∼0.2 μg/g[11] (∼1 μM). Isatin inhibits ANP stimulated guanylate cyclase activity in a dose dependent manner in the brain, heart, and kidney membranes.[14] ANP is mainly responsible for lowering the blood pressure and regulating sodium and water excretion. The IC_{50} value of isatin to the ANP receptor has been measured to 0.4 μM.[6,14]

Increased levels of isatin have been observed in rodents under stress conditions, specifically detected in the serum, brain, and heart.[16,17] Manabe and co-workers have shown that isatin levels vary between individuals and that the levels in plasma correlate with the urinary output levels.[18] A contradictory study performed by Mawatari and co-workers claims that almost no difference in isatin levels can be detected between individuals. Their study was also performed on urine and serum, however, with another detection method.[19]

Only a few methods have been reported for the detection of isatin from serum, urine, and tissue samples. Detection has earlier been performed with gas chromatography−mass spectrometry.[1,20] Furthermore, detection and quantification has been performed from urine and serum samples by high-performance liquid chromatography (HPLC) coupled with absorption spectrometry,[12,18,21] including detection from urine and serum by HPLC using the fluorescence signal of anthranilate.[19] Both MS and HPLC based techniques are well established; however, they remain serial methods that cannot easily be adapted to 96-well plate format to allow routine testing of isatin levels in blood samples. Here, we report the successful application of a bacterial isatin hydrolase (IH), which catalyzes the first part of the reaction from isatin to isatinate, at the interface between a hydrophobic and hydrophilic solvent effectively isolating isatinate. The following decarboxylation by hydrogen peroxide is irreversible and produces the fluorophore anthranilate (Scheme 1). This two-step method results in a highly selective and fast fluorometric quantification assay for isatin in blood samples adaptable to the 96-well plate format. The assay concept is tested with blood from both pigs and from a human. The assay only requires a fluorometer and a tabletop centrifuge alongside common disposable laboratory consumables. This article also includes the necessary biophysical characterization of the isatin hydrolase b (IH-b) from *Labrenzia aggregata* with respect to temperature and pH range of enzymatic activity. The quantification assay serves as a biotechnological application of IH-b for further elucidation of the physiological relevance of isatin in humans.

RESULTS AND DISCUSSION

Biophysical Characterization of IH-b. In order to use IH-b in the isatin quantification assay, a biophysical characterization of the enzyme was necessary. The enzymatic activity as a function of pH, temperature, and cofactor (divalent metal ion) was determined.

The pH dependency of IH-b was determined in the range from pH 5 to 11. An optimum of pH 8.3 was found by fitting a Gaussian function to the measurements of relative activity (Figure 1A). At pH values below 6.0, the enzyme activity is minimal. On the basis of the structure, this is likely a consequence of the protonation of histidine residues in the active site.[22] Above pH 9.5, the activity is also reduced significantly, most likely due to a combination of enzyme instability in an increasingly alkaline environment and the formation of insoluble $Mn(OH)_2$ and MnO, causing sequestration of its essential cofactor through precipitation. Given the clear Mn^{2+} dependent activity of IH-b (Figure 1B) in the presence of excess Mn^{2+}, the biotransformation of isatin declines with the loss of this cofactor. Significant activity is also observed with the divalent ions Cu^{2+} and Ni^{2+} (Figure. 1B). In earlier crystallographic studies, IH-b was crystallized and identified with Mn^{2+} in the active site.[22]

The affinity of IH-b for Mn^{2+} was determined to an approximate K_D of 9.5 μM and only a single Mn^{2+} site. The thermodynamic values determined were $\Delta H = 4.4 \pm 0.3$(kJ/mol) and $T\Delta S = 11.2$(kJ/mol) (Figure 1C). IH-b remained active up to 50 °C. At 60 °C, activity decreased with a half-life $t_{1/2} = 9$ min (Figure 1D).

Assay Protocol with Double Phase Extraction. The formation or the combined enzymatic and chemical synthesis of anthranilate from isatin, which forms the basis of the assay, is shown in Scheme 1. The major stages (I−III) in the assay are visualized in Figure 2. The Roman numbers are referenced in the written protocol.

Initial experiments using only defined buffers show a linear correlation between the initial isatin concentration and the fluorescence response (Supporting Information, Figure 2). To verify a similar correlation in plasma, we developed the initial isatin extraction step. To estimate recovery from each step, the assay was performed with radiolabeled isatin, as summarized in Table 1.

The saturation performed with NaCl in step 6 prevents coagulation that could otherwise reduce the yield of the following isatin extraction after precipitating proteins by HCl in step 7. Keeping the pH low prevents the autohydrolysis of isatin. The isatin recovery was quantified in each step using a radiotracer experiment. Plasma samples were individually spiked with [^{3}H]isatin and processed as described in the Methods section and below. Aliquots of all phases were withdrawn in each step and counted in a liquid scintillation counter. Recovery of isatin was calculated for the major transfer steps as shown in Table 1. There is an average yield of 34.5% (C4−5R2) for isatin from plasma to the supernatant in step 7. It is particularly important that the temperature during centrifugation is kept at 4 °C to ensure that the remaining isatin is preserved in the supernatant.

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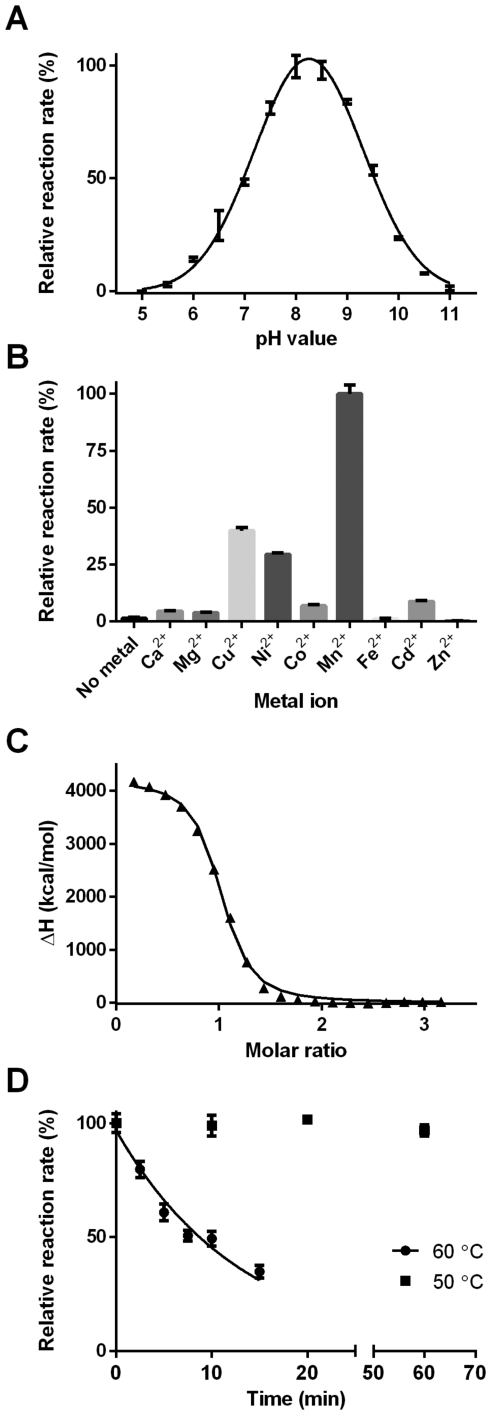

Figure 1. (A) Relative IH-b activity as a function of pH. Optimal catalytic activity is observed at pH 8.3; $N = 3$. (B) Metal dependency of IH-b relative activity. (C) Integrated and fitted data from ITC experiment of IH-b titrated with $MnCl_2$. (D) Thermostability analysis at 50 and 60 °C.

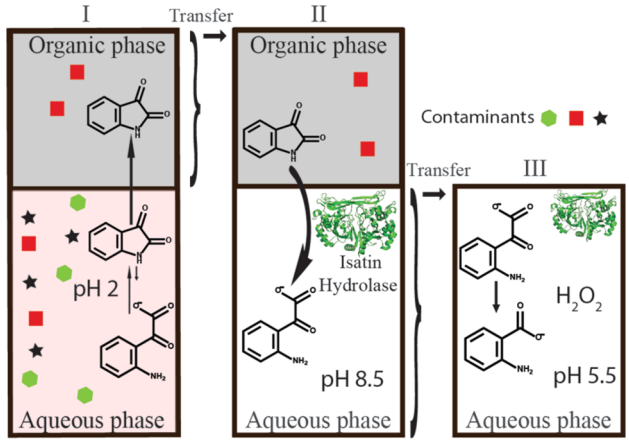

Figure 2. Isatin is extracted from acidified plasma by shaking with ethyl acetate (stage I). The ethyl acetate phase is subsequently transferred to an IH-b containing buffer, which allows the amphiphilic molecule isatin to diffuse across the interface, where it is hydrolyzed by IH-b. This process traps isatinate in the aqueous phase (stage II). IH-b is inactivated by lowering pH to 5.5. The addition of hydrogen peroxide decarboxylates isatinate to anthranilate (stage III). (1) 6 mL heparin-treated whole blood is centrifuged; 15 min, 4,000*g* at 4 °C. (2) Plasma is frozen at −20 °C. Optional step: plasma samples can readily be stored at this point and used for later analysis. (3) Thaw plasma samples at room temperature (RT). Optional step. (4) Aliquot 500 μL of plasma into five 2 mL-Eppendorf tubes at RT. (5) Add final concentrations of 125, 250, and 500 nM isatin to three of the five tubes at RT. Comment: the isatin stock should be kept in 99.8% ethanol to avoid autohydrolysis. The added isatin serves as an internal standard. The following steps are performed on all 5 tubes. (6) Add 0.16 g $NaCl_{(s)}$ to each tube and mix by rotation; 15 min, 200 rpm at RT. Comment: the NaCl will not dissolve completely; this step is to avoid coagulation, and improve the extraction of isatin in steps 7 and 8. (7(I)) Add 33% 4 M HCl (0.16 mL), vortex, and spin; 10 min, 15,000*g* at 4 °C. (8(I) Transfer 350 μL of supernatant (SN) to 600 μL of ethyl acetate and mix by rotation; 10 min, 200 rpm at RT. Comment: avoid the precipitate during transfer. (9II) Transfer 500 μL of ethyl acetate to a fresh tube containing 500 μL of AB1. Incubate sample at 45 min and 200 rpm at 37 °C. Important: add active IH-b to a final concentration of 1 μg/mL (~35 nM) to four of the tubes (0, 125, 250, and 500 nM isatin). The last tube (0 nM isatin) will be kept as a background without IH-b, only containing enzyme buffer. (10(II)) Spin sample; 30 s and 15,000*g* at RT. (11(III)) Transfer 450 μL of the aqueous lower phase to a fresh tube containing 200 μL of AB2. Mix by inverting tubes a few times and spinning for 30 s at 15,000*g* at RT. Incubate with open lids for 120 min at RT. (12) Measure fluorescence on any fluorometer; 600 μL samples are analyzed.

In step 8, isatin is transferred to an ethyl acetate phase, a step also described in an earlier protocol.[23] The radiolabeled isatin shows a reproducible concentration independent yield of 23.5% (C4−5R5) from plasma to ethyl acetate in step 8. This could potentially be optimized by repetitive extraction, but as simplicity is crucial for automation in a potential 96-well plate format, only one extraction was performed. In step 9, the ethyl acetate is transferred to buffer solutions with and without IH-b. With IH-b present, the yield from plasma to AB1 in step 9 is 19.5% (C4−5R7). Without IH-b present, a fraction of the isatin is still extracted to the AB1 phase (C1−2R9); see also Figure 4A. This leads to an underestimation of the endogenous isatin concentration, which can be corrected for by upscaling the determined isatin concentration with the average relative extraction estimate 20.8% (C4−5R9). The double phase extraction in step 8−9 combines the enzyme activity and phase extraction; this ensures both high specificity and high purity of the product. This can only be achieved by exploiting the difference in ethyl acetate−water partition between isatin and isatinate (Figure 2). In step 11, the buffered solution is transferred to a fresh tube and the pH lowered to 5.5, leaving the enzyme inactive during the oxidation of isatinate to anthranilate. To avoid fluorescence variations caused by fluctuations in pH[24] and temperature, the buffers were carefully equilibrated, and special care was taken to keep the samples at a stable temperature. In step 12, hydrogen peroxide is

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Table 1. Radiolabeled Yield Estimation of Isatin[a]

		column (C)				
R	row (R)	125 nM isatin (DPM × 10^6)	500 nM isatin (DPM× 10^6)	relative activity C1/C2 nM (%)	yield 125 nM (%)	yield 500 nM (%)
1	plasma	574 ± 22	2153 ± 33	26.7	100	100
2	pellet (7)	335 ± 7	1219 ± 85	27.5		
3	supernatant (7)	194 ± 7	756 ± 10	25.7	33.8	35.1
4	aqueous (8)	41 ± 1	154 ± 8	26.6		
5	ethyl acetate (8)	89 ± 4	333 ± 19	26.4	23.1	23.9
6	ethyl acetate (9)	14 ± 0.1	55 ± 2	26.4		
7	AB1 (+ IH-b) (9)	67 ± 3	257 ± 15	27.6	19.2	19.7
	isatin extraction in background experiment without IH-b				relative extraction −IH-b	
8	ethyl acetate (9)	70 ± 4	264 ± 5	24.9		
9	AB1 (− IH-b) (9)	19 ± 1	67 ± 3	26.0	21.3	20.2

[a]Columns (C) and rows (R); C1, activity in 10^6 decays per min (DPM) of 20 nM [^{3}H]isatin + 105 nM [^{1}H]isatin. C2: 80 nM [^{3}H]isatin + 420 nM [^{1}H]isatin. C1 and C2 (N = 4). Only activities of the individual transfer steps are directly comparable. C3: Relative activity of C1/C2 nM. C4 and C5: The yields reported have been corrected for sample loss necessary for the liquid scintillation analysis and thus represent expected values following the protocol without radioactively labeled isatin; see Methods. C1R9 and C2R9: The extraction level of isatin without IH-b; this contributes to the background measurement and must therefore be corrected for in the final endogenous determination. The relative extraction levels are given in C4R10 and C5R10. Numbers in parentheses in each row refer to steps in the written assay protocol.

added to complete the irreversible decarboxylation of isatinate to anthranilate.[25] To test whether identical percent-wise yields occurred independent of the initial isatin concentration, the assay was performed with both 20 (105 nM [^{1}H]isatin) and 80 nM (420 nM [^{1}H]isatin) radiolabeled isatin, initially added to the plasma sample. The relative activity was stable in all transfer steps at an average of 26.4% (Table 1 C3) corresponding to a transfer independent of concentration. The deviation from the ideal 25% may arise from error on the pipetting (estimate ∼3%) combined with uncertainty of the measurement. The error in pipetting is often around 2−3%. The total recovery calculated from the radiolabeled experiments is 19.5%, which is lower than the previous published methods.[18,19] This does not influence the final result as the percent-wise yield in each step is independent of concentration and applies to every data point in the internal standard performed on each plasma sample. However, better recovery would potentially decrease the standard deviation on the value obtained for each sample (Figure 4C). The recovery determined by Mawatari et al. is 94.3%; however, this value does not reflect the total recovery as the standard is added after heating and spinning the serum. Manabe et al. estimate a recovery from a serum of 68.2% with UV detection from HPLC and a more thorough extraction.

Linear Correlation between Isatin Concentration and Fluorescence Intensity and Detection Limit. Figure 4A shows the fluorescence spectra of increasing initial isatin concentrations, exemplified by an analysis of a pig plasma sample. At low isatin concentrations, a background peak at ∼405 nm is present; however, as the initial isatin concentration increases, the total spectral peak shifts to a maximum at 398 nm (Figure 4A), which is expected for anthranilate.[19] The individual spectra are clearly distinguishable and exhibit a strong linear correlation (R^2 = 0.998) between the fluorescence intensity and the initial isatin concentration for the entire concentration range (Figure 4A, insert). This linear correlation allows for straightforward absolute quantification. In the initial experiment performed with different amounts of isatin added to a buffer phase (Supporting Information, Figure 2), we were able to significantly distinguish concentration differences down to 3 nM isatin (with an approximate standard deviation of $2\bar{\sigma}$ on the fluorescence intensity differences performed in triplicate). To determine the accuracy and reproducibility of the assay, ten blood samples with and ten without IH-b, all with 500 nM isatin added, were treated using the described assay in identical conditions. The samples without IH-b resulted in a standard deviation of 2.4 nM, and the samples with IH-b resulted in 5.1 nM. The absolute precision of the measurements decreased with increasing isatin concentration, but even for 500 nM isatin with IH-b, the 95% confidence interval was no more than 20 nM isatin (Supporting Information, Figure 3).

Validation of the Assay and Purity by HPLC. RP-HPLC was used to validate the conversion of isatin to anthranilate. A standard run with a known concentration of anthranilate was performed in equivalent conditions to one plasma sample with and one without IH-b; both samples were taken after step 11 in the assay protocol. Anthranilate elutes at a retention time equivalent to the major peak from the conversion assay (at 6.13 min), and it is therefore concluded that anthranilate is formed (Figure 3). The background fluorescence is low and identical

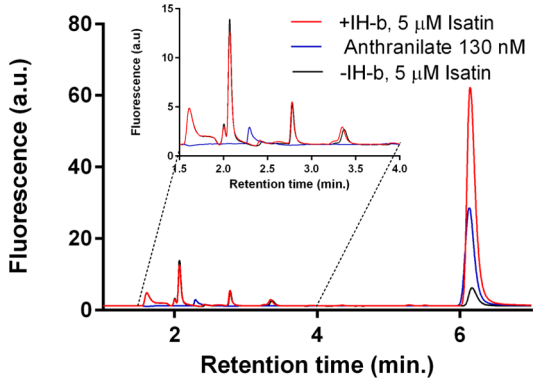

Figure 3. RP-HPLC fluorescence chromatogram. The retention time of anthranilate (blue) and 5 μM isatin added to a plasma sample with IH-b (red) and without IH-b (black).

both with and without IH-b (Figure 3, expanded window). This confirms that the sample without IH-b is a reliable background measure and that IH-b is highly specific with regard to interfering fluorescent species. There might be other substrates and inhibitors in blood, but as long as enzymatic activity is retained, which both the radiolabeled experiments as well as the HPLC data show, and no fluorescent background is observed, this should not interfere with the assay. The conversion also yields a linear increase in fluorescence peak area in the range 0−5 μM isatin (data not shown) as expected. On the basis of the fluorescent signal in Figure 3, we estimate that more than 95% of the fluorescence detected originates from anthranilate.

Quantification of Isatin in Pig Blood. To quantify variations in isatin content between individuals, we collected plasma from 12 different pigs and applied the protocol described above. Data treatment is visualized in Figure 4A. The apparent initial isatin concentration is given by the difference between fluorescence at

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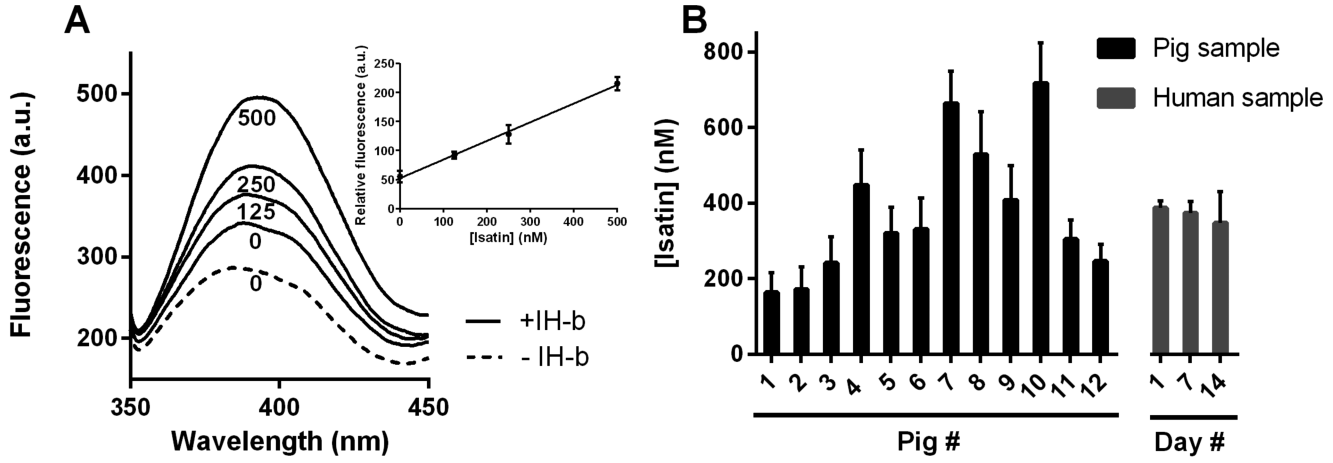

C

Sample	Pig number 1	2	3	4	5	6	7	8	9	10	11	12	One human Day 1	Day 7	Day 14
Corrected isatin level (nM)	197	208	292	541	387	400	802	639	493	867	367	297	469	452	420
Corrected SD (nM)	63	69	81	110	84	98	104	137	109	129	62	54	22	36	99

Figure 4. (A) Plot of averaged fluorescence data for Pig 1 ($N = 3$). The insert represents fluorescence at 398 nm as a function of added isatin; error bars represent 1σ. Given the linear correlation, the apparent endogenous isatin concentration can be found as the intersection with the y-axis converted to isatin concentration. (B) Apparent endogenous isatin concentrations for pig samples 1−12 and one human donor at day 1, 7, and 14. (C) Endogenous isatin concentrations weighted with 20.8% due to the loss in the enzymatic step. All pig plasma measurements are done in triplicate. The standard deviation (SD) on y (σ_y) is calculated from the SD on x (σ_x) through the linear regression as represented in A.

398 nm of plasma samples with and without IH-b and can be calculated using the linear correlation of the internal standard. Figure 4C shows the isatin levels with standard deviations. The mean for all pig samples is 458 ± 91 nM. All measurements are performed in triplicate. The background without IH-b has been subtracted for each point in the analysis. Endogenous isatin levels can be determined by extrapolation of the linear plot of the samples supplied with additional isatin and scaled up with the isatin transfer figures in background samples of 20.8% in the enzymatic step. This is shown in Figure 4C. The apparent endogenous isatin concentrations of the 12 plasma samples before the correction are shown in Figure 4B.

Quantification of Isatin in Human Blood. Human whole blood samples were taken on the same weekday for three consecutive weeks from a healthy donor to determine the variation in plasma isatin over time. The described protocol was applied and isatin concentrations determined as for the pig samples (Figure 4B and C). The endogenous isatin concentrations of the analyzed samples were 459 ± 22 nM, 462 ± 36 nM, and 420 ± 99 nM for the three consecutive weeks (Figure 4C). The samples showed relatively constant concentration with a mean of 447 ± 52 nM.

We report a novel assay for the detection and quantification of isatin in biological samples, exemplified in this paper by quantification in human and pig plasma. The developed protocol is based on a double phase extraction utilizing the substrate specificity of IH-b. The combination of this enzyme specificity and the formation of a fluorophore yield a highly selective and sensitive quantification tool for the analysis of complex samples. The role of isatin in human physiology and, not the least, pathophysiology is far from understood. Previous studies have been based on HPLC and MS methods, which prevented the analysis of large sample arrays. This novel assay presented is, in principle, compatible with detection in a 96-well plate reader format.

In ideal buffer samples, we were able to detect concentrations down to 3 nM (Supporting Information, Figure 2). The standard deviations on each estimation of the endogenous isatin concentrations varies between 54 and 129 nM in pig plasma samples and 22−99 nM in human plasma samples. This is due to the higher background signal in the pig and human samples as compared to that in the buffer samples (Supporting Information, Figure 2A). These values could be improved by increased recovery, decreased manual handling, and automation. The very low detection limit of 3 nM in Supporting Information, Figure 2, highlights the prospective precision of this detection method. Even though current methods show lower detection limits,[19] a more sensitive assay to determine the neurological and physiological role of isatin than the one presented here may not be needed. Preliminary studies suggest that increases in isatin levels are substantial. A 2−6-fold increase in isatin concentration has been reported in bodily fluids and the tissue of rodents in relation to stress.[4]

Manabe et al. have shown that large differences in serum isatin levels occur between individuals (134.2 ± 120.6 μg/L, N = 20) (0.91 ± 0.82 μM) and that the levels between urine and serum correlate.[18] In another study as well on urine and serum, Mawatari et al. showed that the differences in isatin concentrations between individuals seem to be considerably lower: 54.7 ng/mL ± 4.2 ng/mL (N = 6)[19] (0.37 ± 0.02 μM). We determine the average endogenous isatin levels to 458 nM for pigs and 447 nM for one human donor. In the literature, it seems to be unclear whether the serum levels of isatin between different individuals are constant or differ over several concentration units. Our measurements performed on human plasma, from a single individual, suggest that the isatin concentration in plasma is stable over the time scale of weeks. The levels estimated here for pigs and humans are within the range reported in the literature for human serum samples: 0.2−2.9 μM by Manabe and coworkers.[18] The isatin quantification in pig plasma shows that the isatin concentration varies between individuals in agreement

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with Manabe et al.[18] Application of the developed quantification assay on larger sample sizes could reveal the potential of isatin as a biomarker for stress.

IH-b has recently been described as belonging to a novel structural class of metallo hydrolases which also includes bacterial kynurenine formamidase.[22] Although the presence of Mn^{2+} resulted in the highest enzymatic activity, the dissociation constant of the binding was determined to 9.5 μM. Significant activity was also observed in the presence of Cu^{2+} and Ni^{2+}. This observation suggests that this class of enzymes is not strictly manganese dependent but that it may employ a range of metal ions for activity. This could potentially be utilized in a biotechnological setting, e.g., in a reaction that would restrict the use of Mn^{2+}.

The method of double phase extraction directly linked to enzymatic activity is a very strong biotechnical tool which allows for highly selective purification of enzymatic products. Other enzymatic systems have also been shown to function in the presence of an organic phase like the enzyme cholinesterase that remains stable in the presence of hexane and is inhibited by pesticides extracted from the hexane solution.[26] Procedures that involve immiscible phases are also known from enzyme membrane bioreactors used in the pharmaceutical industry.[27] Here, we directly link the double phase extraction with enzymatic conversion. Our extraction and purification procedure represents a simple detection method, using only standard and relatively cheap laboratory equipment, yet high purity of the trapped enzymatic product is achieved. This could be used as a general method for a simple but highly specific purification of enzymatic substrates with chemical properties similar to those of isatin which also changes hydrophobicity once hydrolyzed, e.g., chiral drug synthesis in membrane bioreactor systems.[28] Increased yields in chemical synthesis might be achieved by product removal driving the reaction, as well as separation of substrate and product as in an enantiomeric resolution.[29]

Numerous publications link isatin to stress, and with a simple detection method, isatin has the potential to become a common stress marker. Isatin detection in blood samples could be implemented as a standard procedure during annual medical checkups common for most people today. Whether increased isatin levels will correlate with increased signs of stress in humans is still not completely clear. However, additional statistical validation of the actual isatin levels found in larger cohorts of patients could sharpen patient diagnosis when dealing with stress. A broader screening of patient blood samples using the above assay will ultimately provide the statistical foundation necessary to discover novel correlations of isatin associated disorders and thus could validate isatin as a potential biomarker.

METHODS

Enzyme Expression and Purification. Purification of the isatin hydrolase isoform b (IH-b) (UniProtKB: A0NLY7) from *Labrenzia aggregata*, recombinantly produced in *Escherichia coli*, was performed as described by Bjerregaard-Andersen et al.[22]

Determination of Metal Requirements for Enzyme Activity. IH-b enzymatic reaction rate was monitored and the data analyzed as described in ref 22. IH-b (100 nM) in 25 mM Tris-HCl at pH 8.0 and 25 mM NaCl was pretreated with a 1:1 molar ratio of EDTA (Sigma E5134). EDTA was removed by dialysis (500-fold dilution) against an identical buffer containing no EDTA. The treated IH-b was incubated with 50 μM metal chloride for 30 min prior to activity measurement. The final reaction condition in the cuvette was 25 mM Tris-HCl at pH 8.0 (Sigma T6066), 180 μM isatin (Aldrich 114618), 25 mM NaCl (VWR 27810.295), 10 nM IH-b, and 5 μM metal chloride. Chloride salts of the following ions were tested: Ca^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}, Cd^{2+}, and Zn^{2+} (Sigma). A negative control with no added metal was included.

Determination of IH-b pH Optimum. IH-b with a concentration of 1 μM was incubated for 30 min in 13 tubes each containing 100 mM NaCl and 50 mM of one of the following buffers: MES at pH 5.0, 5.5, 6.0, 6.5, or 7.0; Tris-HCl at pH 7.5, 8.0, 8.5, 9.0, and 9.5; and borate at pH 10.0, 10.5, and 11.0. One minute before measurement, 0.5 mM of $MnCl_2$ was added to the protein. The short incubation time prevented the extensive formation of $Mn(OH)_2$ prior to measurement. To start the reaction, 100 μL of IH-b solution was added to a solution of 500 μM isatin, 100 mM NaCl, 0.5 mM $MnCl_2$, and 50 mM of the enzyme buffer. Final enzyme concentration was 100 nM. Reaction rate measurements were carried out in triplicate.

Determination of the Thermal Stability of IH-b. IH-b was incubated at 50 and 60 °C in a heat block in the presence of 50 mM Tris-HCl at pH 8.0, 100 mM NaCl, and 0.2 mM $MnCl_2$. At time points 0, 2.5, 5, 7.5, 10, 15, 20, and 60 min, samples of IH-b were taken from the heat block and placed on ice. The reaction rate for each time point was determined in the presence of 180 nM isatin, 50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 0.2 mM $MnCl_2$ and a final IH-b concentration of 4 nM.

Determination of Metal Affinity by ITC. Isothermal titration calorimetry (ITC) measurements were used to determine the affinity and stoichiometry of manganese binding to IH-b. The measurements were carried out on a MicroCal VP-ITC at 25 °C. Purified and concentrated IH-b was incubated with 20 mM EDTA followed by dialysis against 1 L of 50 mM HEPES at pH 7.5 (Sigma H3375) and 100 mM NaCl, effectively diluting EDTA concentration 2 × 500-fold. Reducing agents were avoided to prevent the reduction of manganese ions. All buffers were degassed prior to use. The sample cell (1.43 mL) was filled with IH-b concentrated to 350 μM. The syringe was loaded with 290 μL of 5 mM $MnCl_2$ in dialysis buffer. Volumes of 15 μL were sequentially injected into the cell with a spacing of 600 s. The reaction was endothermic and relatively slow (10 min). This delay between injections was necessary for complete re-equilibration of the baseline. Data integration and binding analysis was done using software provided by the manufacturer, with a delay of 10 min between injections. Raw data (Supporting Information Figure 1) were visually inspected, and a good fit of χ^2/DoF = 1685 was produced (DoF, degrees of freedom). From this analysis, binding parameters such as molar binding enthalpy (ΔH) are calculated from the integrated injection peak volumes and stoichiometry of interaction (N), and the association constant (K_A) is calculated by fitting a inding isotherm to the plot of injections volume vs molar ratio. From the $\Delta G = \Delta H - T\Delta S = -RT\ln K_A$ (R is the gas constant and T the absolute temperature), the entropic contribution (ΔS) to binding can be estimated.

Recovery Determination by Tritium Labeled Isatin. [5-^{3}H]-Isatin was purchased from American Radiolabeled Chemicals Inc. in a specific activity of 25 Ci/mmol and a purity >99%. The radiotracer was added in 20 (+105 nM [^{1}H]isatin and 80 nM (+420 nM [^{1}H]isatin) concentration in four replicates to pig plasma and incubated for 5 min. The assay protocol was followed as described in Results and Discussion, and for each step, a sample was taken out for activity determination. The total activity of each step was estimated by multiplying with the factor of the total volume to sample fraction. In the case of the pellet, step 7 (assay protocol), the total sample was used. Ten mL of scintillation liquid was added to each sample, and the activity was read in a liquid scintillation counter, (HIDEX 300 SL). For each sample, the total number of radioactive decays per minute was counted twice for 1 min. Data treatment was performed in MS Excel. Standard deviations in Table 1 represent the difference between the four replicate samples, as the standard deviation resulting from the two readings of the same sample was insignificant in comparison. Values in Table 1 are calculated as follows: C3 = C1/C2·100, C4R1 set to 100%, C4R3 = C1R3/C1R1·100, C4R5 = (C1R5/(C1R5 + C1R4))·C4R3, C4R7 = (C1R7/(C1R7 + C1R6))·C4R5, and C4R9 = C1R9/(C1R9 + C1R8). C5 is calculated in the same way as C4. Part of the samples had to be used for analysis. Because of this, only the percent-wise transfer in each step can be directly compared, and the yields have to be calculated as the transfer

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fraction of each step times the yield of the previous step. The yields presented in the text are the averages of C4 and C5.

Method Validation through HPLC. HPLC analysis was conducted on an Agilent 1260 Infinity HPLC system consisting of a G5611A Bio-Inert Quaternary Pump, G5667A Bio-Inert High Performance Autosampler, G1316C Thermostated Column Compartment, G1365D Multiple Wavelength Detector, G1321B Fluorescence Detector, and a G5664A Bio-Inert Fraction Collector analytical scale. Separation was achieved using a Zorbax Eclipse Plus C_{18} column (150 mm × 4.6 mm, 3.5 μm) particle size. (Matriks AS, Norway). Chromatography was carried out using HPLC grade acetonitrile (ACN) purchased from VWR International (Leuven, Belgium) and 18.2 MΩ purified water. The eluents were filtered using Pall Supor Poly(ether sulfone) membrane disc filters (0.2 μm × 47 mm) purchased from Pall. All samples were filtered using disposable polypropylene syringe filters (0.2 μm × 25 mm) purchased from VWR International.

The mobile phase consisted of 10 mM MES, pH 5.5, (A) and ACN (B) with the following gradient elution: linear gradient elution from 85:15 (A/B) to 78:22 (A/B) over 5 min; isocratic elution at 78:22 (A/B) over 5 min; linear gradient elution from 78:22 (A/B) to 85:15 (A/B) over 2 min. Analysis was conducted at ambient room temperature with a flow rate of 0.7 mL/min and 20 μL of sample injected. Total runtime was 12 min, and 5 min of equilibration time was allowed between consecutive runs. UV detection was carried out at 242 nm for the detection of isatin, and the fluorescence detector was set to excitation at 308 nm and emission at 400 nm for the detection of anthranilate. Detector response times were set to 1 s. The results were processed by Agilent chemstation software. Sample peaks were automatically integrated by the software; peaks with a slope lower than 5 mAU/s (milli Absorbance Units per second) and a peak area lower than 100 mAU (UV detection) and 5 LU (fluorescence detection) were excluded from analysis. All chromatograms were thoroughly inspected and peak integration performed manually if necessary.

Plasma Preparation. Blood was collected in a sealed container supplemented with EDTA (2 mg/mL) at Fatland (Prof. Birkelandsv 30 A, 1081 OSLO, Norway) from 12 different pigs immediately after slaughtering. The gender of the pigs was unknown. A Beckman Coulter Avanti J-26 XP was used to remove cells by centrifugation at 4000*g* for 15 min. The plasma was then frozen and stored at −20 °C. Blood samples from a male human test subject were taken every Tuesday for 3 weeks in heparin containers. After sampling, the whole blood was spun at 4,000*g* for 15 min. The plasma was transferred to a fresh container and frozen at −20 °C.

Isatin Isolation from Plasma and Enzymatic Conversion. The following buffers are referred to in the Results and Discussion section describing the assay: 99.8% EtOH (Sigma 32221), HCl (Merck 37%), ethyl acetate (319902, Sigma-Aldrich), Bis-tris at pH 5.5, (Sigma B9754), and aqueous 30% H_2O_2 (H1009 Sigma-Aldrich). Centrifugations were performed in a Beckmann Coulter tabletop microfuge 22R. Assay buffer 1 (AB1): 50 mM Tris-HCl at pH 8.5 and 100 mM NaCl. Assay buffer 2 (AB2): 0.48 M Bis-tris at pH 5.5, 100 mM NaCl, and 0.12% H_2O_2.

Fluorescence Detection. Pig plasma samples were measured in a Hellma Analytics Quartz SUPRASIL cuvette (10 mm) in a Jasco FP8200 fluorometer. The settings were excitation bandwidth = 2.5 nm, emission bandwidth = 5 nm, excitation wavelength = 308 nm, emission scan interval = 350−450 nm, sensitivity = high, and scan speed = 500 nm/min. A xenon light source was used. The response time was set at 1 s, and the data collection interval was 0.5 nm. Each sample was averaged over two scans (accumulated). For pig plasma, measurements of 3 identical series containing 4 different isatin concentrations (500, 250, 125, and 0 nM) were performed with IH-b. Likewise, measurements of three identical series containing five different isatin concentrations were performed without IH-b. Data treatment and statistics were performed in MS Excel and GraphPad Prism 6. For human samples, one series of one without IH-b and four with IH-b concentrations (500, 250, 125, and 0 nM) was performed per sample.

■ ASSOCIATED CONTENT

Supporting Information

ITC raw data of Mn^{2+} binding to IH-b; enzymatic conversion; and detection limit. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

T.S. performed the assay development including work on the radiolabeled tracer, supervised by P.R. K.B.A. performed biophysical characterization, and J.K.J. supervised ITC measurements. S.M.S. performed HPLC analysis. B.J. and M.E. provided purified IH-b and experimental insight. J.P.M. supervised and designed the experiment with T.S. and K.B.A. J.P.M., T.S., and K.B.A. wrote the paper, and all authors commented on it.

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Notes

The authors declare no competing financial interest.

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