Studies on the Degradation of the mycotoxin Patulin in Blood

Untersuchungen zum Patulinabbau im Blut

M. RYCHLIK

Summary:
The mycotoxin patulin was quantified in blood by the recently developed stable isotope dilution assay (SIDA) using $^{13}$C$_2$-patulin as the internal standard. The application of SIDA revealed a patulin content less than 200 ng/mL in the serum of a volunteer, whose blood was drawn shortly after consumption of a juice containing 50 µg/L patulin. Further in-vitro experiments revealed a rapid degradation of patulin when added to blood. It was concluded, therefore, that even high natural occurring concentrations of patulin in foods are quickly degraded before reaching other tissues than the gastro intestinal tract. The degradation of patulin was attributed to its reaction with glutathione, as several glutathion-patulin adducts were detected in blood by LC-MS.

Keywords: blood, glutathione, patulin; stable isotope dilution assay

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Zusammenfassung:


Kennwörter: Blut, Glutathion, Patulin, Stabilisotopenverdünnungsanalyse

Introduction

The mycotoxin Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one, PAT) is produced by different fungal species, of which the most widespread species, Penicillium expansum, is known to invade different foods such as fruits (1), berries (2), and vegetables (3).

Although it has been discovered as early as in the 1940ies, the toxicology of PAT has still remained a controversial topic. In prior feeding trials its acute and chronic toxicity (4), teratogenicity (5), and immunotoxicity (6) has been shown. Additionally, recent in-vitro studies on cell cultures proved its potency to suppress intercellular communication, to alter intracellular calcium concentrations (7) and to perturb intestinal barrier functions (8). Moreover, in mutagenicity assays the mycotoxin induced single-strand DNA breaks (9) as well as micronuclei (10) and was shown to be a potent carcinogen, whereas orally given PAT produced no tumors (11).

As we reported on the quantification of pantothenic acid (12), of folate vitamins (13) and of the mycotoxin ochratoxin A (14), stable isotope dilution assays (SIDA) exhibit excellent accuracy due to an optimal compensation for losses of the analytes in all analytical steps. Based on the synthesis of $^{13}$C$_2$-PAT (15) we recently developed a SIDA for the mycotoxin (16).

Therefore, the objective of the present study was to follow the fate of PAT in human blood after its consumption with apple juice to help evaluating its toxicokinetic behaviour.

Materials and methods

Materials

The following chemicals were obtained commercially from the sources given in parentheses: N, O-bis-(trimethylsilyl)- trifluoracetamide (BSTFA) metaphosphoric acid (Aldrich, Steinheim, Germany); acetoni-trile, ethyl acetate, hexane, hydrochloric acid, per-fluorokerosene, potassium dihydrogenphosphate, sodium carbonate, sodium hydrogenphosphate, sodium sulfate (Merck, Darmstadt, Germany); 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione, glutathione reductase from baker’s yeast, NADPH (Sigma, Deisenhofen, Germany). $^{13}$C$_2$-PAT was synthesized as reported recently (15).

Glasswares for sample preparation were decontaminated by storing them overnight in phosphate buffer (0.1 mol/L) at pH 12.

Foods

Commercial apple juices were purchased in a retail shop, one home-made juice was produced in Nürnberg, Germany.

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Foods

Commercial apple juices were purchased in a retail shop, one home-made juice was produced in Nürnberg, Germany.
Blood serum
Blood (9 mL) was taken from volunteers and collected into vacutainers, where it was allowed to clot (Sarstedt, Nümbrecht, Germany). Serum was then obtained by centrifuging the clotted blood for 10 min at 2000 g and acidified by addition of hydrochloric acid (1 mol/L, 1 mL).

Consumption assays
The commercial product containing a PAT level of 23.5 µg/L and the home-made juice containing 68 µg/L were chosen as samples for the absorption studies. The two juices were blended to adjust an PAT content of approximately 50 µg/L. Thereafter, the PAT content of the blended juice was checked by SIDA. As a blank, a blood sample of one volunteer (male, age 36 years, weight 70 kg, height 180 cm) was taken immediately before consumption of the juice. Then, the volunteer drank 1 L of the blended apple juice within 15 min and 1 hour after starting the consumption another blood sample was taken. Subsequently, serum was prepared as detailed above. This experiment was repeated twice within two weeks with a break of 4 days between the trials. The consumption trials were approved by the ethics committee of the medical faculty of the Technical University of Munich and carried out in compliance with the ethical rules of the Helsinki Declaration of 1975.

Degradation of PAT in whole blood
Whole blood was taken from the volunteer mentioned above and collected into vacutainers containing heparin as an anticoagulant (Sarstedt, Nümbrecht, Germany). Two different amounts of PAT (7 ng and 100 µg, respectively) were added to the blood (9 mL) and the samples were vortexed for 15 sec. To stop the reaction after one and two min, respectively, the samples were acidified by addition of hydrochloric acid (1 mol/L, 1 mL) and vortexed for 15 sec.

Extraction and clean-up of blood for stable isotope dilution assays
\(^{13}C_2\)-PAT (6 ng and 100 µg, respectively) was added to acidified blood serum (9 mL) or whole blood (9 mL) and the samples were extracted with ethyl acetate (2 x 10 mL). The combined organic solutions were washed with an aqueous solution of sodium carbonate (1.5 % by weight, 2 mL) and the aqueous phase was extracted with ethyl acetate (10 mL). The organic phases were combined, dried over anhydrous sodium sulfate and evaporated to a volume of 0.5 mL in a stream of nitrogen. The concentrated extract was then purified on a Sep-Pak™ silica cartridge (Waters, Milford, MA, USA), preconditioned with hexane (1mL). The column was washed with hexane (4mL) and ethyl acetate/hexane (1+1, v/v; 3mL) Then, PAT was eluted with a further 8 mL of ethyl acetate/hexane (1+1, v/v) followed by evaporating the solvent to dryness in a stream of nitrogen. The residue was then subjected to trimethylsilyl derivatization and the trimethylsilylated fractions were analyzed by HRGC/HRMS for low PAT amounts and by HRGC/MS for higher amounts, respectively.

Preparation of trimethylsilyl derivatives
50 µL of N, O-bis-(trimethylsilyl)-trifluoroacetamide were added to the purified fractions, and the mixture was heated for 10 min at 80 °C in a closed vial. After cooling to room temperature, the solution was evaporated to dryness in a stream of nitrogen. After addition of 100 µL of hexane, the samples were ready for gas chromatography.

Gas chromatography/ mass spectrometry
Gas chromatography/ high resolution mass spectrometry (GC/HRMS) was performed by means of a type 5300 gas chromatograph (Carlo Erba, Hofheim, Germany) using capillary DB-5 (30 m x 0.32 mm fused silica capillary, film thickness of the stationary phase \(d_f = 0.25 \mu m\); Fisons Instruments, Mainz, Germany) coupled to a MAT 95S (FinniganMAT,Bremen,Germany). The samples were applied by the cold on-column technique at 60°C. One min after injecting the sample, the temperature of the oven was raised to 250°C by a rate of 10 °C/min. The flow rate of the carrier gas helium was 2 mL/min. Multiple ion detection (MID) was performed at a resolution of 6000 using perfluorokerosene for calibration. Mass traces for \(^{13}C_2\)-PAT and PAT were \(m/z = 228.0728 \text{ and } 226.0661\), respectively; lock mass was \(m/z = 218.9856\), calibration mass was \(m/z = 230.9856\). Higher amounts of PAT were analyzed by GC/low resolution mass spectrometry (GC/MS) using a type 8000 gas chromatograph (ThermoQuest, Egelsbach, Germany) equipped with a capillary DB-5 (30 m x 0.32 mm fused silica capillary, film thickness of the stationary phase \(d_f = 0.25 \mu m\), Fisons Instruments, Mainz, Germany) coupled to an MD 800 (ThermoQuest). The samples were applied by split injection at 230 °C and a split ratio of 1: 20. After injecting the sample (2µL), the temperature of the oven was held for one min at 150 °C, then raised to 250 °C at a rate of 15 °C/min and held at this temperature for 5 min. The flow rate of the carrier gas helium was 2 mL/min. \(^{13}C_2\)-PAT and PAT were detected in the mass traces at \(m/z = 228 \text{ and } 226\), respectively. Ionisation energy in the electron impact mode was 70 eV.

Analysis of patulin-glutathion adducts
In model experiments, PAT (16 µg) was added to a solution of glutathione (300 µg) in phosphate buffer (1 mL, 100 mmol/l, pH 7.4) and the reaction stopped.
by adding ice-cold metaphosphoric acid (4 mL, 50 g/L). The resulting solution was passed through a syringe filter (0.4 µm, Millipore, Bedford, MA, USA) and subjected to LC-MS. For the blood studies, PAT (16 µg) was added to freshly drawn blood (1mL) and after a reaction time of one hour, ice-cold metaphosphoric acid (4 mL, 50 g/L) was added. The resulting mixture was centrifuged at 13000 rpm for 10 min and the supernatant was subjected to LC-MS.

LC-MS-MS was performed by means of a TSQ Quantum coupled to a photo diode array detector Surveyor and a high performance liquid chromatograph (Thermo electron, Dreieich, Germany) equipped with a Nucleosil C-18 reversed phase column (250 x 3.0 mm; 5 µm, Macherey-Nagel, Düren, Germany). 20 µL of the sample solutions were chromatographed using gradient elution with variable mixtures of aqueous formic acid (0.1 %, solvent A) and methanol (solvent B), at a flow of 0.4 mL/min. A 5-min linear gradient was programmed from 0 to 10 % B followed by a 10-min isocratic period and a further 10-min linear gradient to 30 % B. Then, the concentration of B was raised within 2 min to 100 %, maintained for 5 min and subsequently brought back to the initial mixture for 6 min to allow for column equilibration. During the first 3 min of the gradient programme, the column effluent was diverted to waste to ensure an adequate spray stability. The photo diode array detector was operated between 200 and 500 nm and full scan mass spectra were recorded in a range between 150 and 1500 Da. The mass spectrometer operated in the positive electrospray mode with a spray needle voltage of +3.2 kV. The temperature of the capillary was 350° C and the sheath and auxiliary gas nitrogen nebulized the effluent with flows of 60 and 20 arbitrary units, respectively. The spectrometer was operated at a helium pressure of 10⁻⁶ Torr. MS-MS spectra of the detected glutathione adducts were recorded by applying the collision energies listed in table 1 on the respective protonated molecules.

Quantification of total glutathione in whole blood
Glutathione (GSH) levels in whole blood were determined according to the enzymatic recycling method reported by Richie et al. (17). Briefly, blood samples were deproteinized by addition of metaphosphoric acid and centrifuged. The extracts and calibration solutions of glutathione were then added into a 96-well microtiter plate. Subsequently, GSH was determined by monitoring 2-nitro-5-thiobenzoic acid spectrophotometrically at 405 nm after addition of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione reductase and NADPH to samples and calibrators.

**Figure 1:** Mass traces of gas chromatography/high resolution mass spectrometry of a serum sample after consumption of 49.9 µg patulin reveal no signal in trace m/z = 226.0661 for trimethylsilylated patulin. The trimethylsilyl derivative of the internal standard [¹³C₂]-patulin produces the signal in the trace m/z = 228.0661. RIC reconstructed ion current.
Results

Evaluation of Patulin’s toxicity by means of S.IDAs

A major obstacle to decide whether mutagenic concentrations of PAT can reach any target cells has been the lack of sensitive and accurate analytical methods. Consequently, an application of SIDA to biological samples such as blood or urine was deemed promising.

PAT quantification in blood serum

PAT and its labeled analogue were detected by GC/HRMS which proved to be extremely specific and a hundred fold more sensitive than any existing HPLC method. To test whether sensitivity was sufficient to detect even concentrations below 1 ng/ml, a mixture of the isotopomers was added to a sample of blood serum and analyzed by SIDA. However, no traces of the PAT isotopomers were found in these preliminary studies. As the erythrocytes are known to contain significant amounts of glutathione (17) and this thiol reacts readily with PAT (18), the loss of the mycotoxin was likely to be due to this reaction since residues of GSH stemming from erythrocytes still can occur in blood serum. Because the addition of GSH is assumed to occur via its thiolate anion, it seemed promising to suppress dissociation of GSH by lowering the pH of the samples. This was achieved by adding HCl to the serum, which made, in consequence, PAT detectable in the samples. However, a further problem occurred, which is very common in trace analyses. Due to the sensitivity of SIDA, even small contaminations of PAT in the glasswares used for sample preparation were detectable and, therefore, invalidated the measurements. To solve this problem, we decontaminated the glasses by storing them in a buffered solution at pH 12. Due to its lactone structure, PAT was completely degraded within 8 hours and thereafter no residues of PAT were detectable in system blanks.

To clarify whether PAT was detectable in blood after its oral uptake, an apple juice (1 L) adjusted to a PAT content of 49.9 µg/L was drunk by a volunteer and blood samples were drawn before and one hour after consumption. In three experiments within two weeks with a break of 4 days between the trials, no PAT was detectable by SIDA in the volunteer’s serum before and after consumption.

Figure 2: Mass traces of gas chromatography/low resolution mass spectrometry of whole blood after addition of 100 µg patulin. The residual PAT amount of 5 % is detected in trace m/z = 226 (as TMS-patulin) whereas the internal standard [13C2]-patulin is monitored in the trace m/z = 228 (as TMS-[13C2]-patulin). TIC total ion current.
As can be seen from fig. 1, there was no signal in the mass trace \( m/z = 226.0661 \) of PAT whereas the internal standard produced a discernable peak at \( m/z = 228.0728 \). A possible explanation for this finding is that PAT was degraded when it encountered blood.

**Reaction of PAT with whole blood**

To study the degradation of the mycotoxin in whole blood, a small amount of PAT (7 ng) was added to freshly drawn whole blood (9 mL) and the reaction was stopped after 2 min by addition of hydrochloric acid. But even after this short reaction time, no PAT was detectable by GC/HRMS.

Therefore, it was suggested that a big excess of GSH in whole blood rapidly degrades PAT. This assumption was confirmed by quantification of total GSH in the volunteer’s blood, which contained GSH at a concentration of 1.07 mmol/L and revealed a tremendous excess of GSH (9.63 µmol) compared to PAT (0.045 nmol). Therefore, a further experiment was designed to evaluate the blood’s detoxification capacity by adding a much higher amount of PAT (100 µg).

In this case PAT could be detected by GC coupled to low resolution mass spectrometry (Fig. 2). However, even after adding 100 µg of PAT, only mean residues of 14.0 ± 2.0 % and 6.1 ± 0.5 % (n= 3) could be detected after one and two min, respectively. On the basis of a regression curve combined from two exponential functions, a half-life of 21.2 sec was calculated.

The inactivation of PAT by enzymes and animal blood serum already has been observed by Freerksen and Boenicke (19) who found a 17 % degradation of a PAT solution (0.1 %) in fresh serum within 6 min. However, inactivation of PAT observed by the latter authors was less pronounced than in our experiments, which might be attributed to the negligible extrarrenal content of GSH (0.3 µmol/l), as reported by Wendel and Cikryt, 20) compared to that of whole blood used in our studies.

**Detection of adducts by LC-MS**

To get a more detailed insight into the underlying reaction of PAT degradation in blood, the mycotoxin was first reacted with GSH in a buffer solution at physiological pH. As Fliege et al. (21) suggested that the addition to GSH is the most important reaction to degrade PAT, the resulting mixture was analyzed for adducts already reported by the latter authors. Using a LC-MS-MS equipment we were able to identify several adducts reported by Fliege (21) tentatively by their molecular mass, their MS-MS decomposition behaviour and their UV-spectra (table 1). The CID spectra of the adducts mainly displayed a loss of glutamic acid from the protonated molecule. An exemplary spectrum of the mono adduct is shown in fig 3.

After adjusting the MS conditions for detection of the most adducts, we added PAT to freshly drawn blood and deproteinized the mixture with metaphosphoric acid. The resulting LC-MS chromatogram is displayed in fig. 4 and clearly shows the detection of the respective adducts that were already found in the model experiments.

**Conclusion**

Although we used SIDA, which is currently the most sensitive method for quantifying PAT, no traces of PAT were detectable in the serum of the volunteer after drinking a PAT dose of 49.9 µg. In a recent study on rat stomachs we could show that PAT is quickly absorbed from the gastrointestinal tract (22) and PAT is being partly degraded when diffusing through the mucosa wall. As we furthermore showed that PAT disappears rapidly after added to whole blood, it can be concluded that unmetabolized PAT exerts only local toxicity and cannot evoke systemic effects in other parts of the organism.

<table>
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<th>Adduct no.</th>
<th>([\text{M+H}]^+): m/z</th>
<th>Collision energy in %</th>
<th>Product ion after CID: ([\text{M+H}]^+): m/z</th>
<th>Mass difference in CID Da</th>
<th>Assumed fragment lost during CID</th>
<th>UV maximum nm</th>
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<td>Glutamic acid</td>
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</table>

Table 1: Mass and UV spectral data of glutathione-patulin adducts found in model reactions. CID: collision-induced dissociation
Figure 4: LC-MS chromatogram of patulin-glutathione adducts in whole blood.
The present study is the first investigation to report GSH PAT adducts in blood. Although these products already have been reported to exert only low acute toxicity (18) their final toxicological evaluation is still missing. Furthermore, methods for quantifications of the adducts have to be developed to clarify their role in PAT degradation. As proteins have also been found as reaction partners with PAT (23), the latter reaction products have to be detected and quantified in blood as well.

Figure 3: LC-MS (above) and LC-MS-MS (below) spectrum of the patulin-glutathione mono adduct in a model reaction.

References


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