Structural Characterization of a Case-Implicated Contaminant, "Peak X," in Commercial Preparations of 5-Hydroxytryptophan

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ABSTRACT. ***Objective.*** To determine the chemical structure of a contaminant, X_1, previously found in eosinophilia myalgia syndrome case-implicated 5-hydroxytryptophan (5-OHTrp), and also present in over-the-counter (OTC) commercially available 5-OHTrp.

Methods. Case-implicated 5-OHTrp as well as 6 OTC samples were subjected to accurate mass HPLC-mass spectrometry and HPLC-electrochemical detection, and reacted with reduced glutathione. Peak X_1 was subsequently subjected to HPLC-tandem mass spectrometry (MS/MS), as well as the resulting nucleophilic glutathione product. All these data were compared with analysis carried out under identical conditions on authentic 4,5-tryptophan-dione (Trp-4,5D).

Results. Based on accurate mass, tandem mass spectrometric analysis, and comparision with authentic standard compound analysis, X_1 was determined to be 4,5-tryptophan-dione, a putative neurotoxin. The presence of X_1 in OTC samples varied from 0.5 to 10.3% of the amount of Trp-4,5D present in case-implicated 5-OHTrp.

Conclusion. Peak X_1 was identified as the putative neurotoxin Trp-4,5D. It was found in case-implicated 5-OHTrp as well as 6 OTC samples. This gives some cause for concern in terms of the safety of such commercial preparations of 5-OHTrp. (J Rheumatol 2003;30:89–95)

Key Indexing Terms:
EOSINOPHILIA MYALGIA SYNDROME 5-HYDROXYTRYPTOPHAN ACCURATE MASS HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY TANDEM MASS SPECTROMETRY CASE IMPLICATED CONTAMINANT

There is growing debate regarding the ~$15 billion natural medicine and dietary supplement industry[1-3]. The explosive growth of annual sales and widespread unsupervised usage of such compounds gives rise to some concern. In part this is due to the fact that in the USA these compounds or extracts were deregulated by the 1994 *Dietary Supplement and Health Education Act*. The consequences of the Act were that the purity, safety, and efficacy were (and remain today) no longer evaluated and monitored by the US Food and Drug Administration (FDA). The potential dangers of ingesting such dietary/medicinal supplements were clearly demonstrated in 1989 with the reporting of eosinophilia myalgia syndrome (EMS)[4-7].

The consequences of EMS were that an estimated 30 people died and the disease ultimately affected over 1500 people[8]. Epidemiological studies by state health departments and the US Centers for Disease Control and Prevention indicated that EMS was triggered by the consumption of the dietary supplement L-tryptophan (L-Trp). It was subsequently shown that L-Trp manufactured by one company, Showa Denko K.K. of Japan, caused EMS, and it was suggested that a production contaminant(s) of L-Trp was responsible for the illness[9-11]. The outbreak was essentially curtailed when the suspect L-Trp was removed from the retail market via a recall by the FDA. Analysis of L-Trp by high performance liquid chromatography (HPLC) and HPLC coupled online with mass spectrometry (LC-MS) revealed the presence of numerous contaminants[12-15]. Careful and exhaustive epidemiological studies revealed that at least 6 contaminants were identified as being

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case-associated with the onset of EMS[11,16]. These case-associated contaminants were labeled Peaks UV-5, E, 200, C, FF, and AAA, although the specific component(s) of L-Trp that caused the disease has still not been determined[17]. Analyses of these case-associated contaminants resulted in the identification of Peak E as 1,1'-ethylidenebis[tryptophan][18,19] and Peak UV-5 as 3-(phenylamino)alanine (PAA)[20,21]. This latter compound has been linked to contaminated rapeseed oil implicated in the toxic oil syndrome[22], an epidemic whose symptoms resembled that of EMS[23,24]. Peak 200 has been identified as 2-(3-indolylmethyl)-L-tryptophan using both nuclear magnetic resonance (NMR)[13] and tandem mass spectrometry (MS/MS)[15]. We reported the structures of Peak C as 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-[2,3-b]-indole-2-carboxylic acid and Peak FF as 2-(2-hydroxyindoline)-tryptophan[25]. We have also recently elucidated the structure of Peak AAA, and this will be reported elsewhere.

Due to the EMS outbreak, all L-Trp supplements were subsequently banned by the FDA and the consequences of that decision remain in effect today. An alternative to L-Trp is now strongly advocated by the dietary supplement industry; 5-hydroxytryptophan (5-OHTrp) has been promoted as an even better alternative to L-Trp because it is a later intermediate in the serotonin biosynthetic pathway[26,27]. This compound is freely available over-the-counter (OTC) and is being recommended to overcome "serotonin deficiency syndrome" as well as obesity, headaches, and insomnia[26,27]. Although the efficacy of 5-OHTrp in the alleviation of such maladies is still being evaluated, the purity of both synthetic and naturally derived material is unknown. Indeed, an EMS-like case was reported in 1980 when a patient was taking 5-OHTrp and carbidopa[28]. More recently 3 members of a family that were exposed to a 5-OHTrp supplement also manifested EMS symptoms[29]. The mother was exposed to the 5-OHTrp by inhalation and/or by contact with the skin as she prepared the fine powder without taking any special care, whereas 2 infants were exposed through ingestion. The children developed asymptomatic EMS and mild leukocytosis and their mother eosinophilia, dyspnea, and ankle edema. All symptoms either ceased or improved when the treatment or exposure was stopped. Analysis of the case-implicated product by HPLC with ultraviolet (UV) detection revealed the presence of a unique contaminating peak, denoted Peak X, that eluted very close to the parent 5-OHTrp. Peak X was tentatively identified as 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-ß-carboline by its similar retention time to an authentic standard[29]. Given that Peak X was determined to be a case-implicated contaminant of 5-OHTrp, we analyzed 6 commercially available (OTC) preparations of the supplement. We found that all 6 brand-name 5-OHTrp samples contained significant amounts of Peak X[30]. Further, using different HPLC conditions, we found that Peak X was actually a family of components[31]. We describe the structural characterization of one of the main constituents, namely X_1, using a combination of HPLC-electrochemical detection (LC-ECD), accurate mass–LC-MS, and LC-MS/MS. We report the levels of X_1 present in OTC preparations of 5-OHTrp.

MATERIALS AND METHODS

Materials. Six different 5-OHTrp dietary supplements were obtained from drug stores in the Rochester, Minnesota, and New York City areas. The case-implicated samples of 5-OHTrp were kindly provided by Dr. E.M. Sternberg (Clinical Neuroendocrinology Branch, NIH, Bethesda, MD). HPLC grades of acetonitrile and methanol (MeOH) were purchased from EM Science (Gibbstown, NJ, USA) and ultrapure water from Burdick and Jackson (Muskegon, MI, USA). Acetic acid and 5-OHTrp for synthesis of Trp 4,5-dione were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Synthesis and purification of L-tryptophan-4,5-dione (L-Trp-4,5D). L-Trp-4,5D was prepared by oxidation of 5-OHTrp•2H_2O dissolved in water with potassium nitrosodisulfonate [$(KSO_3)_2$•NO] (Fremy's radical). The resultant solution was then purified by preparative reverse phase HPLC, and pure solid L-Trp-4,5D was isolated by lyophilization. The details of this synthesis of L-Trp-4,5D were identical to those employed to synthesize tryptamine-4,5-dione. Further, spectroscopic evidence for the structure of L-Trp-4,5D prepared in this way was identical to that reported for the compound synthesized by electrochemical oxidation of 5-OHTrp[32]. The 7-S-glutathionyl conjugate of L-Trp-4,5-D was prepared as described[33].

Reaction with glutathione. Addition of nucleophilic glutathione (GSH) to L-Trp-4,5D was carried out in 50 mM potassium phosphate buffer, pH 7.4, at room temperature[32]. The reaction was started by the addition of GSH to a 50 µg/µl buffer solution of either case-implicated 5-OHTrp, commercially available 5-OHTrp supplement, or the synthetic L-Trp-4,5D so that the final ratio of GSH:sample was 1:200 (w/w%). After 10 min, the sample solution was centrifuged for 2 min and immediately analyzed by LC-MS or LC-MS/MS as described below.

HPLC-electrochemical Detection (LC-ECD). LC-ECD with the detector set in reductive mode was performed on a BAS 200B instrument (Bioanalytical Systems Inc., West Lafayette, IN, USA) equipped with a Rheodyne 9125 injection valve, 5 µl sample loop, and parallel thin-layer glassy carbon detector electrodes[32]. The electrodes were set at –50 mV and –300 mV, respectively, versus Ag/AgCl and the detector filter was adjusted to 0.05 Hz. The entire system was purged with ultra high purity He gas for 30 min followed by pressurization at 4 psi prior to use. Samples were separated isocratically using a flow rate of 0.9 ml/min and a 100 × 3.2 mm BAS phase II-ODS reverse phase column (particle size 3 µm) equipped with a 15 × 3.2 mm guard column (particle size 7 µm). The mobile phase consisted of 0.085% (v/v) diethylamine, 0.63 mM Na_2EDTA, 0.258 mM sodium octyl sulfate, 100 mM citric acid in deionized water containing 10% acetonitrile. The solvent was filtered through a 0.22 µm type GVHP membrane (Millipore, Bedford, MA, USA) before use. The background current was typically 1.1 to 3.0 nA and 8 to 12 nA when the electrode was set at –50 or –300 mV, respectively. When the background current exceeded 12 nA, the electrodes were resurfaced using the abrasive method recommended by the manufacturer. Samples were dissolved in deionized water to a concentration of 1 µg/µl followed by sonication for 20 min and centrifugation for 2 min to remove insoluble matter.

Accurate mass LC-MS. HPLC separations for accurate mass determinations were performed on a Hewlett-Packard 1090 (Hewlett-Packard, Palo Alto, CA, USA). Separations were carried out on a 150 × 3.2 mm Phenomenex C18 column (Primesphere HC, 5 µm particle size, 110 Å pore size) equipped with a 30 × 3.2 mm Phenomenex C18 guard column (Primesphere HC, 5 µm particle size) at a flow rate of 350 µl/min. Initial gradient conditions were similar to those used by Michelson, *et al*[29]. To enhance the separation of the Peak X family, we optimized the conditions by using 100% solvent A for 35 min followed by a short, steep gradient elution at 9.5% solvent B/min for 10 min. The solvents used consisted of a 1% aqueous acetonitrile containing 1% AcOH (solvent A) and 89% acetonitrile, 10% MeOH containing 1% AcOH (solvent B). The flow was split 1:5 after the UV-flow cell such that approximately 70

µl were introduced into the MS. Accurate mass determinations were performed on a Finnigan MAT 900 at both resolution 3000 and 5000 (10% valley definition) using exponential scans of the electric sector. The electric sector was calibrated using tetrabutyl ammonium cation (242.28478 Da) and the cetyl trimethyl ammonium cation (284.33172 Da) introduced through the liquid sheath inlet port of the ESI source. The mass references were dissolved at a concentration of 500 pg/µl in 50% aqueous isopropanol containing 1% AcOH and delivered at a flow rate of 20 µl/min. To optimize the ion statistics, the ion monitoring time was increased by dividing the analysis into 2 electric scan experiments including, in each experiment, one reference ion as a lock mass. During the first 11 min of the HPLC run, the electric sector was scanned from m/z 218 to 245 at 30 s per mass decade (1.7 s scan cycle). Spectra were calibrated with Finnigan MAT software using the reference mass in each scan as a lock mass. The calibrated scans from the elution of an LC peak were averaged.

LC-MS/MS. These analyses were performed on a PE Sciex API 365 LC-MS/MS system (Toronto, Ontario, Canada). Separations were carried out on a Shimadzu HPLC system consisting of 2 LC-10 AD pumps and an SCL-10 Avp controller (Shimadzu Scientific Instruments, Columbia, MD, USA), and analytes were detected with an ABI 785A programmable UV detector at 254 nm. All samples were separated at a flow rate of 40 µl/min on a 150 × 1 mm Phenomenex C18 equipped with a 30 × 1 mm Phenomenex guard column. Solvent compositions and gradient conditions were as described above for the accurate mass LC-MS analysis. Mass spectra were acquired in positive ion mode at unit resolution in the m/z range scanned. The spray voltage was 5000 V and the orifice was kept at 17 V. For LC-MS and LC-MS/MS analysis, data were acquired in 0.2 amu steps at a scan rate of 5 and 3 s/scan, respectively. MS/MS analysis was performed by selecting the ion of interest in the first quadrupole analyzer followed by collisional induced fragmentation using argon as collision gas and a collision energy of 25 eV in the connecting octapole. Samples were dissolved to 10 µg/µl in water except if otherwise indicated, sonicated for 2 min, and centrifuged before injection. Data treatment was performed with Multiview 1.3 software.

RESULTS

Separation and structural determination of Peak X_1. Previous analysis of case-implicated 5-OHTrp by LC-UV had revealed the presence of a UV response (254 nm), referred to as Peak X, that was not detected in the parallel analyses of other 5-OHTrp samples[29]. The same case-implicated 5-OHTrp sample in our hands also afforded a similar LC-UV chromatogram[30,31]. In both cases Peak X eluted as a shoulder on the major component, 5-OHTrp (data not shown). However, to facilitate the structural characterization of Peak X, we optimized LC separation conditions to resolve the contaminant away from interfering parent compound 5-OHTrp. A simple LC isocratic separation combined with rapid gradient elution of analytes allowed separation of a cluster of contaminants (including X = X_1) from 5-OHTrp by 10–15 min (Figure 1). Further, using the same LC conditions, the LC-MS analysis of case-implicated 5-OHTrp revealed a family of Peak X contaminants labeled X_1, X_2, X_3, and X_4, shown in Figure 2. All these compounds possessed the same protonated molecular weight (MH^+ = 235), and similar LC hydrophobic properties with retention times of ~40–42 min. The approximate relative amounts of X_1, X_2, X_3, X_4 from their corresponding mass spectrometric responses was estimated to be ~100:5:0.1:0.05, respectively. Accurate mass LC-MS determination of the predominant contaminant X_1 (resolution 5000) afforded a mean value for MH^+ = 235.0719 (SD ± 3.7 ppm). This corresponds to an empirical formula of $C_{11}H_{11}N_2O_4$ with 8 degrees of unsaturation. These data indicate that the previous assertion of Peak X being 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-ß-carboline[29] was incorrect.

To further characterize Peak X_1, we subjected it to online LC-MS/MS. The precursor ion, MH^+ = 235, was subjected to collision induced dissociation (CID) followed by detection of the product ions. The resulting product ion spectrum is shown in Figure 3 and contains a product ion at m/z = 217, indicating a facile loss of H_2O –18 amu. This is due to a partial CID-induced cyclization followed by a further loss of –COOH. This latter process results in a product ion at m/z = 171, and this is shown schematically in the insert of Figure 3. Other prominent ions observed include m/z = 189 (loss of HCOOH) and m/z = 162 (loss of ($NH_2[CH_2]$COOH). These data, along with the LC-MS accurate mass data, are consistent with X_1 being the putative neurotoxin Trp-4,5,D[32].

It has been reported that authentic Trp-4,5D readily reacts with glutathione to afford 7-S-glutathionyl adduct[33]. Hence to confirm the identity of Peak X_1, we incubated case-implicated 5-OHTrp with GSH at pH 7.4 for 10 min at room temperature. The LC-MS analysis of this reaction mixture revealed a conjugate product at a retention time of ~45 min with MH^+ = 540 (Figure 4). This also corresponds to the calculated integer mass of GSH (mol wgt 307) plus Peak X_1 (mol wgt 234), minus 2 hydrogens to form the covalent bond of the adduct. This is also consistent with the measured molecular weight of the previously observed adduct of GSH with Trp-4,5D[33]. An LC-MS/MS product ion spectrum of the case-implicated Peak X_1–GSH adduct (MH^+ = 540) is shown in Figure 5. Analogous to the product ion spectrum of Peak X_1 (Figure 3), an ion at m/z = 522 is observed corresponding to facile loss of H_2O upon CID induced cyclization. Similarly, this compound then decarboxylates to afford the product ion at m/z = 478. The base peak in the product ion spectrum at m/z = 172 corresponds to concomitant loss of both GSH and CO_2(H) from the cyclized product (m/z = 522 in Figure 5, insert). The ions marked with asterisks (Figure 5) correspond to free GSH (MH^+ = 308), as well as its y_1 (m/z = 76), y_2 (m/z = 179), z_2 (m/z = 162), and b_2 (m/z = 233) product ions derived from peptide backbone cleavage[34].

All these data, including elemental composition, degree of unsaturation, and product ion spectra, indicate that Peak X_1 is the oxidation product of 5-OHTrp, namely Trp-4,5D. In addition, the rapid and spontaneous nucleophilic addition of GSH suggested that Peak X_1 was a dione of tryptophan, although it was not possible to determine in which position the oxidation had occurred. Recently, Humphries, *et al*[32] reported that enzyme mediated, as well as electrochemical driven, oxidation of 5-OHTrp at physiological pH resulted in the formation of several oxidation products[33]. Since one of these was identified as Trp-4,5D, we attempted to further identify the case-implicated contaminant by comparative LC-ECD using a synthetic Trp-4,5D that was produced by oxidation of L-5-OHTrp

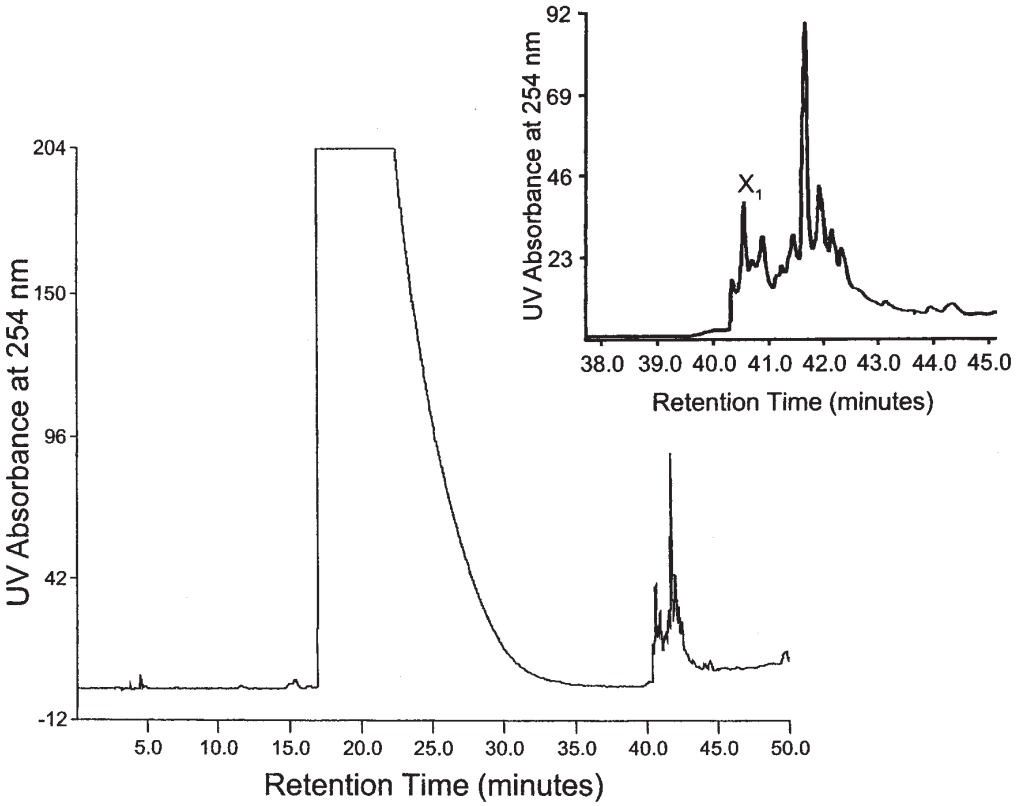

Figure 1. LC-UV chromatogram of case-implicated 5-OHTrp using new optimized conditions for separation: isocratic for 35 min in 100% solvent A (1% aqueous acetonitrile containing 1% AcOH) followed by gradient elution at 9.5% solvent B (89% acetonitrile, 10% MeOH, 1% AcOH)/min for 10 min. The expanded region in which Peak X eluted is shown in the insert labeled X_1.

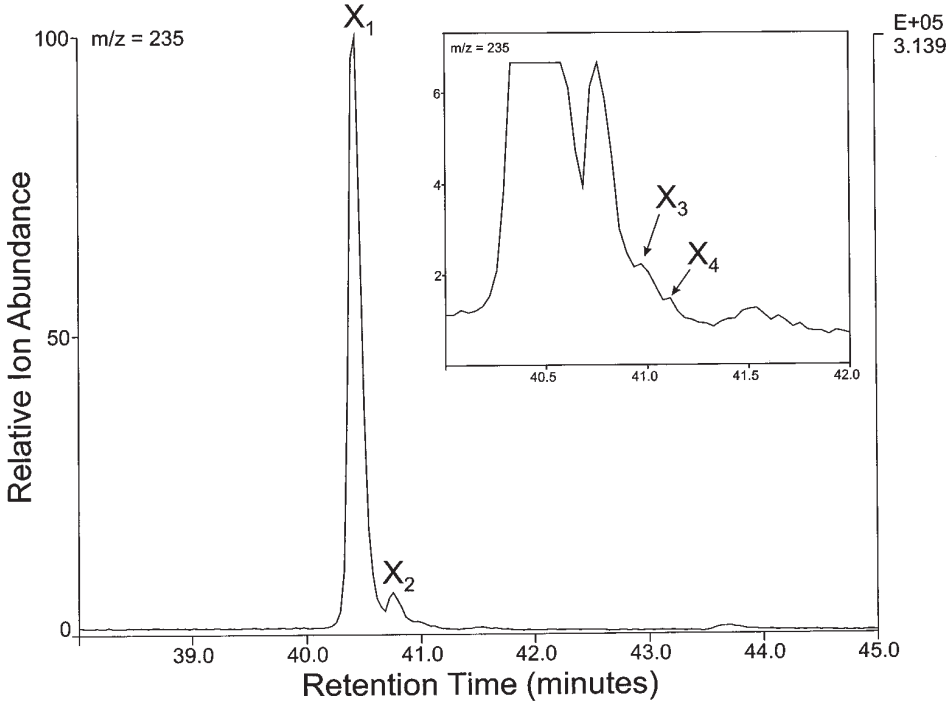

Figure 2. Accurate mass LC-MS analysis of case-implicated 5-OHTrp. Single ion chromatogram of m/z = 235. The same conditions for separation as given for Figure 1 were used and a family of Peak X candidates are observed, X_1, X_2, X_3, X_4.

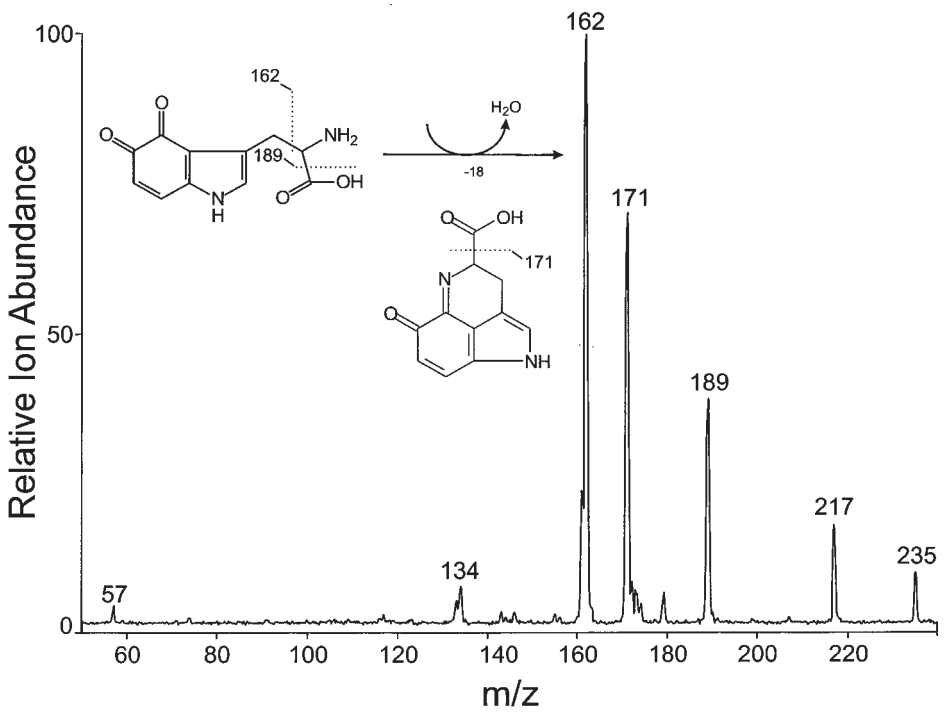

Figure 3. Product ion spectrum of Peak X (m/z 235) from case-implicated 5-OHTrp. Insert shows likely fragmentation pathways to afford product ions detected.

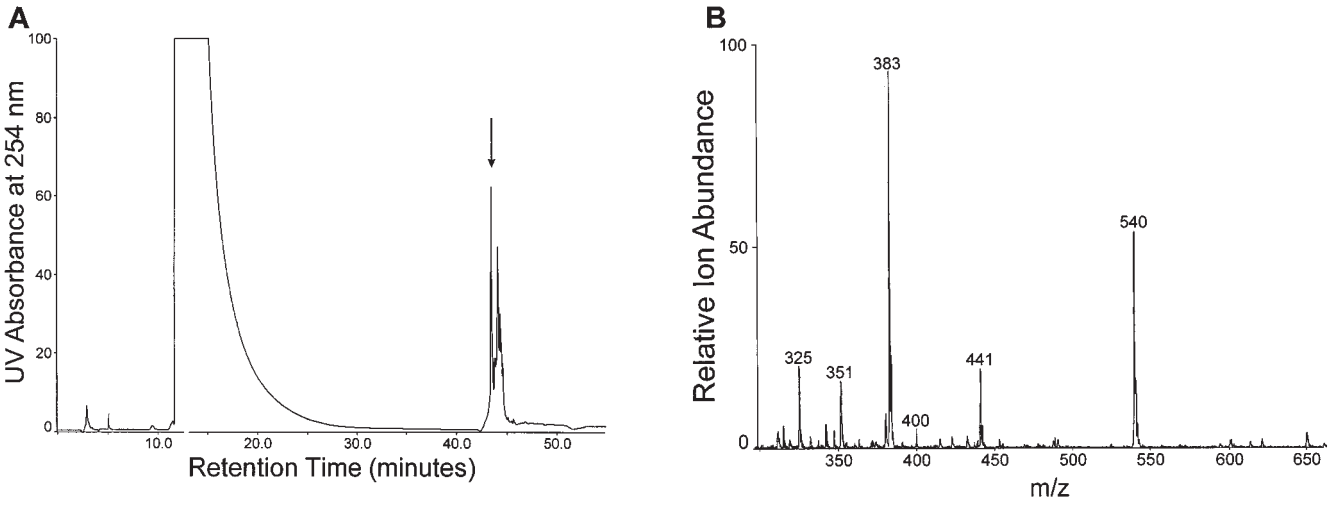

Figure 4. Analysis of case-implicated 5-OHTrp after incubation with GSH. A. LC-UV of the incubation mixture using LC conditions as described for Figure 1. Arrow indicates the peak response shown in Figure 4B. B. LC-MS of the incubation mixture using LC conditions as described for Figure 1. The ion at m/z = 540 corresponds to the GSH-Peak X_1 adduct.

by potassium nitrosodisulfonate as an external standard. By setting the detector in the reductive mode, only compounds that could be reduced at –50 mV were detected. Also, only a very few compounds can be reduced with the LC-ECD conditions utilized. Isocratic LC-ECD analysis of case-implicated 5-OHTrp resulted in the unambiguous detection of an intense peak with a retention time (3.17 min) identical to the external reference compound (data not shown). We could therefore conclude that the contaminant Peak X_1 in case-implicated 5-OHTrp is Trp-4,5D.

Comparison with data obtained for authentic Trp-4,5D. To confirm the identity of Peak X_1, we carried out identical LC-MS and MS/MS analyses on authentic Trp-4,5D and the corresponding glutathione conjugate. The authentic Trp-4,5D preparation was synthesized from electrochemical oxidation of 5-OHTrp followed immediately by preparative purification by HPLC-UV as described[32]. The identity of this preparation of Trp-4,5D has also been verified by NMR[32]. Therefore, we first used this product for evaluation of our accurate mass determination of Peak X_1. Accurate mass LC-MS of the

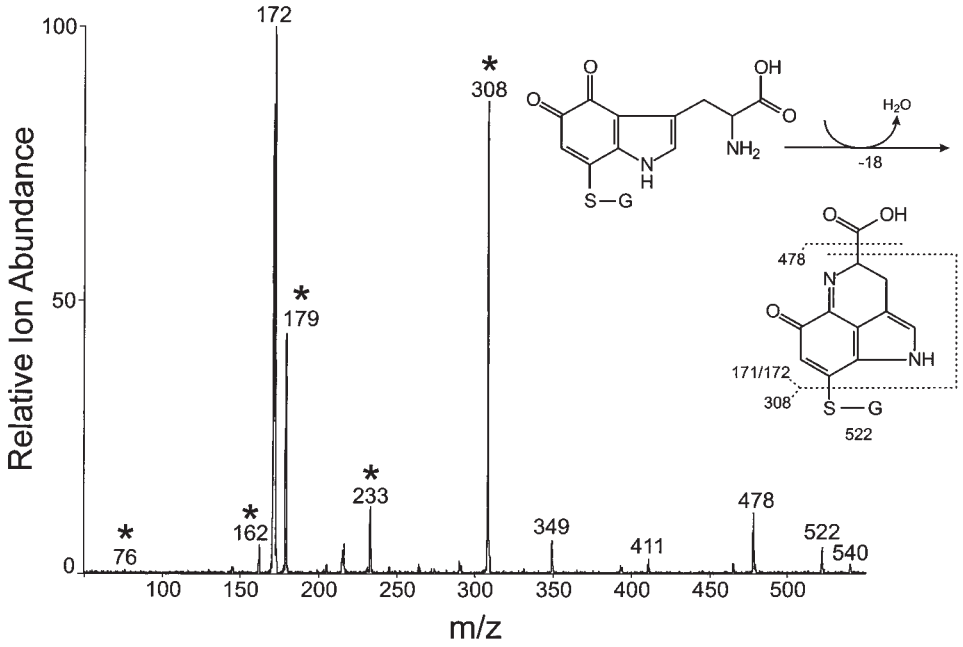

Figure 5. Product ion spectrum of the spontaneously formed glutathione-Peak X conjugate after LC-MS/MS. Glutathione-specific fragment ions are indicated with an asterisk. Insert shows likely fragmentation pathways to afford product ions detected.

authentic Trp-4,5D revealed an identical absorbency maximum of 354 nm, a retention time and a mass of 235.0714 (STD = 3.3 ppm) for the $[M+H]^+$ ion when compared to Peak X_1. Further, LC-MS/MS analysis of the Trp-4,5D and the conjugate derived after reaction with GSH revealed in both cases indistinguishable product ion spectral data, including product ion intensities, compared to the results obtained for Peak X_1 (data not shown). Therefore, based on all the results presented here it can be concluded that Peak X_1 identified in the case-implicated 5-OHTrp supplement is a Trp-4,5D.

Confirmation of presence of Trp-4,5D in commercial preparations of 5-OHTrp. We have reported that Peak X was present in 6 commercially available preparations of 5-OHTrp[30]. However, given that we have separated Peak X into a family of isobaric compounds (MH^+ = 235), it was important to determine the actual amount of Peak X_1, Trp-4,5D, in such preparations. These samples were subjected to accurate mass LC-MS measurements. Among the samples, all 6 products were found to contain detectable amounts of Peak X_1 ranging from 0.5% up to 10.3% of the amount detected in the case-implicated 5-OHTrp batch, as estimated from the relative intensity of the mass spectrometric response. The presence of Trp-4,5D in all of the chosen commercial preparations was confirmed by LC-ECD (data not shown).

DISCUSSION

Following the outbreak of EMS in 1989, several compounds that were present in case-implicated Trp were identified using a combination of LC-UV, LC-MS, and LC-MS/MS techniques[33,34]. In the present investigation we took advantage of these techniques in combination with LC-ECD and accurate mass measurements for the unambiguous characterization of Peak X_1 recently identified in a disease implicated 5-OHTrp sample. Several lines of evidence suggested that Peak X_1 is not 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-ß-carboline, as first proposed, but Trp-4,5D instead. First, the measured accurate mass of the $[M+H]^+$ ion is within 3.7 ppm of the calculated mass of protonated Trp-4,5D. Second, the UV profile in the range 200–400 nm, the retention time, and the fragmentation pattern for Peak X_1 as well as GSH conjugate were all indistinguishable compared to the data obtained for a synthesized authentic Trp-4,5D standard. These data are further supported by the use of optimized HPLC separation conditions in conjunction with electrochemical detection.

Also, using the LC-ECD conditions, Trp-4,5D was found in all 6 commercial preparations of 5-OHTrp in relative amounts ranging from 0.5% to 10% of the amount found in the case-implicated samples. This gives rise to some concern, because, although typical doses recommended are on the order of 10–50 mg of 5-OHTrp/day, doses as high as 300–500 mg/day have recently been suggested for the alleviation of various ailments[27]. The ingestion of such doses can, consequently, bring the total amount of Peak X_1 consumed into the range of the case-implicated sample. Although the direct toxicity of Trp-4,5D is unknown at present, the lethal effects of the corresponding GSH conjugate 7-glutathionyl-Trp-4,5D have recently been documented[5,12]. This study demonstrated that the behavioral response and LD50 of 7-glutathionyl-Trp-4,5D in mice was similar to the one evoked after treatment with the analogous conjugate 7-glutathionyl-tryptamine-4,5-

dione. Other studies have suggested that if the cysteine or GSH conjugates of compounds like Trp-4,5D and tryptamine-4,5-dione are formed *in vivo*, they may play a significant role in the development of the neurodegenerative process that occurs in the brain of patients with Alzheimer's disease[4,12]. Based on such indications, concerns arise about the possible longterm side effects of ingesting large amounts of 5-OHTrp and, perhaps more important, the consequences of continuously ingesting low amounts of Trp-4,5D.

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