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Investigation of the Metabolism of Ergot Alkaloids in Cell Culture by Fourier Transformation Mass Spectrometry

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ABSTRACT: Ergot alkaloids are known toxic secondary metabolites of the fungus *Claviceps purpurea* occurring in various grains, especially rye products. The liver is responsible for converting the ergot alkaloids into metabolites; however, the toxic impact of these end products of metabolism is still unknown. The aim of this study was to analyze the metabolism of ergot alkaloids in colon and liver cell lines (HT-29, HepG2), as well as in human primary renal cells (RPTEC). It was shown that cells in vitro are able to metabolize ergot alkaloids, forming a variety of metabolic compounds. Significant differences between the used cell types could be identified, and a suitable model system was established using HT-29 cells, performing an intensive metabolism to hydroxylated metabolites. The formed substances were analyzed by coupling of high-performance liquid chromatography with fluorescence detection and Fourier transformation mass spectrometry (HPLC-FLD-FTMS) as a powerful tool to identify known and unknown metabolites.

KEYWORDS: ergot alkaloids, metabolism, FTMS, cell lines, HT-29, HepG2, primary cells

INTRODUCTION

Toxicologically relevant food constituents as well as pharmaceuticals undergo metabolism in the human body. Ergot alkaloids are known to have both properties; for example, ergotamine is a drug used for the treatment of migraine[1] as well as to induce toxic effects.[2] Based on the structure of ergot alkaloids, several different drugs were developed to benefit from their effects in the human body.[3] The ergot alkaloids play an important role as potential drugs due to their structural similarity to corresponding neurotransmitters such as dopamine or serotonin.[4] Structural differences of ergot alkaloids have led to a deviation into different groups. According to the European Food Safety Authority (EFSA) six of them are found predominantly in food and feed samples, namely, ergotamine, ergocornine, ergocryptine, ergocristine, and ergosine, belonging to the group of peptide ergot alkaloids, and ergometrine, a lysergic acid amide.[5] For both pharmacologically and toxically relevant compounds, metabolism in the human body plays an important role in terms of activation/deactivation or detoxification. Many metabolites, for example, hydroxylation products, are more water-soluble and easily excreted from the body, but it is also well-known that some of these metabolites are more toxic due to their activation during phase I metabolism.[6] Therefore, it is important to elucidate the metabolism of xenobiotics and in consequence perform studies concerning the toxic relevance of these metabolic products. Metabolism of ergot alkaloids has been reported to occur mainly in the liver.[7] Data concerning metabolism therefore focus on the use of liver microsomes.[8−10] Additional experiments with radiolabeled substances revealed a main excretion in bile and only small amounts in urine.[11] Phase II metabolites such as glucuronides have been discovered in such experiments. Using liver microsomes from different species 8′-hydroxydihydroergotamine was identified as the main metabolite.[8] The main conversion seems to occur in the peptide moiety as the first step, resulting in phase II reactions that resemble glucuronidations.[12] The key step of hydroxylation could also result in the formation of carboxylic acid products.[8] Besides this, it is known that metabolism occurs in both isomeric forms of ergot alkaloids: lysergic 8-(*R*) (-ine) and isolysergic 8-(*S*) (-inine) form (Figure 1), as they are known to easily epimerize.[13] These two epimers are also reported to differ in their biological activities.[14]

In this study we compare a human colon carcinoma cell line (HT-29), a human hepatocellular liver carcinoma cell line (HepG2), and human primary cells (renal proximal tubule epithelial cells, RPTEC) for their ability to metabolize ergot alkaloids. The human hepatocellular liver carcinoma cell line, HepG2, seems to be a very effective model to determine metabolites of ergot alkaloids in cell culture, because they are rapidly metabolized in the liver[12] or by microsomes.[8,9] Additionally, HT-29 cells represent also a good target, because ergot alkaloids are reported to be transported across the gastrointestinal tract.[15] Primary kidney cells were also investigated, because they were previously reported to show a rapid uptake of peptide ergot alkaloids.[16] Three model compounds were used: ergometrine (**1**), ergotamine (**3**), and ergocristine (**5**), all in equilibrium with their corresponding 8-(*S*) isomers (**2**/**4**/**6**). Ergometrine was used as a model compound for lysergic acid amides, and ergotamine and ergocristine were used as model compounds for peptide ergot alkaloids. The occurrence of these two peptide alkaloids appeared to be relatively constant with nearly 60% of the alkaloid content in sclerotia and are therefore quantitatively important substances.[17] A very powerful tool to identify the metabolites, which were formed in only very small amounts, is the coupling of high-performance liquid chromatography with fluorescence detection and Fourier transformation mass spectrometry (HPLC-FLD-FTMS). With exact mass measurements, fragmentation experiments, and comparison to literature

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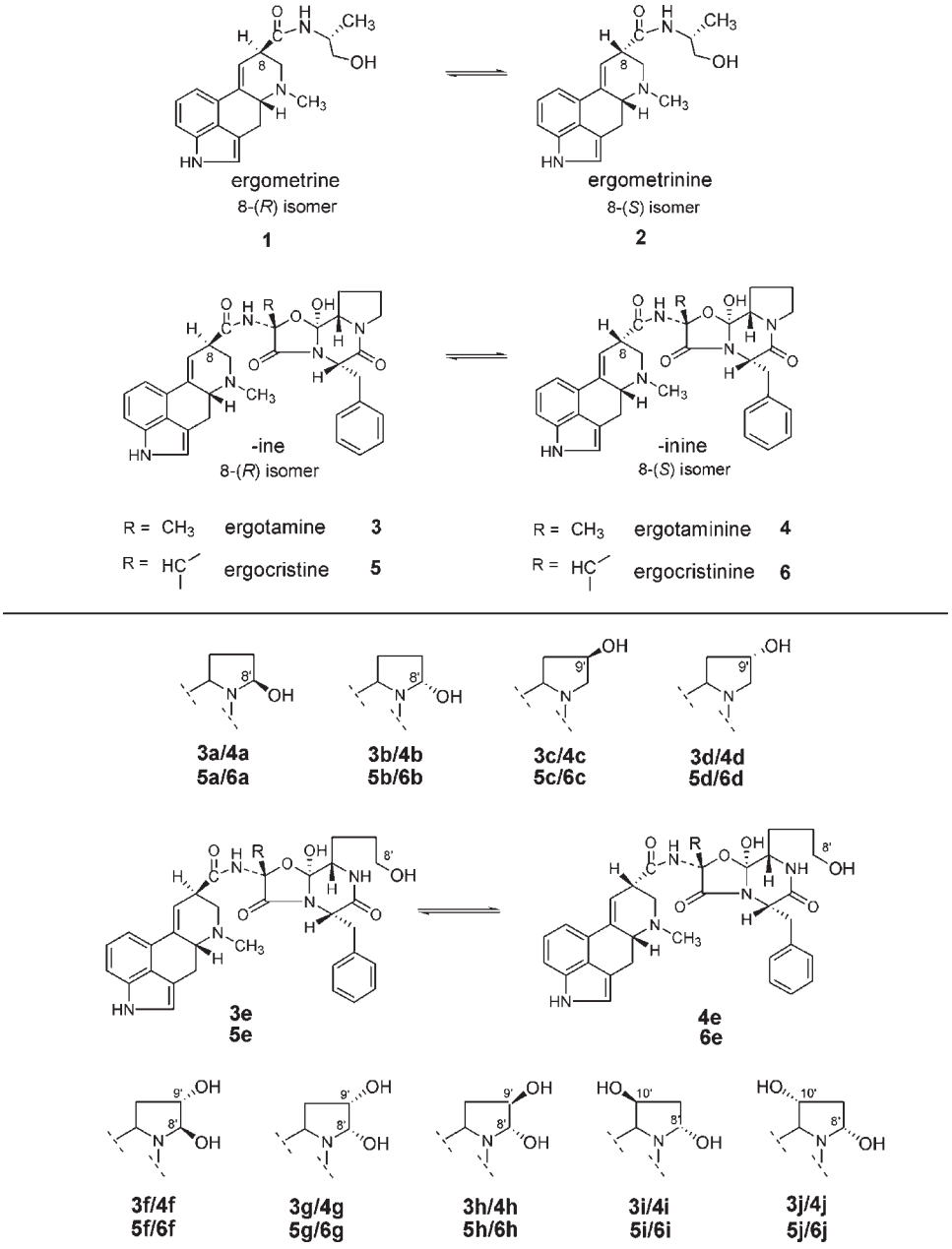

Figure 1. Chemical structures of ergometrine/ergometrinine (**1**/**2**) and peptide ergot alkaloids: ergotamine/ergotaminine (**3**/**4**) and ergocristine/ergocristinine (**5**/**6**). Metabolites are marked as subunits **a**−**j**, with **3**−**6a**−**d** and **3**−**6f**−**j** as known hydroxy and dihydroxy metabolites from the literature and **3**−**6e** as unknown metabolites with suggested structure.

data, the structure elucidation can be carried out, without the use of NMR technique, requiring several milligrams of compounds.

MATERIALS AND METHODS

Chemicals. Standard substances of ergot alkaloids, ergotamine tartrate, and ergometrine maleate were obtained from Sigma-Aldrich (Steinheim, Germany). Ergocristine, ergocristinine, ergotaminine, and ergometrinine were purchased from Alfarma (Černošice, Czech Republic). Internal standard methysergide maleate was ordered from Biotrend (Wangen, Switzerland).

Cultivation medium (DMEM, DMEM/Ham's F-12) and corresponding supplements were obtained from PAA Laboratories (Pasching, Austria), and fetal calf serum (FCS) was from Biochrom AG (Berlin, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma-Aldrich. Purified water was generated by a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany).

Cell Culture. The human colon carcinoma cell line HT-29 and the human hepatocellular liver carcinoma cell line HepG2 (both DSMZ, Braunschweig, Germany) were both cultivated in DMEM, with the following additives: 1% nonessential amino acids, 1% penicillin/streptomycin/glutamine, and 10% FCS. The cells were subcultured after reaching 90% of confluency twice a week with a ratio of 1:10. RPTEC were purchased from Lonza Group AG (Basel, Switzerland) and were cultivated in DMEM/Ham's F-12 with various supplements: 15 mM HEPES buffer solution, 10 μg/L epidermal growth factor (EGF), 5 mg/L apotransferrin, 5 mg/L bovine insulin, 0.5 mg/L hydrocortisone,

5 μg/L sodium selenite, 6.5 μg/L thyroxin, 0.5 mg/L epinephrine, and 1% FCS. The medium was changed twice a week, and the cells were subcultivated every 14−17 days at a ratio of 1:3.

All experiments using ergot alkaloids were performed using DMEM/Ham's F-12 with the addition of 15 mM HEPES as reported previously.[16] The reported calculated correction factors, taking the stability of the two isomers into account, were also used in this study to correct the concentration values of the used ergot alkaloids.[16] Ergot alkaloids were applied from a stock solution (2 mM in ethanol/0.25 g/L tartaric acid (40:60; v/v)) in serum-free medium under red light to exclude any isomerization. During incubation and cultivation steps the cell lines HT-29 and HepG2 were maintained at 37 °C with 8.5% CO_2 atmosphere and RPTEC at 37 °C with 5% CO_2 atmosphere.

Metabolism and Uptake Studies of Ergot Alkaloids. For the identification of ergot alkaloid metabolites 1×10^6 cells were seeded in 100 mm cell culture dishes with at least 10 mL of medium. After 24 h, 5 μM ergotamine/ergotaminine (**3**/**4**) or ergometrine/ergometrinine (**1**/**2**) or 1 μM ergocristine/ergocristinine (**5**/**6**) equilibrium mixture[16] was applied. After 72 h, the medium was removed and centrifuged (10 min, 10000*g*, 4 °C). The supernatants of four dishes were freeze-dried, and the residue was dissolved in 2 mL of acetonitrile/ammonium carbamate buffer (0.2 g/L) (30:70 (v/v)). After an additional centrifugation step (10 min, 10000*g*, 4 °C), the clear supernatant was directly used for HPLC-FLD-FTMS experiments. Remaining cells on the culture dishes were washed twice with phosphate-buffered saline (PBS) and were incubated for 15 min with 1% Triton X-100 solution at 4 °C. The cell lysates of four culture dishes were combined and treated in the same way as the medium to obtain a suitable solution for HPLC-FLD-FTMS analysis. Control samples were substances incubated in only lysis buffer or cell medium, without cells, to exclude chemical degradation.

Relative Response Comparison of Ergot Alkaloid Uptake and Metabolite Formation. For comparison of ergot alkaloids and metabolites 1×10^5 cells were seeded on 24-well plates with at least 300 μL of medium. On the next day 1 μM of the chosen ergot alkaloid was applied, and samples were taken after several time points (5, 10, 20, and 40 min; 1, 2, 4, 6, 8, 24, 48, and 72 h). The samples were collected by removing the cell medium, washing the cells twice with PBS, and performing a cell lysis with 200 μL of 1% Triton X-100 solution. After centrifugation (10 min, 10000*g*, 4 °C), 100−150 μL of the supernatant was mixed with mobile phase (acetonitrile/ammonium carbamate buffer) and measured with high-performance liquid chromatography and fluorescence detection (HPLC-FLD).

Apparatus. *HPLC-FLD Analysis.* For ergot alkaloid comparison the collected samples were separated on a 250 mm × 4.6 mm i.d., 4.6 μm, Varian OmniSpher C18 column (Darmstadt, Germany). The HPLC system consists of a binary pump (Merck-Hitachi L-7100, Tokyo, Japan), an autosampler (Merck-Hitachi AS-2000A), and a fluorescence detector (Merck-Hitachi FLD F-1050). The mobile phase combined acetonitrile (solvent A) and ammonium carbamate buffer 0.2 g/L (solvent B), and the injection volume was 20 μL.

Ergotamine/ergotaminine (**3**/**4**) were separated using a gradient starting at 50% A for 5.5 min increasing to 80% A in 2.5 min and holding this conditions for 2 min. The system was equilibrated again for 4 min with a total run time of 14 min. Ergocristine/ergocristinine (**5**/**6**) were separated similarly to ergotamine/ergotaminine with the exception of starting conditions consisting of 60% A. Ergometrine/ergometrinine (**1**/**2**) were separated using a gradient starting at 27% A. After 2.5 min, the gradient was enhanced to 32% A and maintained for 2 min. After this, the system was equilibrated to the starting conditions for 8.5 min with a total run time of 13 min. The flow rate in all used methods was 1 mL/min, and the substances were detected using fluorescence detection with an excitation wavelength of 330 nm and an emission wavelength of 415 nm. Data analysis was performed using the Merck-Hitachi D-7000 HSM HPLC System Manager. For a comparison, the peak areas of the incubated ergot alkaloids were compared to each other. As a limit of detection a signal-to-noise ratio of 1:3 was used in our experiments. All results referring to uptake in cells were additionally corrected with the cell number and cell volume (measured with CASY model TT (Innovatis AG, Reutlingen, Germany) for every chosen time point). For this at least two different wells were incubated with substance, and instead of screening for ergot alkaloids with HPLC-FLD, the remaining cells were measured with the CASY system.

HPLC-FLD-FTMS Analysis. Analysis of metabolites was carried out using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in positive electrospray ionization mode (ESI^+). Separation of the substances was performed on a 150 mm × 3 mm i.d., 5 μm, Phenomenex Gemini C18 column (Aschaffenburg, Germany) using a gradient consisting of acetonitrile (solvent A) and ammonium carbamate buffer 0.2 g/L (solvent B). The gradient started with 35% A, increased to 60% after 18 min and to 70% after an additional 4 min, and was maintained for 8 min and reequilibrated to 35% in 5 min with a total run time of 35 min. The alkaloids as well as their metabolites were detected by using the FLD-FTMS technique with 10 μL sample injection volume at a flow rate of 0.3 mL/min. The excitation and emission wavelengths were used as described in the previous section. Adjustments of FTMS parameters were as follows: capillary temperature, 275 °C; capillary voltage, 35.00 V; tube lens voltage, 120 V; AGC target setting full MS, 3.0×10^4; multipole 00 offset, −0.75 V; lens 0 voltage, −0.5 V; multipole 0 offset, −4.5 V; lens 1 voltage, −10 V; gate lens voltage, −10.0 V; multipole 1 offset, −4.5 V; front lens, −4.5 V. Product ion spectra with higher energy collision-induced dissociation (HCD) were recorded at an energy level of 30 or 40%. Data analysis was performed using Xcalibur 2.0.7 SP1 (Thermo Fisher Scientific). The fragment ions at m/z 208 and 223 are characteristic of ergot alkaloids and were used for identification.[18]

Statistical Analysis. All results are presented as the mean value ± SD. All comparisons of peak area results were performed with at least two individual experiments in three different passages.

RESULTS AND DISCUSSION

Metabolism of Ergot Alkaloids. To compare uptake properties and metabolic activity, a relative response comparison was performed for the three used cell types. An equal amount of lysate of each cell type was injected into the HPLC-FLD system after 72 h of incubation with **5**/**6** (Figure 2). On the basis of former results,[16] this peptide ergot alkaloid already has shown a high accumulation rate in RPTEC, consequently appearing to be suitable for a comparison between different cell types. As presented, all of the used cells confirm the high cellular uptake of **5**/**6**. In contrast to RPTEC (Figure 2A), additional peaks were obtained for HepG2 and HT-29 cell lines (Figure 2B,C). These metabolites are marked I−VII and were classified as metabolites from **5** and **6** as already reported in the literature for **3** and **4**.[8,9] It has to be highlighted that only traces of the metabolites of **6** (8-(*S*) isomer) were formed in HepG2 cells (Figure 2B), whereas much higher concentrations were detectable in HT-29 cells (Figure 2C). In contrast to the two carcinoma cell lines, RPTEC were not able to metabolize **5**/**6** (Figure 2A), although literature data demonstrating the metabolic capacity of RPTEC are available.[19] For a better comparison between the two cell lines the cellular concentrations of **6** and metabolite peaks I−III are displayed in Figure 3A for HepG2 and in Figure 3B for HT-29 cells. Only the metabolite peaks I−III were taken into account for a comparison because they occur in a detectable range. All values were corrected by cell number and cell volume to give a reliable comparison. Overall, for uptake studies with **6**, a very

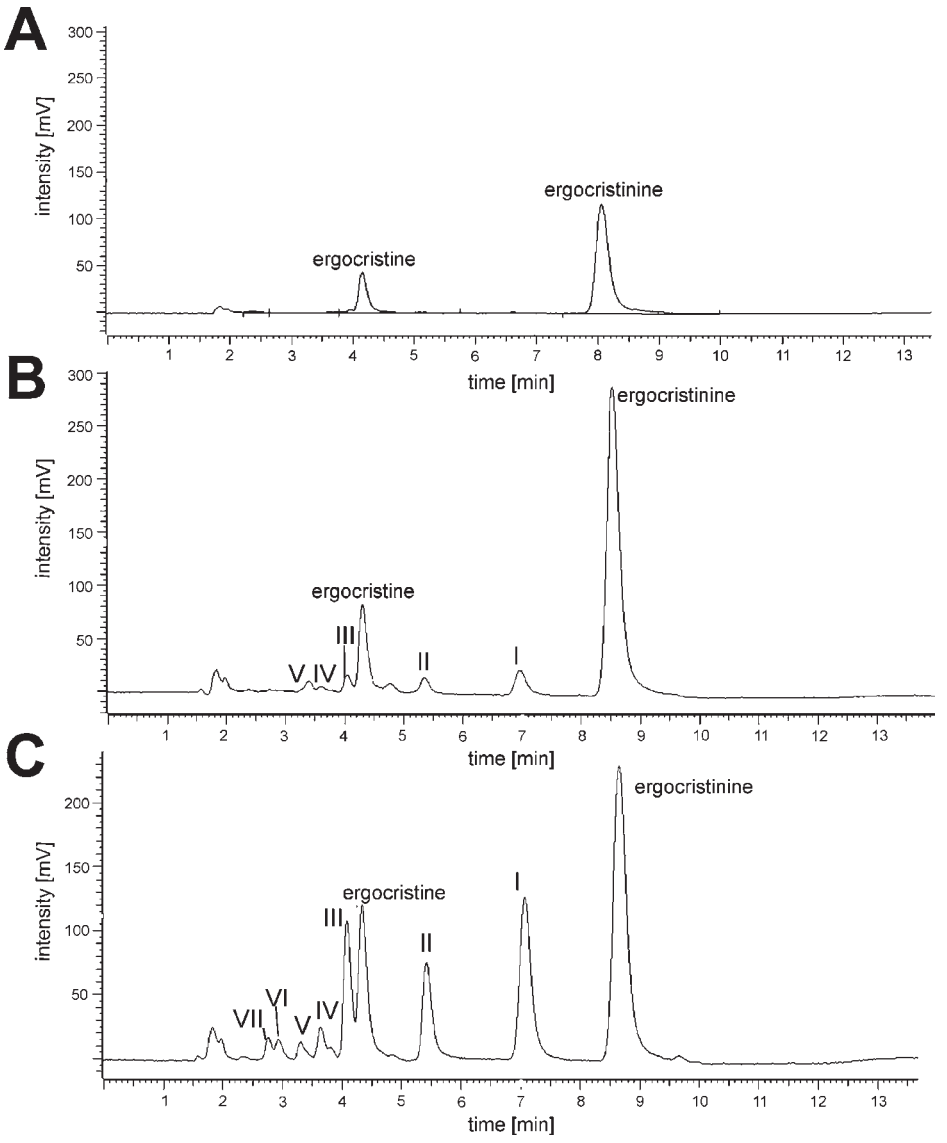

Figure 2. HPLC-FLD chromatograms of cell lysates after incubation with 1 μM ergocristine/ergocristinine (**5**/**6**). Equal amounts of cell lysates are measured after 72 h of incubation of RPTEC (A), HepG2 (B), and HT-29 (C). Incubating substances are indicated, and metabolite peaks are marked I−VII.

similar picture occurred compared to the bioavailability of other known peptide ergot alkaloids.[16,20−22] Ergot alkaloids and metabolites accumulate in the cells after only a few hours and are then slowly excreted. It has to be highlighted that even after a single toxin exposure for 72 h the intracellular concentration of **6** does not return to zero again. This characteristic curve also occurs for blood levels of ergot alkaloids,[21] as well as in primary cells.[16] In addition to these findings, a case study describes the formation of a toxin depot. A patient suffering from ergotism showed an ergotamine plasma level in the range of 10 ng/mL even 3 months after eliminating of every ergot alkaloid source.[23] Consequently, even if the oral bioavailability is low,[24] peptide ergot alkaloids may be able to form a depot in human cells, inflicting chronic toxic effects due to accumulation. All three cell types evaluated, independent of tissue origin or cell type, were able to take up **3**/**4** and **5**/**6** and accumulate them. Cellular uptake of ergot alkaloids results in an additional metabolic step in the two used cell lines. As displayed in the enlarged sections of Figure 3, all resulting metabolites increase over time. Comparing the peak areas shows that the concentrations of formed metabolites differ by >10-fold between the two cell lines used. On the basis of these uptake studies HT-29 cells were chosen as a model cell line to elucidate the structures of the formed metabolites. To achieve a suitable separation of the unknown metabolites shown in Figure 2 the chromatographic conditions were modified (see HPLC-FLD-FTMS). The obtained HPLC-FLD-FTMS chromatograms with the baseline-separated unknown peaks I−VII and I′−VII′ derived from **5**/**6** and **3**/**4** are shown in Figure 4. In comparison with a control (only ergot alkaloids, incubated in lysis buffer without cells) the unknown peaks could be characterized as metabolites, because they only occur during co-incubation with cells and ergot alkaloids. Although a variety of methods for HPLC-MS/MS are available in the literature,[25] the amounts of formed metabolites were not detectable with routine methods. Only with the coupling of fluorescence detection in addition to FTMS (HPLC-FLD-FTMS) were we able to obtain a

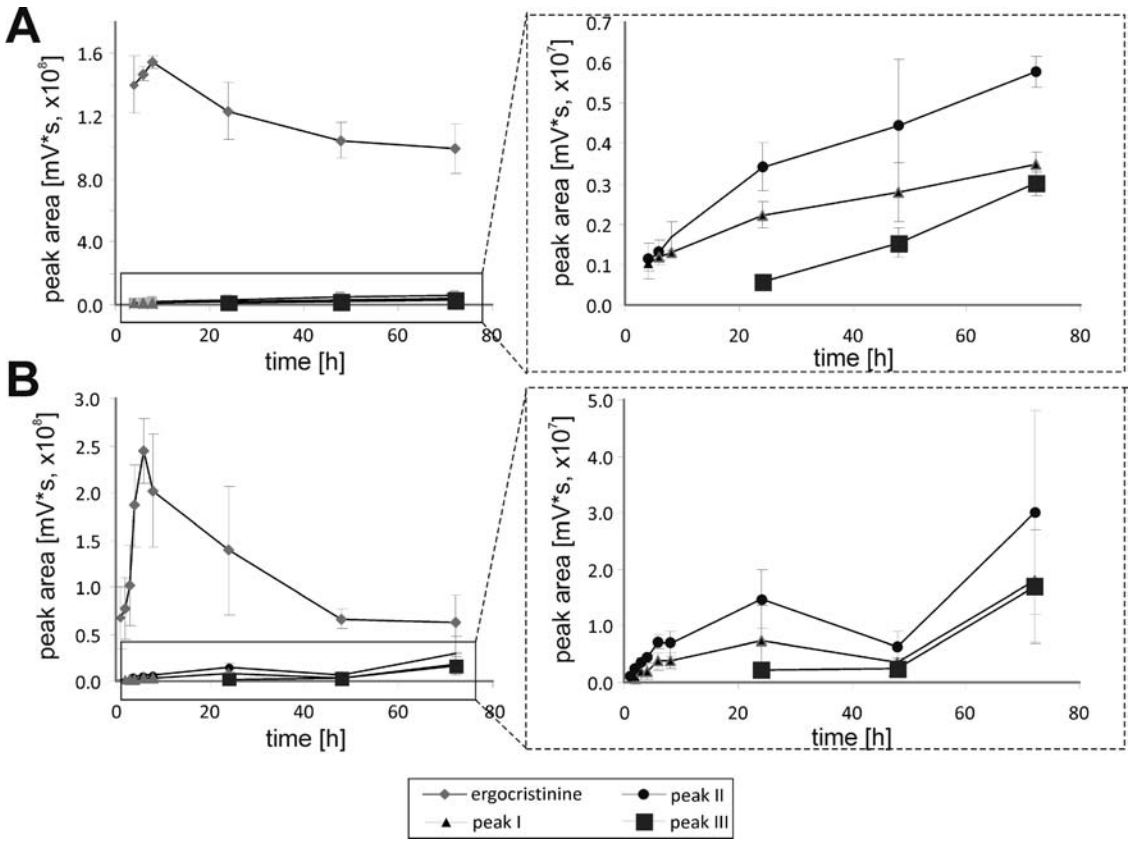

Figure 3. Time-dependent peak area comparison of ergocristinine (**6**) and formed metabolites (peaks I−III) in cell lysate over 72 h (mean ± SD, n = 6): (A) HepG2 cell lysate and (B) HT-29 cell lysate with individual enlargement of metabolites displaying section.

characteristic pattern in the chromatogram for the incubation of **3**/**4** and **5**/**6** (Figure 4). The metabolite peaks are marked I′−VII′ for the incubation of **3**/**4** and I−VII for the incubation with **5**/**6**. Previous papers have identified a similar peak pattern of metabolites.[9,10] We were able to confirm these results by comparison of the exact masses of the formed peaks, occurring in both cell lysate and cell medium of HT-29 cells. The identification was done referring to the characteristic fragments of ergot alkaloids (see HPLC-FLD-FTMS). Figure 4 represents also the extracted ion chromatograms for the newly formed metabolites. In all experiments only **3**/**4** and **5**/**6** had shown an uptake into the cells. As a lysergic acid amide, ergometrine/ergometrinine (**1**/**2**) were not detectable in the cell lysate, and consequently no metabolites could be detected in cell medium or cell lysate, which was also confirmed by literature data.[26] The concentration of **1**/**2** in the incubation medium did not change during the incubation period.

To elucidate the structure of the metabolites formed after peptide ergot alkaloid incubation, detailed FTMS studies were performed using HT-29 cell lysates.

Mass Spectrometric Studies with Metabolite Peaks I′, II′, IV′, and V′ and I, II, IV, and V. Structure elucidation was performed by comparing the mass spectra of the metabolites with the original incubated substances. The ergotamine/ergotaminine (**3**/**4**) metabolite peaks I′, II′, IV′, and V′ resulted in characteristic $[M + H]^+$ signals (±deviation in ppm), which are 16 Da higher compared to **3**/**4**: peak I′, 598.2636 ± 4.01; peak II′, 598.2659 ± 0.13; peak IV′, 598.2650 ± 1.63; peak V′, 598.2649 ± 1.97 (Figure 4A). This leads to the molecular formula of $C_{33}H_{36}O_6N_5$ for $[M + H]^+$ in contrast to the formula of $C_{33}H_{36}O_5N_5$ for **3**/**4**. For ergocristine/ergocristinine (**5**/**6**) analogues signals 16 Da higher were also found: peak I, 626.2963 ± 1.47; peak II, 626.2659 ± 0.13; peak IV, 626.2962 ± 1.68; peak V, 626.2957 ± 2.54 (Figure 4B). This leads to a molecular formula of $C_{35}H_{40}O_6N_5$ in contrast to **5**/**6** ($C_{35}H_{40}O_5N_5$). The characteristic mass difference of 16 Da for metabolite peaks I (I′), II (II′), IV (IV′), and V (V′) gave the first hint for an additional hydroxy group, introduced by phase I metabolism, at different positions of the molecules. For structure elucidation, product ion spectra with higher energy collision-induced dissociation (HCD) were monitored. Panels B.1 and B.2 of Figure 5 show as examples the product ion spectra of metabolites II′ (II) derived from **4** and **6** in comparison to the original substances (Figure 5A.1,A.2). The following fragment ions were obtained for peak II′ (m/z (%)): 208.0755 (42), 223.1228 (100), 268.1442 (47), 313.1180 (14), 562.2435 (6), 580.2544 (12), 598.2649 (16) (Figure 5B.1). For metabolite II the following fragment ions were observed (m/z (%) HCD 30%): 208.0758 (10), 223.1231 (100), 268.1445 (33), 305.1286 (85), 341.1497 (30), 590.2806 (5), 608.2873 (8), 626.2957 (25) (Figure 5B.2) (data for the other metabolites are not shown due to similar fragmentation patterns).

By comparison of these data to already published data[10] analogue results were observed. The hydroxylated products formed by liver microsomes[10] seem to be also formed under cell culture conditions using HT-29 cells. Comparison of the product ion spectra of our measurements clearly showed that the hydroxylation resulting in all of the discussed metabolite peaks in this section occurred in the peptide moiety of the molecule.

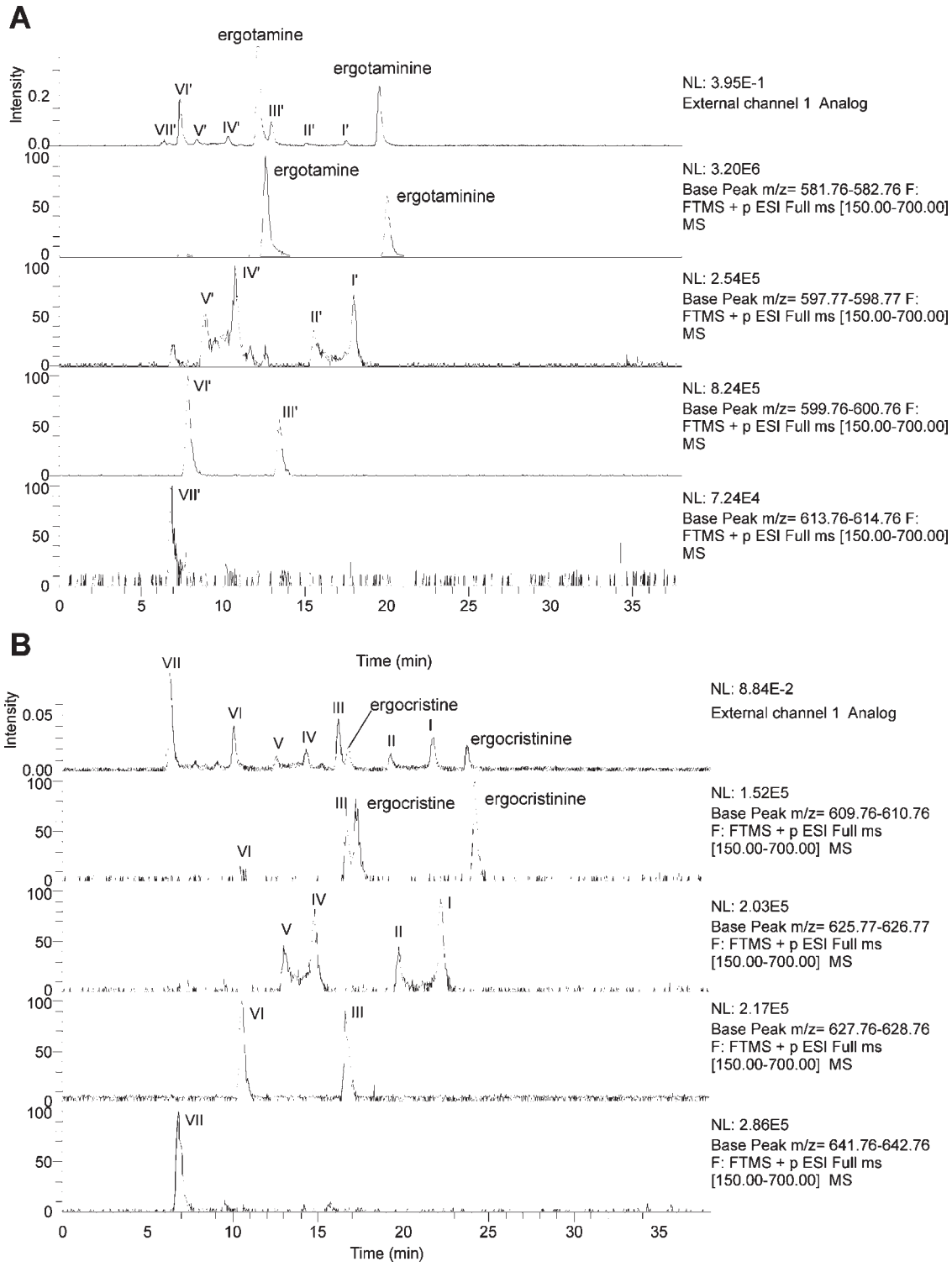

Figure 4. (A) HPLC-FLD-FTMS chromatogram of cell lysate of HT-29 cells after 72 h of incubation with ergotamine/ergotaminine (**3**/**4**). External channel (FLD) indicates the incubating substances and the metabolite peaks I′−VII′. Extracted ion chromatograms of individual peaks are displayed below, with $[M + H]^+$: *m*/*z* 582.27 for **3**/**4**; *m*/*z* 598.27 for peaks I′, II′, IV′, and V′; *m*/*z* 600.28 for peaks III′ and VI′; and *m*/*z* 614.26 for peak VII′. (B) HPLC-FLD-FTMS chromatogram of cell lysate of HT-29 cells after 72 h of incubation with ergocristine/ergocristinine (**5**/**6**). Resulting peaks I−VII are displayed in an extracted ion chromatogram with $[M + H]^+$: *m*/*z* 610.27 for **5**/**6**, additionally showing amounts of metabolite peaks III and VI in this chromatogram; *m*/*z* 626.27 for peaks I, II, IV, and V; *m*/*z* 628.28 for peaks III and VI; and *m*/*z* 642.26 for peak VII.

Suggested fragmentation patterns in the literature indicated the peptide moiety with a characteristic fragment ion at *m*/*z* 297.1232 (Figure 5A.1) for **3**/**4**.[27] This characteristic signal was missing in the formed metabolites, but a 16 Da higher fragment ion at *m*/*z* 313.1180 was observed (Figure 5B.1). Similar data were obtained for the **5**/**6** metabolites with a fragment ion at *m*/*z* 341.1497

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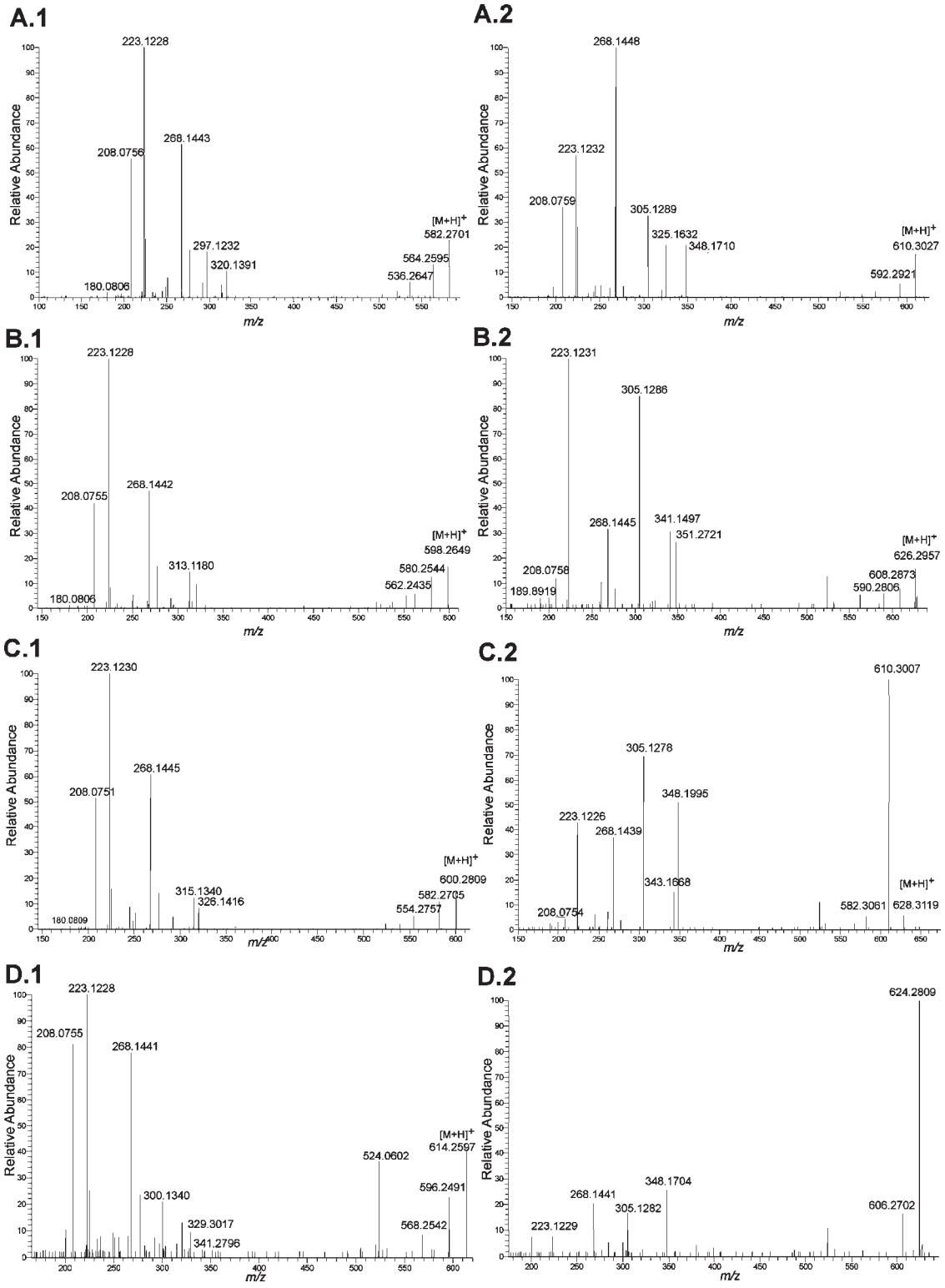

Figure 5. ESI-FTMS product ion spectra of ergotaminine (**3**) (A.1), metabolite peak II′ (B.1), metabolite peak VI′ (C.1), metabolite peak VII′ (D.1), ergocristinine (**5**) (A.2), metabolite peak II (B.2), metabolite peak VI (C.2), and metabolite peak VII (D.2) formed after incubation with HT-29 cells for 72 h (fragmentation energy HCD = 30%).

(Figure 5B.2), which is 16 Da higher than the characteristic fragment at m/z 325.1632 for the peptide moiety (Figure 5A.2). This is in agreement with previous studies,[7,8] in which it was concluded from ^{1}H NMR experiments that the hydroxy group is located in position 8′ or 9′ of the corresponding dihydropeptide ergot alkaloid molecule. Also, different stereoisomers (α and β) are reported for 8- and 9-hydroxyergotamine. From these data we conclude that the same metabolites are also formed under cell

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culture conditions using HT-29 cells. Suggested structures are presented in Figure 1 with **3**−**6**/**a**−**d** depending on the incubated ergot alkaloid. Due to the toxic properties of ergot alkaloids, only low concentrations can be applied in cell culture experiments. Therefore, only small amounts of metabolites are formed, and it is not possible to isolate them in larger amounts to elucidate the exact position of the hydroxy group and the stereochemistry by detailed NMR studies. Nevertheless, from our FTMS measurements together with literature data the metabolite peaks I (I′), II (II′), IV (IV′), and V (V′) are all hydroxylation products **3**−**6**/**a**−**d** formed in phase I reactions. The characteristic peak pattern for the hydroxylated metabolites shown in Figure 4 is also in agreement with literature data.[9] Our results clearly demonstrate the occurrence of the hydroxylated metabolites of the -ine and -inine forms of peptide ergot alkaloids. Besides ergotamine (**3**), which is commonly used in most studies, we also demonstrated that the hydroxylation also occurs during ergocristine (**5**) metabolism.

Mass Spectrometric Studies with Metabolite Peaks III′ and III and Peaks VI′ and VI. FTMS measurements in cell lysate of HT-29 cells with metabolites III′, III, VI′, and VI revealed the molecular formula $C_{33}H_{38}O_6N_5$ for the metabolites derived from **3**/**4** (peak III′, 600.2795 ± 3.53; peak VI′, 600.2809 ± 2.10; *m*/*z* (±ppm) with HCD 30%) and $C_{35}H_{42}O_6N_5$ for the metabolites derived from **5**/**6** (peak III, 628.3148 ± 1.42; peak VI, 628.3119 ± 2.96, *m*/*z* (±ppm) with HCD 30%). Both metabolites are 18 Da higher compared to individually incubated ergot alkaloids and lead to the same structure, as they could be referred to their corresponding -ine and -inine form. The resulting fragment ions for **3**/**4** metabolite peak VI′ in the HCD mode (30%) were *m*/*z* (%) 208.0751 (49), 223.1230 (100), 268.1445 (61), 315.1340 (12), 554.2757 (5), 582.2705 (11), and 600.2809 (15) (Figure 5C.1) and for **5**/**6** metabolite peak VI *m*/*z* (%) 208.0754 (5), 223.1226 (44), 268.1439 (39), 305.1278 (75), 343.1668 (15), 348.1995 (53), 582.3061 (5), 610. 3007 (100), and 628.3119 (4) (Figure 5C.2). The other occurring peaks III (III′) gave the same fragment ions and are not shown. The product ion spectra again clearly demonstrate a change in the peptide moiety because the characteristic *m*/*z* of 297.1232 is missing and is replaced by *m*/*z* 315.1340 (peak VI′) (Figure 5C.1) or *m*/*z* 343.1668 (peak VI) (Figure 5C.2). In contrast to the previously mentioned hydroxylated metabolites, only one loss of water from the molecular ion peak could be detected, compared to the original substance. To gain additional structural information of this metabolite an enhanced product ion spectra (MS^3) of the peptide moiety in the HCD mode (30%) was measured. The resulting fragment ions of VI were compared to the original alkaloid **6** and metabolite peak II: peak VI (peptide moiety *m*/*z* 343.1652) *m*/*z* (% intensity) 131.0378 (100), 200.1889 (42), 245.1286 (35), 277.9842 (16), 315.1717 (15), 343.1652 (61); original alkaloid **6** (peptide moiety *m*/*z* 325.1536) *m*/*z* (% intensity) 131.0165 (70), 200.2656 (22), 215.1177 (91), 243.1131 (100), 297.1597 (25), 325.1536 (34); peak II (peptide moiety *m*/*z* 341.2693) *m*/*z* (% intensity) 131.0563 (100), 200.1864 (39), 241.0969 (24), 259.1077 (61), 323.1387 (57), 341.2693 (17). On the basis of these fragment ions, obtained by MS^3 from the peptide moiety, no loss of water could be detected in the metabolite peak VI, analogue to the original ergot alkaloid **6**, although an 18 Da higher exact mass was observed. The loss of water is very dominant in the product ion mass spectra of the metabolite peak II but missing in peak VI metabolite. From the fact that the modification took place in the peptide moiety, the mass difference of 18 Da and the polar chromatographic properties, only the structures **4e**/**6e** are possible for metabolite peak VI (VI′) and **3e**/**5e** for peak III (III′) (Figure 1). The obtained mass spectrometric fragmentation for this metabolite is in agreement with literature data, showing that primary hydroxy group may not easily be cleaved.[28]

As described in the literature the carbon atom at position C8′ could also be metabolized to a carboxylic acid group.[7,8] In our cell culture studies these metabolites were not detectable, but our suggested structures **3e**−**6e**, as an open ring structure, present a so far unknown precursor for this molecule, which could be oxidized to the described carboxylic acid at position 8′. Additionally, the suggested structure is underlined by the different fragmentation pattern of the hydroxylated metabolites (**3**−**6**/**a**−**d**) and **3e**−**6e**, as for these metabolites no loss of water could be detected. Also, the chromatographic properties confirmed the suggested structure of metabolites **3e**/**4e** and **5e**/**6e** as only one peak with a shorter retention time, indicating a higher polarity was detectable. Due to the limited amount of metabolites formed under cell culture conditions, confirmation by NMR was not possible. Nevertheless, it is the first time a possible precursor and unknown metabolite is described for cell lines, which seems not to occur after incubation with liver microsomes.[8−10]

Mass Spectrometric Studies with Metabolite Peaks VII′ and VII. For the metabolite peak VII (VII′) *m*/*z* (±ppm) values of 614.2598 ± 1.88 for **3**/**4** incubation and 642.2918 ± 0.65 for **5**/**6** incubation were found by FTMS (HCD 40%). This results in the molecular formula $C_{33}H_{36}O_7N_5$ for peak VII′ and $C_{35}H_{40}O_7N_5$ for peak VII, indicating that the molecular weight is 32 Da higher compared to **3**/**4** and **5**/**6**. Resulting fragment ions in the HCD mode (40%) were *m*/*z* (%) 208.0755 (82), 223.1228 (100), 268.1441 (82), 329.3017 (9), 568.2542 (8), 596.2491 (25), and 614.2597 (40) for metabolite VII′ derived from **3**/**4** (Figure 5D.1) and *m*/*z* (%) 223.1229 (7), 268.1444 (20), 305.1282 (18), 348.1704 (26), 606.2702 (17), and 624.2809 (95) for metabolite VII derived from **5**/**6** (Figure 5D.2). Because only one peak occurs, a differentiation of whether the metabolite is formed of the 8-(*R*) isomer (**3**, **5**) or 8-(*S*) isomer (**4**, **6**) is not possible.

The obtained mass difference of 32 Da could be referred to two possible metabolites: dihydroxy-ergot alkaloid metabolites or carboxylic acid ergot alkaloid metabolites.[8,10] The changes again occurred in the peptide moiety and were identified in the same way as described for other metabolites. Due to the fact that no CO_2 loss was detectable and a small signal indicating an additional loss of water occurred from the metabolite VII (VII′), dihydroxy metabolites were concluded from the FTMS data. Although again the exact position of the hydroxyl groups and the stereochemistry could not be elucidated due to the limited amount of compound, the substances corresponding with previous publications as dihydroxylated metabolites were also occurring in liver microsomes.[7,9] The suggested structures are summarized as **3**−**6**/**f**−**j** in Figure 1.

In summary, all ergot alkaloid metabolites identified in mice liver microsomes,[9] could also be found in a human cell line in our study. In addition, a new unknown metabolite, the possible precursor for the 8′-carboxylated metabolites, was identified for the first time.

Comparison of Different Cell Types. All described metabolites were characterized via HPLC-FLD-FTMS in HT-29 cells. For HepG2 cells the metabolites were identified through retention time, exact mass measurements, and comparison to the structure elucidation data from HT-29 cells. All metabolites derived from **3**/**4** and **5**/**6** could be confirmed, with the exception of peak VII (VII′), referring to dihydroxy metabolites, which

was not detectable in HepG2 cells. Peak VI (VI′), with the structure **3e**/**5e**, was not detectable during metabolite screening (Figure 2B) but could be identified after enrichment of cell lysate (see Metabolism and Uptake Studies of Ergot Alkaloids). In RPTEC no metabolites were detectable at all, although a high cellular uptake of ergot alkaloids was measured in previous studies and characterized as an accumulation.[16]

Referring to the biological activity, Figure 3 shows much less metabolic activity for HepG2 cells compared to HT-29 cells. The seemingly lower ability to metabolize ergot alkaloids of HepG2 cells is in agreement with previous results. The metabolic activity of HepG2 cells cultured in DMEM was characterized with only 10−20% of the P450 monooxygenese activity in contrast to freshly isolated hepatocytes.[29] Additionally, it was also shown that HepG2 cells showed a low expression of phase I enzymes, but a high expression of phase II enzymes.[30] Because the metabolism of peptide ergot alkaloids occurs with the introduction of a hydroxy group as key step during phase I metabolism, the weak metabolism of this cell line could easily be explained by the low P450 enzyme activity. In contrast, HT-29 cells appeared in our experiments as a very good in vitro model system for metabolism studies in human cells, forming rapidly a variety of metabolites of ergot alkaloids. Whether the observed metabolism of ergot alkaloids is a detoxification process or this accumulation takes place in storage depots in the body needs to be elucidated in further studies. On the one hand, the alkaloids are metabolized to more polar and hydrophilic substances facilitating their excretion, but on the other hand, the receptor relevant part of the ergoline system remained unchanged. The ergoline system is responsible for several receptor interactions and, as reported already for the main metabolite of dihydroergotamine, this modified ergot alkaloid had shown a similar receptor interaction property.[8] Further experiments concerning the toxic aspects of the detected metabolites are necessary to determine the toxic impact on human cells.

Nevertheless, we could demonstrate a metabolism of the peptide ergot alkaloids ergotamine/ergotaminine (**3**/**4**) and ergocristine/ergocristinine (**5**/**6**) using HPLC-FLD-FTMS in HepG2 and HT-29 cells. Several metabolites have been identified in cell lysates and cell media, corresponding to literature data as hydroxylation products. Ergometrine/ergometrinine (**1**/**2**) as a lysergic acid amide was not able to enter cells, which is also in agreement with literature data. In our experiments neither in cell medium nor in cell lysate were metabolites of this lysergic acid amide detectable. Human primary kidney cells (RPTEC), in contrast to colon carcinoma and hepatocellular liver carcinoma cells, were not able to perform a metabolism, although they have shown an immense ability for uptake of peptide ergot alkaloids. For the first time we were able to show an in vitro metabolism of peptide ergot alkaloids in cell culture and we were able to show that HT-29 cells are a very effective model to study ergot alkaloid metabolism in human cells. With the use of these cells in combination with HPLC-FLD-FTMS coupling, unknown products of ergot alkaloid metabolism could be identified.

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REFERENCES

(1) Tfelt-Hansen, P.; Saxena, P. R.; Dahlof, C.; Pascual, J.; Lainez, M.; Henry, P.; Diener, H.; Schoenen, J.; Ferrari, M. D.; Goadsby, P. J. Ergotamine in the acute treatment of migraine: a review and european consensus. *Brain* **2000**, *123* (Part 1), 9–18.

(2) Griffith, R. W.; Grauwiler, J.; Hodel, C.; Leist, K. H.; Matter, B. Toxicologic considerations. In *Ergot Alkaloids and Related Compounds*; Berde, B., Schild, H. O., Eds.; Springer Verlag: Berlin, Germany, 1978; pp 805−851.

(3) Schiff, P. L. Ergot and its alkaloids. *Am J. Pharm. Educ.* **2006**, *70*, 98.

(4) Eich, E.; Pertz, H. Ergot alkaloids as lead structures for differential receptor systems. *Pharmazie* **1994**, *49*, 867–877.

(5) EFSA Opinion of the scientific panel on contaminants in food chain on a request from the commission related to ergot as undesirable substance in animal feed. *EFSA J.* **2005**, *225*, 1−27.

(6) Testa, B.; Kramer, S. D. The biochemistry of drug metabolism − an introduction: part 5. Metabolism and bioactivity. *Chem. Biodivers.* **2009**, *6*, 591–684.

(7) Eckert, H.; Kiechel, J. R.; Rosenthaler, J.; Schmidt, R.; Schreier, E. Biopharmaceutical aspects, analytical methods, pharmacokinetics, metabolism and bioavailability. In *Ergot Alkaloids and Related Compounds*; Berde, B.; Schild, H. O., Eds.; Springer Verlag: Berlin, Germany, 1978; pp 719−803.

(8) Maurer, G.; Frick, W. Elucidation of the structure and receptor binding studies of the major primary, metabolite of dihydroergotamine in man. *Eur. J. Clin. Pharmacol.* **1984**, *26*, 463–470.

(9) Duringer, J. M.; Lewis, R.; Kuehn, L.; Fleischmann, T.; Craig, A. M. Growth and hepatic in vitro metabolism of ergotamine in mice divergently selected for response to endophyte toxicity. *Xenobiotica* **2005**, *35*, 531–548.

(10) Moubarak, A. S.; Rosenkrans, C. F., Jr. Hepatic metabolism of ergot alkaloids in beef cattle by cytochrome P450. *Biochem. Biophys. Res. Commun.* **2000**, *274*, 746–749.

(11) Nimmerfall, F.; Rosenthaler, J. Ergot alkaloids: hepatic distribution and estimation of absorption by measurement of total radioactivity in bile and urine. *J. Pharmacokinet. Biopharm.* **1976**, *4*, 57–66.

(12) Slaytor, M. B.; Wright, S. E. The metabolites of ergometrine and lysergic acid diethylamide in rat bile. *J. Med. Pharm. Chem.* **1962**, *91*, 483–491.

(13) Komarova, E. L.; Tolkachev, O. N. Medicinal plants: the chemistry of peptide ergot alkaloids. Part 1. Classification and chemistry of ergot peptides. *Pharm. Chem. J.* **2001**, *35*, 504–513.

(14) Stadler, P. A.; Sturmer, E. Comparative studies on the pharmacological properties of stereoisomers of ergotamine and dihydro-ergotamine. *Naunyn Schmiedebergs Arch. Pharmakol.* **1970**, *266*, 457–458.

(15) Shappell, N. W.; Smith, D. J. Ergovaline movement across Caco-2 cells. *In Vitro Cell Dev. Biol. Anim.* **2005**, *41*, 245–251.

(16) Mulac, D.; Humpf, H. U. Cytotoxicity and accumulation of ergot alkaloids in human primary cells. *Toxicology* **2011**, *282*, 112–121.

(17) Franzmann, C.; Wachter, J.; Dittmer, N.; Humpf, H. U. Ricinoleic acid as a marker for ergot impurities in rye and rye products. *J. Agric. Food Chem.* **2010**, *58*, 4223–4229.

(18) Uhlig, S.; Vikoren, T.; Ivanova, L.; Handeland, K. Ergot alkaloids in norwegian wild grasses: a mass spectrometric approach. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1651–1660.

(19) Königs, M.; Mulac, D.; Schwerdt, G.; Gekle, M.; Humpf, H. U. Metabolism and cytotoxic effects of T-2 toxin and its metabolites on human cells in primary culture. *Toxicology* **2009**, *258*, 106–115.

(20) Aellig, W. H.; Nuesch, E. Comparative pharmacokinetic investigations with tritium-labeled ergot alkaloids after oral and intravenous administration man. *Int. J. Clin. Pharmacol. Biopharm.* **1977**, *15*, 106–112.

(21) Sanders, S. W.; Haering, N.; Mosberg, H.; Jaeger, H. Pharmacokinetics of ergotamine in healthy volunteers following oral and rectal dosing. *Eur. J. Clin. Pharmacol.* **1986**, *30*, 331–334.

(22) Ala-Hurula, V.; Myllyla, V. V.; Arvela, P.; Heikkila, J.; Karki, N.; Hokkanen, E. Systemic availability of ergotamine tartrate after oral, rectal and intramuscular administration. *Eur. J. Clin. Pharmacol.* **1979**, *15*, 51–55.

(23) Stange, K.; Pohlmeier, H.; Lübbesmeyer, A.; Gumbinger, G.; Schmitz, W.; Baumgart, P. Vaskulärer Ergotismus durch Getreidestaubinhalation. *Dtsch. Med. Wschr.* **1998**, *123*, 1547–1550.

(24) Little, P. J.; Jennings, G. L.; Skews, H.; Bobik, A. Bioavailability of dihydroergotamine in man. *Br. J. Clin. Pharmacol.* **1982**, *13*, 785–790.

(25) Lehner, A. F.; Craig, M.; Fannin, N.; Bush, L.; Tobin, T. Electrospray[+] tandem quadrupole mass spectrometry in the elucidation of ergot alkaloids chromatographed by HPLC: screening of grass or forage samples for novel toxic compounds. *J. Mass Spectrom.* **2005**, *40*, 1484–1502.

(26) Klotz, J. L.; Kirch, B. H.; Aiken, G. E.; Bush, L. P.; Strickland, J. R. Bioaccumulation of ergovaline in bovine lateral saphenous veins in vitro. *J. Anim. Sci.* **2009**, *87*, 2437–2447.

(27) Mohamed, R.; Gremaud, E.; Tabet, J. C.; Guy, P. A. Mass spectral characterization of ergot alkaloids by electrospray ionization, hydrogen/deuterium exchange, and multiple stage mass spectrometry: Usefulness of precursor ion scan experiments. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2787–2799.

(28) Zaia, J. Mass spectrometry of oligosaccharides. *Mass Spectrom. Rev.* **2004**, *23*, 161–227.

(29) Doostdar, H.; Duthie, S. J.; Burke, M. D.; Melvin, W. T.; Grant, M. H. The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived Hep G2 cell line. *FEBS Lett.* **1988**, *241*, 15–18.

(30) Yoshitomi, S.; Ikemoto, K.; Takahashi, J.; Miki, H.; Namba, M.; Asahi, S. Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology. *Toxicol. In Vitro* **2001**, *15*, 245–256.