RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2010; **24**: 791–800
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4451

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Identification of NTBC metabolites in urine from patients with hereditary tyrosinemia type 1 using two different mass spectrometric platforms: triple stage quadrupole and LTQ-Orbitrap

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Received 5 December 2009; Revised 10 January 2010; Accepted 11 January 2010

The objective of our work was to identify known and unknown metabolites of the drug NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) in urine from patients during the treatment of hereditary tyrosinemia type 1 (HT-1) disease, a severe inborn error of tyrosine metabolism. Two different mass spectrometric techniques, a triple stage quadrupole and an LTQ-Orbitrap (Fourier transform mass spectrometry (FTMS)), were used for the identification and the structural elucidation of the detected metabolites. Initially, the mass spectrometric (MS) approach consisted of the precursor ion scan detection of the selected product ions, followed by the corresponding collision-induced dissociation (CID) fragmentation analysis (MS^2) for the targeted selected reaction monitoring (SRM) mode. Subsequently, accurate and high-resolution full scan and MS/MS measurements were performed on the possible metabolites using the LTQ-Orbitrap. Final confirmation of the identified metabolites was achieved by measuring commercially supplied or laboratory-synthesized standards. Altogether six metabolites, including NTBC itself, were extracted, detected and identified. In addition, two new NTBC metabolites were unambiguously identified as amino acid conjugates, namely glycine-NTBC and β-alanine-NTBC. These identifications were based on their characteristics of chromatographic retention times, protonated molecular ions, elemental compositions, product ions (using CID and higher-energy C-trap dissociation (HCD) techniques) and synthesized references. The applied MS strategy, based on two different MS platforms (LC/MS/MS and FTMS), allowed the rapid identification analysis of the drug metabolites from human extracts and could be used for pharmaceutical research and drug development. Copyright © 2010 John Wiley & Sons, Ltd.

NTBC, which is administrated as Orfadin® (Swedish Orphan Int. AB), is a specific inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase and still the unique efficient drug for a successful treatment of hereditary tyrosinemia type 1 (HT-1) in combination with a tyrosine- and phenylalanine-restricted diet.[1] This disease has an incidence of approximately 1/100 000 neonates in Europe and the United States and is the most severe of the tyrosine catabolic pathways. HT-1 is due to inherited deficiency of the enzyme fumarylacetoacetate hydrolase that catalyzes the cleavage of fumarylacetoacetate to acetoacetate and fumarate. This enzyme defect results in an accumulation of toxic metabolites, which are formed upstream of the enzyme block. Accumulation of such highly reactive electrophilic metabolites (fumarylacetoacetate or succinylacetoacetate) leads to severe liver and kidney disease.[2] Additionally, the generated diketone compound succinylacetone (4,6-dioxoheptanoic acid), a reactive derivative of fumarylacetoacetate, is considered as a potent inhibitor of δ-aminolevulinic acid dehydratase, the second enzyme in the heme biosynthesis pathway. Increasing levels of δ-aminolevulinic acid, a well-known neurotoxin, lead to neurological dysfunctions in patients with HT-1.[3] Before the introduction of NTBC in therapy of this fatal inborn error of metabolism, liver transplantation was the only therapeutic alternative for children affected with HT-1. NTBC has also been used successfully for the treatment of the disease alkaptonuria, which results from the deficiency of the enzyme homogentisate 1,2-dioxygenase in the tyrosine catabolism pathway.[4]

Recently, we have demonstrated a sensitive and selective liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the determination of the NTBC content in plasma of HT-1 patients for optimal individual dose adaptation, drug response and pharmaco-/toxicokinetic studies.[5] An interpatient variability in drug metabolism could clearly be observed confirming the necessity of drug monitoring application during therapy. NTBC is registered as an orphan drug and knowledge of its metabolites present in urine is important to provide a better understanding of its metabolic fate. Generally, once a drug is administrated to an

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organism, it is absorbed and distributed to its site of action, where it interacts with targets (e.g., receptors and enzymes), undergoes metabolism, and finally is excreted. Pathways of drug metabolism are classified as either phase I reactions (e.g., hydrolysis, oxidation, and reduction) or phase II, conjugation reactions (e.g., methylation, hydroxylation, sulfation, and glucuronidation). Hence, both types of reaction convert relatively lipid-soluble drugs into relatively more water-soluble metabolites.[6]

One- or two-dimensional nuclear magnetic resonance (NMR) spectroscopy is for many drug metabolism scientists the method of choice for structural elucidation of drug metabolites. These experiments can be performed on a relatively small quantity of purified material (approx. 5–10 mg). In the majority of cases, labor-intensive purification of adequate amounts of metabolites for NMR experiments from complex biological matrices is not feasible due to their very low abundance. In addition, mass spectrometers became routine tools for pharmaceutical metabolite identification due to their excellent selectivity and sensitivity in the nano- to picomolar range. These days, more sophisticated MS scan techniques, such as precursor ion scan, product ion scan, neutral loss scan, accurate mass measurement, and multistage MS^n, are available facilitating identification of drug metabolites in complex biological matrices such as plasma or urine.[7] Furthermore, accurate mass MS/MS data using different fragmentation sources such as collision-induced dissociation (CID) and higher-energy C-trap dissociation (HCD) deliver tremendous structural information for identification and confirmation of existing and novel drug metabolites.[8]

So far, we developed a MS-based strategy for direct detection and structural elucidation of known and novel NTBC metabolites in patient urine. The key to structure identification approaches is based on the fact that metabolites generally retain most of the core structure of the parent drug, in this case 'the substituted aromatic ring of NTBC'. In this paper, we used two different mass spectrometric platforms with different types of fragmentation cells, the triple stage quadrupole and the LTQ-Orbitrap. In the literature, three NTBC metabolites, including NTBC itself, were described and identified in HT-1 urine samples by means of ^{1}H- and ^{19}F-NMR techniques.[9] In this work, two further novel metabolites were found and unambiguously identified and their structural assignment was ascertained through low abundance standards which were synthesized in our laboratory.

EXPERIMENTAL

Materials

Pure NTBC (Orfadin®) of pharma grade was supplied by Swedish Orphan (Stockholm, Sweden) as solid material. LC/MS grade acetonitrile and water were supplied from Fisher Scientifics. Formic acid (LC/MS grade) and ethyl acetate were obtained from Fluka and trifluoroacetic acid from Merck.

Sample preparation procedure and synthesis of standards

Volumes of 10 mL of the collected and blank urine samples were acidified with 6 N hydrochloric acid (pH 2) and than extracted twice with 10 mL ethyl acetate. The organic layer was dried over Na_2SO_4, evaporated and the residue was dissolved in 300 μL of acetonitrile/water (60:40, v/v). Urine samples were collected from HT-1 patients that had been treated with NTBC (n = 5) and stored immediately at −80°C until extraction and analysis.

Appropriate standards were synthesized by mixing NTBC (0.1 M) with the corresponding amino acids (glycine, β-alanine or alanine; 0.1 M) in water/acetonitrile solution (2 mL; 50:50) containing 200 μL of 2 N NaOH. The reaction was stirred gently at room temperature for 1 day and left under the same conditions for a further 3 weeks. The produced minor products of the desired standards were subjected to LC/MS/MS analysis.

Conditions for LC/MS/MS

LC/MS/MS analyses of the samples were performed using a Waters Quattro micro triple quadrupole system equipped with an electrospray ionization (ESI) probe and a high-performance liquid chromatography (HPLC) system (Waters 2795 Alliance). Chromatographic separation was performed at room temperature with a Gemini-NX C18 Phenomenex column (150 mm × 2 mm × 3 μm) attached to a Phenomenex C18 Gemini-NX guard column and ran isocratically for 7 min with 40% A and 60% B. Eluent A consisted of water/formic acid (0.1%)/TFA (0.01%) and eluent B of acetonitrile. The solvent flow rate was 0.2 mL/min and the injection volume 10 μL. MS/MS analysis was performed in selected reaction monitoring (SRM) mode and positive or negative ionization mode was used. The following mass transitions for NTBC *m/z* 330 → 218 and *m/z* 330 → 126 were used. The CID-MS/MS spectra were recorded in positive ion mode. In the case of precursor ion scan mode we used the product ions of NTBC at *m/z* 218 and 126. LC/MS/MS instrument settings were as follows for ESI-pos: capillary, 3200 V; source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 80 L/h; desolvation gas flow, 660 L/h; cone voltage, 22; CID, 20/35 eV; dwell time, 100 ms. Settings for ESI-neg were: capillary, 3000 V; source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 60 L/h; desolvation gas flow, 660 L/h; cone voltage, 20; CID, 16/25 eV; dwell time, 100 ms.

The software MassLynx version 4.0 (Micromass, Waters) was used to accomplish data acquisition and analysis as well as to control the mass spectrometer and all peripheral components. Peak integration and calculation were performed using the software QuanLynx 4.0.

Conditions for the LTQ-Orbitrap

High-resolution full scan and MS/MS spectra were obtained with the LTQ-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). The mass spectrometer was operated in positive ion mode (1 spectrum s^{-1}; mass range: 90–340 *m/z* for MS/MS and 250–600 *m/z* for full scan) with a nominal mass resolving power of 7500 (MS/MS) or 60000 (full scan) at *m/z* 400 and a scan rate of 1 Hz. Automatic gain control and an internal calibration; bis(2-ethylhexyl)phthalate: *m/z* 391.284286; were used to provide high-accuracy mass measurements within 2 ppm. MS/MS experiments were performed by CID (collision-induced dissociation, 20/35 eV) and HCD (higher-energy C-trap dissociation, 35/50 eV) modes

DOI: 10.1002/rcm

using the following parameters: spray voltage 5 kV, capillary temperature 260°C, tube lens 70 V. In the case of negative ionization mode an external mass calibration was used with the following settings: spray voltage −5 kV, capillary temperature 260°C and tube lens −70 V. Nitrogen was used as sheath gas (5 arbitrary units) and helium served as the collision gas. The mass spectrometer was equipped with a Dionex Ultimate 3000 HPLC system (Sunnyvale, CA, USA), which contained a pump, UV-vis detector, flow manager, and autosampler (injection volume 0.5 μL). The measurements were conducted by direct injection of the sample into an isocratic flow of water/acetonitrile containing 0.1% formic acid (50:50) at a flow rate of 4 μL min^{-1}.

RESULTS AND DISCUSSION

In a previous study we developed a rapid, sensitive and selective LC/MS/MS method for the determination of the NTBC content in plasma of HT-1 patients.[5] For this method the same isocratic elution mode was used consisting of 60% acetonitrile and 40% water (0.1% formic acid, 0.01% TFA). The total chromatographic run time on the C18 NX column (Phenomenex) was 7 min. Mesotrione, a structurally similar compound, was used as internal standard for quantitative analysis based on standard calibration curves. Further LC/MS basic criteria which concerned the method validation such as recovery, stability, linearity, and reproducibility and matrix effects were also fulfilled and discussed in the mentioned reference.

Here, we present a LC/MS-based strategy for the identification of the NTBC drug metabolites in urine of HT-1 patients in order to further elucidate drug metabolism.

Mass spectrometry (LC/MS/MS and LTQ-Orbitrap)

The precursor ion scan technique is a precious and helpful tool for the rapid confirmation of targeted compounds or for the detection of non-targeted compounds (e.g., unidentified metabolites) bearing common product ions. MS/MS analysis of the precursor protonated molecule obtained from NTBC gave, as was reported in the previous study, two specific product ions at *m/z* 218 (CID 20 eV) and 126 (CID 35 eV).[5] To assign the correct molecular structure of both detected product ions, high-resolution accurate mass MS/MS measurements were performed in positive ion mode with the LTQ-Orbitrap mass spectrometer. As was expected, the first product ion at *m/z* 218 corresponds to the well-known acylium cation and was formed by the loss of cyclohexanedione moiety ($C_6H_8O_2$), with an elemental composition of $C_8H_3O_3NF_3^+$. The second product ion at *m/z* 126 had an elemental composition of $C_6H_2NF_2^+$ and it was assigned as an aryne derivative with a triple bond in the aromatic cycle. Both main product ions contain unequivocally fluorine groups (CF_3 or NF_2) and hence are specific product ions for the detection of NTBC and its existing CF_3 metabolites. While the natural content of fluorine atoms in human biological fluids is marginally small, a detection of these ions can only be generated with the utmost probability from NTBC compound. In the first part of this study our interest was focused on recognition of any NTBC modifications in urine samples using precursor ion scan technique.

In general, the identification of a certain metabolite is based on a mass shift of its molecular weight and its product ions from the corresponding molecular weight and product ions of the parent ion (unchanged drug). The proposed structure of a product ion can be confirmed by the chemical formula obtained from the accurate mass measurement using the LTQ-Orbitrap mass spectrometer. This mass analyzer is capable of providing high resolution up to 100 000 at *m/z* 400 and a mass accuracy of ≤2 ppm using internal mass calibration and lock masses.[10,11] The use of high resolution in drug discovery is primarily needed for distinguishing isobaric product ions which result from multiple fragmentation pathways. The chemical formula and the unsaturation value (number of rings and double-bond equivalents) can also be calculated from the accurate mass of precursor and product ions which lead to correct assignment of the metabolite structures. The strategy used in this work for novel metabolite identification of NTBC is illustrated in Fig. 1. The first part of this strategy was to detect all possible precursor ions giving a same set of product ions as the precursor ion NTBC. In the second step, full scan and product ion mass spectra of the possible metabolites were recorded by tandem mass spectrometry. Generation of accurate full scan and MS/MS mass spectra (CID and HCD) of the corresponding metabolites by means of the LTQ-Orbitrap was the third part of the strategy. While in the fourth and last steps the identification of the metabolites was ascertained by a chemically, even though in a low amount, synthesized or commercially supplied standard.

Analysis of NTBC metabolites

NTBC-containing urine samples and blank urines were collected, extracted, analyzed and compared by LC/MS in positive (five metabolites) and negative (one metabolite) ion modes. Figure 2(A) shows the precursor ion scan of the selected product ion of NTBC at *m/z* 218 using positive ion mode from a urine sample of a patient treated with NTBC.

Identification of the urinary metabolite NTFA

In the negative ion full scan mass spectrum, the compound NTFA (2-nitro-4-trifluoromethylbenzoic acid) was observed as a deprotonated molecular ion $[M–H]^-$ at *m/z* 234 with a retention time of 3.4 min. The molecular structure of NTFA is depicted in Fig. 2(B). The ESI-neg MS^n (n = 2–4) product ion mass spectra of NTFA contained three characteristic product ions at *m/z* 190, 160 and 132 (Supplementary Fig. S1, see Supporting Information). The product ion at *m/z* 190 was formed by the neutral loss of CO_2 (44 Da) from the $[M–H]^-$ ion. The product ion at *m/z* 160 was formed by the loss of CO_2+NO (74 Da) yielding an epoxide derivative with radical properties. The product ion at *m/z* 132 was formed by the loss of 102 Da, and its formation was attributed to a loss of a further CO group, which corresponded to $[M–H–CO_2–NO–CO]^{-\bullet}$ radical ion. The fragmentation pathway occurred via a ring opening and a subsequent intramolecular cyclization to yield the five-ring molecule containing a double and triple bonds (aryne derivative). Figure 3(A) illustrates the proposed molecular structures of

DOI: 10.1002/rcm

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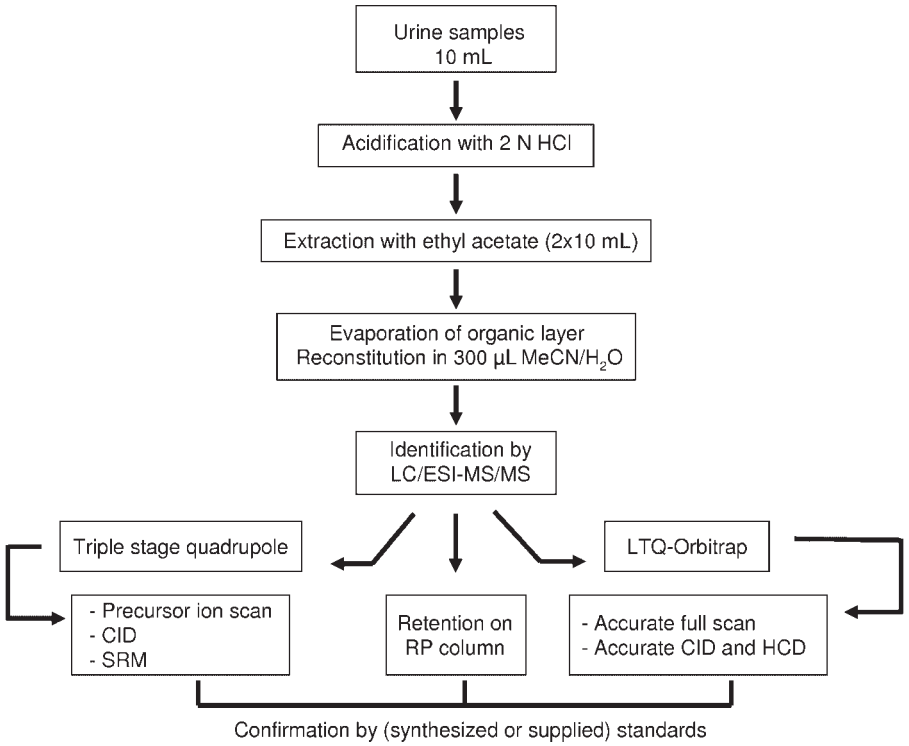

Figure 1. Flow diagram for identification of NTBC metabolites.

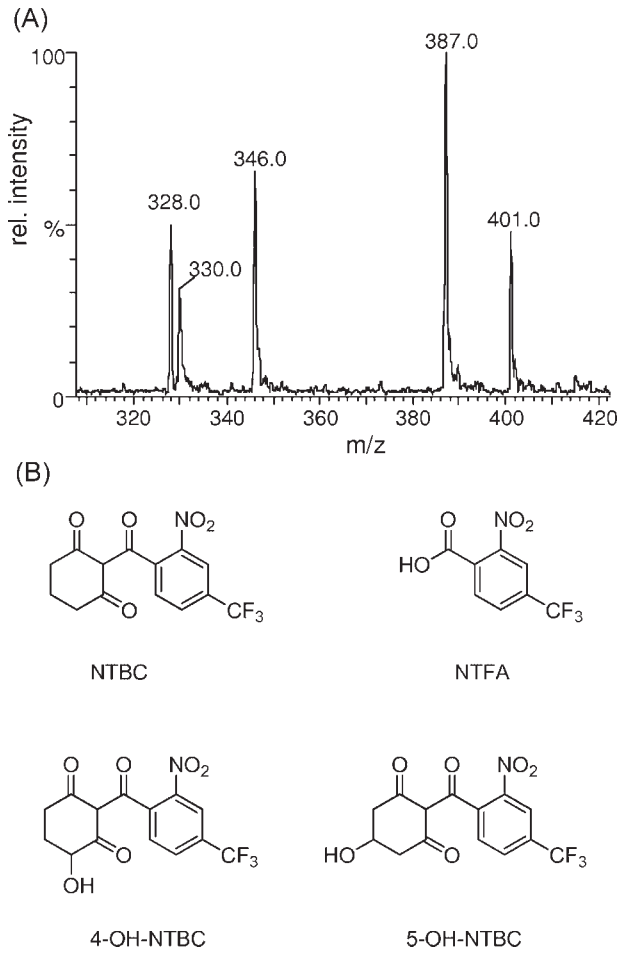

Figure 2. (A) Precursor ion scan (ESI-pos) of the ion at *m/z* 218 extracted from the NTBC metabolites in HT-1 urine samples. (B) Chemical structures of the metabolites NTBC, NTFA, 4-OH-NTBC and 5-OH-NTBC.

the fragmentation ions of NTFA. Interestingly, the CF_3 group did not appear to give any additional fragmentation, whatever the energy was used for CID in MS/MS experiments. All three product ions were detected on both instruments. However, the product ion at *m/z* 132 showed a very low intensity on the LTQ-Orbitrap instrument in both CID and HCD experiments. The elemental compositions found for the above-mentioned product ions corresponded to the proposed molecular structures shown in Fig. 3(A), with a mass error <5 ppm for external calibration mode and without using lock masses (Table 1). Hence, these three product ions were selected for SRM (selected reaction monitoring) mode to identify NTFA in urine extracts of HT-1 patients. Based on the above MS/MS data and additionally confirmed by comparison with a commercially available standard, the metabolite NTFA was unambiguously identified in the extracts. The transformation of NTBC into NTFA was consistent with published findings on the metabolism of mesotrione, a structurally similar compound, in plants, which is due to a hydrolytic cleavage of the substituted benzoyl group of the triketone compound.[12]

Identification of the urinary metabolite NTBC

NTBC, as depicted in Fig. 2(B), was easily identified in positive ion mode as a protonated molecular ion at *m/z* 330 on the basis of its retention time and typical fragmentations.[5] MS/MS experiments gave two main product ions (*m/z* 218 and 126), which were observed and used for SRM mode (Supplementary Figs. S2(A) and S2(B), see Supporting Information). The first product ion at *m/z* 218 corresponded to the expected acylium cation by a loss of $C_6H_8O_2$, a cyclohexanedione moiety. The second intense product ion at *m/z* 126 represented an aryne derivative. The assignment of the molecular structures of these product ions was described

DOI: 10.1002/rcm

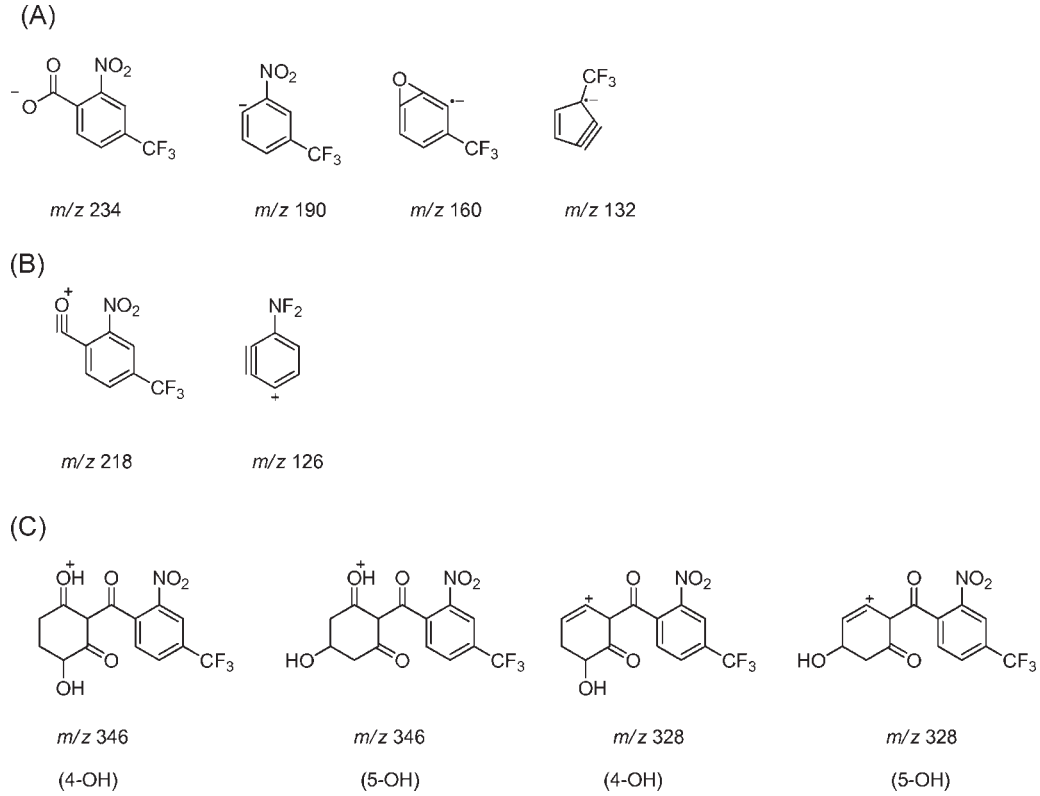

Figure 3. Proposed molecular structures for the product ions of (A) NTFA, (B) NTBC, and (C) 4-OH-NTBC/5-OH-NTBC.

in detail in our previous work.[5] However; it is still surprisingly to find the NTBC parent drug in the urinary extracts. This is probably due to the various polar functions of the drug, e.g. CF_3, NO_2 or keto/enol moieties, which consequently makes NTBC more hydrophilic for renal excretion. The proposed structures for the product ions based on accurate mass MS/MS data are illustrated in Fig. 3(B).

Table 1. Accurate mass measurements of NTFA, 4(5)-OH-NTBC and NTBC as determined using the LTQ-Orbitrap in MS and MS/MS mode using two different collision cells (CID[a] and HCD[b])

Product ion	Elemental composition	Calculated *m/z*	Measured *m/z*	Relative error (ppm)	DBE[c]
1) NTFA[d]					
$[M-H]^-$	$C_8H_3O_4NF_3$	234.00087	234.00207	1.202	6.5
CID/HCD					
m/z 190	$C_7H_3O_2NF_3$	190.01104	190.01154	2.318	5.5
m/z 160	$C_7H_3OF_3$	160.01305	160.01347	2.619	5.0
m/z 132	$C_6H_3F_3$	132.01814	132.01861	3.588	4.0
2) NTBC[e]					
$[M+H]^+$	$C_{14}H_{11}O_5NF_3$	330.05838	330.05838	−0.008	9.5
CID/HCD					
m/z 218	$C_8H_3O_3NF_3$	218.00595	218.00571	−1.107	6.5
m/z 126	$C_6H_2NF_2$	126.01498	126.01490	−0.640	5.5
3) 4-OH-NTBC[e]/5-OH-NTBC[e]					
$[M+H]^+$	$C_{14}H_{11}O_6NF_3$	346.05330	346.05321	−0.267	9.5
CID/HCD					
m/z 328	$C_{14}H_9O_5NF_3$	328.04273	328.04267	−0.188	9.5
m/z 218	$C_8H_3O_3NF_3$	218.00595	218.00579	−1.707	6.5

[a] CID = collision-induced dissociation.
[b] HCD = higher energy C-trap dissociation.
[c] DBE = double-bond equivalents.
[d] = negative ion mode and external mass calibration.
[e] = positive ion mode and internal mass calibration.

DOI: 10.1002/rcm

Identification of the urinary metabolites 4-OH-NTBC and 5-OH-NTBC

In the precursor ion scan of the product ion at *m/z* 218 extracted from NTBC, we noted the presence of an oxidized metabolite of the drug as major peak at *m/z* 346 in ESI-pos mode. The ion was thus 16 Da higher than NTBC demonstrating that this ion was presumably a hydroxylation conjugate of the drug. This precursor ion (*m/z* 346) yielded product ions at *m/z* 218 and 328 (Supplementary Fig. S2(C), see Supporting Information). The formation of the product ion at *m/z* 218 indicated that the aromatic ring remained unchanged and the hydroxylation reaction occurred at the cyclohexanedione ring. The presence of the hydroxylated NTBC was consistent with previous studies and was assigned as 4-OH-NTBC (Fig. 2(B)). Interestingly, the total ion current (TIC) profile showed a major peak with a retention time of 3.8 min which corresponded to the above-mentioned 4-OH-NTBC metabolite and it was followed by a low abundance peak at a retention time of 4.3 min having the same mass set. The observation of this second metabolite is indicative for a hydroxylation reaction at the C5 position of the cyclohexanedione and this was identified as 5-OH-NTBC (Fig. 2(B)), consistent with a previous report.[13] Under acidic and CID conditions, these precursor metabolites ions showed the dehydrated product ions $[M+H–H_2O]^+$ at *m/z* 328 which were consistent with hydroxylation products (Supplementary Fig. S2(C), see Supporting Information). Both product ions were also observed via in-source fragmentation (cone voltage 22 V). The presence of the OH-NTBC metabolites was also confirmed by using the LTQ-Orbitrap. High-resolution and accurate MS and MS/MS measurements in ESI positive ion mode were performed. The following elemental compositions with mass error <1 ppm were found: *m/z* 346 ($C_{14}H_{11}O_6NF_3$, −0.267 ppm) and 328 ($C_{14}H_9O_5NF_3$, −0.188 ppm). Figure 3(C) illustrates the molecular structures of the corresponding metabolites with their major product ions. In Table 1, calculated and observed *m/z* values for OH-NTBC including their errors and elemental compositions are presented.

Identification of the urinary metabolite gly-NTBC

In the precursor ion scan mass spectrum of the ion at *m/z* 218, a major metabolite at *m/z* 387 was detected in the HT-1 urine samples (Fig. 2(A)). This ion appeared at 57 mass units higher than the parent compound NTBC (*m/z* 330). A difference of 57 Da is in the majority of cases an indication for a glycine residue, thus an amino acid conjugation. A formation of Schiff base derivatives is obviously favourable in the case of NTBC due to the presence of the ketone groups. This metabolite was consequently detected with the LTQ-Orbitrap operated at a resolution power of 60 000. The protonated molecular ion at *m/z* 387.07972 gave a molecular formula of $C_{16}H_{14}O_6N_2F_3$ with a mass error of −0.329 ppm and a double-bond equivalent (DBE) of 9.5 (Table 2). Based on the data of this MS analysis, we concluded a molecular structure of gly-NTBC, an imine derivative, for this unknown ion. The CID product ion mass spectrum (ESI-pos mode) at 35 V of this metabolite showed two characteristic product ions at *m/z* 341 and 156, as depicted in Fig. 4(A). The first product ion at *m/z* 341 corresponded to the $[M–H_2O–CO]^+$ structure and the second at *m/z* 156 was attributed to a carbamic acid derivative (see Fig. 5(A)). All other significant product ions are listed in Supplementary Table S1 (see Supporting Information) with their protonated molecule formulae, calculated and measured *m/z*, and mass accuracy and DBE. The HCD experiment at 50 V gave the same

Table 2. Accurate mass measurements of gly-, ala- and β-ala-NTBC as determined using the LTQ-Orbitrap in MS and MS/MS mode (CID[a] 35 V)

Product ion	Elemental composition	Calculated *m/z*	Measured *m/z*	Relative error (ppm)	DBE[b]
gly-NTBC					
$[M+H]^+$	$C_{16}H_{14}O_6N_2F_3$	387.07961	387.07972	−0.329	9.5
m/z 369	$C_{16}H_{12}O_5N_2F_3$	369.06928	369.06862	−1.795	10.5
m/z 341	$C_{15}H_{12}O_4N_2F_3$	341.07437	341.07415	−0.639	9.5
m/z 282	$C_{14}H_{11}O_2NF_3$	282.07364	282.07324	−1.434	8.5
m/z 218	$C_8H_3O_3NF_3$	218.00595	218.00571	−1.107	6.5
m/z 185	$C_8H_{11}O_4N$	185.06826	185.06826	0.004	4.0
m/z 169	$C_8H_{11}O_3N$	169.07334	169.07328	−0.383	4.0
m/z 156	$C_7H_{10}O_3N$	156.06552	156.06548	−0.254	3.5
ala-NTBC					
$[M+H]^+$	$C_{17}H_{16}O_6N_2F_3$	401.09550	401.09497	−1.315	9.5
m/z 355	$C_{16}H_{14}O_4N_2F_3$	355.09002	355.08949	−1.487	9.5
m/z 282	$C_{14}H_{11}O_2NF_3$	282.07364	282.07317	−1.665	8.5
m/z 170	$C_8H_{12}O_3N$	170.08117	170.08102	−0.881	3.5
m/z 124	$C_7H_{10}ON$	124.07569	124.07569	−0.004	3.5
β-ala-NTBC					
$[M+H]^+$	$C_{17}H_{16}O_6N_2F_3$	401.09550	401.09504	−1.140	9.5
m/z 383	$C_{17}H_{14}O_5N_2F_3$	383.08493	383.08473	−0.529	10.5
m/z 282	$C_{14}H_{11}O_2NF_3$	282.07364	282.07323	−1.453	8.5
m/z 218	$C_8H_3O_3NF_3$	218.00595	218.00571	−1.120	6.5
m/z 183	$C_9H_{13}O_3N$	183.08899	183.08896	−0.190	4.0
m/z 170	$C_8H_{12}O_3N$	170.08117	170.08106	−0.645	3.5

[a] CID = collision-induced dissociation.
[b] DBE = double-bond equivalents.

DOI: 10.1002/rcm

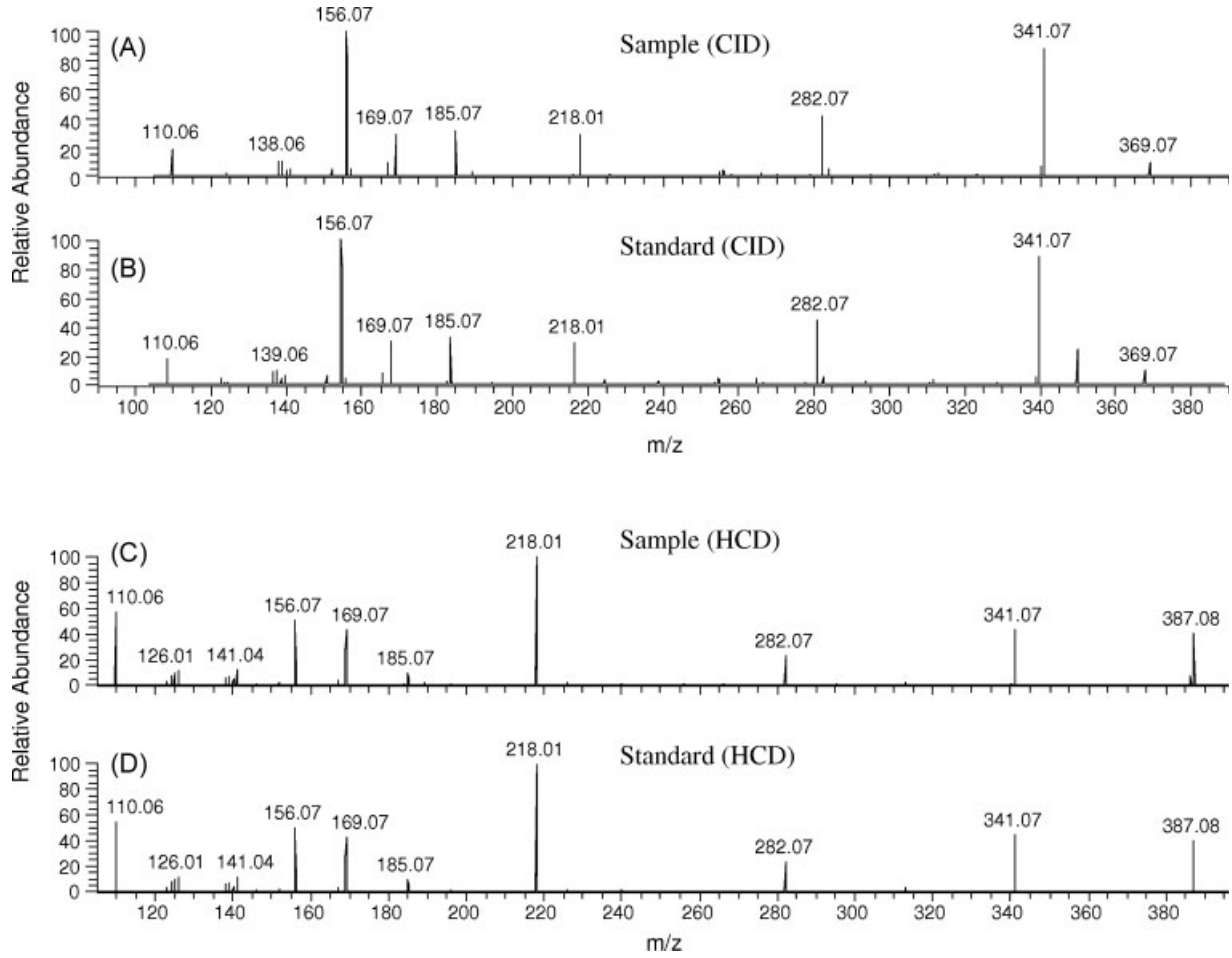

Figure 4. Product ion spectra in positive ion mode of the urinary metabolite gly-NTBC at CID 35 V (a) and of the corresponding synthesized standard at CID 35 V (B). Product ion spectra in positive ion mode of the urinary metabolite gly-NTBC at HCD 50 V (C) and of the corresponding synthesized standard at HCD 50 V (D).

fragmentation pattern as the above-mentioned CID spectrum (Fig. 4(C)). In addition, a comparison of both product ion scans (CID and HCD) indicated considerable differences with regard to the peak intensity distributions. The major peak with highest intensity in the HCD spectrum was the product ion at *m/z* 218 which corresponded to the acylium cation which was formed by the loss of $C_6H_{11}O_3N$ (169 Da) from the precursor ion. The proposed molecular structures of some significant product ions are shown in Fig. 5(A). Finally, a synthesized standard confirmed the postulated metabolite. Both dissociation experiments (CID and HCD) were performed with the standard compound and their product ion scan spectra showed exactly the same fragmentation pattern and intensity values as the product ions found in the urine samples (Figs. 4(B) and 4(D)). Interestingly, the protonated molecular ion (*m/z* 387) of the matrix-free standard solution showed only by CID experiments (35 V) a loss of two water molecules at *m/z* 369 and 351 (Fig. 4(B)) instead of one as in the real sample, while, by performing HCD experiments (50 V), no elimination of water molecules was observed (Fig. 4(D)). The higher concentration of the standard in the matrix-free solution may cause this fragmentation process. Based on these results, the unknown metabolite at *m/z* 387 was identified as gly-NTBC. The proposed molecular structures for all intense fragmentation ions are depicted in Supplementary Fig. S3 (see Supporting Information).

Identification of the urinary metabolite β-ala-NTBC

In the precursor ion scan mass spectrum of the ion at *m/z* 218, a metabolite at *m/z* 401 was detected in HT-1 urine samples. This ion appeared 71 mass units higher than the unmodified drug NTBC (Fig. 2(A)). Accurate mass measurements gave a molecular formula of $C_{17}H_{16}O_6N_2F_3$ at *m/z* 401.09504 with a mass error of −1.140 ppm and a DBE of 9.5. At first, this information seemed to point to a conjugation of an amino acid moiety, namely alanine. The corresponding product ion spectrum under CID conditions at 35 V gave rise to major product ions, which are listed in Table 2. As illustrated in Fig. 6(A), a main product ion at *m/z* 170 was observed with the molecular formula of $C_8H_{12}O_3N$. Under HCD (50 V) conditions, the product ion spectrum showed the same product ions as in CID experiments but having different abundances. The major product ion in the corresponding HCD spectrum was at *m/z* 183 and yielded a molecular formula of $C_9H_{13}O_3N$ (Fig. 7(A)). To confirm the chemical structure of the postulated metabolite, which was composed of NTBC and alanine through an iminization reaction, we have synthesized the standard as described in the Experimental section. The accurate mass measurement revealed that the

DOI: 10.1002/rcm

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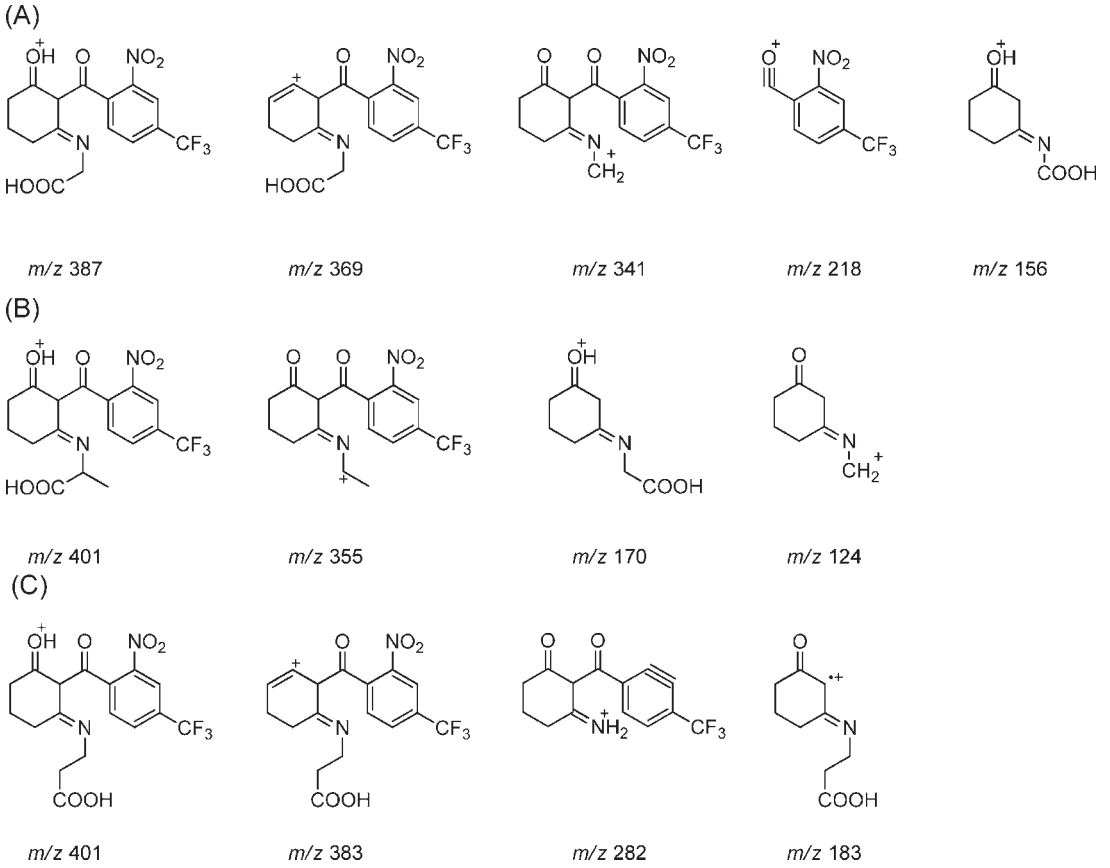

Figure 5. Proposed chemical structures of the most dominant product ions in positive ion mode of the urinary metabolite gly-NTBC (A), ala-NTBC (B) and β-ala-NTBC (C).

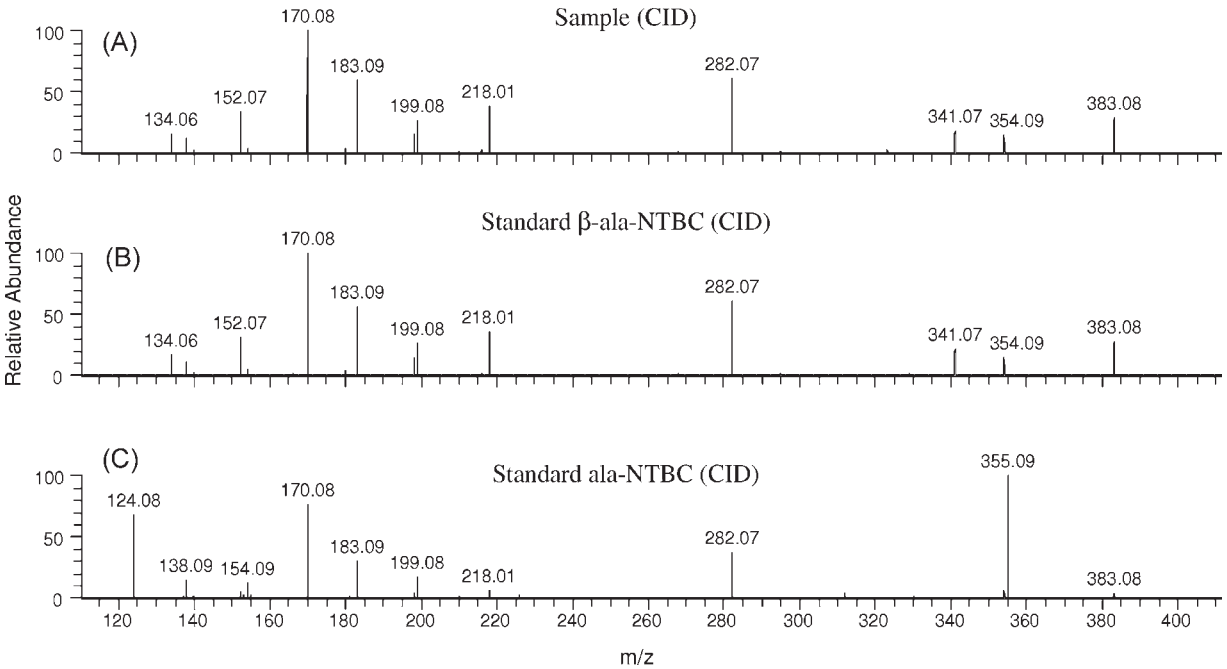

Figure 6. Product ion spectra in positive ion mode of the protonated molecule $[M+H]^+$ obtained from the urinary metabolite gly-NTBC at CID 35 V (A), of the synthesized standard with β-alanine at CID 35 V (B) and of the synthesized standard with alanine at CID 35 V (C).

DOI: 10.1002/rcm

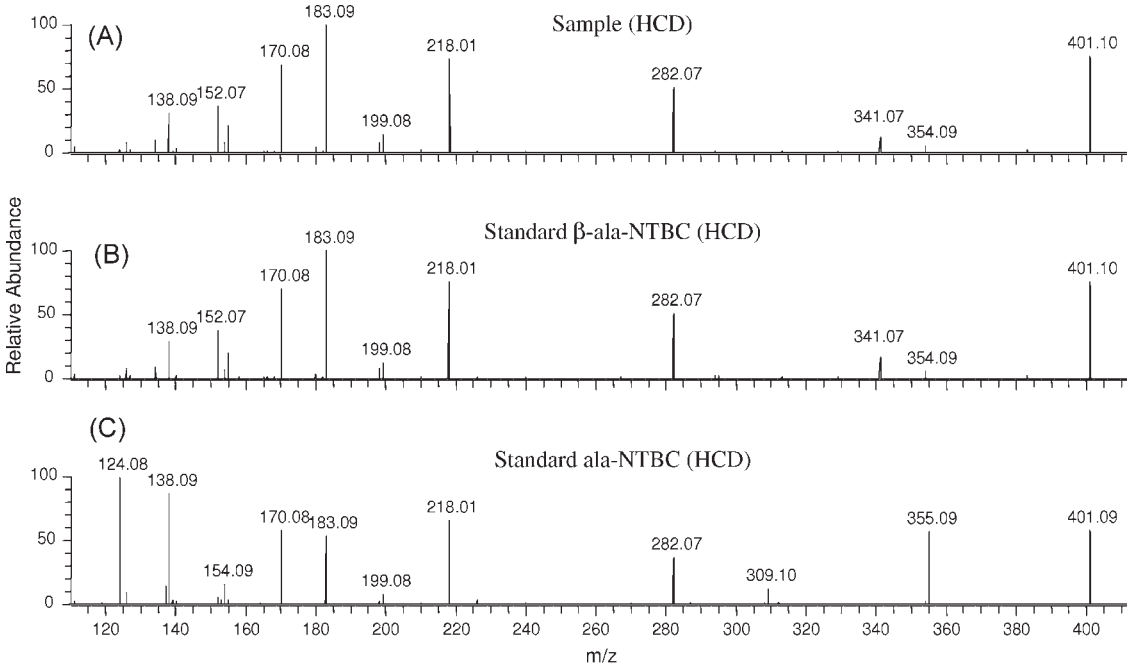

Figure 7. Product ion spectra in positive ion mode of the protonated molecule $[M+H]^+$ obtained from the urinary metabolite gly-NTBC at HCD 50 V (A), of the synthesized standard with β-alanine at HCD 50 V (B), and of the synthesized standard with alanine at HCD 50 V (C).

elemental composition of the molecular ion of the standard was the same as that was found in the urine matrix with a mass error of < -1.134 ppm. The formation of this protonated molecule $[M+H]^+$ corresponded to the loss of an oxygen from the cyclohexane ring of NTBC, as a water molecule, via the iminization step with the amino acid alanine. On the other hand, both CID and HCD product ion spectra of the standard showed a different fragmentation pattern than was obtained for the postulated metabolite (Figs. 6(C) and 7(C)). In this case, the highest abundance peak in the HCD spectrum was at *m/z* 124, and not at *m/z* 183, with the molecular formula of $C_7H_{10}ON$. The retention time of the standard (3.4 min) on the reversed-phase (RP) column was different than that which was found in urine matrix (3.0 min). The most intense product ion in the CID spectra (35 V) of the ala-NTBC standard was at *m/z* 355 showing an elemental composition of $C_{16}H_{14}O_4N_2F_3$ (-1.487 ppm). This ion corresponded to the elimination of H_2CO_2 (46 Da) of the amino acid residue and is illustrated in Fig. 5(B). In contrast, the CID (35 V) spectrum of the urinary metabolite displayed a major product ion at *m/z* 170 with the elemental composition of $C_8H_{12}O_3N$. The proposed chemical structure for this ion is given in Fig. 5(B). Based on these findings, the molecular structure of the modified NTBC (ala-NTBC) could not be ascertained. In the search for another compound with similar physicochemical properties, we chose β-alanine, which contained the same mass units differences (71 Da) and the same elemental composition as alanine. Indeed, after synthesizing the β-ala-NTBC standard as a Schiff base derivative, we found that no differences were observed either in the fragmentation pattern (CID and HCD) or in the retention time (3.0 min) between the metabolite in urine matrix and the standard (Figs. 6(B) and 7(B)). The proposed molecular structures for the fragmentation ions of β-ala-NTBC are shown in Fig. 5(C). Therefore, the unknown metabolite was unambiguously identified as β-alanine-NTBC. All significant MS^2 spectral data of both alanine and β-alanine derivatives are listed in Table 2. The proposed molecular structures of the most significant individual product ions and their corresponding accurate mass MS^2 (CID/HCD) data are shown in Supplementary Figs. S4 and S5 and in Supplementary Tables S2 and S3 (see Supporting Information).

Finally, a SRM chromatogram of the detected metabolites in positive ion mode, based on the corresponding major product, is displayed in Fig. 8. In the case of NTFA, all three product ions were selected in the negative ion mode for performing qualitative and quantitative analysis.

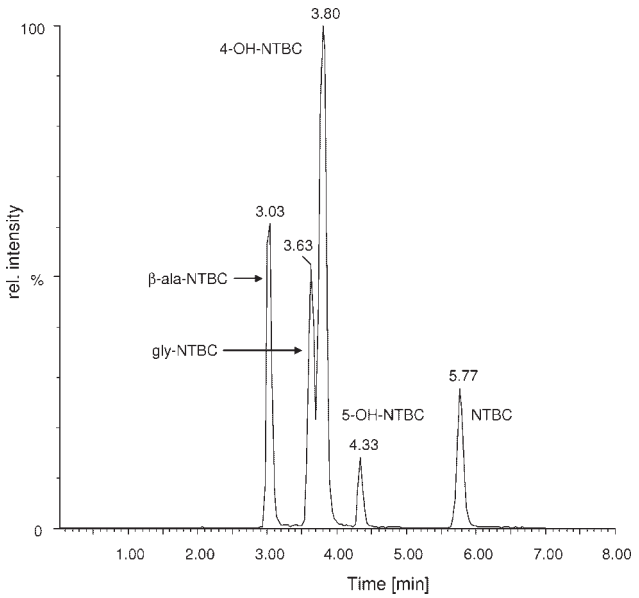

Figure 8. SRM-ESI-pos chromatogram of the five detected metabolites in urine of HT-1 patients.

DOI: 10.1002/rcm

Urine blank samples

We analyzed 11 urine blank samples from healthy persons using the same extraction procedure and LC/MS/MS method. As expected, no detection of the above-mentioned metabolites was observed. So, we concluded from these measurements that the detected metabolites could only have originated from the metabolism of the drug NTBC in HT-1 patients.

CONCLUSIONS

Two powerful mass spectrometric techniques using a triple stage quadrupole (LC/MS/MS) and a LTQ-Orbitrap were applied for the identification of the NTBC metabolites in urine of HT-1 patients. The aim of this study was to identify the known and unknown metabolites based on different MS and MS/MS techniques in order to further elucidate the various drug metabolisms.

Therefore, NTBC and its five formed metabolites were identified in urine of HT-1 patients. Two of these metabolites (gly-NTBC and β-ala-NTBC) have never been described before and were unambiguously identified by their retention times, elemental compositions, MS/MS fragmentation (CID and HCD) and ascertained by synthesized standards. These two novel metabolites were identified as amino acids conjugates, otherwise known as Schiff base derivatives. These results indicated that the drug NTBC can be subjected to three metabolic pathways (hydrolysis, hydroxylation and amino acid conjugation) *in vivo*, which could be of primordial importance for the further pharmacological study of dose-dependent NTBC effectiveness. Using this MS strategy and extraction procedure no glucuronidation conjugation of the drug was observed. Hence, the combination of tandem mass spectrometry and high-resolution accurate mass measurement increases the confidence of the proposed structures. The relative errors of precursor and product ions as determined with ESI positive ion mode were below 2 ppm using internal lock mass calibration; while in negative ion mode the errors were without lock mass correction <5 ppm. The results obtained in this work represent an important step in monitoring metabolism of NTBC in HT-1 patients.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

We would like to thank the Swedish Orphan International AB for providing us pure NTBC standard for this work.

REFERENCES

1. Lindstedt S, Holme E, Lock EA, Hjalmarson O, Strandvik B. *Lancet* 1992; **340**: 813.
2. Moran GR. *Arch. Biochem. Biophys.* 2005; **433**: 117.
3. Strife CF, Zuroweste EL, Emmett EA, Finelli VN, Petering HG, Berry HK. *J. Pediatr.* 1977; **90**: 400.
4. Phornphutkul C, Introne WJ, Perry MB, Bernardini I, Murphey MD, Fitzpatrick DL, Anderson PD, Huizing M, Anikster Y, Gerber LH, Gahl WA. *N. Engl. J. Med.* 2002; **347**: 2111.
5. Herebian D, Spiekerkötter U, Lamshöft M, Thimm E, Laryea M, Mayatepek E. *J. Chromatogr. B* 2009; **877**: 1453.
6. Weinshilboum R. *N. Engl. J. Med.* 2003; **348**: 529.
7. Wang PG, Zhang J, Gage EM, Schmidt JM, Rodila RC, Ji QC, El-Shourbagy TA. *Rapid Commun. Mass Spectrom.* 2006; **20**: 3456.
8. Koulman A, Woffendin G, Narayana VK, Welchman H, Crone C, Volmer DA. *Rapid Commun. Mass Spectrom.* 2009; **23**: 1411.
9. Szczecinski P, Lamparska D, Gryff-Keller A, Gradowska W. *Acta Biochim Pol.* 2008; **55**: 749.
10. Herebian D, Zühlke S, Lamshöft M, Spiteller M. *J. Sep. Sci.* 2009; **32**: 939.
11. Lim H-K, Chen J, Sensenhauser C, Cook K, Subrahmanyam V. *Rapid Commun. Mass Spectrom.* 2007; **21**: 1821.
12. Mitchell G, Bartlett DW, Fraser TEM, Hawkes TR, Holt DC, Townson JK, Wichert RA. *Pest Manag. Sci.* 2001; **57**: 120.
13. Lock EA, Ellis MK, Gaskin P, Robinson M, Auton TR, Provan WM, Smith LL, Prisbylla MP, Mutter LC, Lee DL. *J. Inher. Metab. Dis.* 1998; **21**: 498.

DOI: 10.1002/rcm