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Article

Simple and Fast Quantification of Nitisone (NTBC) using Liquid Chromatography–Tandem Mass Spectrometry Method in Plasma of Tyrosinemia Type 1 Patients

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Tyrosinemia type 1, which is caused by a deficiency in fumarylacetoacetate hydrolase, is successfully treatable with nitisone (NTBC), an inhibitor of 4-hydroxyphenyl pyruvate dioxygenase. The recommended average dose of NTBC is 1 mg/kg per day. A rapid liquid chromatography (LC) coupled with negative electrospray ionization tandem mass spectrometry method was developed and validated for the quantification of NTBC in heparinized human plasma. The plasma samples were prepared by precipitation in acetonitrile. NTBC and the internal standard (IS) were chromatographed on a BEH C18 column. Gradient elution was done with a mixture of 10 mM ammonium acetate and methanol. The analyte was analyzed by LC–tandem mass spectrometry with only 2 min run time. Selected reaction monitoring modes for detection of NTBC and the IS were achieved by using m/z 328 > 281 and 234 > 190, respectively. The LC retention times for NTBC and IS were 0.99 and 0.93 min, respectively. The method was linear in the concentration range of 0.75–150 μM with $r \geq 0.998$. Thus, this method is suitable for follow-up of patients treated with NTBC, because the current therapeutical concentrations range from 20 to 120 μM.

Introduction

Hereditary tyrosinemia type 1 (HT1; MIM number 276700), the most severe disease of the tyrosine catabolic pathway, is caused by a deficiency in fumarylacetoacetate hydrolase and is characterized by progressive liver damage, renal tubular dysfunction and increased urinary excretion of toxic metabolites such as succinylacetone (SA) and delta-aminolevulinic acid (δ-Ala). Dietary treatment with phenylalanine and tyrosine restriction is insufficient to improve the disease; if left untreated, most patients die during childhood unless they receive a liver transplant. Since 1992, nitisone [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclo-hexanedione (NTBC)] has become an effective pharmacological treatment for HT1 by inhibiting 4-hydroxyphenyl pyruvate dioxygenase (1, 2). Nitisone can prevent the development of liver disease and significantly reduce the risk of developing hepatocellular carcinoma. The recommended average dose of NTBC is 1 mg/kg per day (2). Efficiency is controlled with urinary SA and δ-Ala concentrations, which can cause steep drops in serum alphafetoprotein (AFP), normalization of synthetic liver functions, reduction in phosphate loss in urine and healing of active rickets.

Nitisone seems to have few adverse effects, primarily thrombocytemia, leukopenia, cutaneous disorders and various visual disorders, attributable to secondary hypertyrosinemia (2). The follow-up of plasma NTBC concentrations in HT1 treated patients in concert with urinary SA and δ-Ala, serum AFP and tyrosinemia should help to determine the efficient therapeutic range of NTBC.

The detection of NTBC in plasma by high-performance liquid chromatography (HPLC) with ultraviolet (UV), fluorescent or tandem mass spectrometric (MS-MS) detection has been reported (3, 4, 5). This work describes a further improvement in the chromatographic process that allowed for a fast 2-min assay of NTBC without compromising the specificity and sensitivity. This was achieved by the use of ultra-performance liquid chromatography (UPLC), which combined a special column packed with very small particles (1.7 μm) and very high back pressures, coupled to MS-MS detection.

Materials and Methods

Chemicals

2 nitro,4-(trifluoromethyl)benzoic acid as internal standard (IS) and LC–MS grade ammonium acetate were purchased from Fluka Chemie (Buchs, Switzerland). LC–MS-grade acetonitrile and methanol were purchased from Sigma Aldrich (St Louis, MO). Water for the LC–MS was purchased from J.T. Baker (VWR, Batavia, IL). NTBC (Orfadin) of pharma grade was supplied by Swedish Orphan Biovitrum (Stockholm, Sweden) as a solid compound.

Sample and pretreatment procedure

This study was carried out according to the Declaration of Helsinki for biomedical research involving human subjects and approved by the local ethical committee. Blood samples from patients with HT1 treated with NTBC were collected into lithium-heparin tubes and centrifuged for 10 min at 2,000 *g*. Plasma was stored at −20°C until use. Plasma samples or calibrators (20 μL) were precipitated with 250 μL of acetonitrile containing the IS at a constant concentration of 6 μM. The tubes were thoroughly mixed for 45 s and left for 10 min before being centrifuged at 10,000 *g* for 5 min. Then, 25 μL of the supernatant was added into wells of the autosampler plate (96 well-plate, Waters) that contained 475 μL of a mixture of ammonium acetate 10 mM and methanol (v/v, 60/40). Two microliters were subjected to analysis by UPLC–MS-MS.

LC and MS systems

The UPLC–MS-MS consisted of a Waters Acquity and a triple quadrupole tandem mass spectrometer. Chromatographic

separation was performed on an Acquity UPLC BEH C18 column [2.1 × 50 mm (i.d.), 1.7 μm particule size, Waters] protected by a guard column of the same material and designed to withstand 15,000 psi. Colomn temperature was maintained at 40°C. For separation of the compounds, we used a mixture of 10 mM ammonium acetate (eluent A) and methanol (eluent B) at a flow rate of 0.5 mL/min in a gradient mode. The following linear elution gradient was used: 0–0.4 min, 70% A/30% B; 0.4–0.6 min, 70% to 5% A; 0.6–1.3 min, 5% A/95% B; 1.3–1.7 min, 5% to 70% A. Between runs, the system was recalibrated with 70% A for 0.3 min. UPLC–M-MS analysis required 2 min. To reduce the amount of solvent gaining entry into the mass spectrometer, the LC eluent was diverted to waste during time intervals 0–0.5 and 1.2–2 min. NTBC and IS were detected by multiple reaction monitoring using the following transitions of mass to charge (m/z): NTBC, 328 > 281 (quantifier ion) and 328 > 239 (qualifier ion); IS, 234 > 190 (quantifier ion). The electrospray ionization source was operated in the negative ion mode at a capillary voltage of 2.8 kV. The ion source and the desolvatation temperature were maintained at 120 and 450°C, respectively. Other LC–MS-MS instrument settings were as follows: cone gas flow, 50 L/h; desolvatation gas flow, 1,100 L/h; cone voltage, 20 V (NTBC and IS); collision energy, 10/30 eV (for both analytes as quantifier/qualifier ions); collision gas, argon; dwell time, 90 ms. All LC–MS-MS peak areas were integrated by use of Waters QuanLynx software.

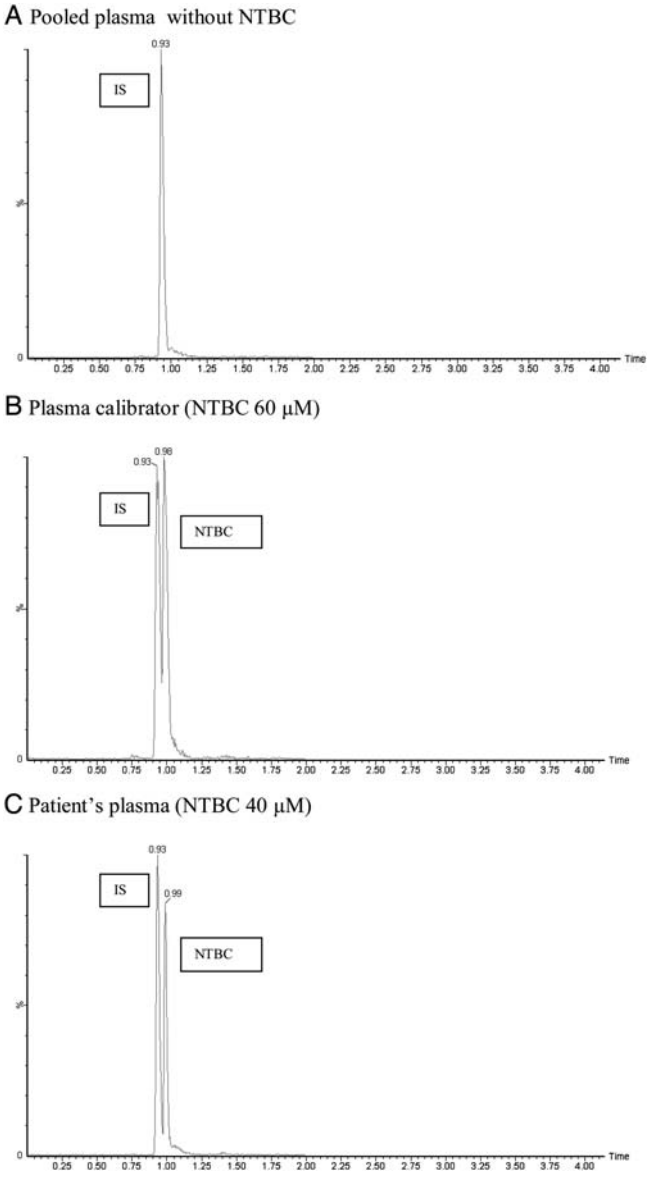

Figure 1. Total ion chromatograms of (A) a pooled plasma without NTBC (6 μM IS); (B) a plasma calibrator (60 μM NTBC and 6 μM IS); (C) a plasma sample of an HT1 patient (40 μM NTBC and 6 μM IS).

Method validation

To determine the linear range, standard solutions of NTBC were prepared via serial dilution of a 0.9 mM stock solution in acetonitrile. A pooled plasma sample without NTBC was used as a biological matrix. Plasma calibrators were prepared by adding 100 μL of acetonitrile or each NTBC calibrator to 500 μL of pooled plasma. Nine-point calibration standard solutions ranging from 0.75 to 152 μM were used to determine the linear range. Pooled plasma without added NTBC was also included in the set of calibrators. These sample calibrators were stored at −80°C until analysis. Intra-day ($n = 10$) and inter-day ($n = 10$) variations were assessed by analyzing calibrators at nine different concentrations over a period of 10 working days. The data were also used to calculate the recovery based on the following equation: Recovery (%) = 100 × found concentration/added concentration.

Results and Discussion

The ideal IS for this analytical method is isotopically labelled NTBC, which is not commercially available. Therefore, 2 nitro,4-(trifluoromethyl)benzoic acid was used, which presents a similar chromatographic separation to NTBC. Figure 1 shows a mass chromatogram obtained by UPLC–MS-MS for a plasma calibrator containing NTBC and IS. As shown, NTBC elutes at 0.99 min and IS at 0.93 min.

Validation of the method

The performance data of this UPLC–MS-MS method are presented in Table I. One set of plasma calibrators was run with

Table I
Recovery of NTBC and Intra-Day and Inter-Day Precision of the Method

	Intra-day ($n = 10$)			Inter-day ($n = 10$)		
NTBC (μmol/L)	Mean (μmol/L)	CV (%)	Recovery (%)	Mean (μmol/L)	CV (%)	Recovery (%)
0.75	0.72	12	96	0.73	20	97
1.5	1.6	11	106	1.6	16	104
7.5	7.6	12	101	8.1	9	107
15	14.9	11	99	15.4	9	101
30.5	33.8	11	110	30.7	7	101
61	63.7	5	104	63.9	7	105
91.2	92.9	5	102	92.2	6	101
122	125	4	102	120	3	99
152	145	4	95	147	3	97

each batch of samples and linearity was monitored for 10 working days. Measured data were subjected to linear regression analysis of NTBC to IS peak area ratio against the concentration (μM). The linearity of the method was found to be in the range of 0.75–152 μM. Calibration plots were reproducible with the following linear regression equation: $y = 0.014x + 0.002$ ($r > 0.998$). The corresponding standard deviations were ±0.002 for the slope and ±0.002 for the intercept. The lower limit of quantification (LLOQ) at a signal-to-noise ratio (S/N) of 20 was 0.75 μM, whereas the detection limit (S/N = 3) was 0.15 μM (Figure 2). Both the intra-assay and inter-assay variations for the pooled plasma samples were satisfactory with a maximum coefficient of variation (CV) of 12 % in the intra-day study and 20% in the inter-day study. The overall recoveries of NTBC from the plasma sample were calculated at different concentrations and found to be in the range of 95–110%, as shown in Table I. Although a previously reported LC–MS-MS assay for the analysis of NTBC in plasma was linear up to 40 μM (4), our developed method was linear up to 152 μM. This linearity seems to be more convenient for NTBC measurements in the plasma of treated HT1 patients, because NTBC concentrations range from 20 to 100 μM (4).

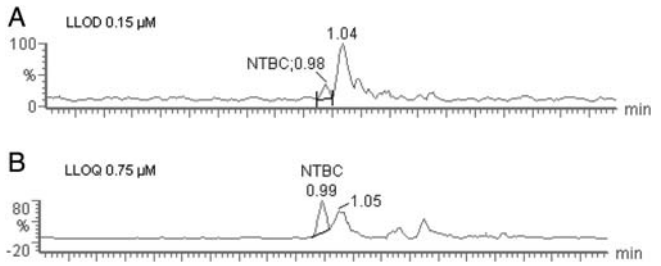

Figure 2. LC-ESI-MS/MS extracted ion chromatogram for (A) the lower limit of detection (LLOD) of NTBC and (B) the lower limit of quantification (LLOQ) of NTBC.

Table II
NTBC Concentrations in Plasma of 25 HT1 Patients

Patient	Age (year, month)	Weight (kg)	NTBC dose (mg/kg/day)	Plasma NTBC (μmol/L)	Urinary SA (μmol/L)
1	7 mo	8	1	25	<0.5
2	1 yr, 2 mo	9	1.1	40	<0.5
3	2 yr	11	1	38	NA
4	3yr, 6 mo	14	1	44	<0.5
5	3yr, 10 mo	16	1	64	<0.5
6	4yr, 3 mo	18	1	75	<0.5
7	4yr, 6 mo	16	1	32	<0.5
8	4yr, 7 mo	18	1	38	NA
9	5yr, 5 mo	16	1	50	2.3
10	6 yr, 5 mo	20	1	57	<0.5
11	6 yr, 5 mo	20	1	37	<0.5
12	5 yr, 3 mo	21	1	42	1.2
13	6 yr, 1 mo	24	1	64	<0.5
14	7 yr, 5 mo	24	1	50	<0.5
15	8 yr	22	1	42	<0.5
16	9 yr	30	1	50	<0.5
17	10 yr, 10 mo	30	1.1	60	<0.5
18	12 yr, 11 mo	46	1	21	<0.5
19	14 yr, 11 mo	38	1	21	<0.5
20	14 yr	68	0.9	94	0.7
21	18 yr, 4 mo	86	0.7	58	<0.5
22	4yr, 7 mo	19	1.4	52	<0.5
23a	7 yr, 1 mo	22	Not taken for a few days	1.4	1.1
23b	7 yr, 2 mo	22	1.4	57	<0.5
24	9 yr	30	2	122	<0.5
25	3yr, 7mo	19	2	90	NA

NA = not available.

Analysis of patients' samples

NTBC concentrations were determined in the plasma of 25 HT1 patients. In all patients, NTBC was orally given twice daily and blood for NTBC measurement was collected 12 h after NTBC intake. With a half-life of 54 h, NTBC concentrations are expected to be stable during the day, as previously reported (4). As shown in Table II, patients were usually treated with an NTBC dose of 0.9 to 1.1 mg/kg per day ($n = 20$). NTBC concentrations ranged from 21 to 94 μM, with 70% between 30 and 60 μM. These NTBC concentrations in plasma are usually sufficient to inhibit the production of hepatotoxic metabolites, because urinary SA excretion was found to be below 0.5 μM in all but three patients. To reach the therapeutic range of NTBC (30–60 μM), one patient had a lower NTBC intake (0.7 mg/kg) and two had higher NTBC intake (1.4 mg/kg). Thus, NTBC metabolism and distribution differences between patients and NTBC measurements are essential to define individual NTBC posology. Two patients that presented with hepatic nodules and high AFP levels needed higher NTBC intake (2 mg/kg per day) and their NTBC concentrations reached 90–122 μM. One patient (number 23) stopped NTBC treatment for few days and plasma NTBC concentration decreased to 1.4 μM. In this condition, urinary SA increased above 1 μM, indicating that a low NTBC concentration in plasma is insufficient to efficiently inhibit SA production.

Conclusion

We developed a novel analytical method based on UPLC–electrospray ionization–MS-MS for NTBC determination in plasma with a run time of 2 min and with good selectivity and sensitivity. This rapid method also presents a good linearity up to 150 μM, which is suitable for NTBC treatment follow-up.

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