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Monitoring tyrosinaemia type I: Blood spot test for nitisinone (NTBC)

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ABSTRACT

Background: Quantification of nitisinone, 2-(nitro-4-trifluoromethylbenzoyl)1,3-cyclohexanedione (NTBC) has been repeatedly described. Nevertheless monitoring of NTBC has not yet become part of routine therapy surveillance in tyrosinaemia type I (OMIM 276700).
We developed a blood spot test to facilitate collection and transport of samples. Furthermore, the test material can be used for determination of other parameters like tyrosine and succinylacetone.
Method: For quantification of NTBC in blood spots filter paper discs of 3.2 mm diameter were extracted with 150 μL methanol containing mesotrione as internal standard (IS). Analysis was done by UPLC–MS/MS on a Xevo mass spectrometer (ESI+), (MRM). Parent ions were 330.05 for NTBC and 340.05 for IS, daughter ions were *m/z* 217.95 and *m/z* 125.95 for NTBC, and *m/z* 227.95 and *m/z* 103.95 for IS.
Results: The calibration curve for NTBC in blood spots was linear from 0.1 μmol/L to 100 μmol/L. Recovery exceeded 73.1%, CV intraday and interday were below 9.6%. Instrumental run time was 2.5 min. Sensitivity of the method was 0.1 μmol/L. NTBC concentrations in plasma were higher than in blood spots by a factor of 1.56 ± 0.13.
Conclusion: As demonstrated in patients with tyrosinaemia type I quantification of NTBC by UPLC–MS/MS in blood spots is feasible and gives valuable information for monitoring NTBC treatment.

1. Introduction

Nitisinone (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) has been the standard therapeutic regimen in hepatorenal tyrosinaemia (Tyr I) (OMIM 276700) for more than 10 years [1–5]. Before it had been introduced, the prognosis of this inborn error of metabolism was extremely poor, except for those patients who successfully underwent liver transplantation [6–8]. NTBC is a potent inhibitor of 4-hydroxyphenylpyruvate dioxygenase [9] and binds tightly to the enzyme, thus reducing the flux of metabolites through the catabolic tyrosine pathway. The formation of toxic products accumulating proximal to the deficient fumarylacetoacetate hydrolase (FAH) is reduced (substrate reduction). In order to avoid undue accumulation of tyrosine, NTBC therapy has to be accompanied by a low-protein diet supplemented with a tyrosine-free amino acid mixture.

In most patients, NTBC therapy is initiated with a dose of 1–2 mg/kg per day, given in 2 or 3 doses. This dosage has been chosen based on clinical and biochemical observations. The aim of therapy is to improve liver function by suppressing the production of toxic compounds. Succinylacetone (SA) is commonly used as a surrogate parameter for toxicity. There are no published data regarding bioavailability and cellular uptake of NTBC. A few anecdotal patients were reported who did well on lower doses of NTBC. El-Karaksy et al. [10] described 4 patients in whom doses as low as 0.55 to 0.65 mg/kg per day were sufficient to prevent liver dysfunction.

Biochemical monitoring of NTBC therapy in Tyr I patients is usually done by testing urine for SA excretion and/or by measuring tyrosine and SA concentrations in serum or plasma. Dried blood spots on filter paper have also been used for quantification of tyrosine and/or SA. Determination of NTBC blood levels has not generally been part of clinical practice, although a liquid chromatographic method has shown sufficient sensitivity [11]. Cansever et al. [12] demonstrated clinical utility of capillary electrophoresis of NTBC with a quantification limit of 10.6 μmol/L using UV detection at 278 nm. Herebian et al. [13] recently described the quantification of NTBC by LC–MS/MS suitable for plasma samples down to 100 μL. Linearity of the method was shown within a range of 2.5 to 40 μmol/L. Interfering substances have not been found in vivo.

It was our aim to improve monitoring of NTBC treatment by measuring NTBC levels in dried blood spots. In particular, patients living in remote areas in developing or newly industrialized countries could benefit from this procedure. Even blood collection at home is possible. Dried blood on filter paper is the optimal material for easy

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handling. It can easily be sent over long distances to the few laboratories providing specialized techniques. However, as reference values for NTBC treatment have been established in serum and plasma only, it was necessary to relate blood spot values to plasma or serum in the same patients. As there are no published data on the distribution of NTBC in blood between serum and erythrocytes a theoretical approach is not possible. Blood spot levels had to be directly compared with plasma/serum concentrations in the same patient.

2. Material and methods

NTBC was supplied by Swedish Orphan (Langen, Germany). Internal standard (IS) was mesotrione purchased from Fluka (Deisenhofen, Germany). All other chemicals were commercially obtained at the highest purity grade available. Filter paper for blood spots was 2.460.00015 Q, Lot Nr. 08-150 from Munktell-Filtrak, Baerenstein, Germany.

2.1. Standards and calibrators

Heparinized venous blood for all experimental work as well as for standards and calibrators was collected from a healthy male volunteer. The donor blood did not show detectable amounts of NTBC. The hematocrit was 42.8%. Blank and spiked whole blood were spotted on filter paper cards. Each spot contained a volume of 25 μL. Spot diameter was 9 mm. After air drying at ambient temperature for at least 4 h the material was ready for immediate use or for storing at −20 °C. The calibration curve was established with blood spots containing 0, 0.1, 1, 5, 20, 40, 60, 80 and 100 μmol/L NTBC. A similar calibration curve was also established for plasma. Quality control blood spot samples were adjusted to 5, 25 and 100 μmol/L. These were also used for quantifying intraday (11 measurements per concentration) and interday (1 measurement per day for 20 days) precision.

2.2. Patient samples

Venous blood spotted on filter paper cards (plus plasma in 11 patients) was sent to our laboratory to monitor NTBC therapy. In addition to NTBC, tyrosine and succinylacetone (SA) were determined. The latter was analyzed using standard methods described elsewhere [14,15].

2.3. Sample preparation

For NTBC quantification, fresh 3.2 mm diameter blood spots were extracted with 150 μL methanol containing the internal standard mesotrione (0.14 μmol/L) in a 96 well plate by shaking the plate for 30 min at room temperature. The extract was dried under a gentle stream of nitrogen at 65 °C. The residue was resolved in 200 μL acetonitrile/water 30/70 (by volume), and injection volume was 7.5 μL.

For plasma, the same method was used, however equal volumes of plasma calibrator and sample were used instead of blood spots.

2.4. UPLC–MS/MS analysis

Quantification of NTBC was performed on a Xevo MS/MS instrument coupled to an ultra high pressure liquid chromatography equipment (UPLC) both from Waters (Eschborn, Germany). Chromatographic separation was achieved on an AQUITY UPLC BEH C18 1.7 μm (2.1×50 mm) column at 40 °C. A constant flow of 450 μL/min of aqueous acetonitrile 50/50 (by volume) containing 0.1% formic acid and 0.01% trifluoroacetic acid was used. Separation was isocratic, after every 50 samples the column was washed with pure acetonitrile. The ionization parameters were a capillary voltage of 1.5 kV and source and desolvation temperatures of 150 and 600 °C respectively. Cone and desolvation gas (N_2) were fixed at 50 and 1000 L/h respectively. Collision gas (Ar) flow was 0.15 mL/min. Results were generated in the positive ion mode with cone energy set at 22 V, collision voltage at 31 V (17 V for qualifiers) and dwell time at 0.032 s. The analysis was performed in the multiple-reaction monitoring (MRM) mode using MassLynx 4.1 (Waters Corp.) software for automatic data processing. We measured two mass specific transitions for NTBC (m/z 330.05→217.95 and 330.05→125.95) and two transitions for mesotrione (m/z 340.05→227.95 and 340.05→103.95). Signals of IS and NTBC were of comparable relative intensity. Instrumental run time was 2.5 min.

3. Results

Analytical method validation for NTBC was accomplished following standard guidelines for industry [16]. The regression equation for the calibration curve for blood spots was based on eight calibration points with eightfold measurements ($y=0.1891x-0.0019$ with weighting $1/x$). The calibration curve of dried blood spot calibrators was linear over the total range measured with a regression coefficient of 0.995. The lower limit of quantification (signal to noise ratio 10:1) was 0.100 μmol/L, and the limit of detection was 0.019 μmol/L. Absolute recovery of IS was 92%. Recovery and imprecision for NTBC are shown in Table 1.

To determine ion suppression by whole blood, an extract of blood spots was injected into a flow of NTBC and mesotrione solution (30 μmol/L) inserted by a syringe pump at a rate of 20 μL/min. Decreased intensity of the baseline in the mass transition of NTBC and IS occurred between 0.15 and 0.30 min. No further ion suppression appeared at any later point of time. Neither the NTBC peak at 0.53 min nor the IS peak at 1.24 min was influenced by ion suppression. The isocratic separation of NTBC and IS in a patient sample is shown in Fig. 1.

4. Patient samples

4.1. Comparison of blood spot to plasma values

In 11 patients we had the opportunity to analyse blood spots and plasma collected simultaneously. Values obtained in blood spots were lower than in plasma by a factor of 1.56±0.13.

4.2. Follow-up of patients by blood spot testing

As an example of successful NTBC treatment we describe findings in two brothers suffering from Tyr I. Patient 1 has been treated with NTBC for about 8 years, his younger brother (patient 2) for more than five years. Therapy had been started with 1 mg/kg per day but the daily dose was reduced later on. Despite low NTBC dosage in both patients no excretion of SA in urine nor any positive blood spot test for SA was found. Psychomotor development as well as liver function in these children is normal. Compliance was good in both patients. Monitoring of NTBC concentrations was possible beginning at an age of 82 months for the older patient and at 51 months for the younger one. Results are shown in Table 2.

Patient 3 is shown as an example of unsuccessful treatment (Fig. 2). The girl was 8 months old when she was hospitalized due to severe liver disease. She suffered from hepato-splenomegaly,

Table 1
Recovery of NTBC from dried blood samples and precision data.

Concentration [μmol/L]	Recovery [%]	Intraday CV [%]	Interday CV [%]
5.0	75.3	6.4	9.6
25.0	73.1	5.3	8.7
100.0	75.4	2.5	9.3