Journal of Chromatography B, 879 (2011) 671–676

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Comparison of plasma and dry blood spots as samples for the determination of nitisinone (NTBC) by high-performance liquid chromatography–tandem mass spectrometry. Study of the stability of the samples at different temperatures

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ARTICLE INFO

Article history:
Received 8 October 2010
Accepted 20 January 2011
Available online 2 February 2011

Keywords:
Tyrosinemia
Tandem mass spectrometry
Nitisinone
Plasma
Dry blood spots
Stability

ABSTRACT

Tyrosinemia is an inborn error of metabolism characterized by the accumulation of tyrosine as well as toxic by-products. NTBC or nitisinone is a drug currently used for the treatment of tyrosinemia that avoids the formation of these toxic substances. This paper presents the determination of NTBC in plasma and dry blood spots by high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry. The concentration of NTBC in matched plasma-dry blood spots was compared and the study of degradation of NTBC in plasma and dry spots at different temperatures is presented. Method: For sample preparation, plasma proteins were precipitated with acetonitrile and 3-mm discs were extracted with methanol. ESI(+) was used as inozation method and the analytes were detected by multiple reaction monitoring using the transitions 330 > 218 for NTBC and 340 > 228 for mesotrione, used as internal standard. Results: There is good correlation between concentrations obtained in dry blood spots and plasma (r^2 = 0.83), although values are 2.4 times higher in plasma samples. NTBC in plasma is stable at least for 45 days frozen at −30 °C and refrigerated at 4 °C. However, it shows slow decomposition at room temperature, approximately 30% after 45 days. The method shows good precision, accuracy and linearity and the detection limit is 50 nmol/L and paper samples are appropriate for the monitorization of NTBC.

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1. Introduction

Hereditary tyrosinemia type I (OMIM 276700) is the most common of the three known diseases caused by defects in tyrosine metabolism. This type of tyrosinemia is inherited as autosomal recessive disease caused by a mutation in the gene coding for fumarylacetoacetate hydrolase, the last enzyme in the tyrosine catabolism. This metabolic blockage induces the accumulation of metabolites of the upper part of the metabolic path, such as maleylacetoacetate and fumarylacetoacetate, and the activation of secondary metabolic paths producing succinylacetoacetate and succinylacetone. These toxic metabolites are responsible, among others, for liver and kidney damage [1]. Fortunately, early diagnoses by means of the determination of succinylacetone during neonatal screening are becoming increasingly available thanks to tandem mass spectrometry [2].

Treatment consists in the pharmacological inhibition of 4-hydroxyphenylpyruvate dioxygenase, one of the enzymes that participate in the formation of secondary toxic metabolites, in addition to the restricted diet free of the offending amino acids. The drug, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC or nitisinone), was originally developed as an herbicide, but it found a new application in the treatment of hereditary tyrosinemia type I [3]. NTBC has replaced liver transplantation as the first-line treatment for this rare condition [4].

Plasmatic NTBC monitoring is necessary, because patients need surveillance of concentration for optimal dosage adaptation and for pharmacokinetic studies [5,6]. NTBC is rapidly absorbed with an approximate half life of 54 h. Therapeutic plasmatic concentrations are 15–40 μmol/L and Spanish guidelines recommend its determination every three months, together with methionine, tyrosine and succinylacetone [7]. The analysis of NTBC is important in order to avoid over dosage in patients with impaired metabolism of the drug, or to avoid the production of toxic metabolites in case of a low dose of the drug is present in plasma.

The determination of this drug in plasma has been carried out by a number of methods, including HPLC with photometric detection in plasma, using a precolumn that elutes most polar compounds before transferring the analyte, retained on it, to the chromato-

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doi:10.1016/j.jchromb.2011.01.031

graphic column [8], and most recently capillary electrophoresis with photometric detection [9]. Nowadays, tandem mass spectrometry is becoming more available in clinical laboratories. This technique provides high sensitivity and very high selectivity; thus, it is the technique of choice since it normally requires a very quick and simple sample preparation, usually with easy method development [10]. In addition, mass spectrometry and the study of the fragments produced by the parent ions have allowed the detection and structural elucidation of several NTBC metabolites and conjugates in urine [11]. Elucidation of unchanged NTBC and its metabolites in plasma has also been attempted by nuclear magnetic resonance [12]. Very recently, a method for the determination of NTBC in blood spots has been published [13].

Due to the low incidence of this disease, it is estimated that approximately 1/100,000 neonates in Europe suffer from it, there are very few centres performing with this kind of analysis. Consequently, plasma samples must be generally sent to reference laboratories. For this reason, it is important to facilitate the handling of the sample, either for staff or for patients, who could even take the samples themselves at home by the use of blood spots on paper cards. The aim of this work is the evaluation of plasma and blood spots as samples for the measurement of NTBC by tandem mass spectrometry. The stability of the drug in these matrixes under different storage temperatures is also studied in order to select the simplest method of shipment or storage.

2. Materials and methods

2.1. Reagents and solutions

Pure NTBC (Orfadin®) was a kind gift from Swedish Orphan (Stockholm, Sweden). Mesotrione, a structurally similar insecticide used as internal standard, was supplied by Sigma–Aldrich (Madrid, Spain).

General reagents and solvents used in the experimental (methanol, acetonitrile and formic acid) were supplied by Merck (Madrid, Spain) of the purest available quality (gradient HPLC).

1 mmol/L stock solutions of both, NTBC and mesotrione, were prepared in acetonitrile and stored in dark at 4 °C. They were stable for at least four months. Working solutions were prepared monthly by appropriate dilution of the stock solution in acetonitrile for the analysis of plasma samples (final concentration 50 μmol/L), or in methanol for the analysis of dry blood spots (final concentration 2 μmol/L).

2.2. Subjects and sampling

The study was approved by the ethics committee of Cruces Hospital, and it was carried out according to the Declaration of Helsinki for biomedical research.

All patients treated with tyrosinemia type I in our hospital were invited to participate in the study. Volunteers, 3 male and 3 female, were 2–16 years old, and they themselves or their tutors signed written informed consent. Other hospitals of the area kindly participated in the study by sending three matched plasma-dry blood spots of their patients, but unfortunately, data about these patients are unknown. Blood was drawn from an antecubital vein and collected in EDTA tubes after overnight fasting; it was immediately centrifuged at 4 °C to obtain the plasma fraction. Blood from the same tube – obviously before centrifugation – was used to stain the paper cards (Whatman 903), using a Pasteur pipette. Spots were allowed to dry at room temperature for approximately 24 h before use.

2.3. Sample preparation

The clean-up procedure for plasma samples consists of protein precipitation with acetonitrile. 100 μL of plasma were mixed with 50 μL of the internal standard mesotrione in acetonitrile (50 μmol/L) in an Eppendorf tube. 150 μL of acetonitrile was also added. The tube was kept at 4 °C for 20 min, the protein precipitate centrifuged for 5 min and the clear supernatant, ready for injection, transferred to a clean injection vial.

As for dry blood spots, a 3 mm of diameter disc was punched by means of a cutting tool (Sigma–Aldrich) and placed in a 96-well plate. 50 μL of mesotrione in methanol (2 μmol/L) and 50 μL of pure methanol were added (final volume of the liquid phase 100 μL). The plate was shaken at 500 rpm at room temperature for 20 min. The methanolic solution with the extracted components is ready for its injection in the chromatographic system.

2.4. Calibration and standards

The calibration standards for plasma samples were prepared using 100 μL of a blank pooled plasma, to which 50 μL of 50 μmol/L mesotrione in acetonitrile were added as internal standard, and different amounts of 50 μmol/L of NTBC in acetonitrile (between 10 and 100 μL, corresponding to calibration points between 5 and 50 μM). Different volumes of pure acetonitrile were added to the sample in order to obtain a homogeneous total volume of 300 μL for all calibrators (between 140 and 50 μL).

For blood spots, a 3 mm paper disc stained with blood free of NTBC was used. Blood volume in the sample was considered to be 3.1 μL. To all blank blood spots, 50 μL of 2 μmol/L mesotrione in methanol was added and different amounts of 2 μmol/L NTBC in methanol (between 5 and 40 μL, corresponding to calibration points between 3.2 and 25.8 μM). Different amounts of methanol were added to make up a total volume of 100 μL for all calibrators (between 45 and 10 μL). The plate was shaken and treated according to the instructions for unknown samples.

Calibration curves were obtained by the least-square method of relative responses (areas) against concentration of NTBC, and concentrations of problem samples were calculated by inverse prediction.

2.5. Chromatographic and instrumental conditions

The chromatographic system consisted of an Agilent 1100 series equipped with an Agilent Zorbax C8 column (150 mm × 4.6 mm, 5 μm). Injection volume was 5 μL for plasma samples and 10 μL for dry blood discs. Owing to differences in polarity between NTBC and the internal standard mesotrione, a gradient had to be used. Eluent A consisted in 0.1% (v/v) formic acid, and eluent B was pure methanol. The chromatographic run started with a proportion of methanol/formic acid 60/40 (v/v) during the first 4 min. The proportion of methanol was gradually increased up to 80/20 (v/v) at 4.5 min. This mobile phase was maintained for 3.5 min, and then the proportion of methanol decreased to the initial composition between 8 and 8.5 min. This mobile phase containing methanol 60% was kept until the end of the run (12 min). Flow rate was maintained at 0.5 mL/min during the whole chromatographic run.

The tandem mass spectrometer (Agilent 6440 triple quad) used electrospray as ionization source. The drug and the internal standard were detected in multiple reaction monitoring (MRM) mode, selecting the most intense and selective transitions for each molecule for quantitative purposes, m/z 330 > 218 for NTBC and m/z 340 > 228 for mesotrione in the positive mode. Two additional transitions were used as qualifiers, m/z 330 > 316 and 340 > 104 for NTBC and mesotrione, respectively. For both compounds, the ionization potential was set at 3200 V and collision energy was

20 eV. Other parameters were: dwell time 100 ms, fragmentor 100, gas temperature 350 °C, gas flow 11 L/min and temperature of the quadrupoles 100 °C. Due to the higher sensitivity required for the analysis of blood spots, an extra potential of +300 mV had to be applied to the photomultiplier. Mass Hunter version B01.04 (Agilent) was the software used for acquiring and processing the chromatograms.

2.6. Reproducibility, repeatability and quantitation limits

Reproducibility was tested by performing five extractions at three concentration levels on three different days. Results were calculated by analysis of variance ANOVA. Repeatability was calculated at the same concentration levels by consecutively injecting 5 times the same sample. All data are presented as percentages, relative of standard deviation (RSD).

Detection and quantitation limits were defined as the amount of NTBC that produced a signal to noise ratio of 3 and 10, respectively.

2.7. Comparison of plasma with dry blood spots

We obtained matched plasma-dry blood spot samples of 9 patients. Plasma concentrations were set in the abscises axis and concentration measured in blood spots in the *y*-axis. A lineal regression was performed, obtaining the slope and regression coefficient r^2.

2.8. Sample storage and stability

Three spiked plasma samples of different concentrations were prepared and separated in three fractions. Their concentrations were studied for a period of one month. A fraction of each of the three samples was aliquoted and frozen at −40 °C; a second fraction was stored refrigerated in the fridge at 4 °C and finally, the third fraction was kept at room temperature (20–25 °C). Stability was also tested with several real plasma samples obtained from patients under treatment with NTBC.

Paper cards with blood spots were kept tightly closed in a plastic bag at room temperature. Measurements were repeated in a period of a month and a half and quantitativer results compared with those of the first day.

3. Results and discussion

3.1. Chromatography

NTBC is an acidic molecule (pK_a 4.4). Therefore, the retention on the column varies according to the pH of the mobile phase. Several nonpolar columns were tested, all C18 and C8. It turned out that Zorbax C8 produced the most symmetric peaks and acceptable retention. C18 columns needed a very high content of organic modifier due to the strong retention of the analytes by the stationary phase. Mesotrione, a pesticide, was used as internal standard due to the structural analogies with NTBC and the lack of commercially available NTBC marked isotopically. The effect of pH of the mobile phase was studied using formic acid/ammonium formate and acetic acid/ammonium acetate buffers. The effect of using methanol or acetonitrile as organic modifiers was also studied. Methanol and low pH (formic acid 0.1%, v/v) produced the most sensitive peaks, since analytes are almost completely protonated at this pH value and this fact favours ionization in the source. The addition of trifluoroacetic acid at very low concentration to the mobile phase was also investigated, since the previous paper on the subject by Herebian et al. [10] described that it enhanced retention and peak shape of the analytes. Under our chromatographic conditions, the addition of TFA did not produce any improvement, and thus it was discarded.

We also investigated possible peak enhancement or loss of sensitivity due to ion suppression. This phenomenon is more likely to happen in multicomponent samples of high concentration, competition for either space or charge most likely is occurring and, in turn, suppression of signal is observed. Ion suppression was observed at the beginning of the chromatogram by Herebian et al. A full mass scan of the sample revealed that most plasma components were poorly retained (especially in a mobile phase with such high percentage of methanol) and eluted during the first minutes of the chromatogram [14]. Thus, it is important to separate mesotrione from endogenous compounds eluting in the front of the chromatogram, which led us to develop a gradient in order to increase the retention time of mesotrione without delaying the elution of NTBC. Nevertheless, in order to evaluate the matrix effect, we spiked five blank plasma samples after sample preparation and mobile phase with identical amounts NTBC and mesotrione. Peak areas were not significantly different in all cases, so there was no influence of the matrix. The same experiment was applied to dry blood spots extracts. Ion suppression should be less important in the analysis of dry blood spots due to dilution. Under the selected chromatographic conditions, mesotrione eluted at 4.9 min and NTBC at 10.7 min (Fig. 1).

3.2. MS/MS detection

The MS/MS parameters of NTBC and the internal standard were optimized by direct infusion of the analytes in the mass spectrometer in a phase containing 0.1% formic acid. Both compounds exhibited a sensitive parent ion corresponding to $[M+H]^+$, m/z 330 and 340 for NTBC and mesotrione, respectively, and an intense fragment at m/z 218 for NTBC and 228 for mesotrione (Fig. 1). These fragments correspond to the cyclohexanedione moiety [10]. The spectra also show fragments at m/z 316 and 104 for NTBC and mesotrione, respectively, that were used as qualifiers. The instrumental parameters of our model of spectrometer, such as fragmentor, temperatures and collision energy, were optimized in order to produce the maximum sensitivity.

3.3. Sample pretreatment

For plasma samples, the addition of acetonitrile is a frequently used procedure to remove proteins by precipitation, so it was not further investigated [10]. As far as dry blood spots are concerned, we used methanol because it is normally used for extractions, as well as a solvent where these molecules are very soluble. The selected time of extraction was 20 min, as further shaking did not produce higher recovery.

3.4. Recovery

The recovery was assayed by comparing the peak area of NTBC of a spiked blank plasma sample and a standard sample in acetonitrile:formic acid 2:1 (v/v). Results were close to 100%. The experimentally obtained recovery is slightly above 100%, but this figure is influenced by several factors, such a decrease of the volume of the sample after precipitation of the proteins. Nevertheless, this recovery above 100% is in agreement with that obtained by the authors who previously wrote on the subject [10].

The recovery of blood spots was calculated using samples from patients under treatment with NTBC. The discs were extracted once with methanol, and the solvent removed after 20 min. The discs were allowed to dry for 1 h, and re-extracted in a different well of the plate with 100 μL of methanol. The peak areas of the second extraction ranged between 3 and 6% of the peak areas obtained in the first extraction of the blood spot. Most probably, these small

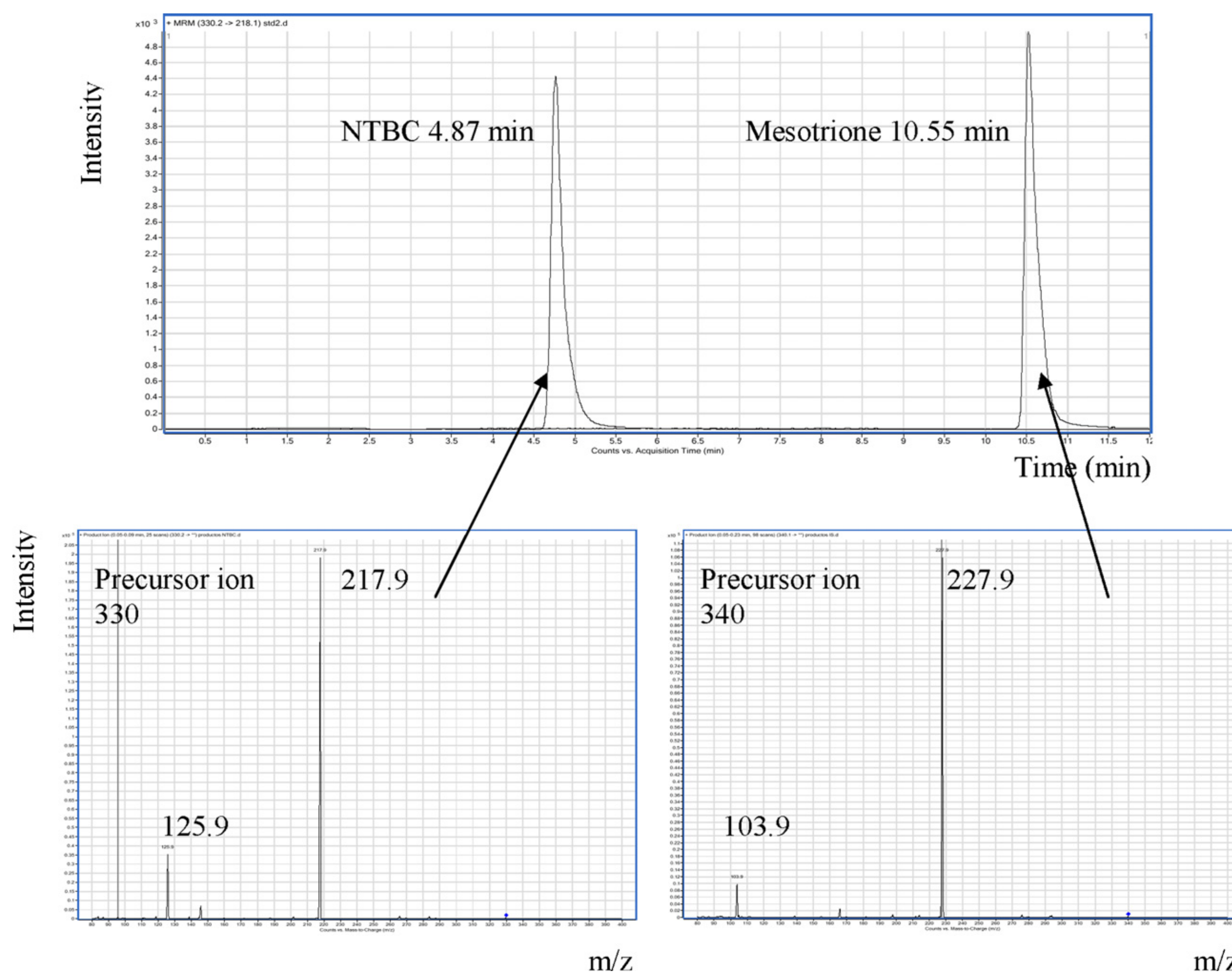

Fig. 1. Chromatogram of a blank plasma sample spiked with standard solutions of NTBC and mesotrione. Final concentration 25 μmol/L. Product ion spectra of standard solutions of NTBC and mesotrione.

peaks are due to the fact that it is impossible to remove methanol used for the first extraction, since the paper is soaked with it, and it does not mean that NTBC was incompletely dissolved.

3.5. Statistics, reproducibility, repeatability and detection limits

The calibration plots yielded a straight line with correlation coefficient always >0.995. Some values for statistical parameters can be viewed in Table 1. As can be seen, both, intra-day and inter-day reproducibility is very good for all concentrations in plasma, and intra-day reproducibility is better than inter-day.

In the case of blood discs, the inter-day reproducibility is better than intra-day reproducibility. This lack of precision can be the result of a lack of homogeneity in the blood stain on the paper, whereas the plasma sample is homogenous. For instance, acylcarnitine concentrations vary with hematocrit and the distance

Table 1
Recovery, precision and detection limits of NTBC in plasma and dry blood spots.

	Plasma	Dry blood spots
% Recovery	100.6 ± 1.56; 103.9 ± 1.24[a]	100 (estimate)
Intra-day reproducibility (RSD)	4.13; 3.62; 2.61[b]	10.38; 6.56; 4.20[c]
Inter-day reproducibility (RSD)	6.12; 7.28; 2.77[b]	5.97; 3.34; 11.74[c]
Repeatability (5 injections) (RSD)	1.24	1.55
Linear range of calibration (μmol/L)	At least up to 100	At least up to 50
Equation of the calibration plot y expressed in μmol/L	$y = 0.091x - 0.088$ $r^2 = 0.999$	$y = 0.052x - 0.046$ $r^2 = 0.998$
Detection limit (S/N=3) (μmol/L)	0.053	0.046
Quantitation limit (S/N = 10) (μmol/L)	0.18	0.15

[a] For mesotrione and NTBC, respectively.
[b] Five replicates, three days. Concentration of spiked plasma samples 2.5, 11 and 41 μmol/L, respectively.
[c] Five replicates, three days. Calculated concentration of the blood samples 10.3, 15 and 26 μmol/L, respectively.

Table 2
Study of the degradation of NTBC at different temperatures.

	Plasma RT (20–25 °C)	Plasma refrigerated (4 °C)	Plasma frozen (−30 °C)	Dry blood spots (RT)
Initial concentrations (μmol/L)	4.24; 14.29; 48.09	4.24; 14.29; 48.09	4.24; 14.29; 48.09	22.55; 43.66
Mean concentration of the period ± SD (μmol/L) (45 days)		4.27 ± 0.07; 14.61 ± 0.40; 46.78 ± 0.93	4.25 ± 0.09; 14.39 ± 0.51; 47.31 ± 0.68	22.71 ± 0.29; 41.89 ± 1.73
% Degradation after 30 days	20.8; 22.0; 24.1			
% Degradation after 45 days	30.7; 29.9; 31.2			

to the centre of the disc. As the blood drop extends by capillarity, it acts as a chromatographic system that affects homogeneity [15]. As a matter of fact, we performed an experiment to measure if the concentration of NTBC changed from the border to the centre of the stain. Concentration gradually increased towards the centre, and the difference was around 15% (mean of three experiments). However, when several determinations of the same stain are performed (five in our case) we can estimate a good value of concentration, which is repeated between days. In the case of plasma samples, inter-day is worse than intra-day reproducibility, as normally expected, since plasma samples each day are homogeneous and always have the same concentration.

As for repeatability, the repeated injection of the same sample produced very good results in all cases, either for dry blood spots on paper or for plasma, with relative standard deviations always below 1.5%.

Detection limits are well below therapeutic doses of NTBC. The slightly lower detection limits obtained for paper discs are due to the more sensitive method used (gain of the photomultiplier +300 mV) and higher injection volume, but sensitivity is partially spoilt by a higher baseline noise. Detection limits are approximately one half of those published by Herebian et al. [10].

3.6. Comparison between concentrations obtained in plasma and in dry blood spots

Matched plasma and paper samples were obtained for 9 patients suffering from tyrosinemia (we obtained two samples of one of the patients at different times). Recommended therapeutic concentration of NTBC is around 35 μmol/L [16]. By this reason, most patients under treatment had rather similar plasma concentrations of the drug. This makes correlations and drawing a straight line across the points difficult because most points are concentrated in the same area.

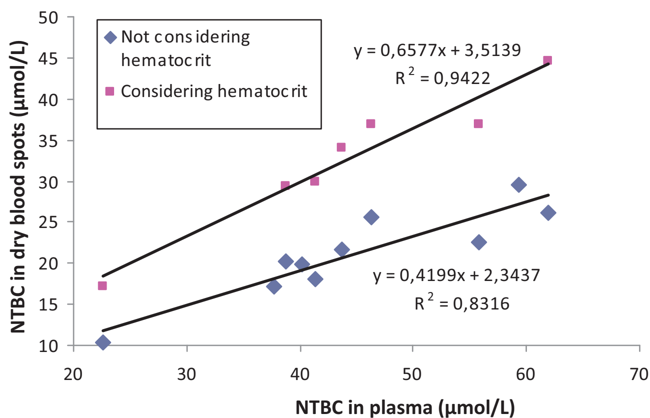

Fig. 2. Comparison between the concentrations of NTBC of matched plasma and dry blood spot samples. One of the graphs shows the direct comparison of results, the second graph shows the comparison after subtracting to the blood volume contained on the paper spot the volume of hematocrit.

As can be observed in Fig. 2, the comparison between concentration of NTBC in plasma and in dry blood spot discs renders a correlation coefficient of $r^2 = 0.832$, thus indicating a good agreement of concentrations between both kinds of matrixes. However, the slope of the line is not 1, as one should expect from these kinds of comparisons. Similar results were reported in the other publication that compared plasma and dry blood spots, obtaining that concentrations in plasma were 1.56 times higher [13]. We assume that the blood volume contained in the 3 mm paper discs is 3.1 μL, according to the manufacturers of kits for acylcarnitine analyses. The reason for this apparent disagreement is that NTBC is mainly

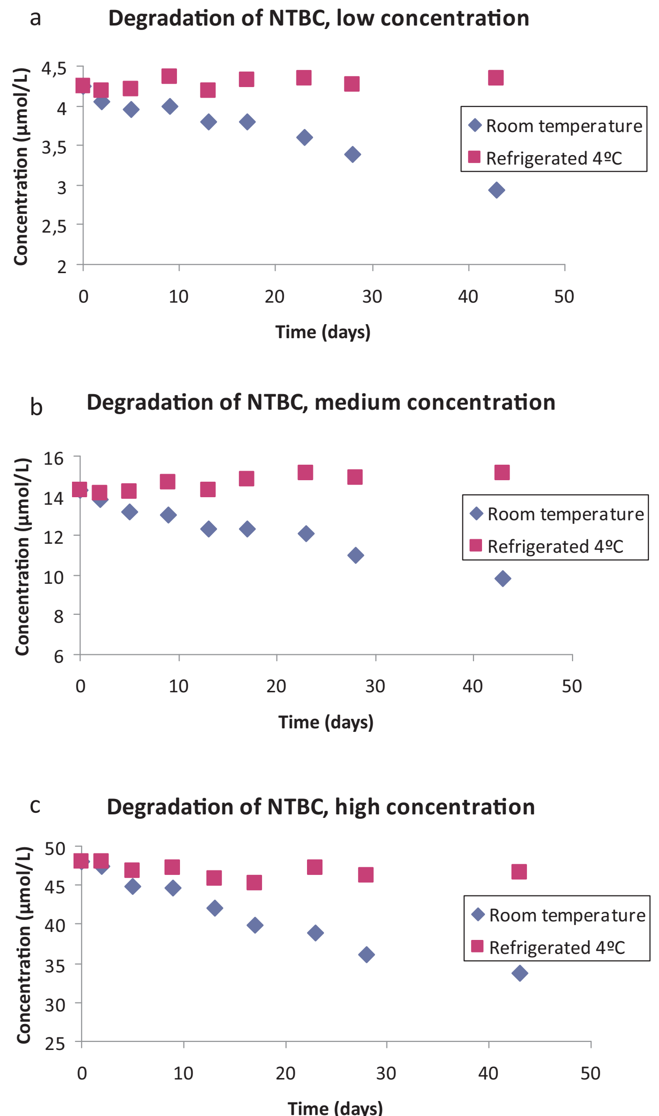

Fig. 3. Study of the degradation of NTBC in spiked plasma samples at room temperature or refrigerated at 4 °C. Initial concentrations of NTBC: 4.27; 14.61; 46.78 μmol/L.

contained in plasma. Although we have not found data on distribution of this drug between plasma and red blood cells, we analysed 100 μL of RBC of some patients under treatment with the drug. The procedure used was same as for plasma, but erythrocytes were sonicated, mixed, and crushed, obtaining very low concentration of NTBC in these cells (less than 9% than in plasma). For this reason, although the volume of blood stained on the paper can be 3.1 μL, the volume of plasma is lower, taking into account that the average hematocrit is close to 40%, or 50% in neonates. Therefore the real volume of plasma contained in the disc is around 1.9 μL, and depends on the person. Fig. 2 also shows the results taking into account hematocrit of each patient. The experimental result previously obtained by direct comparison with the calibration plot was multiplied by a factor of 100/(100 − hematocrit), which takes into account the real volume of plasma contained in 3.1 μL of total blood. In this case, the slope of the straight line increases up to 0.72, and the regression coefficient up to 0.942.

3.7. Stability

The stability of these compounds was investigated. Herebian et al. [10] had already studied the stability of the plasma extracts (after protein precipitation) at different temperatures. They concluded that plasma extracts were stable at least for 5 days at 15 °C and 10 days at 4 °C.

NTBC in plasma is stable at 4 °C at least for the studied period of a month and a half. However, there is a slow degradation at room temperature and it reaches a degradation of around 3% after only two days. Results are collected in Table 2. The graphical representation of the results along this period is presented in Fig. 3. Data of frozen samples are not represented for clarity. From these data, we can conclude that samples can be shipped at room temperature if they are supposed to arrive within the following 48 h. If not, refrigeration with cooling agents or refrigerated transport would be advised, but dry ice is not necessary.

Paper with dry blood spots was kept at room temperature in order to investigate if ordinary mail is appropriate for these samples. The concentration of NTBC remains constant at least during a month. However, it is worth mentioning that in the beginning, we used blood without EDTA to stain the cards. In this case, a slow decay of the concentration is observed with time (percentage of change −36% after a month, mean value of two samples).

4. Conclusions

We have developed a simple method for the determination of NTBC in plasma and dry blood spots, comparing quantitative results with the two kinds of matrixes. We found good agreement between the concentrations found in paper discs and in plasma, and therefore dry blood spots can be used in the daily practise, unless very good accuracy is required. In addition, we studied the degradation of NTBC in plasma, finding that it is stable in the refrigerator for at least one month and a half, but that concentration slowly decreases at room temperature. The decision on the shipment conditions depends on the estimated time of arrival of the sample, although the use of dry ice is not necessary. This procedure shows that the use of dry blood spots is useful for the optimization and monitorization of the dosage of the drug. This kind of sample is always easier to obtain from paediatric patients, and can be easily sent to the reference laboratories.

Acknowledgements

Authors thank Swedish Orphan for their gift of NTBC. Research in this paper was partially financed by the SAMID network (Spanish Ministry of Health)

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