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Determination of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione in plasma by direct injection into a coupled column liquid chromatographic system

Margareta Bielenstein[a,*,1], Leif Astner[a,1], Staffan Ekberg[b,c]

[a]*Central Laboratory, The National Corporation of Swedish Pharmacies, 105 14 Stockholm, Sweden*
[b]*Swedish Orphan AB, Drottninggatan 98, 111 60 Stockholm, Sweden*
[c]*RCEM, Gröna Stråket 8, Department of Internal Medicine, Sahlgrenska University Hospital, 413 45 Göteborg, Sweden*

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Abstract

The chemical substance 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) is in clinical use for the treatment of hereditary tyrosinemia type 1. In the present study, the plasma concentration of NTBC was determined by a coupled column liquid chromatographic method. A 20-μl volume of plasma was diluted with phosphate buffer, pH 2, and injected into a small precolumn (BioTrapAcid C_{18}) with a mobile phase containing sulfuric acid. The precolumn was based on the restricted access principle, i.e., retention of NTBC within the lipophilic pores, while polar and large endogenous compounds were eluted with the void volume. NTBC was transferred to the analytical column using a mobile phase with a high content of acetonitrile. The compound was monitored by UV detection at 278 nm. The standard curve was linear between 0.3 and 69 μ*M*, and the between-day precision (RSD) was 3% ($n=6$ days) at 13.8 μ*M* and 14% ($n=6$ days) at 0.3 μ*M* NTBC in plasma. The quantitation limit was approximately 0.3 μ*M* using 20 μl of plasma.

Keywords: Direct injection; Coupled columns; 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

1. Introduction

NTBC, which is a member of the triketone class of compounds (Fig. 1) [1], inhibits the activity of 4-hydroxyphenylpyruvate dioxygenase (HPD), an enzyme involved in an early step of the tyrosine catabolic pathway [2,3]. NTBC's ability to block HPD has led to its development as the first effective pharmacological therapy for hereditary tyrosinemia type 1 (HT-1) [2,4–6]. HT-1 is caused by a deficiency of fumarylacetoacetase, the last enzyme in tyrosine degradation [7], leading to accumulation of toxic tyrosine metabolites primarily in the liver and

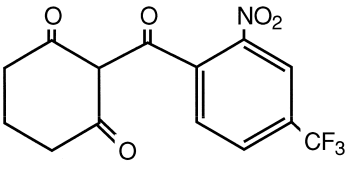

Fig. 1. Chemical structure of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC).

*Corresponding author.
[1]Present address: Astra Pain Control AB, 151 85 Södertälje, Sweden.

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kidney. Acute hepatic failure and chronic liver disease with cirrhosis and liver carcinoma frequently develop in patients with HT-1, and fatal outcome at a young age is common [8]. Restriction of phenylalanine and tyrosine intake has previously been the only standard treatment of HT-1. The diet may improve liver and kidney function, but does not prevent a progressive course of the disease [8], making liver transplantation the ultimate treatment. The preliminary results of a prospective, international study on the efficacy and safety of NTBC treatment in HT-1 have been presented [5,6]. Daily NTBC treatment seems to prevent the accumulation of toxic degradation products, and significant biochemical and clinical improvement of liver and kidney function has been observed. The short-term results demonstrate that NTBC treatment is superior to dietary treatment alone [2,5,6,8], and the use of NTBC has reduced the number of patients who are in urgent need of liver transplantation due to acute liver failure.

So far, no method has been published for the determination of NTBC in plasma. In order to determine the pharmacokinetics of different formulations of NTBC, a liquid chromatographic method was developed. The method is based on direct injection of plasma into a coupled column liquid chromatographic system with UV detection. The precolumn used in this system, BioTrapAcid C_{18}, has a biocompatible external surface that is covered with α_1-acid glycoprotein and it has a hydrophobic surface within the pores. The precolumn has previously been used for determination of the plasma concentration of carbamazepine, phenytoin, ibuprofen and naproxen [9]. Furthermore, a second generation of BioTrap columns has been introduced after the development of this method. The new generation enables injection of larger volumes of plasma, i.e., 1 ml [10]. In two recently published articles, various types of columns based on principles of restricted access were reviewed [11,12].

2. Experimental

2.1. Chemicals

NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione] was supplied by Swedish Orphan (Stockholm, Sweden). Acetonitrile (LiChrosolv quality), sulfuric acid (ultra-pure quality), sodium dihydrogen phosphate monohydrate and *ortho*-phosphoric acid (85%) were received from Merck (Darmstadt, Germany).

2.2. Chromatographic system and conditions

A scheme of the coupled column system is shown in Fig. 2. The chromatographic system consisted of a CMA 200 microsampler (Microdialysis, Stockholm, Sweden), with a loop volume of 20 μl. The six-port switching valve, V1 (Valco, Houston, TX, USA), was equipped with an electric actuator and was controlled by the autosampler. Two pumps were used, a Gynkotek M480G gradient pump (Gynkotek, Munich, Germany) for the analytical column (pump A) and an LKB 2150 (Pharmacia, Stockholm, Sweden) for the precolumn (pump B). The precolumn, a BioTrapAcid C_{18}, 10×3 mm (Chrom-Tech, Hägersten, Sweden) was inserted in the back-flush mode into the valve. The mobile phase for the precolumn was 0.01 *M* sulfuric acid and the flow-rate was 1.0 ml/min. The analytical column was a YMC basic, 3μm, 150×3 mm, (YMC, Kyoto, Japan) equipped with a guard column (Upchurch CN, 5μm, 10×2 mm; Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase for the analytical column was 50% acetonitrile in phosphate buffer, pH 2 (ionic strength=0.01) and the flow-rate was 0.4 ml/min. The detector was a Gynkotek UVD320S (Gyn-

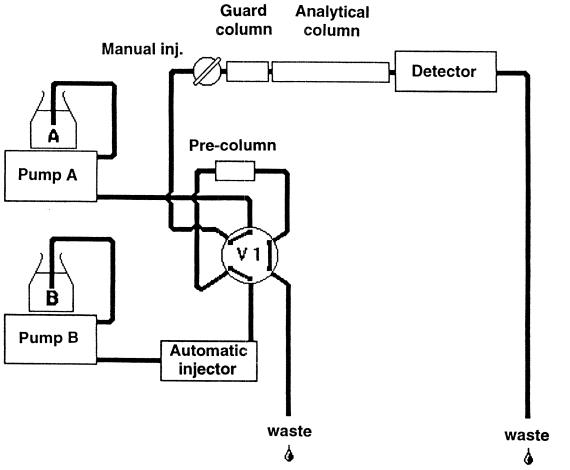

Fig. 2. Scheme of the coupled column system.

kotek), diode array detector used at 278 nm. Data were collected and processed by a Gynkosoft integrating system version 5.30 (Gynkotek).

2.3. Preparation of plasma samples

Blood samples were collected, centrifuged and the plasma was stored at −20°C. After thawing, the plasma samples were mixed and centrifuged at 7000 *g* for 5 min. A 20-μl volume of plasma was diluted with 20 μl of phosphate buffer, pH 2 (ionic strength=0.5). A volume of 20 μl was injected into the BioTrapAcid C_{18} column.

2.4. Preparation of a standard curve and quality control samples

A standard curve containing one, zero and eight different concentrations of NTBC between 0.3 and 69 μ*M* was used. The standard samples were prepared each day by adding 10 μl of various concentrations of NTBC, dissolved in phosphate buffer, pH 7.4, to 190 μl of plasma. Quality control samples in plasma were prepared at two different concentrations of NTBC, i.e., 0.3 and 13.8 μ*M*, and the samples were stored at −20°C. Two separate stock solutions of NTBC were used for the standard samples and the quality control samples. The standard samples and the quality control samples were diluted with phosphate buffer, pH 2 (ionic strength= 0.5) before injection, in a similar way to that used for the plasma samples. Each analytical batch included one standard curve, two quality control samples at each level and about 50 samples. Furthermore, a standard control sample was injected every tenth injection to ensure the stability of the chromatographic system.

2.5. Purification of plasma on the precolumn

The time schedule for the switching events is shown in Table 1.

The plasma sample was injected into the precolumn with acidic mobile phase. The endogenous polar plasma components were eluted to waste, while NTBC was adsorbed within the hydrophobic pores. After 2 min, the valve switched the flow direction on the precolumn and NTBC was desorbed and eluted into the analytical column with the mobile phase containing a high content of acetonitrile and phosphate buffer, pH 2, ionic strength=0.01. After another 8 min, the two columns were disconnected and the precolumn was reequilibrated over 6 min with sulfuric acid (0.01 *M*) before the next injection.

Table 1
Switching events

Time (min)	Valve position	Event
0.0	Load	Injection of diluted plasma into the precolumn. NTBC is adsorbed within the pores and plasma components are eluted to waste.
2.0	Inject	The precolumn is connected in series with the analytical column. NTBC is eluted into the analytical column.
10.0	Load	The precolumn and the analytical column are disconnected. The precolumn is equilibrated with sulfuric acid
16.0		End. Next injection

3. Results and discussion

3.1. Chromatography

3.1.1. Analytical column

NTBC is lipophilic and has weak acid properties due to the hydrogen in the first position in the cyclohexane ring (Fig. 1). At pH 2, when NTBC is neutral, the retention of NTBC was increased compared to that found at pH 7. A content of about 20% acetonitrile at pH 7 gave about the same retention time as 50% acetonitrile at pH 2. A high content of acetonitrile on the second column has the advantage of giving desorption from the precolumn in a small volume. A further enrichment will be achieved on the top of a hydrophobic analytical column, such as YMC basic. Furthermore, the efficiency on the analytical column was higher at a low pH, as well as on a base-deactivated column compared to an ordinary reversed-phase column.

3.1.2. Precolumn

The mobile phase on the precolumn was acidic in order to increase the enrichment on the precolumn and to decrease the protein binding of NTBC (see 'Dilution of plasma' below). The precolumn was used in the backflush mode, which gave about a 10% contribution to the band-broadening of the peak. In the foreflush mode, the band-broadening was considerably higher.

The times for the switching events should be kept as short as possible to give as many injections as possible per hour. However, during the loading time, the polar compounds and the proteins should be eluted to waste in order to protect the analytical column. The loading time was optimized by comparing the width of the front disturbances on the analytical column. Neither the width of the front disturbances nor the recovery of NTBC was affected by changing the loading time between 2 and 10 min. A loading time of 2 min was chosen. The desorption time influenced the carry-over between the injections. By increasing the desorption time from 2 to 8 min, the contamination decreased, from 3% to less than 0.9%, when a plasma sample containing 69 μ*M* NTBC was injected before a blank plasma sample. However, the carry-over cannot be neglected, when the concentration range is between 0.3 and 69 μ*M*. A careful examination has to be performed of the concentration of the previous sample in the autoinjector, before the actual concentration in the next sample is accepted.

The recovery from the precolumn depended on the injected volume of plasma. Plasma volumes of up to 200 μl, diluted 1:1 (v/v) with phosphate buffer, pH 2, could be injected with a recovery higher than 90%. In this study, the injection volume of diluted plasma was only 20 μl. The lifetime of the precolumn is dependent on the number of injections and the total volume of plasma injected. A continuously shorter retention-time of NTBC was obtained on the precolumn (BioTrapAcid C_{18}), probably due to irreversible adsorption of small amounts of endogenous compounds. The breakthrough was initially investigated on two precolumns. In this system, the breakthrough of the precolumn came after about 100 injections of 20 μl of diluted plasma. The breakthrough was not preceded by an increase in backpressure on the precolumn or an increased peakwidth (a change in efficiency) on the analytical column. Therefore, the performance of the precolumn was controlled by evaluating the peak area on the analytical column of the 'standard control sample' injected every tenth injection. Normally, one batch containing about 80 injections of 20 μl of plasma was completed before the precolumn was discharged and replaced. The analytical column was stable for more than 1000 injections.

3.2. Dilution of plasma

The recovery of NTBC from plasma samples was tested at one concentration level, e.g. 2.7 μ*M* NTBC. The recovery increased on diluting the plasma before injection into the precolumn. Undiluted plasma gave a recovery of 60% compared to dilution to pH 2. Dilution to pH 5 gave a recovery of 80% compared to dilution to pH 2. This is probably a combined effect of less binding of NTBC to the plasma proteins at the low pH and an increased enrichment on the precolumn.

3.3. Adsorption

The absolute recovery of NTBC from the precolumn was determined by comparing the peak areas on the analytical column after injection of plasma samples on the precolumn, with the peak areas of buffer samples injected directly on the analytical column. The samples were diluted 1:1 (v/v) with phosphate buffer, pH 2. A slightly higher recovery of NTBC was obtained from diluted plasma compared to phosphate buffer, pH 7.4. A possible explanation is a higher adsorption to the walls in the vials and the tubes when the compound is dissolved in phosphate buffer compared to plasma, which contained a lot of competing agents. An adsorption effect has previously been shown for other compounds in two similar types of systems [9,13]. However, in routine analysis, the adsorption is not a problem as long as the standard samples are prepared in plasma.

3.4. Selectivity, sensitivity, repeatability and linearity

A typical rat plasma sample chromatogram before dose and 24 h after intravenous administration of

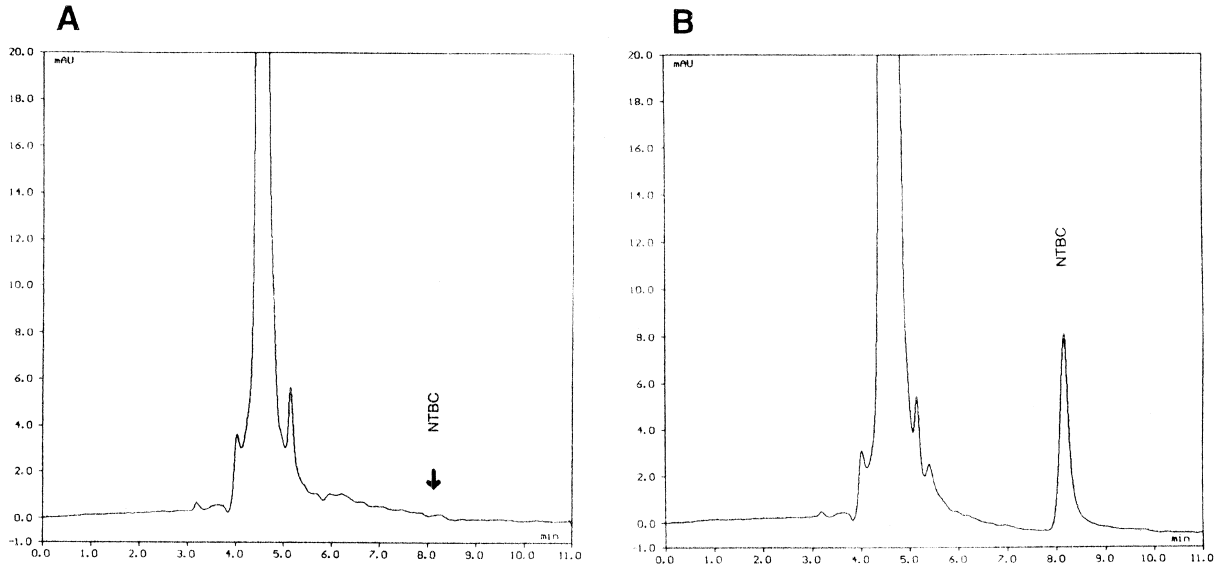

Fig. 3. Typical chromatograms of rat plasma before dose (A) and 24 h after an intravenous dose of 10 μmol/kg NTBC (B), respectively. The plasma concentration was 8.8 μ*M* NTBC.

NTBC (10 μmol/kg) is shown in Fig. 3A and B, respectively. The rat plasma sample 24 h after a single dose had a concentration of 8.8 μ*M* NTBC. The within-day precision was initially determined at two different concentrations using spiked blank plasma samples. The between-day precision over six days was determined using spiked plasma samples at two different concentration levels. The limit of quantitation was 0.3 μ*M* when 20 μl of diluted plasma were injected. The within-day and the between-day precision are shown in Table 2. The concentrations of the between-day quality control samples and the standard curve were chosen to cover the expected concentrations of the rat plasma samples. The standard curve was calculated by $1/X$ weighted linear regression analysis of the concentration of NTBC versus the peak height. The back-calculated values are shown in Table 3. The calibration curve was linear within the range 0.3–69 μ*M*. No decrease in the concentration of NTBC (6.4 μ*M*) in rat plasma was observed during storage at −20°C for at least one year.

Table 2

Added concentration (μ*M*)	RSD (%)	*n* (days)	*n* (quality control samples)
Within-day precision			
2.5	1.5		8
61	3.0		8
Between-day precision			
0.3	13.5	6	12
13.8	3.0	6	12

3.5. Time-course of NTBC in plasma

Fig. 4 shows a time course of the plasma concentration of NTBC in one rat 0–48 h after a single intravenous dose of NTBC (10 μmol/kg). The

Table 3
Back-calculated values of the standard curve for NTBC in plasma

Standard concentration (μ*M*)	Back-calculated concentration (μ*M*)	Deviation (%)
0.28	0.27	−4.4
1.37	1.17	−14.9
2.76	2.35	−14.7
6.93	6.66	− 3.9
13.9	13.2	− 4.8
25.4	26.0	2.2
34.7	34.9	0.6
69.3	70.0	1.0

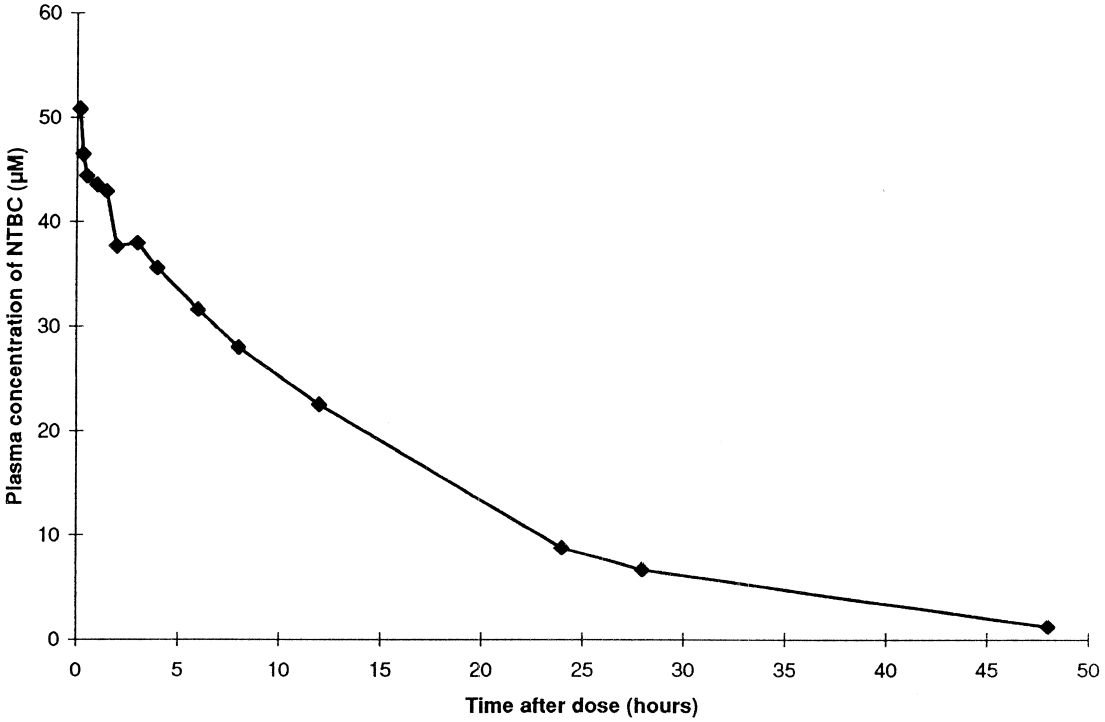

Fig. 4. Time course of the plasma concentration of NTBC in one rat 0–48 h after a single intravenous dose of NTBC (10 μmol/kg).

results of a pharmacokinetic study of NTBC in the rat are currently being evaluated.

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