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Identification of 2-[2-nitro-4-(trifluoromethyl)benzoyl]-cyclohexane-1,3-dione metabolites in urine of patients suffering from tyrosinemia type I with the use of ^{1}H and ^{19}F NMR spectroscopy★

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Organic extracts of six urine samples from children treated with nitisinone, a medicine against tyrosinemia type I, were investigated by ^{1}H and ^{19}F NMR spectroscopy. The presence of unchanged 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC), 6-hydroxy-2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC-OH) and 2-nitro-4-trifluoromethylbenzoic acid (NTFA) as well as a few other unidentified compounds containing CF_3 group was documented.

Keywords: NTBC metabolites, tyrosinemia, NMR

INTRODUCTION

Several compounds belonging to the 2-acylcyclohexane-1,3-dione family have been found to be very efficient herbicides (Secor, 1994; Mitchell *et al*., 2001). They are presently in use to prevent the growth of a wide range of broad-leaved and grass weeds in maize. Extensive studies have shown that the mode of action of those herbicides is the inhibition of (4-hydroxyphenyl)pyruvate dioxygenase (HPPD), the enzyme which catalyzes the conversion of (4-hydroxyphenyl)pyruvate to homogentisate. In plants this step is essential for the synthesis of plastoquinone and α-tocopherol. In animals, analogous HPPD-catalyzed transformation takes part in tyrosine catabolism, the final products of which are fumarate and acetoacetate excreted in urine. One of the hereditary diseases caused by the malfunction of fumarylacetone hydroxylase (FAH), the enzyme active at the last step of the tyrosine catabolism pathway, is tyrosinemia type I (Russo *et al*., 2001). This defect results in the accumulation of abnormal products of tyrosine degradation which severely damage patients' liver and kidneys. It has been shown that several triketone herbicides inhibiting HPPD, an enzyme active at a tyrosine catabolism step prior to that catalyzed by FAH, preclude the formation of the poisonous compounds. One of those triketones, 2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC), has appeared to be an excellent life-saving medicine against tyrosinemia type I (Linstedt & Holme, 1992; Pronicka *et al*., 1996; Lock *et al*., 1998; Holme & Linstedt, 1998). One can find some works on modeling the NTBC–HPPD interaction (Kavana & Moran, 2003; Brownlee *et al*., 2004; Neidig *et al*., 2005), but to our knowledge there is no informa-

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Abbreviations: FAH, fumarylacetone hydroxylase; HPPD, (4-hydroxyphenyl)pyruvate dioxygenase; HRMS, high resolution mass spectrometry; HSQC, heteronuclear single quantum correlation; NTBC, 2-[2-nitro-4-(trifluoromethyl)benzoyl]-cyclohexane-1,3-dione; NTBC-OH, 6-hydroxy-2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione; NTFA, 2-nitro-4-trifluoromethylbenzoic acid; TLC, thin layer chromatography.

tion about the NTBC transformation in the human organism. Thus, in this study we decided to check if it would be possible to identify NTBC and/or its metabolites using NMR spectroscopy in urine of patients cured with this medicine. One may expect that ^{19}F NMR will be useful in such an investigation, as NTBC possesses a rather stable CF_3 group and the natural content of fluorine in the human body fluids is negligibly small. Moreover we established experimentally that nitisinone contains no other fluorine possessing compounds than NTBC and that the procedure used for the separation of NTBC and its putative metabolites from urine (see Materials and Methods) applied to water solution of nitisinone does not cause NTBC degradation.

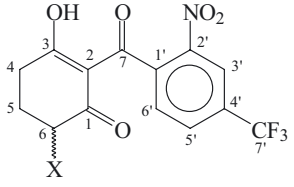
MATERIALS AND METHODS

All investigated urine samples, collected at The Children's Memorial Health Institute, Warszawa, were of 24 h collections. Their volumes varied between 250 ml and 900 ml. Each sample was acidified with 2 M hydrochloric acid (pH about 2) and thoroughly extracted with methylene chloride (6 × 80 ml). After drying ($MgSO_4$) and solvent evaporation (rotary evaporator, diminished pressure, 30°C) the residue was dissolved in $CDCl_3$. NMR spectra of the solution were measured using a Varian VNMRS spectrometer operating at 11.7 T.

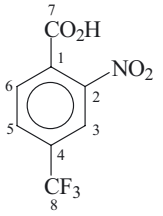
NTBC-OH was prepared by four-step synthesis starting from 1,2,4-benzenetriol. Details of this synthesis as well as NMR and HRMS parameters of NTBC-OH and intermediate compounds are given in Supplementary Materials (www.actabp.pl).

RESULTS AND DISCUSSION

The chemical structures of the compounds under investigation and their carbon atom numbering are shown below. It has been established (Szczeciński *et al.*, 2007) that for NTBC (X = H) the tautomeric form presented prevails overwhelmingly. The structure of the main NTBC-OH tautomer (X = OH) was assumed arbitrarily.



X = H - NTBC
X = OH - NTBC-OH



NTFA

In our pilot investigations urine samples from two patients (girl and boy) affected by tyrosinemia type I and cured with NTBC were investigated. NMR spectra of organic extracts were taken using a spectrometer operating at 200 MHz for protons. For both samples two signals of arene-bound CF_3 groups were observed in ^{19}F NMR spectra (δ = –63.46 and –63.47 p.p.m.). One of these fluorine-containing compounds was then separated in a tiny amount from both combined samples using preparative TLC (Silicagel, acetone as eluent). On the basis of its 1D ^1H NMR and 2D ^1H-^1H correlated spectra one may suspect this compound to be 6-hydroxy-2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC-OH). However, not all of the expected signals in 1D ^{13}C NMR and 2D HSQC spectra were identified because of the small amount of the investigated compound available. Therefore we decided to undertake further investigations which would definitely disclose the identity of the both compounds giving the mentioned fluorine signals. Six new, 24-hour-collected urine samples were obtained. Their descriptions are given in Table 1. Patients 1, 2, 3, 4, and 6 were treated with 0.72 mg and patient 5 with 1 mg of NTBC per 1 kg of body mass per day. All urine samples were treated as described in Materials and Methods and ^1H and ^{19}F NMR spectra of $CDCl_3$ solutions of the extracted mixtures were obtained using a spectrometer operating at 500 MHz for protons. Similarly as in the preliminary investigations the two most intensive CF_3 signals were observed in the fluorine spectra of all the samples. In each case the signal at –63.47 p.p.m. was more intensive and the relative intensity of the signal at –63.46 p.p.m. was about 40% of the former. Apart from those two, several other signals were observed in the spectra. Their intensities varied among the samples. The data for some of the more intensive signals are collected in Table 2. Out of those additional signals the presence of two at –63.72 p.p.m. (sample 2) and –63.69 p.p.m. (sample 5) should be noticed because of their significant intensities.

The phenyl ring of NTBC is substituted with three electron-withdrawing groups. For this reason the aromatic protons, and especially proton H3′, are strongly deshielded. The signal of proton H3′ (δ=8.48

Table 1. Characteristics of investigated urine samples

Sample number	Patient's sex	Patient's age (years)	Sample volume (ml)
1	boy	5	700
2	boy	5	400
3	boy	9	900
4	girl	12	900
5	girl	3	250
6	girl	7	900

Table 2. Chemical shifts[a] and relative intensities of fluorine (CF_3) and proton (H3′) NMR signals observed in investigated urine samples.

For proton spectra residual signal of $CHCl_3$ (δ = 7.26 p.p.m.) and for fluorine spectra signal of CCl_3F (δ = 0 p.p.m.) were used as chemical shift reference

Sample	Metabolite										
	Unknown		Unknown[a]	NTBC		NTBC–OH[b]		NTFA		Unknown	
	δ_F –63.25	$\delta_{H3'}$ 8.45	δ_F –63.45	δ_F –63.46	$\delta_{H3'}$ 8.48	δ_F –63.47	$\delta_{H3'}$ 8.51	δ_F –63.69	$\delta_{H3'}$ 8.15	δ_F –63.72	$\delta_{H3'}$ 8.33
1	1	4	4	39	47	100	100	–	2	3	–
2	5	4	–	60	52	100	100	34	29	119	111
3	13	18	5	35	42	100	100	3	2	3	4
4	2	3	–	47	43	100	100	–	–	–	–
5	1	3	4	36	33	100	100	85	95	11	8
6	trace	3	–	[c]	35	[c]	100	trace	–	–	–

[a]Proton H3′ signal for this metabolite was not identified. [b]Intensities of CF_3 and H3′ for NTBC-OH were arbitrarily assumed as 100. [c]Signals not resolved because of line broadening.

p.p.m.), because of its spin-spin interactions with the other ring protons as well as with the CF_3 fluorine atoms, has the characteristic form of a broadened pseudo-singlet. It is observed in the spectrum region in which only few, if any, weak signals of other compounds extracted from urine are present. Therefore one may expect that the fluorine-containing NTBC metabolites should give similar signals in this region. Indeed, it was possible to find proton H3′ signals corresponding to all the most intensive fluorine ones. Their chemical shifts and intensities are collected in Table 2. It is worth noticing that the relative intensities measured from the proton spectra are in qualitative agreement with those found from the fluorine spectra.

The chemical shift differences between metabolite signals are small and the proton and fluorine chemical shift values for particular NTBC metabolites differ a little from sample to sample because of the different concentrations and compositions of the investigated solutions. Therefore the identification of a metabolite by comparing its signal position in the spectrum of the investigated sample with the signal position in the spectrum of a pure model compound could be misleading. Thus, we decided to base the metabolite identification on a comparison of the spectrum of the original sample with that obtained after the addition of the substance the presence of which was suspected (sample spiked with an internal standard). Three such compounds were taken into consideration: 2-nitro-4-trifluoromethylbenzoic acid (NTFA), NTBC, and NTBC-OH. NTFA is commercially available. NTBC and NTBC-OH were prepared as described in Supplementary Materials (www.actabp.pl).

The ^1H and ^{19}F NMR measurements of appropriate mixtures of urine extracts with the model compounds were performed. Addition of any of the model compounds did not cause a new signal to appear, but changed the relative intensities of those

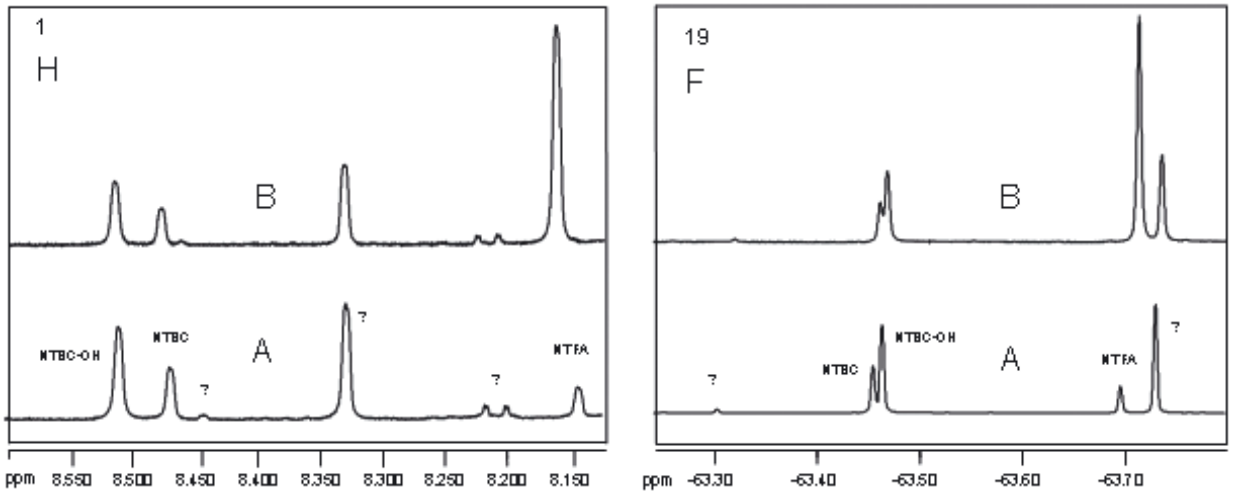


Figure 1. Proton H3′ and fluorine CF_3 NMR spectra of urine sample 2 before (A) and after (B) NTFA addition.

observed in the original sample. An example of such an experiment is illustrated in Fig. 1. The spectra presented there concern sample 2 before and after addition of NTFA. Although the signal positions at –63.69 p.p.m. (CF_3) and 8.15 p.p.m. (H3′) moved a little after addition of the acid, the experiment proved that those signals originated from NTFA. Such a procedure allowed ascribing the CF_3 and H3′ signals observed in the spectra of the investigated samples to the appropriate metabolites. On the basis of their signal intensities the relative concentrations of the identified metabolites could be estimated (Table 2). We could definitely ascertain the presence of NTBC-OH and NTBC in all the investigated samples. The concentrations of both those compounds in relation to the other observed fluorine-containing compounds are high and that for NTBC-OH is the highest in all but one (sample 2) samples. An elevated concentration of NTFA was found in samples 2 and 5.

Our finding concerning the transformation of NTBC into NTBC-OH and NTFA is in agreement with the studies on the metabolism of benzoylcyclohexanediones in plants which have revealed two routes of that process: hydroxylation at position 4 of the cyclohexanedione and hydrolytic cleavage of the benzoyl group (Mitchell *et al*., 2001). It is also worth noting that compounds analogous to NTBC-OH, usually with an aliphatic acyl group, have been found in plants and other organisms (Zaitsev *et al*., 1994; Cheng *et al*., 2003). NMR spectra of sample 2 indicate the presence of another NTBC metabolite whose relative concentration is even higher than that of NTBC-OH. Attempts to identify this compound are under way. Another unidentified metabolite is present, in a small amount, in sample 3.

In the next step we checked whether it is possible to confirm the presence of NTBC metabolites directly in unprocessed urine samples. For three such samples ^{19}F NMR spectra were measured. In all the cases, just after 2-h accumulation of FID signal, two signals originating from NTBC and NTBC-OH could be observed with the signal-to-noise ratio high enough for their reliable integration. Adding a known amount of a standard compound, preferably containing CF_3 group, one may determine the absolute concentration of the investigated metabolites. In our opinion, the information obtained in this way could be very useful for the control of NTBC therapy, for example for medicine dose optimization.

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