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Determination of diethylcarbamazine, an antifilarial drug, in human urine by ^{1}H-NMR spectroscopy

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Abstract

^{1}H-NMR spectroscopy is a convenient method for determination of diethylcarbamazine (DEC) in urine, and can be used to monitor medication with the drug. Urine samples were mixed with 10% of deuterium oxide as a spectrometer field frequency lock, which is the only sample pretreatment required. Tailored excitation with the $1\bar{3}3\bar{1}$ pulse was used for water peak suppression. The quantification of DEC was carried out with the triplet of the N-ethyl group, for which the T_1 relaxation time was 1 s. In aqueous solutions, amounts below 1 μg ml^{-1} of DEC could be easily detected. In urine, the detectability depended on the level of chemical noise but was better than 10 μg ml^{-1}. The accuracy and precision of the method were better than 15%. Analysis of urine from volunteers receiving a single therapeutic dose of DEC (6 mg kg^{-1} body weight orally) showed that the drug was eliminated in unchanged form during 2 days, in agreement with earlier results. The concentration of DEC in urine several hours after the intake exceeded 100 μg ml^{-1} making the ^{1}H-NMR assay rapid and easy. No significant amounts of the N-oxide of DEC could be detected.

Keywords: DEC; Quantitative ^{1}H-NMR; Solvent suppression; $1\bar{3}3\bar{1}$ Pulse; Urine analysis; Drug excretion; Drug metabolism; Diethylcarbamazine N-oxide

1. Introduction

Diethylcarbamazine, or DEC (Fig. 1), is the drug of choice for treatment of lymphatic filariasis, a disease which affects people in the tropical areas of the world [1–3]. It is estimated that over 80 million people are infected worldwide [1]. Lymphatic filariasis results from infection with the parasitic roundworms *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. The infection is transmitted by mosquitoes. The adult parasites (macrofilariae) live in the lymphatics, whereas the larval stages (microfilariae) circulate in the bloodstream. Chronic manifestations of the disease in-

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clude hydrocele, lymphoedema and elephantiasis, which may affect more than 50% of the adult population in affected areas. Mass treatment with DEC is recommended for control of lymphatic filariasis [1], and in this connection there is a need for an assay which could be used for the purpose of medication control.

Several methods applicable to DEC analysis in body fluids have been described. These include spectrophotometry [4–7], polarography [8], liquid chromatography [9,10], and gas chromatography [11–14]. In this paper, we report that the determination of DEC in urine can be conveniently achieved with ^{1}H-NMR spectroscopy.

2. Experimental

2.1. General

Diethylcarbamazine citrate was obtained from Sigma Chemical Co. NMR spectra were recorded on a Bruker AMX 400 WB spectrometer at 400.13 and 100.6 MHz for ^{1}H and ^{13}C respectively, using 5 mm tubes. Statistical analysis was performed using the Ultrafit v. 2.11 program from Biosoft, Cambridge, UK.

2.2. NMR spectroscopy

^{1}H-NMR spectra were acquired with aqueous solutions containing 10% of D_2O using the $1\bar{3}3\bar{1}$ pulse [15], or in chloroform-*d*. The FIDs were zero-filled from 32 k to 64 k computer points to give the digital resolution in the frequency domain of 0.124 Hz per data point. ^{13}C{^{1}H}-NMR spectra were obtained using composite pulse (WALTZ16) decoupling of protons. COSY and NOESY (400 ms) spectra were recorded using the phase-sensitive mode. Heteronuclear ^{13}C, ^{1}H correlations were obtained with ^{13}C-detection and proton decoupling using the BIRD pulse [16]. Longitudinal ^{1}H relaxation times (T_1) were measured by the inversion recovery method. The spectra were standardized to internal tetramethylsilane or sodium 3-(trimethylsilyl)propanesulfonate set to δ 0.0. All spectra were recorded at 25°C.

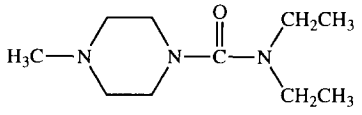
2.3. Urine analysis

Urine was collected from two male and three female volunteers before and after receiving a single oral dose of 6 mg of DEC per kilogram body weight (total dose 380–480 mg, urine collection times 4 h, 8–10 h, 16–18 h, 30 h and 42–44 h post-dose). After addition of 10% vol. of D_2O, urine samples were analyzed directly by ^{1}H-NMR. DEC exhibited signals at δ 1.112 (t, $J = 7.1$ Hz, $NCH_2\underline{CH_3}$), δ 2.916 (s, NCH_3), δ 3.260 (q, $J = 7.1$ Hz, $NC\underline{H_2}CH_3$), δ 3.09–3.77 (piperazine ring). The corresponding signals of DEC N-oxide appeared at δ 1.108 (t, $J = 7.1$ Hz, $NCH_2\underline{CH_3}$), δ 3.253 (s, NCH_3), δ 3.250 (q, $J = 7.1$ Hz, $NC\underline{H_2}CH_3$), δ 3.45–3.55 (piperazine ring).

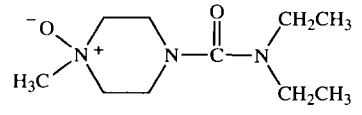
2.4. Assay validation

Urine was spiked with DEC at three concentration levels, preparing four samples at each concentration. ^{1}H-NMR spectra of the 12 solutions were recorded, and the concentrations determined from peak heights using a calibration curve. The calibration curve was obtained from three concentrations of DEC in urine in the concentration region 33–169 μg ml^{-1}, with four individually prepared samples for each concentration. A through-zero-forced, proportionally weighted regression line ($r = 0.996$) of peak heights vs. concentration was used.

The concentration in the spiked solutions was determined by the standard addition method.



(1)



(2)

Fig. 1. Structures of diethylcarbamazine (**1**) and its main metabolite, the N-oxide (**2**).

Table 1
Accuracy and precision of ^{1}H-NMR spectroscopic determination of DEC in urine

Parameter	Concentration added (μg ml^{-1})		
	22.3	81.7	122.6
Measured concentration[a] (μg ml^{-1}) (mean ± SD, $n=4$)	19.6 ± 0.6	82.1 ± 1.4	124.9 ± 2.6
Relative stantard deviation[b] (%)	2.9	1.7	2.1
Accuracy[c] (%)	−11.9	0.4	1.9
Measured concentration[d] (μg ml^{-1}) (mean ± SD, $n=4$)	19.2 ± 1.8	69.3 ± 3.8	122.4 ± 9.1
Relative standard deviation[b] (%)	9.3	5.5	7.4
Accuracy[c] (%)	−14.0	−15.2	0.2

[a] Using calibration curve.
[b] (SD/mean) × 100%.
[c] [(Measured concentration − added concentration)/added concentration] × 100%.
[d] Using standard addition method.

Thus, aliquots of a DEC standard were added to each solution, ^{1}H-NMR spectra were recorded again, and the original DEC concentration was calculated from the increase of the DEC peak height. The accuracy of the method was calculated based on the differences between the mean found and nominal (added) concentrations, while precision was expressed as relative standard deviation. The results are collected in Table 1.

2.5. Synthesis of DEC N-oxide (N, N-diethyl-4-methyl-1-piperazinecarboxamide 4-oxide

Diethylcarbamazine (free base obtained by extraction of an alkaline solution of the citrate with dichloromethane and evaporation) was oxidized with 35% hydrogen peroxide [17] and purified by chromatography on silica gel using a step gradient of methanol in ethyl acetate; yield 58% of oily product. ^{1}H-NMR ($CDCl_3$): δ 1.13 (t, $J=7.1$ Hz, $NCH_2\underline{CH_3}$), δ 3.13 (br d, $J=11.5$ Hz, H3 equatorial), δ 3.23 (s, NCH_3), δ 3.23 (q, $J=7.1$ Hz, NCH_2CH_3), δ 3.31 (dt, $J=3.4$ and 11.5 Hz, H3 axial), δ 3.49 (br d, $J=13.9$ Hz, H2 equatorial), δ 3.88 (m, $J=2.8$, 11.5 and 13.9 Hz, H2 axial). ^{13}C-NMR ($CDCl_3$): δ 13.16 ($NCH_2\underline{CH_3}$), δ 41.76 ($N\underline{CH_2}CH_3$ and C2), δ 60.84 ($N\underline{CH_3}$), δ 65.13 (C3), δ 163.61 (CO). The corresponding ^{13}C-NMR signals of DEC (free base in $CDCl_3$) appeared at δ 12.90 ($NCH_2\underline{CH_3}$), δ 41.46 ($N\underline{CH_2}CH_3$), δ 45.84 (NCH_3), δ 46.59 (C2), δ 54.52 (C3), δ 164.09 (CO).

3. Results and discussion

Although NMR spectroscopy generally suffers from lower sensitivity compared to mass spectrometric or chromatographic methods, DEC is a particularly suitable candidate for analysis by ^{1}H-NMR. The compound does not contain any highly absorbing chromophores, and thus HPLC analysis must rely on derivatization, which makes the procedure more difficult and potentially less accurate. Gas chromatographic methods employ uncommon detectors, internal standards that are not readily available, and troublesome extraction steps [11–14]. Although the GLC/MS methods, when carefully adapted, will eventually detect nanogram quantities of DEC, the high doses of the drug used (typically 6 mg kg^{-1} body weight per day or more), and its rapid elimination in unchanged form in the urine [3], make the high sensitivity an unimportant issue in the case of urine analysis. The only pretreatment of urine required for the analysis by NMR is addition of 10% of D_2O for the internal frequency lock.

DEC contains two magnetically equivalent N-ethyl groups, and thus the high-field triplet at δ 1.11 (in water) is detected with the same sensitivity as the less characteristic singlet of the N-methyl group which appears at δ 2.92. Heteroatom-bound ethyl groups are uncommon in the biological environment, and the DEC triplet occ-

urs in the region of the spectrum with a relatively low level of chemical noise [18,19]. The N-ethyl group triplet was therefore considered to be the most appropriate for the analysis. With the equipment used in this study, about 1 μg ml^{-1} of DEC could be detected in an aqueous solution without difficulty (Fig. 2). As little as about 5 μg ml^{-1} of DEC could be observed in urine (Fig. 3).

Water suppression was achieved using the binominal $1\bar{3}3\bar{1}$ pulse sequence [15]. The water peak signal of urine samples could be typically shimmed to half-height line widths of 4–5 Hz, and was effectively suppressed by the $1\bar{3}3\bar{1}$ pulse. Although this method gives non-uniform excitation over the whole spectral range, the flat region of maximum excitation can be conveniently adjusted [15] to observe the region of interest (here, 1450 Hz from the center frequency set at the water resonance). The T_1 relaxation time of the triplet in diluted aqueous solutions was found to be 1.06 ± 0.03 s, and was used to optimize the acquisition parameters. For fully relaxed spectra, optimal shimming, constant acquisition and processing parameters, and unchanged T_2 relaxation time, the peak heights are adequate measures of the content of DEC. Thus, in the concentration range from 1.1 to 161 μg ml^{-1} the height of the central line of the triplet increased linearly with the concentration with correlation coefficient

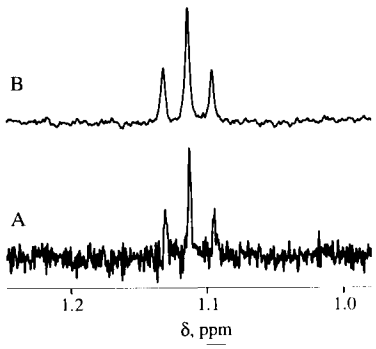


Fig. 2. Detectability of DEC in water by 400 MHz ^{1}H-NMR using the triplet resonance of the N-ethyl group. The sample contained 1.12 μg of DEC in 1 ml of H_2O–D_2O (9:1, v/v). (A) 1024 transients acquired using 90° $1\bar{3}3\bar{1}$ pulse with repetition period of 6 s; the data set was zero-filled from 32 k to 64 k computer points and Fourier-transformed without apodization. (B) Same sample; 6144 transients transformed with 0.5 Hz line broadening.

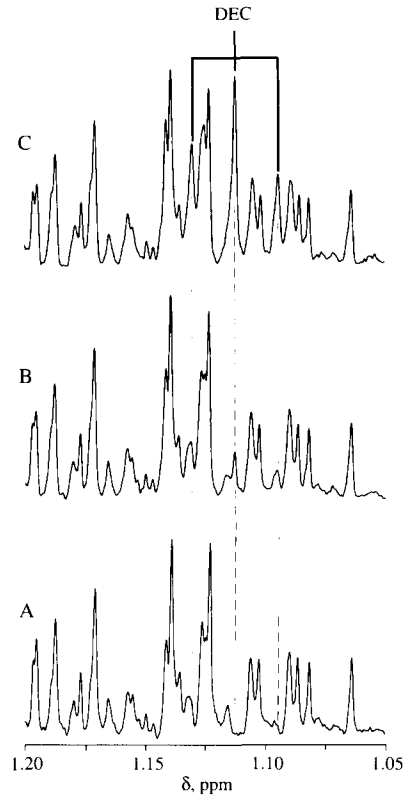


Fig. 3. Illustration of detectability of DEC in urine by 400 MHz ^{1}H-NMR. (A) Urine sample before addition of DEC. (B) The same urine sample to which 5.25 μg ml^{-1} of DEC was added. (C) The latter sample to which 21.7 μg ml^{-1} of DEC was added. All urine samples contained 10% of D_2O. The spectra were obtained with 90° $1\bar{3}3\bar{1}$ pulse using 6 s repetition period, and acquiring 1024 transients. The data set was zero-filled from 32 k to 64 k computer points and Fourier-transformed using the matched filter; 4th order polynomial was applied for baseline correction.

0.999 and random residuals (aqueous solutions of DEC). Precision of the quantification of DEC in urine was excellent, and accuracy better than 15% when using a calibration graph (Table 1). Quantification using standard addition, based on two spectra of the same solution recorded before and after spiking with a standard, is somewhat less accurate but still very satisfactory and fast (Table 1).

Analysis of urine from persons exposed to a single therapeutic dose of DEC (6 mg kg^{-1} of body weight) revealed the presence of the characteristic triplet of DEC, which showed the expected

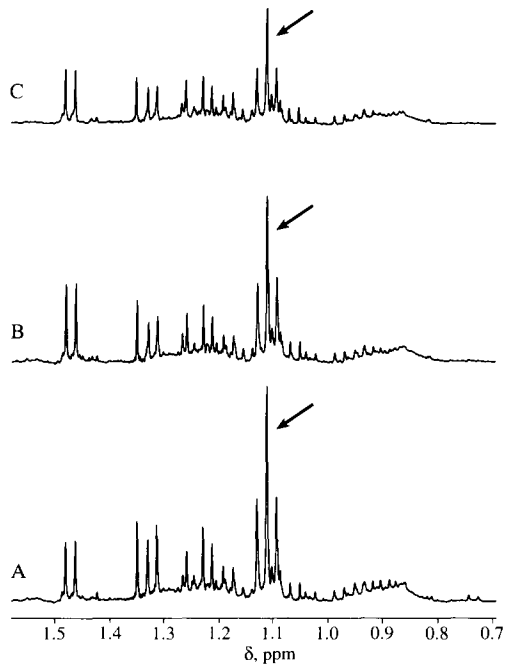


Fig. 4. 400 MHz ^{1}H-NMR spectra of urine showing elimination of DEC after intake of a single dose of 6 mg per kilogram body weight. The arrows point to the N-ethyl group triplet of DEC. The urine was collected 4 h (A), 8–10 h (B) and 16–18 h (C) after administration of DEC. D_2O (10%) was added to the urine samples; spectral conditions as in Fig. 3.

decrease with time (Fig. 4). Determination of DEC urine concentration gave elimination profiles exemplified by Fig. 5. The identity of the

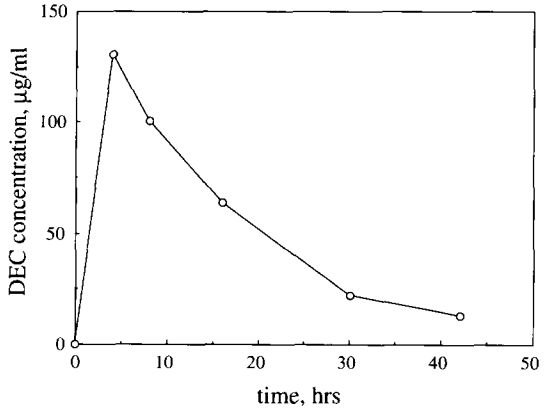


Fig. 5. Changes in concentration of DEC in urine determined by 400 MHz ^{1}H-NMR after a single oral dose of 6 mg per kilogram body weight (data for one subject).

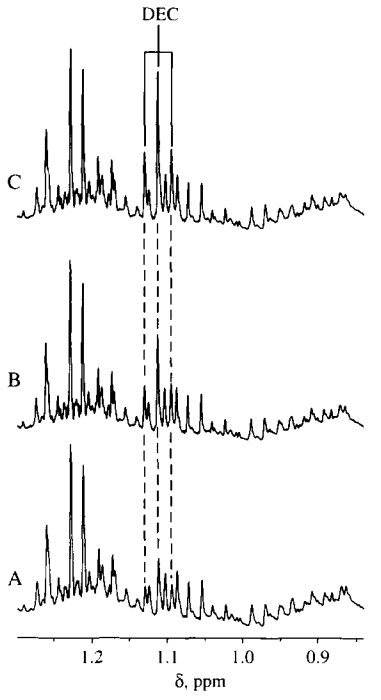


Fig. 6. Spiking of DEC signal in urine. (A) 400 MHz ^{1}H-NMR spectrum of urine collected 42–44 h after intake of 6 mg per kilogram body weight of the drug. (B) The same urine sample after addition of 13.7 μg ml^{-1} of DEC. (C) Previous sample with additional 16.2 μg ml^{-1} of DEC added. The calculated amount of DEC in (A) is 18.7 $\pm$ 1.3 μg ml^{-1}. D_2O (10%) was added to the urine samples; spectral conditions as in Fig. 3.

triplet appearing in the urine spectra was confirmed by the presence of the remaining resonances of DEC, and by spiking with authentic DEC. Thus, addition of increasing, known amounts of DEC to the urine sample caused the expected increase in the signal height, providing quantification independent of external calibration curves (Fig. 6).

Although most of DEC is excreted in the urine in unchanged form, about 10% is converted to the N-oxide (Fig. 1) [20]. The metabolism of DEC is markedly species-dependent, and in some animals the drug is excreted mainly as the N-oxide [21–24]. It was therefore of interest to determine whether the N-oxide can be detected in the ^{1}H-NMR spectra of human urine.

The N-oxide was synthesized from DEC by oxidation with hydrogen peroxide, and its struc-

ture confirmed by ^{1}H and ^{13}C spectra assigned with the help of two-dimensional experiments. Thus, a ^{13}C, ^{1}H-shift correlation spectrum allowed identification of piperazine ring protons attached to the same carbon, and at the same time resolved overlapping ^{13}C-NMR peaks. The assignment of the ring protons was confirmed by COSY and NOESY spectra, the latter showed intramolecular nuclear Overhauser effects (NOEs) between the protons attached to C2 and the methylene groups of the N-ethyl groups.

Although the ethyl triplet of the N-oxide could be resolved from that of DEC when the amounts of the two compounds were similar (Fig. 7), the small difference in resonance frequencies at 400 MHz prevented observation of small amounts of the metabolite in the presence of a large excess of DEC, as expected in the case of human urine. Moreover, the N-methyl group signal of the N-oxide (δ 3.25) appeared in the region of the spectrum with a relatively high level of chemical noise [18,19], and the compound could not be identified in the urine samples investigated in this work. This confirms previous results of the low

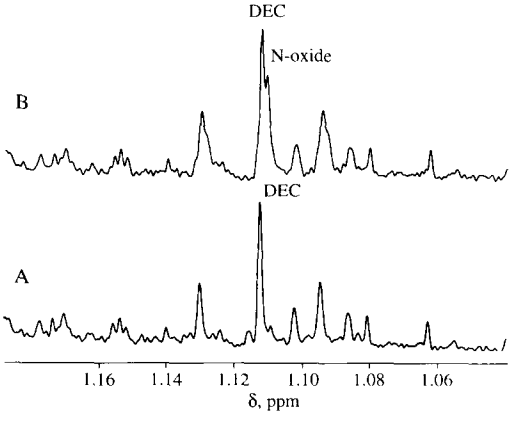


Fig. 7. Detectability of DEC N-oxide in the presence of DEC by 400 MHz ^{1}H-NMR. (A) Spectrum of urine collected 42–44 h after intake of 6 mg per kilogram body weight of the drug. This urine contained 34 ± 4 $\mu g\ ml^{-1}$ of DEC as determined by spiking. (B) The same urine sample after addition of 20.1 $\mu g\ ml^{-1}$ of the N-oxide. The difference in resonance frequencies of the two triplets is 0.7 Hz. Spectral conditions as in Fig. 3; the spectra were resolution-enhanced by Lorentz–Gauss transfomation.

amount of the N-oxide of DEC excreted in human urine. However, it can be expected that ^{1}H-NMR can be useful for determination of the N-oxide in urine of animals which convert most of the DEC to this metabolite [21–24].

We conclude that high-field ^{1}H-NMR is a convenient means of determination of DEC in urine, and that the sensitivity and the accuracy of the method are more than sufficient to monitor treatment with DEC. The method can also be used to investigate factors that affect the rate of DEC elimination, such as parasitism [25] and urine pH [26]. Although ^{1}H-NMR (at 60 MHz) has previously been used to determine the amount of DEC in tablets [27], this is the first report on the determination of DEC in a biological fluid by NMR spectroscopy. The results further emphasize the usefulness of ^{1}H-NMR as a quantitative analytical tool for urine analysis.

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