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Diagnosis of a Case of Acute Chloroquine Poisoning Using ^{1}H NMR Spectroscopy: Characterisation of Drug Metabolites in Urine

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Analysis of biological fluids by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) is a promising tool in clinical biology. We have used this method for a rapid toxicological screening in the case of a suicide attempt. A urine sample was analysed at 300 and 600 MHz by 1D and 2D sequences (J-resolved and TOCSY) in a short experimental time. Quantification was realized by peak integration of the 1D spectrum. The results showed the presence of chloroquine and its major metabolite monodesethylchloroquine at concentrations of 462 and 140 mg/L, respectively. Ethanol was also detected in the spectrum. It can be concluded that ^{1}H NMR provides many advantages as a tool for clinical diagnosis in a case of acute intoxication. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Despite many reported cases of poisoning, chloroquine (Fig. 1) is the major drug used in the chemotherapy and chemoprophylaxy of malaria[1] and is also prescribed for autoimmune disorders.[2] Chloroquine poisoning is extremely dangerous because of the narrow margin between therapeutic and toxic doses.[3, 4] For the determination of chloroquine in biological fluids, the Dill-Glazko test,[5] the Haskin's test,[6] the Wilson-Edeson test,[7] thin-layer chromatography[8] and spectrophotometric methods are not very sensitive and not very selective. The reference analytical method is HPLC, the best sensitivity being obtained with fluorescence detection.[9] All these methods require an extraction step which leads to a time consuming and laborious assay. Immunoassay procedures, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA),[10] are simple and avoid any major sample preparation such as extraction but, compared with the HPLC method, are less specific.

High resolution NMR spectroscopy is increasingly used to analyse a variety of physiological fluids.[11–13] Numerous studies have demonstrated its usefulness in the toxicological field. NMR spectroscopy showed modifications of endogenous compounds following administration of toxins, especially nephrotoxins[14, 15] and hepatoxins,[16] and improved the undersanding of toxicological processes. The analysis by NMR of body fluids led also to the identification of unknown metabolites.[17] Finally, NMR spectroscopy was

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Abbreviations used: ELISA, enzyme-linked immunosorbent assay; FID, free induction decay; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; RIA, radioimmunoassay; TMSP-d_4, 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionic acid; TOCSY, total correlation spectroscopy.

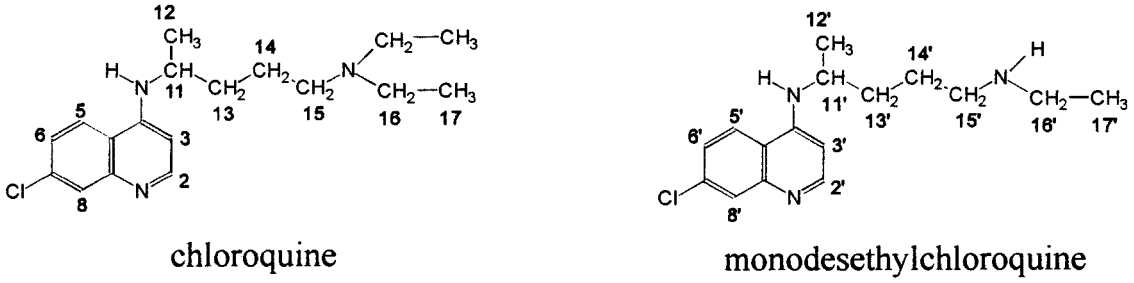

Figure 1. Structure of chloroquine and its major metabolite monodesethylchloroquine.

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applied to overdose cases that suggested its usefulness in routine clinical toxicology.[18, 19] In the present paper, we report the diagnosis of an acute chloroquine poisoning by ^{1}H NMR analysis at 300 and 600 MHz of a urine sample taken from an intoxicated patient.

MATERIALS AND METHODS

Case history

A urine sample was obtained from a 41-year-old man who had attempted suicide. A toxicological screen revealed a plasma chloroquine level of 890 mg/L. The sample was immediately stored at −20 °C until required for NMR measurements.

Sample preparation

The sample was directly used for ^{1}H NMR analysis. 20 μL of deuterium oxide (Euriso-top, Saint Aubin, France) providing an internal field-frequency lock and containing 3 - (trimethylsilyl) - 2, 2′, 3, 3′ - tetradeuteropropionic acid (TMSP-d_4) (Euriso-top, Saint Aubin, France), the internal chemical shift reference (δ=0.00 ppm), were added to 500 μL of the urinary sample. The sample was adjusted to pH = 7.0 and introduced into a 5 mm diameter NMR tube.

Proton NMR spectroscopy

Spectra were recorded at 300 MHz on a Bruker AC300 spectrometer (L.A.R.M.N., Facualté des Sciences Pharmaceutiques et Biologiques de Lille II) at ambient probe temperature. All chemical shifts were referenced with respect to the TMSP-d_4 signal set at δ=0 ppm.

Spectral assignments of endogenous compounds in urine were performed by consideration of literature values of chemical shifts in biological fluids,[12, 20–22] spin–spin coupling patterns and constant values obtained from the urine sample of a normal patient. Prior to Fourier transform, an exponential apodization function was applied to the FID, corresponding to a line broadening of 0.3 Hz.

One dimensional spectra of crude urine were obtained by operating in the pulsed Fourier-transform mode with quadrature detection. Application of a gated secondary irradiation field at the water resonance frequency during the relaxation delay gave insufficient reduction of the water signal. In order to obtain a good solvent suppression, the selective reduction in the T_2 of the water protons by chemical exchange with ammonium chloride (0.8 M)[23] was combined with saturation of the water signal. Free induction decays (FID) were collected into 16 K computer data points with a spectral width of 3200 Hz.

Two-dimensional *J*-resolved spectra of the same urine sample were obtained at 300 MHz with the following pulse sequence: relaxation D (90°x-t_1-180°x-t_1) acquisition, where the delay of relaxation D was 1 s and t_1 was an incremented variable delay to allow modulation of the spin–spin couplings. Reduction of the water signal was achieved by the same conditions as for the one dimensional spectra.

For each sample, 128 free induction decays (FID) were collected into 8 K computer data points wih a spectral width of 3200 Hz and 25 Hz for the F_1 dimension. Prior to the double Fourier transform, the data were apodized by means of a sine-bell function in t_1 and t_2. The spectra were tilted by 45° to provide orthogonality between the chemical shift and coupling constant axes.

Table 1. δ and *J* values of chloroquine (CQN) and monodesethylchloroquine (MDCQN)

Standard	Proton	Multiplicity[a]	δ (ppm)	*J* (Hz)
CQN	H-2	d	8.29	j_{2-3} = 7.02
	H-5	d	8.13	J_{5-6} = 9.21
	H-8	d	7.71	J_{8-5} = 1.97
	H-6	dd	7.54	J_{6-5} = 9.21
				J_{6-8} = 2.10
	H-3	d	6.83	J_{3-2} = 7.02
	H-11	m	4.13	
	H15-16	m	3.22	
	H13-H14	m	1.87	
	H-12	d	1.46	J_{12-11} = 6.28
	H-17	t	1.29	J_{17-16} = 7.33
MDCQN	H-2′	d	8.28	$J_{2'-3'}$ = 7.02
	H-5′	d	8.12	$J_{5'-6'}$ = 8.99
	H-8′	d	7.69	$J_{8'-5'}$ = 2.09
	H-6′	dd	7.52	$J_{6'-5'}$ = 8.99
				$J_{6'-8'}$ = 1.97
	H-3′	d	6.82	$J_{3'-2'}$ = 7.02
	H-11′	m	4.13	
	H15′-16′	m	3.13	
	H13′-H14′	m	1.87	
	H-12′	d	1.45	$J_{12'-11'}$ = 6.25
	H-17′	t	1·31	$J_{17'-16'}$ = 7.34

[a] d, doublet; dd, doublet of doublets; t, triplet; m, multiplet.

The TOCSY experiments were carried out at 298 K using a Bruker DMX600 spectrometer operating at 600 MHz on a 5 mm triple nucleus inverse geometry self-shielded gradient probe. The water peak was suppressed by pre-irradiation with a selective pulse of 1 s duration. The 2D-TOCSY spectra of urine were obtained with the following pulse sequence: D (90°x-t_1 − spin lock) acquisition, where D = 1 s, t_1 was an incremented variable delay to allow modulation of the spin-spin couplings and spin-lock with a mixing time of 54 ms. For the one dimensional selective experiment, the first ninety degrees pulse is replaced by a Gaussian shaped selective pulse (4 K points, truncated at the 1% level, 62.8 ms of duration was used for the multiplet inversion) and the spin-lock employed the MLEV-17[13] sequence with different mixing time. For 1D selective TOCSY experiments typical acquisition and processing parameters utilized 32 K time domain points filled by 16 K zeroes before Fourier transformation, an acquisition time of 2.02 s, a spectral width of 8090 Hz, four dummy scans, and line broadening weighting factors of 0.25 Hz.

^{1}H NMR spectra of the two standards chloroquine and monodesethylchloroquine were recorded at 600 MHz in the same conditions as for urine sample and completely assigned: δ and *J* values are reported in Table 1. At 300 MHz, in presence of ammonium chloride (0.8 M), the δ and *J* values are the same except for H-5 (δ=8.25 ppm).

RESULTS AND DISCUSSION

NMR Experiments at 300 MHz

A one pulse ^{1}H NMR spectrum of the urine sample is shown in Fig. 2. The major metabolites are assigned according to the literature.[12, 20–22] Compared with the urine of a normal subject, the most noticeable differences are the presence of unusual and intense peaks between 1.2 and 1.5 ppm and around 8.3 ppm. Because the interpretation of the 1D spectrum is complicated by the presence of many over-

lapped peaks, the assignments have been made principally through the use of 2D *J*-resolved map. This method is an effective aid to peak assignment, as chemical shift and coupling constant information can be easily extracted.[12, 18]

Expansions of the ^{1}H-^{1}H *J*-resolved map of the sample are shown in Fig. 3 In the aliphatic expansion (Fig. 3A), the signals of major endogenous metabolites are visible: lactate (doublet at 1.33 ppm), alanine (doublet at 1.45 ppm) and 3-hydroxybutyrate (doublet at 1.22 ppm). In the same expansion, the unusual signals which severely overlap on the 1D spectrum are resolved on the F2 skyline projection: especially one doublet at 1.40 ppm (J=6.5 Hz) and a triplet at 1.25 ppm (J=7.4 Hz) are observed. The presence of ethanol is also revealed by a triplet at 1.20 ppm and a quartet at 3.56 ppm. This compound was not expected by the clinicians at admission and suggests that the patient probably drank some alcoholic beverage at the time of the suicide attempt. Detailed examination of the expanded aromatic region (Fig. 3B) shows three doublets at 8.25 ppm (J=7.05 Hz), 8.24 ppm (J=9.15 Hz) and at 6.82 ppm

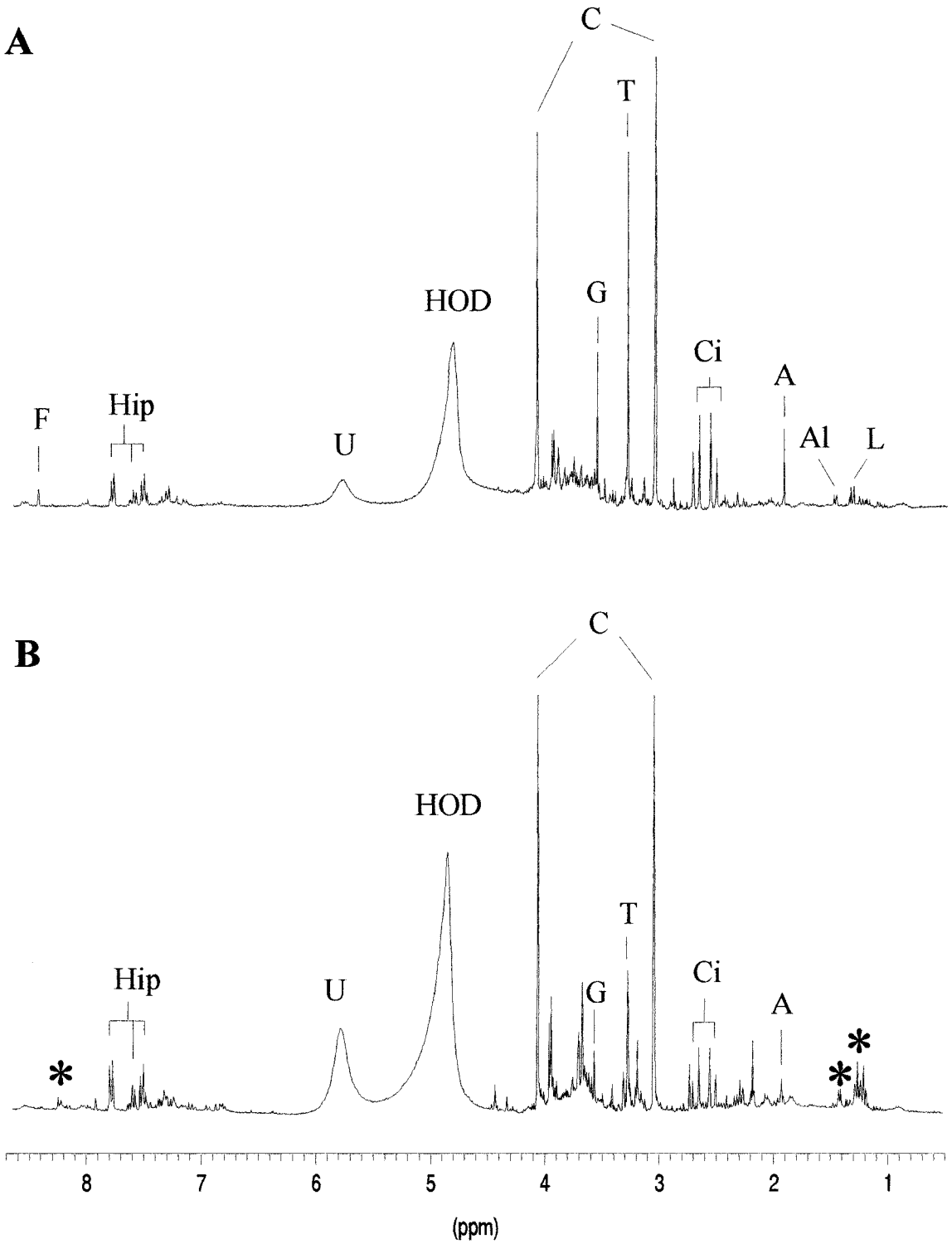

Figure 2. 300 MHz ^{1}H NMR spectrum of crude urine (pH 7.0; NH_4Cl 0.8 M) from a healthy subject (A) and from the intoxicated patient (B). The assignments of resonances are as follows: A, acetate; Al, alanine; C, creatinine; Ci, citrate; G, glycine; F, formate; Hip, hippurate; HOD, residual water peak; L, lactate; T, trimethylamine-N-oxide; U, urea; *, unusual resonances.

(J = 7.20 Hz). Hippurate is the major aromatic metabolite and clearly shows separated resonances from the two previous doublets.

The δ and J values of the unusual resonances correspond to those of the H-2, H-3, H-5, H-12 and H-17 protons of chloroquine described in Table 1.

These results suggest the interest for NMR analysis of urine sample in poisoning cases using the concerted application of single pulse and homonuclear J-resolved sequences. In order to confirm the presence of chloroquine, we have repeated the NMR experiments at very high frequency (600 MHz).

600 MHz Analysis

No addition of NH_4Cl (0.8 M) was needed as the water signal could be completely suppressed by a field gradient. As is made clear by Figs 4 and 5, unusual peaks are

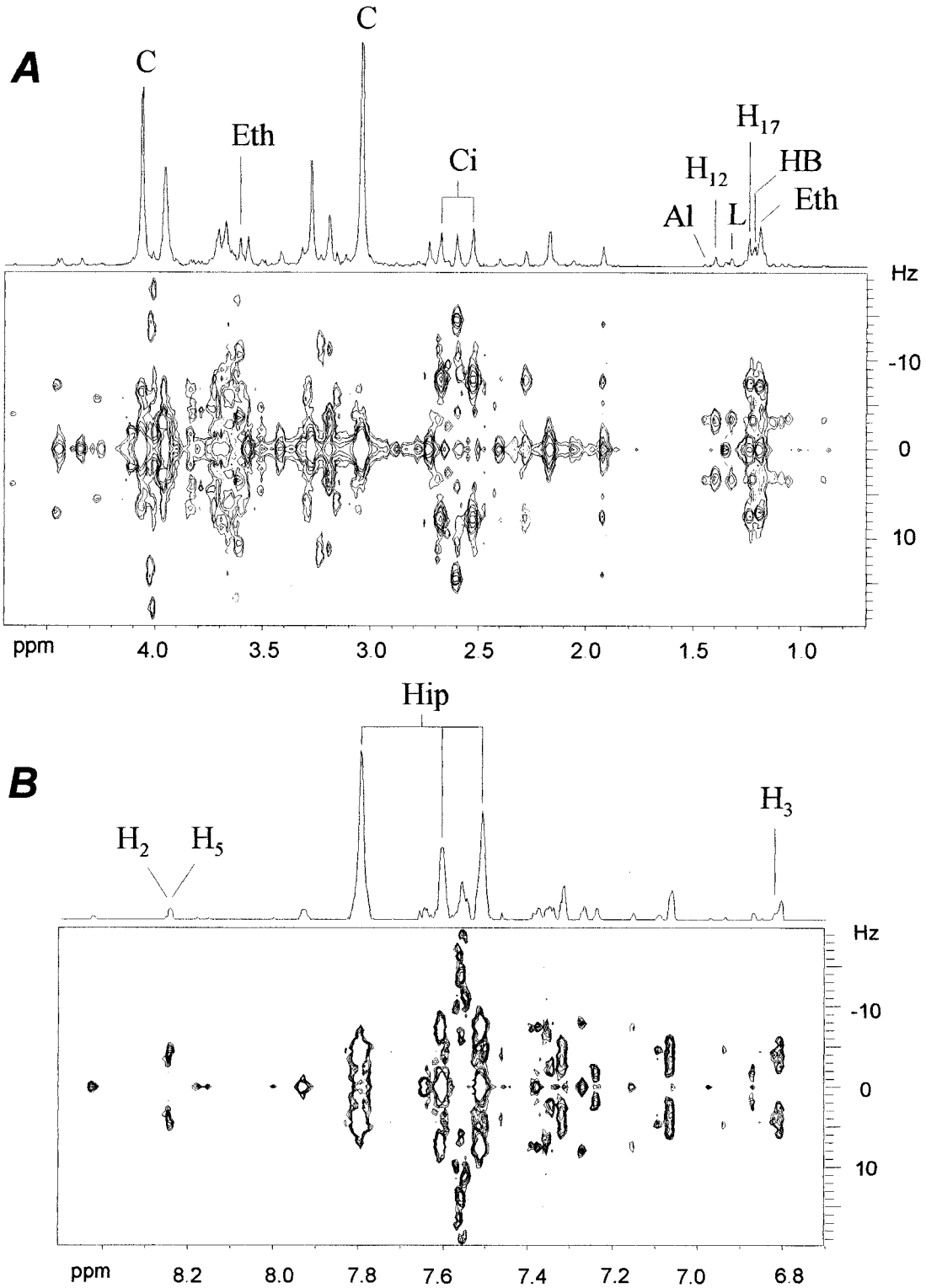

Figure 3. Expansion of the aliphatic region (0.7–4.7 ppm) of the 300 MHz J-resolved spectrum (A). The assignments of resonances are as follows: 3-HB, 3-hydroxybutyrate; Eth, ethanol; H_{12} and H_{17}, protons of chloroquine. Expansion of the aromatic region (6.7–8.5 ppm) of the 300 MHz J-resolved spectrum (B). The assignments of resonances are as follows: H-2, H-3 and H-5, protons of chloroquine.

observed in the 600 MHz single pulse spectrum consistent with the chloroquine resonances. The observed chemical shifts are 1.25 (t, H-17); 1.40 (d, H-12); 6.83 (d, H-3); 8.20 (d, H-5) and 8.27 ppm (d, H-2). In addition, the identification of chloroquine is supported by the observation of expected signals for H-13-14 at 1.84 ppm. The H-15, H-16, H-11 and H-6, H-8 protons would be hidden by large endogenous resonances. The resolution at 600 MHz is very much improved compared to 300 MHz and allows one to trace out less intensive unusual signals which partly overlap those of chloroquine H-12, H-3, H-5 and H-12 protons and could correspond to monodesethylchloroquine resonances.

In order to assign all the chloroquine resonances, we have used the TOCSY 2D experiment (Figs 4 and 5) which results in off-diagonal peaks for all the chemical shifts along an unbroken chain of proton-proton couplings. The side-chain $-CH_2$ group of H-12 protons at 1.40 ppm shows a connectivity to the other coupled protons along the chain H-11, H-13, H-14 and H-15 (Fig. 4). In return, the H-17 proton is coupled to H-16. On Fig. 5, the H-2 proton shows a connectivity to a doublet at 6.83 ppm corresponding to the H-3 proton of chloroquine. Finally, H-8 and H-6 chloroquine protons can be easily assigned through the two cross-peaks which show that they are coupled to the H-5 proton. On the same TOCSY experiment (Fig. 5) it is possible to observe cross peaks which partially overlap those of chloroquine and can be assigned to H-2′, H-3′ and H-5′ monodesethylchloroquine protons.

Use of TOCSY 1D sequences by applying the selective $\pi/2$ pulse to the less shielded δ1.40 doublet of the chloroquine H-12 proton yields a spectrum in which only chloroquine ^{1}H-resonances should be present. The result is depicted in Fig. 6. With a short mixing time (10.3 ms), the spectrum traces out the proton H-11. If the mixing time is increased, the connectivity to the more remote protons appears and for a long mixing time (145 ms) the spectrum shows all the proton coupling pathway, that is H-11, H-13, H-14 and H-15. The results confirm those obtained by the TOCSY 2D experiment. The advantage of the selective 1D experiment is rapidity and economy of disk space.

Quantification of metabolites and comparison to classical procedures

Quantification of chloroquine and monodesethylchloroquine was achieved at 600 MHz by peak integration of the classical 1D spectrum after Gaussian deconvolution. The

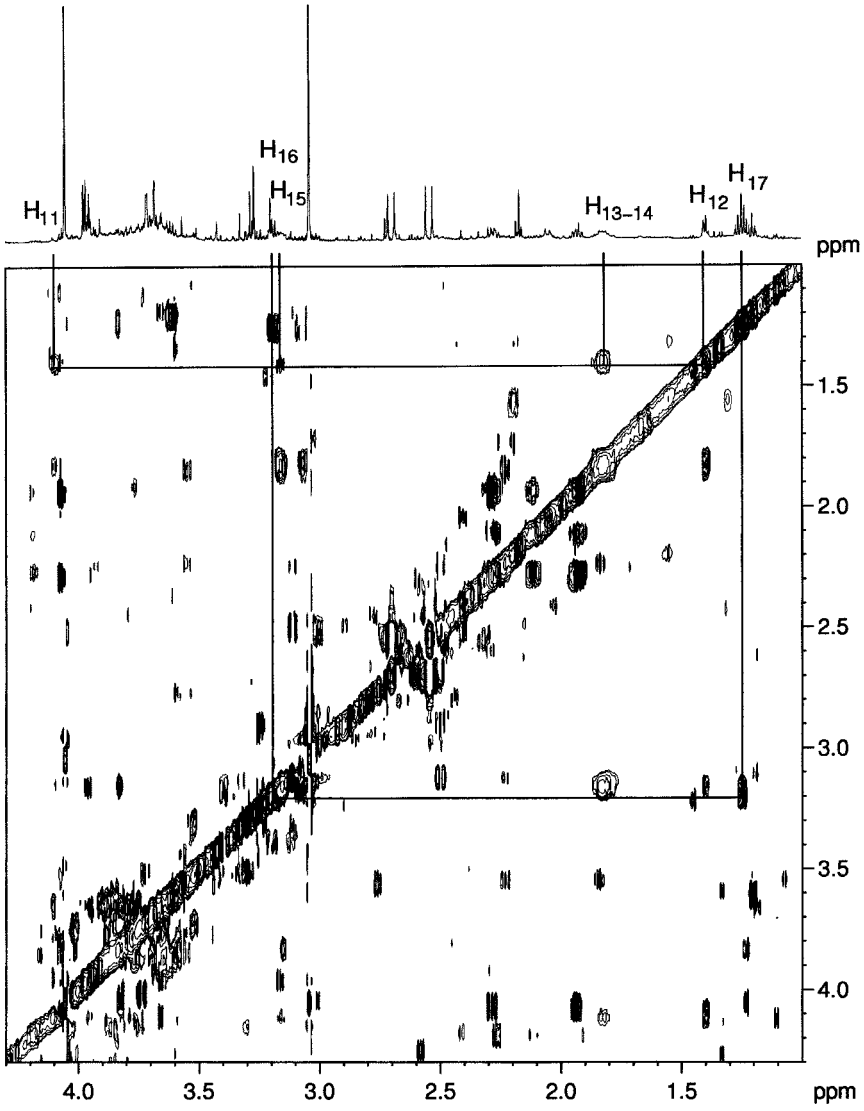

Figure 4. Expansion of the aliphatic region (1.0–4.4 ppm) of the 2D 600 MHz TOCSY map. The assignments are as follows: H-11, H-12, H-13, H-14, H-15, H-16 and H-17: chloroquine protons.

concentrations of chloroquine and monodesethylchloroquine determined by NMR spectroscopy are respectively 1.25 mM (462.5 mg/L) and 0.48 mM (140.2 mg/L). The results show that most of the chloroquine dose is excreted unchanged (72%) and that the main metabolite is monodesethylchloroquine which amounts 28% of the urinary fraction. These values are equivalent to those described in the literature and obtained with classical biochemical methods.[24, 15] Further metabolites represent less than 7% of the excreted dose and cannot be detected in urine samples by ^{1}H NMR spectroscopy.

CONCLUSION

The use of high field ^{1}H NMR spectroscopy has enabled the identification of unusual compounds corresponding to chloroquine, monodesthylchloroquine and ethanol in the urine sample of an intoxicated patient. Apart from these unusual resonances, the spectrum of the intoxicated patient didn't show any difference compared with that of a healthy subject except the physiological variabilities in the excretion of endogenous metabolites. These results are in agreement with the clinical picture of the patient who presented with only minor problems of cardiac rhythm and recovered rapidly.

High resolution ^{1}H NMR spectroscopy has several advantages compared to classical biochemical methods: it doesn't alter the sample and allows the determination of many endogenous and exogenous metabolites without preselection of the analytes. The clinician, facing several possible diagnosis, has no need to make any assumption before the biological analysis. The single pulse spectrum can be obtained within a few minutes and the 2D pulse sequences used for our work combine a relatively low total spectral acquisition time (about 2 h) with high sensitivity and dispersion. This type of analysis is very well suited for a rapid toxicological screening and routine clinical diagnosis. For this purpose, it would be necessary to measure by NMR spectroscopy the major xenobiotics involved in poisonings and generate a database of structures and ^{1}H NMR parameters. In addition, it is possible to observe on the same spectra toxicological informations especially concerning nephrotoxicity and hepatotoxicity.

The choice of the 1D and 2D experiments differed with the field strength of the available spectrometer. In this case, the accurate identification of chloroquine was only possible

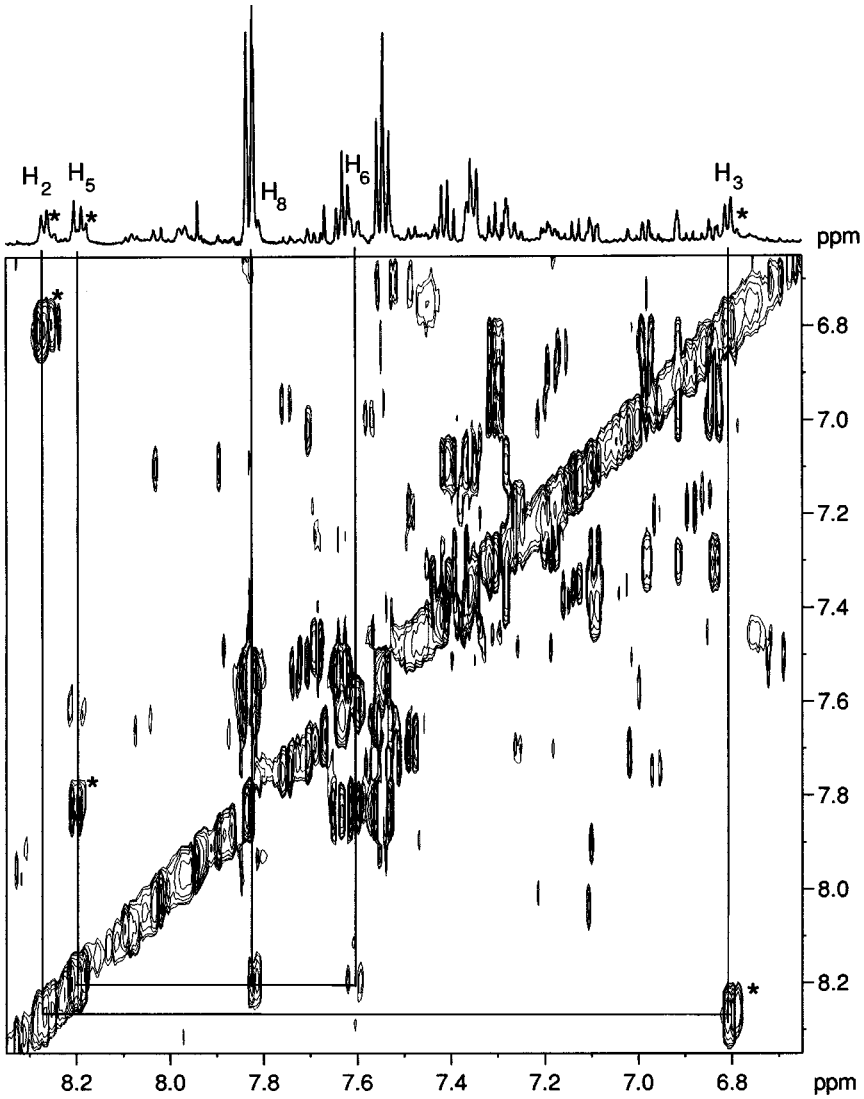

Figure 5. Expansion of the aromatic region (6.65–8.35 ppm) of the 2D 600 MHz TOCSY map. The assignments are as follows: H-2, H-3, H-4, H-5 and H-6, chloroquine protons. ∗: monodesethylchloroquine H-2′, H-3′, H-6′ protons and their respective cross-peaks.

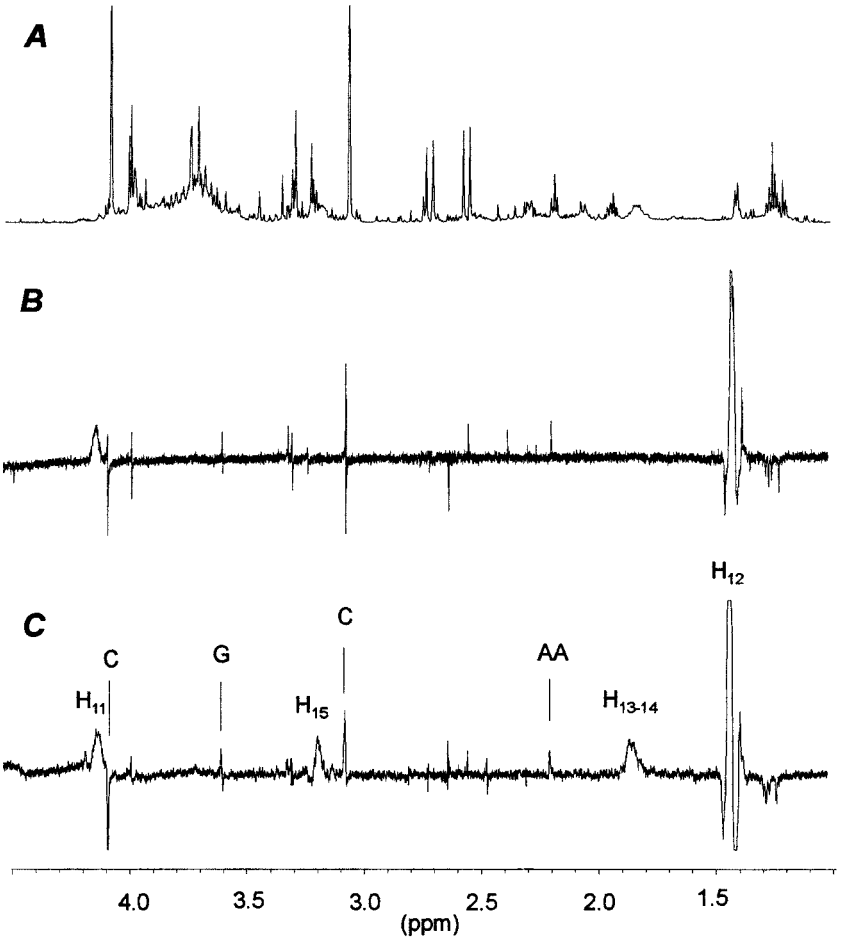

Figure 6. Spectrum of the aliphatic region (1.0–4.5 ppm) of the 600 MHz one dimensional spectrum (A) and the selective 1D-TOCSY experiment at different mixing times (B: 10.3 ms and C: 145 ms). The assignments are as follows: H-11, H-12, H-13 and H-14 and H-15, chloroquine protons, C, creatinine, G, glycine and AA acctoacetate.

at 600 MHz, the resolution being more effective and the detection more sensitive than at 300 MHz. Sometimes the use of 300 MHz can be sufficient as we have described it previously for an acute salicylate poisoning.[16] The use of very high frequencies is required especially in situations where the overdose is moderate (as in this case); in more confusing, mixed drug poisonings or for the detection of unusual resonances in the most crowded region of the spectrum (δ3–4.5 ppm).

A sample of urine is more convenient for toxicological analysis by ^{1}H NMR spectroscopy. Analysis of plasma sample results in very informative but very complex spectra resulting in delayed identification of unusual resonances since no automatic recognition exists. After having rapidly identified the xenobiotic in the urine sample, the drug can be quantified in blood by a specific analytical method.

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