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Filtering methods for selection of singlet and doublet signals in NMR spectra of DNA oligomers

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SUMMARY

The base proton (purine H8 and pyrimidine H6) resonances are key signals for the assignment of the proton resonances of DNA oligomers. They are classified into two groups, i.e., cytosine H6 signals, observed as *doublets*, and the other base proton signals, observed as *singlets*. Here we propose some experiments for distinguishing the cytosine H6 signals from the other base proton signals. Moreover, the ability of signal selection and the sensitivity as to signal detection were compared for all experiments, and the optimum conditions for spectral measurements were surveyed. Some of the experiments were employed as the NOESY detection pulse. Previously proposed experiments, such as HOENOE and HAL, were also used in the comparison.

INTRODUCTION

Proton resonance assignments for DNA oligomers are mainly performed by using NOE connectivities (Wüthrich, 1986; Hosur et al., 1988; Van de Ven and Hilbers, 1988; Feigon et al., 1992), since the through-bond connectivity obtained by COSY or TOCSY can only be used for limited purposes, such as simple identification of the sugar and base protons. It should be noted that no sequential connectivity to neighboring nucleotide residues can be obtained from this through-bond connectivity, and thus ambiguity remains as to the sequential assignments for nucleic acid oligomers. Such aspects of assignments were previously reviewed and explained in detail (Wüthrich, 1986; Hosur et al., 1988; Van de Ven and Hilbers, 1988; Feigon et al., 1992).

Recently, Sklenář and Feigon (1990) introduced a novel experiment HOENOE, which reduces the ambiguity of the assignments by selecting cytosine H6 doublets from the base proton region (7–8.5 ppm) in the NOESY spectrum. Though several experiments for the detection of multiplets

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were proposed previously, none of them, except HOENOE, was designed for the detection of doublet signals of nucleic acids. For example, experiments involving the use of double-quantum transitions, i.e., DQF-COSY, 2Q spectroscopy and DQ-NOESY (Van de Ven et al., 1985), were applied to nucleic acids, but were mainly employed for the assignment of the complex sugar proton resonances, not for the selection of *doublets* in the base proton region. Selected and simplified information, similar to that in the HOENOE experiment, can be obtained by means of other types of experiments. For example, the HAL experiment proposed by us selects *singlets* instead of *doublets* (Kojima and Kyogoku, 1993). The HAL and HOENOE experiments allow simplification of the base proton region in the NMR spectra of DNA oligomers and thus result in easy spectrum analysis.

Here we compare several 1D filtering experiments, including newly proposed ones. The procedures for the selection are classified into three categories, doublet selection, singlet selection, and selection of both but with opposite signs. For simplicity, we call cytosine H6 signals doublets and the other base proton (thymine H6, guanine H8 and adenine H8 and H2) signals singlets. Furthermore, we extend some of the new techniques to NOESY-type 2D experiments, and compare the abilities of the various experiments for singlet and doublet selection in practice.

THEORETICAL BACKGROUND

Classification of experiments

In Fig. 1, the pulse trains of seven experiments are given. SGP (Fig. 1A) is the conventional single-pulse experiment, given as a reference for the other six experiments (Figs. 1B–G). Two experiments (Fig. 1B and C) were designed for the selection of singlets. (B) is the HAL (**HA**hn-echo and spin-**L**ocking) experiment proposed previously by us (Kojima and Kyogoku, 1993). SL in the figure indicates a spin-locking pulse, but a purging pulse is actually used. (C) is the SQF experiment newly proposed in the present paper. SQF comprises single-quantum filtering, achieved by phase cycling. Both experiments (B) and (C) have the same delay time, Δ, which is set to 1/4J for the separation of singlets and doublets. A more detailed explanation was given in the previous paper (Kojima and Kyogoku, 1993).

Three experiments, D–F, were designed for the detection of doublets. (D) is a DQF experiment, regarded as 1D DQF-COSY (Piantini et al., 1982; Rance et al., 1983; Shaka and Freeman, 1983; Müller et al., 1986). (E) is the HOE experiment, which is a part of the HOENOE experiment (Sklenář and Feigon (1990). (F) is the HARD (**HA**hn-echo **R**efocused **D**QF) experiment newly proposed here, which is regarded as a 1D 2Q experiment (Bax et al., 1981; Sørensen et al., 1983). These three experiments will be discussed in more detail later.

(G) is a HAHN experiment (Hahn, 1950). This experiment has a delay time, Δ, which is set to 1/4J. Singlets and doublets are observed with opposite signs, positive and negative.

Optimized parameters for the doublet selection experiments

For singlet detection experiments, there is no parameter which should be adjusted to get the best efficiency. This is also true for the HAHN experiment. In contrast, the experiments designed for doublet detection should be optimized by searching for the best efficiency in detection.

A scheme of the HOE experiment is shown in Fig. 1E. In this experiment, cytosine H5 resonances (5.3–6 ppm) are selectively excited and followed by means of the isotropic mixing pulse.

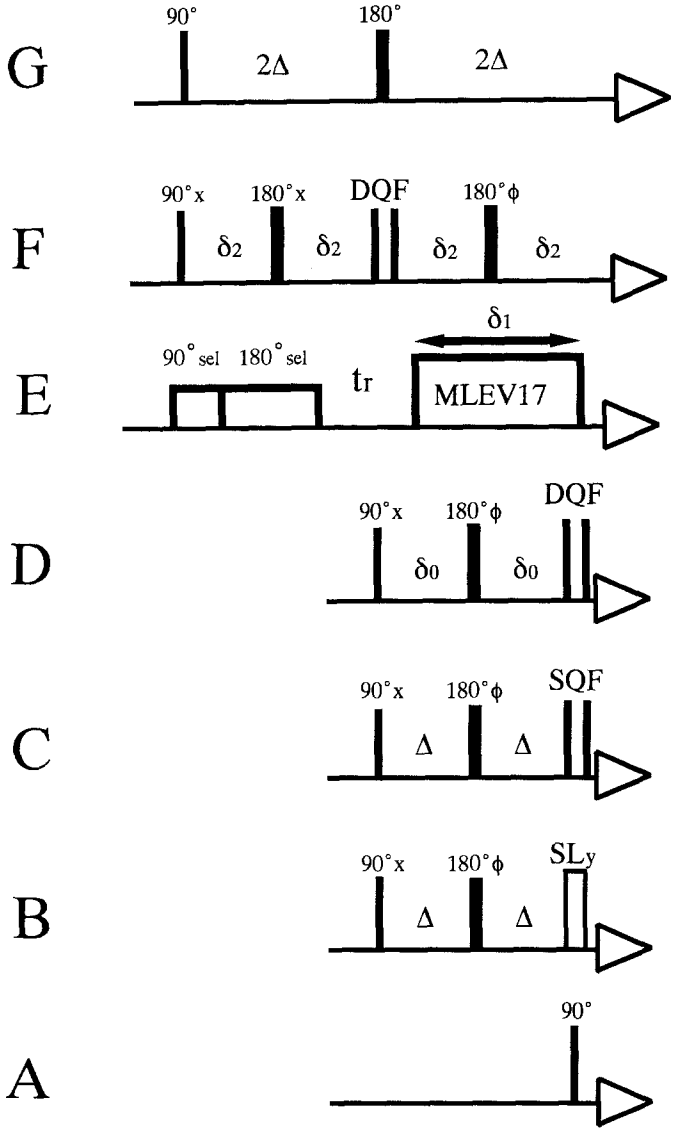

Fig. 1. (A–G) Pulse sequences used for several types of 1D experiments. The experiments are the following: (A) SGP; (B) HAL; (C) SQF; (D) DQF; (E) HOE; (F) HARD; and (G) HAHN. All the sequences were designed for specific purposes, i.e.: (A) reference; (B,C) singlet signal selection; (D–F) doublet signal selection; and (G) both singlet and doublet signal detection but with opposite signs. Except for the conventional experiment (A) and the echo experiment (G), the phase cyclings were as follows: ϕ = x, SQF (first pulse phase = 4(x),4(−x); second one = x,y,−y,−x,−x,−y,y,x), Acq. = x,y,−y,−x for (C); ϕ = x, DQF (first pulse phase = 4(x),4(−x), second one = x,y,−y,−x,−x,−y,y,x), Acq. = x,−y,y,−x for (D); DQF (first pulse phase = 4(x),4(−x), second one = x,y,−y,−x,−x,−y,y,x), ϕ = x, Acq. = x,−y,y,−x for (F). In (E) 'sel' indicates the selective excitation pulse. Phase cycles for (B) and (E) were given previously (Sklenář and Feigon, 1990; Kojima and Kyogoku, 1993).

The total signal transfer efficiency, F, of the HOE experiment relative to that of SGP was calculated as the product of two factors. As pointed out previously (Braunschweiler and Ernst,

1983), one is the conversion factor under isotropic mixing conditions and the other is the relaxation factor. Thus, the total efficiency is described by Eq 1:

$$F = \sum_i \left[\frac{1}{2}(1 - \cos(2\pi J_i \cdot \delta_1)) \cdot \exp(-\delta_1/T1\rho_i)\right] = \sum_i \left[\sin^2(\pi J_i \cdot \delta_1) \cdot \exp(-\delta_1/T1\rho_i)\right] \quad (1)$$

where δ_1 is the mixing time, J the coupling constant of a doublet, and T1ρ the relaxation time in the rotating frame. The subscript i denotes the specified cytosine H6 resonance. In the case of an aqueous solution of a DNA oligomer, the value of T1ρ is nearly equal to that of T2 (Freeman, 1988), and all of the cytosine H6 resonances have almost constant T2 and ^3J values, which are given as $T2_{av}$ and J_{av}. Then, the signal transfer efficiency of the specified H6 is approximated with Eq. 2:

$$F(\delta_1, T2_{av}) \cong \sin^2(\pi J_{av} \cdot \delta_1) \cdot \exp(-\delta_1/T2_{av}) \quad (2)$$

Equation 2 means that the total signal transfer efficiency is a simple function of two time parameters, i.e., the mixing time and $T2_{av}$. As the averaged coupling constant, J_{av}, is fixed at 7.5 Hz for DNA oligomer duplexes, the total signal transfer efficiency can be calculated to be as plotted in Fig. 2A. The ridge line in Fig. 2A indicates the condition that gives the maximum efficiency with a certain $T2_{av}$ value. Accordingly, the mixing time, δ_1, is uniquely determined by the $T2_{av}$ value of an individual sample, viz., the δ_1 value is a function of the $T2_{av}$ value under efficiency-maximized conditions. The averaged transverse relaxation time, $T2_{av}$, is about 120 ms for a DNA dodecamer (Lane et al., 1991), so the δ_1 value was determined to be 59 ms from Fig. 2A.

For the DQF experiment in Fig. 1D, the delay time, δ_0, should be shorter. The total signal transfer efficiency depends on the delay time and on individual transverse relaxation times, $T2_i$. In practice, the $T2_i$ values are replaced by the averaged $T2_{av}$, as mentioned above. These parameters are related through Eq. 3:

$$F(\delta_0, T2_{av}) \cong \sin(\pi J_{av} \cdot 2\delta_0) \cdot \exp(-2\delta_0/T2_{av}) \quad (3)$$

The HARD experiment, shown in Fig. 1F, exhibits similar relations among the following three parameters; the delay time, δ_2, the averaged transverse relaxation time, $T2_{av}$, and the total signal transfer efficiency, F:

$$F(\delta_2, T2_{av}) \cong \sin^2(\pi J_{av} \cdot 2\delta_2) \cdot \exp(-4\delta_2/T2_{av}) \quad (4)$$

The optimum conditions for the DQF and HARD experiments were investigated in similar ways as for HOE. Three-dimensional graphical expressions for the total efficiency are displayed in Fig. 2B for DQF and in Fig. 2C for HARD, using 7.5 Hz as the J_{av} value in the calculations with Eqs. 3 and 4.

The optimized delay time, total delay time, and total efficiency were calculated by using J_{av} (7.5 Hz) and $T2_{av}$ (120 ms) for three doublet selection experiments, HOE, DQF and HARD, and are given in Table 1. The total delay time is the sum of the optimized delay times in each experiment (Fig. 1), and the total efficiency is the relative intensity compared to that of SGP.

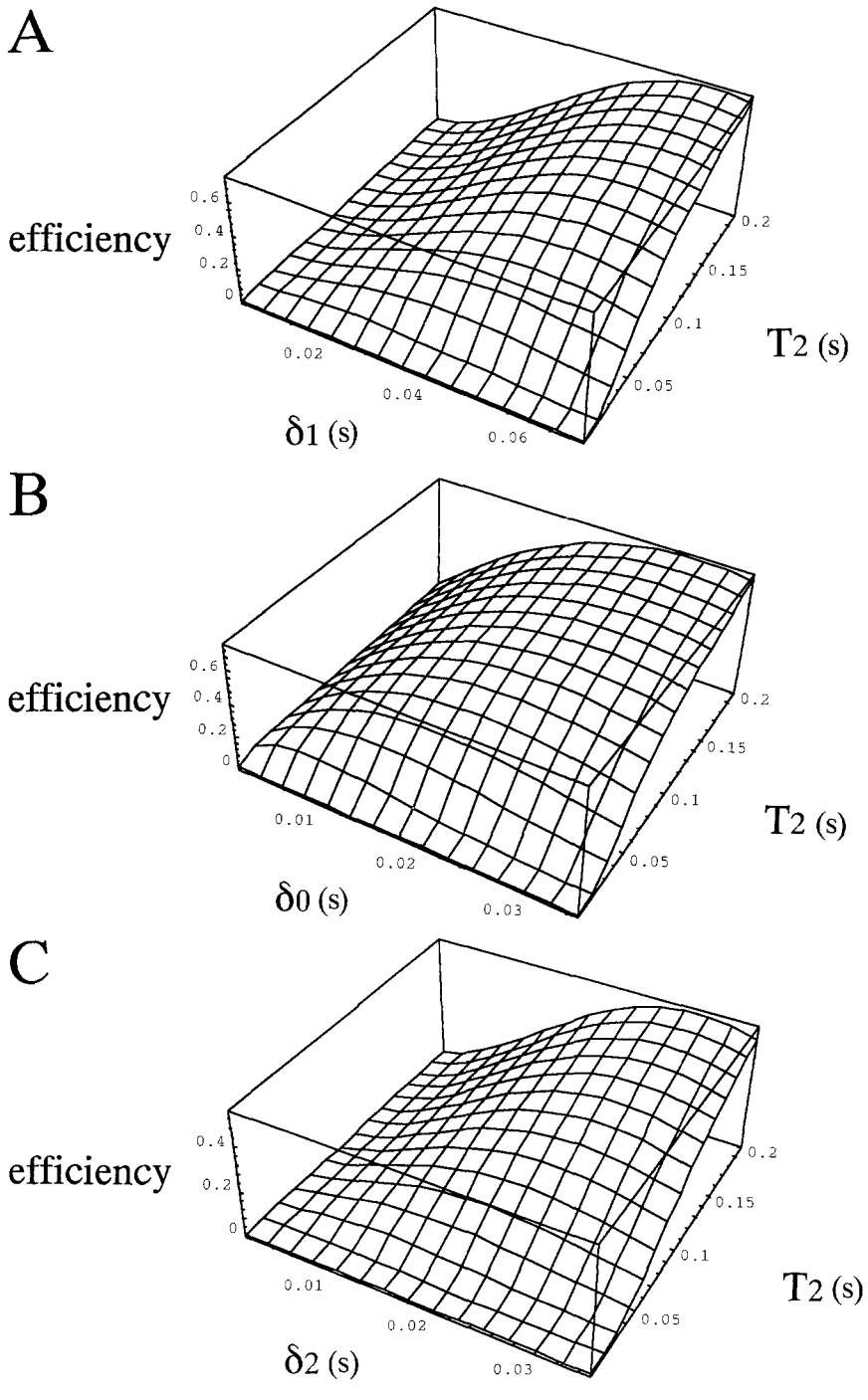

Fig. 2. (A) Three-dimensional graphic display of the calculated signal transfer efficiency for the HOE experiment as a function of the averaged T2 value, $T2_{av}$, and the mixing time, δ_1. (B) DQF and (C) HARD: the parameters are the same as those in (A), except for the delay time, δ_0 and δ_2. These graphics show the optimum measurement conditions with various T2 values.

Two-dimensional experiments

The experiments shown in Fig. 1 can be extended to two dimensions by combining them with the NOESY experiment. As mentioned above, the HOE experiment has already been combined with NOESY as HOENOE (Sklenář and Feigon, 1990). Similarly, the NOESY detection pulse is replaced by HAL, SQF or DQF. These 2D experiments are named NOESY-HAL, NOESY-SQF and NOESY-DQF, respectively. The HARD and HAHN experiments may be combined with NOESY, but these experiments require longer total delay times and thus may result in lower S/N ratios. Actually, the calculated efficiency of the HARD experiment is worse than those of the other doublet selection experiments, DQF and HOE, as shown in Table 1.

The replacement of the detection pulse in NOESY with HAL, SQF or DQF gives the ω_2-filtered NOESY spectrum. The ω_1-filtered spectra are also obtained by replacing the preparation pulse in the NOESY sequence, just like in the case of HOENOE. Since all the experiments in Fig. 1, except for HOE and HARD, do not give in-phase absorption spectra in the spectral region of the sugar protons, NOESY-type experiments combined with these experiments do not give cross peaks with the sugar proton signals on ω_1 filtering. Moreover, the point resolution of the ω_2 axis is higher than that of the ω_1 axis, and thus ω_2 filtering is preferable for the analysis of the base proton resonances.

EXPERIMENTS

The experimental conditions, except for the pulse sequences, were the same for all experiments. The NMR measurements were performed with a JEOL GSX500 spectrometer, operating at 500 MHz and the temperature was set at 303 K.

The material used for these experiments was a double-stranded DNA oligomer, d(CGCGAATTCGCG)$_2$. The solution comprised 3 mM duplex in 500 µl buffer, 20 mM sodium phosphate and 50 mM sodium chloride, at neutral pH.

For the HAL, SQF, NOESY-HAL and NOESY-SQF experiments, the delay time was set to 1/4J, 34.5 ms. The DQF, HOE, HARD and NOESY-DQF experiments were performed using the delay times listed in Table 1. The 90° pulse width was 10 µs for high-power excitation, 23.5 µs for MLEV17 mixing, and 1 ms for selective excitation. The spin-lock pulse width was 500 µs at the high power level in the HAL experiment. For all spectra, 128 hyper-complex points for t1 and 1024 complex points for t2 were recorded, and 128 scans for each t1 increment were used.

TABLE 1
CALCULATED OPTIMUM TIMES BETWEEN PULSES AND THE ESTIMATED EFFICIENCY FOR THREE DOUBLET SELECTION EXPERIMENTS

Experiment	δ_i (ms)	Total (ms)	Efficiency
DQF	26	52	0.61
HOE	59	59	0.59
HARD	26	104	0.37

J was fixed at 7.5 Hz. δ_i: mixing or delay time; total: total time from the first pulse to the detection pulse.

RESULTS AND DISCUSSION

One-dimensional experiments

The spectra in Fig. 3 were obtained by performing the respective experiments given in Fig. 1. All the signals in these spectra were assigned previously (Hare et al., 1983; Lane et al., 1991). The signal intensities of the spectra in Fig. 3, relative to SGP, are listed in Table 2.

Selectivity. The ability of signal selection in each experiment can be checked by referring to Fig. 3. As to singlet detection experiments, HAL and SQF showed slightly incomplete selection of the signals. Both spectra still contained small doublet peaks. These experiments were performed with a 500-μl sample volume. When the sample volume was reduced to 400 μl, these small peaks disappeared. Thus, it can be concluded that they reflect B_1 inhomogeneity. Consequently, the signal selection should be influenced by the imperfection of the 90° and 180° pulses. To overcome this problem, the optimum sample volume should be searched for. Another solution to this problem is the use of the gradient-enhancement technique (Hurd et al., 1992) or the composite-pulse technique (Ernst et al., 1987; Freeman, 1988), or simply a micro cell.

In the case of the doublet detection experiments, the four cytosine H6 signals at 7.25, 7.32, 7.46 and 7.62 ppm were clearly selected in all experiments. The two weak signals at 7.10 and 7.36 ppm represent the singlets from thymine H6. The HOE experiment shows relatively worse selection, because the ROE (rotating frame NOE) part in HOE is difficult to adjust. In particular, the peak at 8.09 ppm exhibits negative intensity.

Sensitivity. The calculated sensitivities are given in Table 1 as efficiency, and the experimental ones are listed in Table 2. It should be noted that the optimized parameters in Table 1, the theoretically expected values, are close to those obtained experimentally from the 1D NMR spectra in Fig. 4.

As judged by comparison among the doublet selection experiments, the DQF experiment should exhibit the best sensitivity. The theoretically expected sensitivity of the HOE experiment was better than that of the HARD experiment, but the observed sensitivity was the worst. This fact may be explained in two ways. One is the neglect of some factors in the theoretical calculation. For example, the relaxation during the selective excitation and refocusing periods, and the efficiency of selective excitation were not taken into account. The other explanation is the experimental difficulty, namely, the determination of the 90° pulse for the selective excitation without signal dephasing, and the complete adjustment of the refocusing delay time. Imperfection of the former condition causes lower sensitivity and the latter introduces signal decay. Thus, the experimentally obtained sensitivity of HOE may not be the best obtained under given conditions.

Total evaluation for practical use. For practical use of these experiments, comparison in view

TABLE 2
OBSERVED RELATIVE SIGNAL INTENSITIES

Selected signals	SGP	HAL	SQF	DQF	HOE	HARD	HAHN
Singlets	1.00	0.42	0.40				0.19
Doublets	1.00			0.53	0.06	0.21	0.13

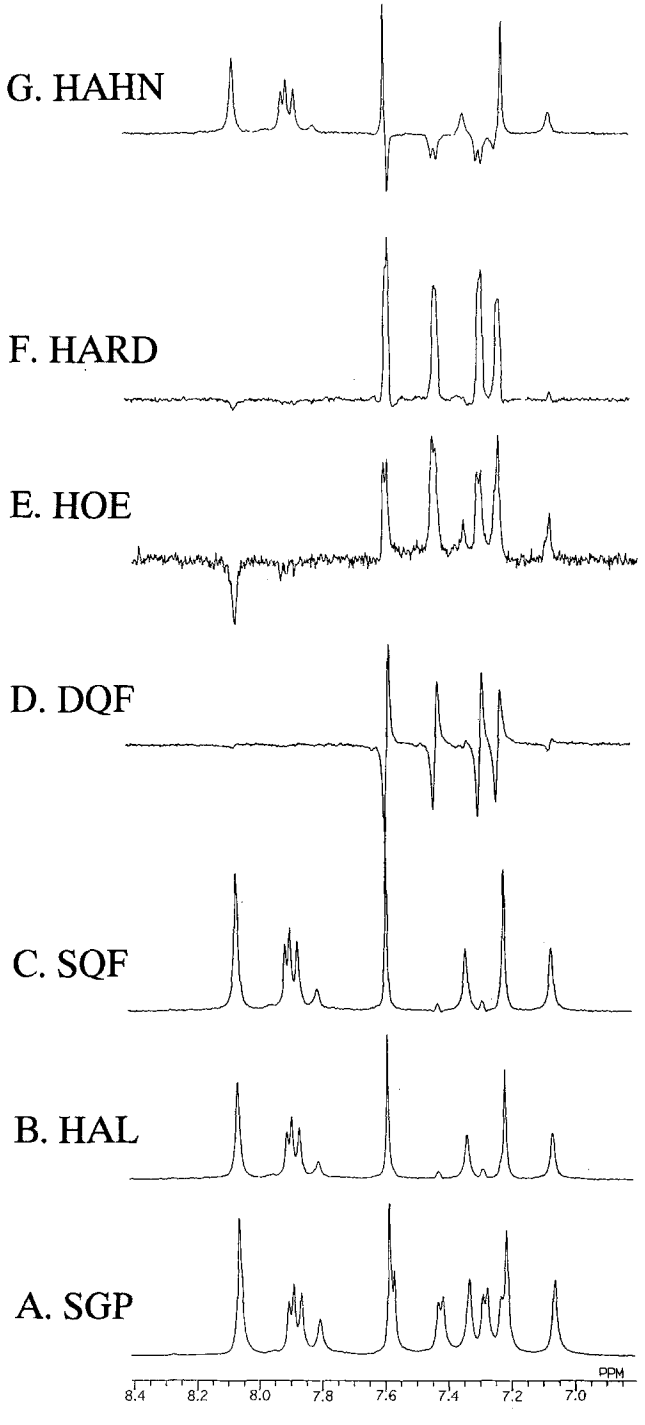

Fig. 3. (A–G) One-dimensional spectra obtained with the pulse sequences shown in Fig. 1. The experiments are identical to those in Fig. 1. In all the spectra, the base proton region is shown (6.9–8.4 ppm).

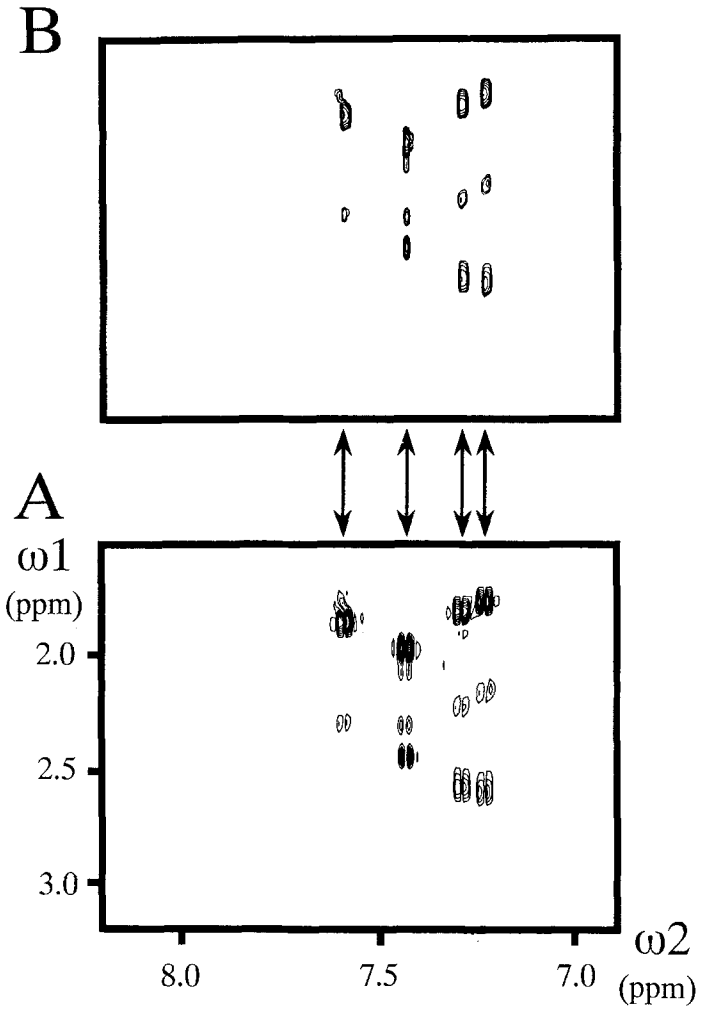

Fig. 4. NOESY-DQF spectra with a different zero-order phase value for the ω2 dimension; (A) 0°; and (B) 90°. ω1 (1.8–3.0 ppm) is the frequency region of the sugar 2',2" protons and ω2 (7.0–8.2 ppm) that of the base protons. The arrows indicate the resonance positions of cytosine H6 doublets (Hare et al., 1983; Lane et al., 1991).

of convenience is important. In this sense, the HOE experiment has problems. It includes soft and semi-soft pulses, which should be adjusted properly, as mentioned above. For the use of these experiments as 1D NMR, HARD and HAHN experiments show the best performance, and are useful for such cases as the titration of DNA–drug or DNA–protein systems. For the assignment of signals, the DQF, HAL and SQF experiments may be chosen because of their high sensitivity.

NOESY-type 2D experiments

The NOESY-DQF spectra are presented in Fig. 4. The displayed region is the cross section between the sugar 2',2" proton region (1.5–3.2 ppm) and the base proton region (6.9–8.2 ppm).

All the peaks of these spectra were assigned previously (Hare et al., 1983; Lane et al., 1991). The arrows in the figures indicate the positions of cytosine H6 signals.

The difference between the two spectra (A) and (B) arises from the condition of the zero-order phase value in the ω2 dimension: 0° for (A) and 90° for (B). The peak shape in Fig. 4A represents the antiphase doublet in ω2. This shape is favorable for the identification of doublet peaks, but unfavorable for the assignment of signals in a complicated pattern. The experiment giving the spectrum in Fig. 4B was designed to overcome this disadvantage. The peak appears as a dispersive antiphase doublet, though it appears as a single peak at low resolution (Pelczer et al., 1991). The sensitivity of the NOESY-DQF experiment is so high that the spectrum in Fig. 4B retains a high S/N ratio. The NOESY-DQF spectrum gives cross peaks between the unfiltered resonances in ω1 and the filtered cytosine H6 resonances in ω2. However, NOESY-DQF has another advantage. The DQF part selects not only the cytosine H6 resonances but also the evolved multiquantum coherence from the thymine H6 resonance coupled with the methyl protons. This coherence gives a cross peak between the intrabase protons in the thymine ring, i.e., the methyl and H6 protons. Such cross peaks are hardly ever observed, even in COSY, because of the small coupling constant between the protons.

Comparison of available information. NOESY-DQF and HOENOE experiments are classified as doublet selection experiments. For the selection of the base proton resonances, there is no difference between them except for the peak shape, i.e., antiphase for NOESY-DQF and in-phase for HOENOE. In other regions, like the sugar proton region, HOENOE also gives in-phase peaks, but NOESY-DQF does not give simple antiphase peaks. This means that HOENOE and conventional NOESY give the same information in the sugar proton region. The 3D NOESY-TOCSY experiment (Piotto and Gorenstein, 1991) provides more information on peak connectivity than conventional NOESY. This 3D experiment covers the information from NOESY-DQF, but does not allow clear signal selection because of its still crowded and overlapped spectrum. Another eperiment, DQ-NOESY (Van de Ven et al., 1985), is similar to NOESY-DQF. DQ-NOESY is a kind of ω1-filtered NOESY. This means that the chemical shift term in ω1 evolves under the double-quantum coherence. Though this experiment was designed to separate the sugar proton resonances, the selection in the base proton region is better than in the case of NOESY-DQF. As DQ-NOESY needs a refocusing delay like HARD in Fig. 1F, its sensitivity is lower than that of NOESY-DQF.

CONCLUSIONS

We compared several 1D filtering experiments for doublet (cytosine H6 signal) and singlet (other base proton signals) selection. Some of them are newly proposed here and others were previously reported. The ability of signal selection is so high for each experiment that no problem is found at this point, except in the case of signal cancellation of opposite signals. As far as sensitivity is concerned, the DQF and SQF experiments showed better performance. Thus, like the previously reported NOESY-HAL, they are applicable to the NOESY detection pulse, maintaining a high S/N ratio, and giving NOESY-DQF and NOESY-SQF. Moreover, they have such a high selectivity that they can be practically used for the assignment of nucleic acid signals and the confirmation of the signal assignments in crowded signal regions.

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