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Ultraselective, Ultrahigh Resolution 1D TOCSY

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Solution state ^{1}H NMR spectroscopy provides valuable insights into molecular structure and conformation. However, when the spectrum exhibits severe signal overlap, it hampers the extraction of key structural information. Here, an ultraselective, ultrahigh resolution TOCSY method is introduced that greatly reduces spectral complexity, allowing the extraction of previously inaccessible spectral information. It combines the recently developed GEMSTONE excitation with homonuclear decoupling to provide highly simplified through-bond correlation 1D ^{1}H NMR spectra, showing all signals within the selected spin system as singlets. The new method can greatly facilitate the analysis of mixtures, as shown here for a mixture of *Cinchona* alkaloids (popular catalysts in asymmetric synthesis) and a mixture of glucocorticoids (used for treating conditions such as asthma).

Introduction

Multidimensional, *n*D, NMR spectroscopy is routinely used for structure elucidation and molecular assignment, as well as to gain insight into molecular conformation and dynamics.[1,2] Signals are dispersed into multiple dimensions, alleviating the signal overlap commonly observed in ^{1}H NMR spectra due to the narrow chemical shift range and the presence of signal multiplicity. Although very valuable, *n*D NMR techniques can be time-consuming and unnecessary for determining molecular structure. Instead, targeted analysis of specific signals can sometimes provide sufficient information about molecular structure, conformation, and geometry in a much shorter time. Selective 1D NMR experiments enable targeted analysis by choosing a single part of the spectrum and propagating coherence from a (preferably) single signal in the selected region to nearby nuclei, by either through-bond or through-space mechanisms.[3] The selection of a signal in conventional 1D methods uses a shaped radiofrequency pulse to excite a narrow range of frequencies. In the ideal scenario, an isolated diagnostic signal is available, but in densely populated spectra, where signal overlap is an issue, the use of conventional selective methods typically leads to the selection of both the targeted signal and any other multiplets that overlap with it. In such cases, ultraselective NMR methods are employed, which enable selection of single signals even in regions of multiplet overlap.

The ultraselective method GEMSTONE (gradient-enhanced multiplet-selective targeted-observation NMR experiment)[4] has been successfully applied to a number of 1D selective experiments. Both through-bond (COSY and TOCSY)[5,6] and through-space (NOESY and ROESY)[4,7] mechanisms have been exploited to map diagnostic interactions in molecules with complex NMR spectra. However, even though GEMSTONE selective experiments provide greatly simplified spectra of individual spin systems from densely populated spectra, simple unambiguous analysis can still be prevented where the signals that are

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coupled to the selected signal overlap. In these cases, spectra need to be further simplified to reveal the individual chemical environments present in the molecule.

Ultrahigh resolution pure shift methods are well established NMR tools for the simplification of complex spectra.[8–11] Pure shift experiments report only the chemical shifts of the observed nuclei; multiplet structures are collapsed and spectral resolution is greatly increased. The previously reported 2D pure shift TOCSY experiment has shown great utility in sample analysis, achieving ultrahigh resolution correlation information, although requiring an experiment time of multiple hours.[12,13] Here, the pure shift and GEMSTONE approaches are combined to provide ultrahigh resolution 1D GEMSTONE-TOCSY spectra. The new experiment allows the acquisition of simplified spectra containing only chemical shift information for targeted spin systems from within densely populated spectral regions. For concision, the new method is termed TREASURE (TOCSY relayed excitation in acutely selective ultrahigh resolution experiments).

Results and Discussion

Application of the TREASURE method to the analysis of an alkaloid mixture of quinine and cinchonidine (Figure 1) demonstrates its benefits. Natural alkaloids have a wide range of chemical uses, including anti-malarial treatment[14] and as catalytic agents in asymmetric synthesis.[15] Both structure and molecular conformation influence chemical properties and functions, so it is essential that tools exist to tackle the analysis of intact complex mixtures.

The alkaloids quinine and cinchonidine, naturally found in the bark of the *Cinchona officinalis*,[16] are structurally similar molecules which present with a high degree of spectral overlap causing difficulties in analysis by ^{1}H NMR (Figure 1a). Analogous multiplet signals in quinine and cinchonidine overlap, which prevents the use of conventional selective NMR methods for analysis. GEMSTONE allows selection of an individual multiplet from each alkaloid (shown with selection of H16a in cinchonidine, Figure 1c and 1d), and associated signals from the selected spin system are then found by TOCSY transfer. The simplification achieved in the GEMSTONE-TOCSY subspectrum for cinchonidine (Figure 1c) is still insufficient to identify each signal in the system, this only being possible when TREASURE is used to suppress multiplet structure. While the PSYCHE (pure shift yielded by chirp excitation) pure shift spectrum (Figure 1b) offers increased resolution compared with the conventional ^{1}H spectrum, it is not possible to identify the signals responsible for each spin system. Combining targeted analysis with the increased resolution afforded by pure shift acquisition enables the identification of the geminal H13 protons, which are hidden in the GEMSTONE-TOCSY spectra by the more intense H14 and H15a multiplets. Each chemical environment is cleanly identified for cinchonidine in the final pure shift spectrum (Figure 1d), enabling the extraction of the desired information from this complex mixture.

The proposed TREASURE method shows further utility in the analysis of a glucocorticoid mixture. Glucocorticoids are

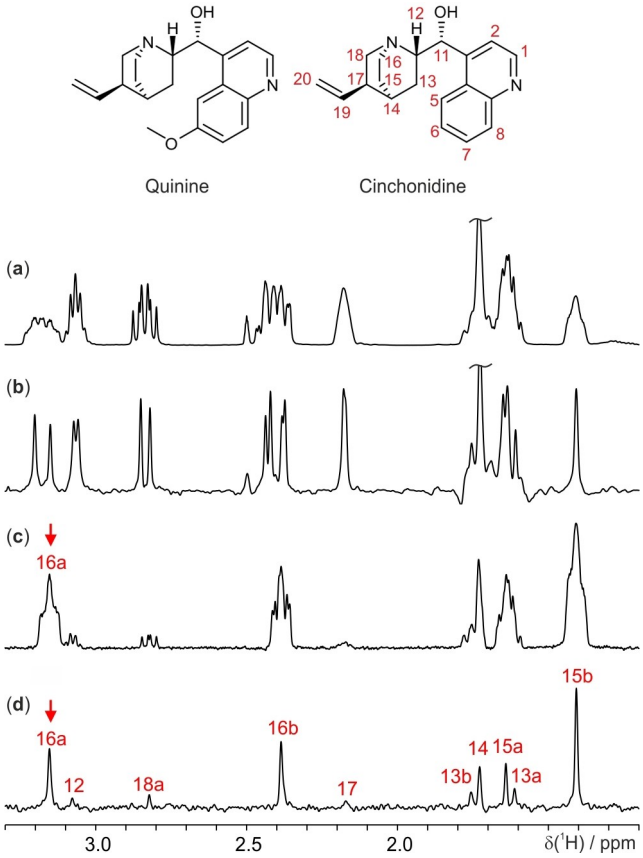

Figure 1. 500.13 MHz ^{1}H NMR spectra of a mixture of quinine and cinchonidine in DMSO-d_6 (100 mM each). (a) Conventional ^{1}H 1D spectrum, (b) pure shift PSYCHE spectrum, (c and d) GEMSTONE-TOCSY and TREASURE spectra, respectively, selecting H16a in cinchonidine (3.16 ppm). 32 and 128 scans were acquired for the GEMSTONE-TOCSY and TREASURE spectra with experiment times of 3 min (with a recycle time of 4.6 s) and 2 hr (with a recycle time of 3.1 s), respectively. Full experimental details are given in the Supporting Information.

important pharmaceuticals, often used as anti-inflammatory agents, for asthma treatment, and as immunosuppressants for organ transplantation.[17,18] In recent years they have also proved vital in the treatment of COVID-19.[19,20] Synthetic glucocorticoids, such as prednisone, prednisolone and methylprednisolone (Figure 2), were developed to increase biological half-life, prolong therapeutic effects, and boost the potency of glucocorticoid drugs *in vivo*. However, careful administration of these drugs is required to avoid serious side effects. To understand the pharmacokinetic properties of these molecules, the influence of structural changes needs to be identified and understood.

The NMR analysis of a mixture of prednisone, prednisolone and methylprednisolone is shown in Figure 2. From the ^{1}H NMR spectrum (Figure 2a), the need for GEMSTONE is immediately made apparent by the spectral overlap, which limits the use of conventional selective excitation. A comparison between a selective-TOCSY pure shift experiment[21,22] and TREASURE is shown in Figure S3; in this instance, the selective-TOCSY-PSYCHE method was unable to select a single signal, resulting in a spectrum containing signals from multiple spin systems.

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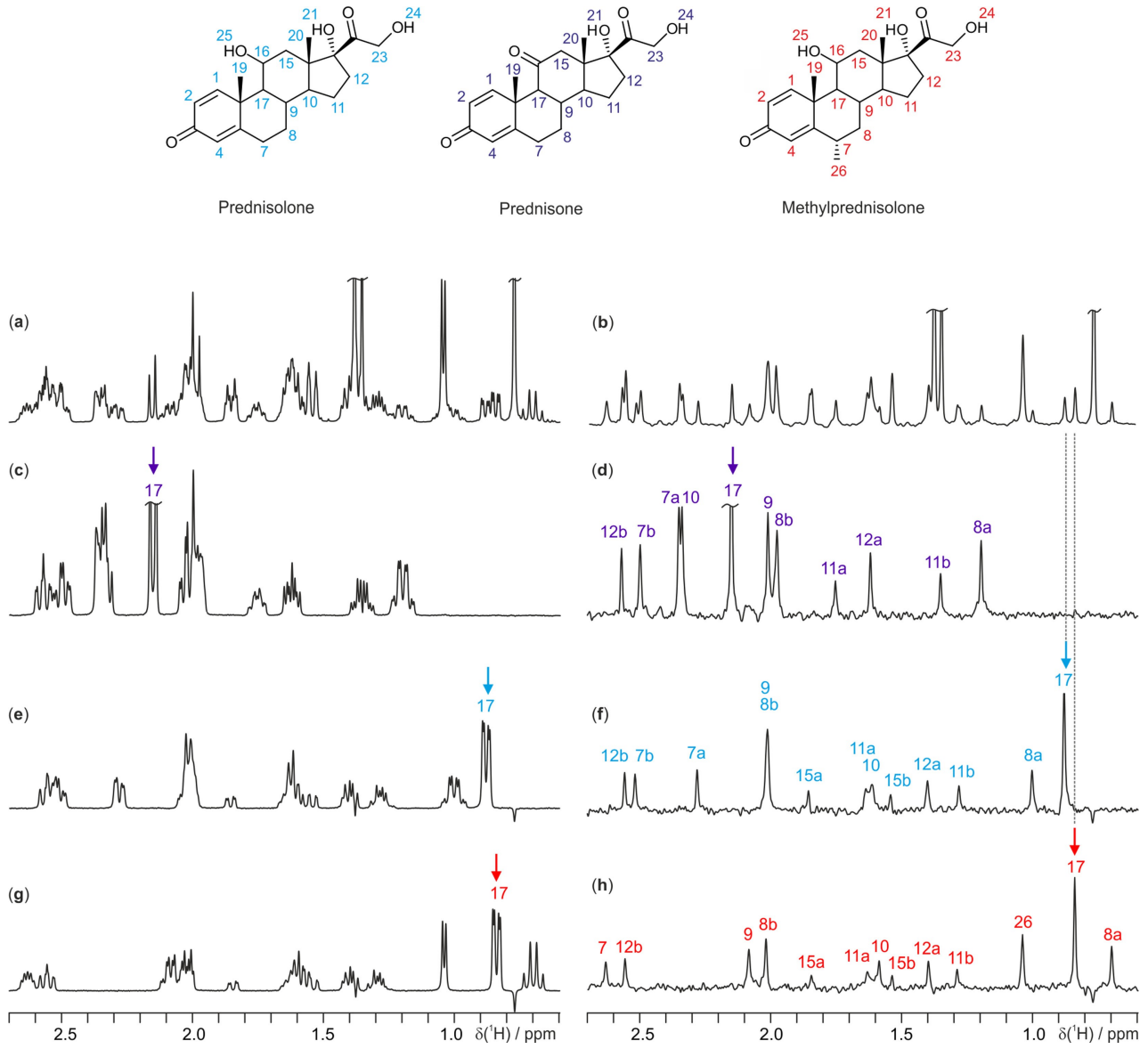

Figure 2. 500.13 MHz ^{1}H NMR spectra of a mixture of prednisone, prednisolone, and methylprednisolone in DMSO-d_6 (100 mM each). (a) Conventional ^{1}H 1D spectrum, (b) PSYCHE spectrum, (c, e, g) GEMSTONE-TOCSY and (d, f, h) TREASURE spectra selecting H17 in prednisone (2.15 ppm), prednisolone (0.89 ppm) and methylprednisolone (0.85 ppm), respectively. All TOCSY spectra were acquired using a spin-lock mixing period of 80 ms. Spectra (c–h) were all acquired with 128 scans. GEMSTONE-TOCSY and TREASURE spectra were acquired in 9 min (with a recycle time of 3.6 s) and 1 hr 30 min (with a recycle time of 2.1 s), respectively. Spectra (d, f, h) were scaled to match the signal intensity of (c, e, g). Further experimental details are given in the Supporting Information.

To enable structure assignment of each individual spin system, GEMSTONE-TOCSY was used to target a single multiplet in each molecule (H17). Although simplified subspectra were obtained (Figures 2c, 2e and 2g), some spectral regions (e.g. H10 and H11a in prednisolone and methylprednisolone) in the GEMSTONE-TOCSY spectra are difficult to analyse, even when acquired with a range of mixing times, due to the TOCSY correlations having a high degree of signal overlap. Increased spectral resolution is required to allow unambiguous structure assignments. The TREASURE spectra for each spin system are shown in Figures 2d, 2f and 2h, demonstrating the high spectral resolution of the experiment. The removal of signal multiplicities produces an ultrahigh resolution spectrum that enables clear identification of signals in each targeted spin system, allowing the core moiety of each glucocorticoid to be established.

A schematic representation of the TREASURE pulse sequence is shown in Figure 3. The sequence has three elements: a GEMSTONE ultraselective element, a TOCSY spin-lock, and a pure shift element with interferogram (pseudo-2D) acquisition. GEMSTONE was chosen as the ultraselective element due to its ability to achieve selection of a single signal in a single scan, in contrast to the previously state-of-the-art CSSF (chemical shift selective filter)[23,24] method which requires multiple acquisitions to achieve the same selectivity, leading to an increased experiment time.

The choice of a suitable pure shift method to use in TREASURE is not trivial. The interferogram approach, while

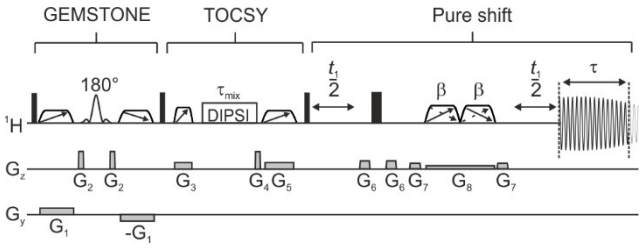

Figure 3. Schematic representation of the 1D TREASURE pulse sequence. Narrow and wide black vertical bars correspond to hard 90° and 180° pulses, respectively. Open trapezoids with diagonal arrows correspond to swept-frequency pulses. The pulse shape labelled "180°" indicates a selective 180° pulse. The rectangle labelled 'DIPSI' corresponds to a DIPSI-2[32] spin-lock, for TOCSY mixing. Trapezoids with two diagonal arrows correspond to low-power saltire[33] chirp pulses of flip angle β (typically 20° on resonance). The interferogram acquisition mode uses an evolution time t_1 incremented in steps of the chunk duration τ. Shapes $G_{1\text{-}8}$ represent field gradient pulses applied either along the *z* or the *y* direction. Further details of the pulse sequence are given in the Supporting Information.

more time-consuming than the real-time counterpart, allows the use of a wider range of active spin refocusing (ASR) elements and provides higher spectral quality (narrower linewidths and lower intensity chunking artefacts).[25] Regarding the ASR element chosen to refocus homonuclear scalar couplings, two criteria need to be fulfilled. First, it must provide a broadband pure shift spectrum, to ensure that all coherences generated by the TOCSY element are seen. Second, the ASR element must not interfere with the spatiotemporal averaging used by the GEMSTONE element.

ASRs used in pure shift experiments include homonuclear band-selective decoupling,[26,27] bilinear rotation decoupling (BIRD),[28,29] Zangger-Sterk (ZS),[30] and PSYCHE.[31] The first criterion for the ASR rules out band-selective decoupling, as it is not a broadband technique. The second requirement is more challenging, because GEMSTONE, zero-quantum coherence suppression in TOCSY, and PSYCHE all rely on spatiotemporal averaging. Although with judicious choice of gradient amplitudes it is possible to acquire acceptable TREASURE spectra with *z* gradients only, where triaxial gradients are available they should always be used as higher spectral quality is achievable (Figure S4). For the ZS element, triaxial gradients are essential to avoid interference with the GEMSTONE selection. While a higher SNR per unit time is achievable when real-time acquisition is used, PSYCHE is not compatible with this approach. Instead, the BIRD ASR can be used in real-time acquisition, although it fails to decouple geminal protons.

Although relaxation, diffusion and convection do limit the sensitivity of the GEMSTONE element, the main sensitivity penalty of TREASURE is caused by the ASR element. When convection causes large sensitivity losses, the new GEMSTONES[34] approach, or suitable variant, could be employed (pulse sequence provided in Section S3.3 of the Supporting Information); in the results shown here, a single GEMSTONE loop was used as GEMSTONES gave only marginal sensitivity improvement (data not shown). For general use the PSYCHE element (Figure 3) is preferred because of its versatility and often superior sensitivity; if only a narrow frequency range is of interest the use of the ZS element (Figures S5 and S6) might be more convenient, to maximize sensitivity.

Conclusions

In conclusion, a new ultraselective, ultrahigh resolution 1D TOCSY experiment has been introduced that can be used to perform targeted analyses of spin systems where spectral overlap prohibits the use of conventional selective methods. The ultrahigh resolution nature of the experiment enables unambiguous identification of chemical environments within the targeted spin system. TREASURE has great potential in simplifying spectral analysis of mixtures of structurally analogous molecules, common in many synthetic pathways and natural product extractions, which pose difficulties in analysis due to their structural similarities. The proposed method enables chemists to access previously unattainable information.

Supporting Information

The authors have cited additional references within the Supporting Information.[35,36] All experimental data for all the spectra shown, pulse program codes and experimental parameters are freely available at DOI: 10.48420/25606086.

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Conflict of Interests

Work contributed by J.R.D.M. and P. K. was conducted while they were postdoctoral fellows, and work contributed by M.J.S., and D.A.T. was conducted while they were PhD candidates, at the University of Manchester.

Data Availability Statement

The data that support the findings of this study are openly available in Figshare at https://doi.org/10.48420/25606086, reference number 25606086.

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