Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 164–169

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Application of diffusion-edited NMR spectroscopy for the structural characterization of drug metabolites in mixtures

Smriti Khera[a,*], Mark Grillo[b], Paul Schnier[c], Steve Hollis[d]

[a] Department of Molecular Structure, Amgen Inc., 1120 Veterans Blvd., South San Francisco, CA 94080, United States
[b] Pharmacokinetics and Drug Metabolism, Amgen Inc., 1120 Veterans Blvd., South San Francisco, CA 94080, United States
[c] Department of Molecular Structure, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799, United States
[d] Department of Molecular Structure, Amgen Inc., One Kendall Square, Bldg 1000, Cambridge, MA 02139, United States

ARTICLE INFO

Article history:
Received 16 April 2009
Received in revised form 6 July 2009
Accepted 7 July 2009
Available online 15 July 2009

Keywords:
Diffusion-edited spectroscopy
DOSY
Solvent suppression
Ethacrynic acid
Mefenamic acid
Metabolite identification

ABSTRACT

Diffusion-edited NMR spectroscopy is used to enable the structural characterization of low level metabolites in the presence of endogenous compounds, and organic solvents. We compared data from standard one-dimensional (1D) ^{1}H, 1D NOESY-presaturation, and 1D diffusion-edited experiments run on 20 μg and 100 μg samples of ethacrynic acid glutathione thioether (EASG) and a previously unreported metabolite of mefenamic acid, mefenamic acid glutathione thioester (MSG). The 1D NOESY-presaturation technique gave spectra with the best signal-to-noise (S/N) ratio, approximately three times that observed with the standard ^{1}H experiment, with respect to the metabolite signals. However, it was not selective for solvent signals as overlapping metabolite signals were also suppressed by this technique. In some cases, these signals were key to determining the site of glutathione attachment on the parent molecule. 1D NOESY-presaturation spectra also produced baseline distortions and inconsistent integration values. By comparison, 1D diffusion-edited experiments were found to selectively and simultaneously remove multiple solvent signals, resolve overlapping metabolite signals, and provide more uniform integration for metabolite signals overlapping with or proximal to solvent peaks, without producing baseline distortions. However, the diffusion-edited experiments caused significant signal attenuation of the metabolite signals when compared with a standard ^{1}H spectrum. Partially purified metabolites isolated from biological matrices were also characterized by using two-dimensional diffusion-ordered spectroscopy (DOSY). DOSY spectra acquired on a sample of EASG purified from rat bile proved useful in 'separating' the signals of EASG, from those of a co-eluting bile acid and parent drug ethacrynic acid (EA) in the diffusion-dimension in regions where there was no spectral overlap. In the low-field regions of high overlap, the DOSY experiment did not effectively separate the signals from the individual components. Diffusion based experiments provide a way to determine the total number of components that are present in a metabolite sample as well as an ability to identify them based on the chemical shift information, without the need for laborious chromatography on small samples.

1. Introduction

Reactive metabolites of many pharmaceutical agents are associated with idiosyncratic toxicities and adverse drug reactions that can lead to product recalls and costly labeling changes [1]. Many reactive metabolites are produced by electrophilic or free radical reactions that occur during Phase I metabolism and these intermediates are thought to cause toxicity by covalently modifying essential cellular components. Pharmaceutical discovery efforts have become increasingly focused on characterizing and identifying reactive metabolites early on in the discovery process in order to produce drug candidates with better safety profiles. One popular strategy for the identification of reactive metabolites is through 'trapping' the metabolites by conjugation with nucleophilic agents such as glutathione [2]. This technique takes advantage of the natural process for the detoxification of xenobiotics during Phase II metabolism. *In vitro* experiments involving the incubation of a drug candidate with liver microsomes in the presence of glutathione are conducted to produce conjugates of chemically reactive molecules. These glutathione conjugates are often more stable then the reactive intermediates, and thus are generally amenable to structure determination and the identification of site(s) of metabolic liabilities in parent molecules. With this structural information, medicinal chemists can attempt to design better molecules that bypass undesirable bio-activation and/or block labile sites on the original molecule.

* Corresponding author. Tel.: +1 650 244 2375; fax: +1 650 837 9369.
E-mail address: Skhera@amgen.com (S. Khera).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.07.010

In order to determine structures of unknown metabolites, it is often necessary to collect and analyze ^{1}H NMR data and compare it to the data acquired on the parent compound. The ^{1}H NMR spectrum provides rich structural information through chemical shifts, coupling interactions, and peak integration data and this information can be used to assign the structure of drug metabolites and the sites of conjugation with glutathione or other 'trapping' agents. There are numerous examples in the literature where the ^{1}H NMR alone has provided sufficient information to determine the structure of unknown drug metabolites [3–5]. Because the quantity of metabolites obtained from *in vitro* or *in vivo* experiments are usually limited (sub-milligram), their ^{1}H NMR spectra are typically dominated by large signals arising from the organic solvents used in chromatographic mobile phases, in addition to water and/or residual non-deuterated solvent. These undesirable signals can cause dynamic range issues and complicate the assignment of metabolite peaks, especially when they are obscured by the solvent peaks.

Differences in the molecular diffusion properties of the solute and solvent molecules, have been utilized previously for the purpose of solvent suppression in NMR [6,7], as with the DRYCLEAN approach to suppress water signals in aqueous solutions of macromolecules [8]. More recently, an application of 1D diffusion-editing has been reported for the suppression of water and residual non-deuterated solvent signals from ^{1}H NMR spectra of organic compounds [9] and two-dimensional DOSY has also been used to suppress solvent signals in neat ionic liquids [10]. DOSY has also been reported to monitor the production of lovastatin in the presence of other co-metabolites and impurities during the fermentation process [11].

In this study we use 1D diffusion-editing techniques to suppress solvent and other signals from small-molecular weight impurities in the ^{1}H NMR spectrum of metabolites isolated in microgram quantities. We present a comparison of data obtained from standard ^{1}H, 1D NOESY-presaturation, and 1D diffusion-edited experiments, using 20 μg and 100 μg samples of a glutathione adduct of ethacrynic acid and a new glutathione conjugate of mefenamic acid. We also evaluate the use of DOSY as a means of 'separating' NMR signals belonging to a metabolite, purified from rat bile, from those of endogenous co-eluting impurities.

2. Experimental

The glutathione conjugate of ethacrynic acid is well known and studies of this compound have been widely reported [12–14]. The *S*-acyl-glutathione conjugate of mefenamic acid (MSG) is a previously unreported metabolite which was first detected by LC/MS/MS techniques in extracts from incubations of mefenamic acid with freshly isolated rat hepatocytes (data not shown). However, structurally dissimilar, thioether-linked glutathione conjugates of mefenamic acid that were formed by P450-mediated metabolism of the drug have also been reported [15]. Standards for ethacrynic acid glutathione thioether (EASG, 1) and mefenamic acid glutathione thioester (MSG, 2) were synthesized in-house as described below (Fig. 1). The proton NMR spectra of these standards were fully assigned using 2D NMR experiments and these assignments were found to be consistent with those reported previously for EASG [13]. This report presents the first proton chemical shift assignments for MSG.

2.1. Chemicals

Ethacrynic acid (EA), mefenamic acid, glutathione (GSH), potassium hydrogen carbonate ($KHCO_3$), hydrochloric acid (HCl), tetrahydrofuran (THF), ethyl acetate, acetone, ethyl chloroformate, triethylamine (TEA), formic acid, and phosphoric acid were obtained from Sigma–Aldrich (St. Louis, MO). All solvents used for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis were of chromatographic grade. DMSO-d_6 (99.9%) and CD_3OD (99.8%) used for the NMR experiments were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

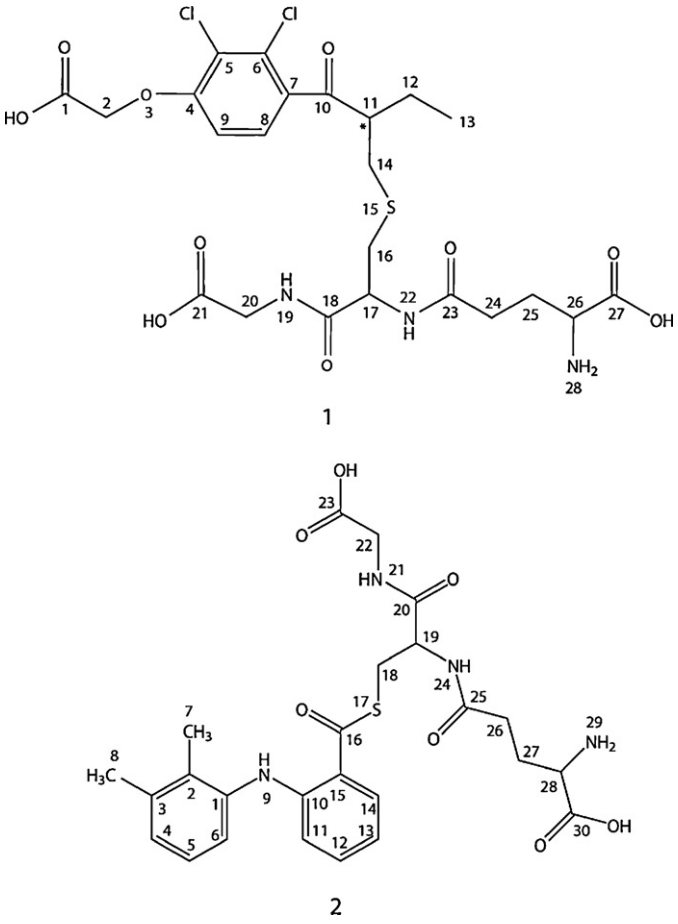

Fig. 1. Ethacrynic acid glutathione thioether (EASG) (1), and mefenamic acid glutathione thioester (MSG) (2).

2.2. Preparation and identification of ethacrynic acid glutathione thioether (EASG)

EASG was synthesized by adding GSH (6.5 mmol, 2 g) to EA (1.7 mmol, 500 mg) dissolved in 100 ml of 1:1 H_2O/THF, containing $KHCO_3$ (8 mmol, 0.8 g, pH 8.1). The mixture was stirred for 24 h at room temperature after which 20 drops of HCl (concentrated) were added. The THF was evaporated off under reduced pressure to afford an aqueous mixture containing precipitated EASG. The precipitate was isolated by filtration through a glass filter where it was washed with acidic water (0.1N HCl; 4× 50 ml, pH 3) followed by washing with ethyl acetate (4× 50 ml). The washed precipitate was dried under a stream of N_2 gas at room temperature to provide a white solid where the yield was ~80%.

The Tandem CID MS/MS spectrum in the positive ion mode of EASG was as follows: (CID of MH^+ ion at *m/z* 610), *m/z* (%): *m/z* 535 ([M+H–glycine]$^+$, 7%), *m/z* 481 ([M+H–pyroglutamic acid]$^+$, 5%), *m/z* 378 ([ethacrynic-*S*-$CH_2CH{=}NH_2$]$^+$, 5%), *m/z* 335 ([ethacrynic-S]$^+$, 100%), *m/z* 308 ([glutathione + H]$^+$, 0.5%), *m/z* 303 ([M+H–GSH]$^+$, 4%), *m/z* 232 ([cys-γ-glu]$^+$, 1%) *m/z* 178 ([cys-gly]$^+$, 16%), *m/z* 76 ([glycine]$^+$, 2%).

^{1}H NMR of re-synthesized EASG consists of signals from two diastereomers formed during the conjugation reaction. In the aro-

matic region the signals from the two diastereomers were well resolved but in the aliphatic region they were broad and overlapped, consistent with a previous report [13]. The spectrum also contains signals from parent EA and GSH as a result of degradation of EASG in solution. This gives rise to several broad and overlapped signals in the aliphatic region of the spectrum. To get accurate integration values for the EASG NMR peaks, data from diffusion-edited ^{1}H NMR and ^{1}H–^{13}C HSQC experiments were used complementarily.

^{1}H NMR (in DMSO-d_6, 500 MHz): δ8.56 (1H, br t, J = 5 Hz, H-19), 8.40 and 8.33 (1H, 2 d, J = 8 Hz, H-22 iomer A and B), 7.74 and 7.66 (1H, 2 d, J = 9 Hz, H-8 isomer A and B), 7.08 and 7.01 (1H, 2 d, J = 9 Hz, H-9 isomer A and B), 4.76 (2H, br s, H-2), 4.46 (1H, br m, H-17), 3.73 (2H, br s, H-20), 3.61 and 3.56 (1H, 2 br t, J = 6 Hz, H-11 isomer A and B), 3.47 (1H, ov m, H-26), 2.90 (2H, ov m, H-14b and H-16b), 2.76–2.55 (2H, ov m, H-14a and H-16a), 2.27 (2H, br m, H-24), 1.91 (2H, br m, H-25), 1.63 (1H, sex, J = 7 Hz, H-12b), 1.52 (1H, br m, H-12a), 0.83 (3H, br t, J = 7 Hz, H-13).

2.3. Preparation and identification of mefenamic acid glutathione thioester (MSG)

MSG was synthesized by a conventional method employing ethyl chloroformate in a manner as described previously for the synthesis of clofibryl-*S*-acyl-glutathione thioester [16] and provided the glutathione thioester derivative as a white solid with a yield of 63%. Thus, MSG was synthesized by dissolving 1.6 mmol (386 mg) of mefenamic acid in 25 ml of anhydrous THF at room temperature and adding 1.6 mmol (220 μl) of triethylamine while the solution was stirred. Next, 1.6 mmol (160 μl) of ethyl chloroformate was added and the mixture stirred for an additional 30 min. The precipitate was removed by filtration through glass wool and the filtrate added directly to 25 ml of H_2O/THF (1:1.5) containing GSH (3.3 mmol, 1 g) and $KHCO_3$ (1.6 mmol, 100 mg). The resulting mixture was stirred for 1 h at room temperature under nitrogen, at which time the reaction was terminated by the addition of 8 drops of HCl (concentrated). The THF was removed under vacuum, and the remaining aqueous phase was extracted four times with ethyl acetate (50 ml). The product, MSG, was precipitated at the interface between the aqueous and organic layers. The MSG was recovered and washed four times with H_2O (50 ml) to remove GSH. The resulting product was then washed four times with acetone (50 ml) to remove mefenamic acid. The final precipitate was dried under a stream of nitrogen. The resulting MSG was pure as determined by reversed-phase LC/MS analysis in the positive ion scan mode.

The Tandem CID MS/MS spectrum in the positive ion mode of MSG was as follows: (CID of MH^+ ion at m/z 531), m/z (%): m/z 384 ([M+H–pyroglutamic acid–H_2O]$^+$, 1%), m/z 325 ([mefenamyl-*S*-$CH_2CH(NH)C{=}O]^+$, 0.6%), m/z 308 ([glutathione + H]$^+$, 0.5%), m/z 224 ([M+H–GSH]$^+$, 100%), m/z 130 ([pyroglutamic acid]$^+$, 1%).

^{1}H NMR (in DMSO-d_6, 500 MHz): δ9.32 (1H, s, H-9), 8.84 (1H, br s, H-21), 8.56 (1H, d, J = 8 Hz, H-24), 7.92 (1H, d, J = 8 Hz, H-14), 7.36 (1H, dd, J = 7, 8 Hz, H-12), 7.05–7.16 (3H, ov, H-4, H-5, and H-6), 6.76 (1H, dd, J = 7, 8 Hz, H-13), 6.64 (1H, d, J = 8 Hz, H-11), 4.52 (1H, td, J = 4, 9 Hz, H-19), 3.71 (2H, d, J = 5 Hz, H-22), 3.61 (1H, dd, J = 4, 13 Hz, H-18b), 3.25 (1H, t, J = 7 Hz, H-28), 3.13 (1H, dd, J = 10, 13 Hz, H-18a), 2.33 (2H, ov m, H-26), 2.29 (3H, ov s, H-8), 2.09 (3H, s, H-7), 1.92 (1H, m, H-27b), 1.80 (1H, m, H-27a).

2.4. Purification of EASG from rat bile

Ethacrynic acid was administered to a 300–330 g bile duct-cannulated rat as a 20 mg PO dose (dissolved as suspension in distilled water with final pH ~7). Approximately 4 ml bile was collected for 4 h into a tube containing 400 μl of phosphoric acid (pH 3) on wet-ice post-administration. Collected bile then was treated with 4 ml of acetonitrile (containing 3% formic acid) followed by purification by reversed-phase HPLC to collect the major metabolite observed by UV detection at 226 nm. Chromatographic resolution was achieved with a Shiseido Capcell Pak-AQ C-18 column (4.6 mm × 250 mm, 3 μm, Tokyo, Japan). Mobile phases consisted of 0.1% aqueous formic acid (solvent-A) and acetonitrile with 0.08% formic acid (solvent-B) run at a constant flow rate of 1 ml/min. The solvent gradient was initially held at 0% solvent-B for 5 min, and increased linearly to 70% solvent-B over another 45 min, kept at 70% solvent-B for an additional 4 min, then immediately dropped to 0% solvent-B over 0.1 min where it was held constant at 0% solvent B for 6 min for re-equilibration.

2.5. NMR instrumentation

All 1D NMR experiments were acquired on a Bruker Avance 500 MHz spectrometer operating at a resonance frequency of 500.13 MHz for proton (Bruker BioSpin Corporation, Billerica, MA) equipped with a 5 mm QNP cryoprobe with a *z*-axis gradient. DOSY spectra were acquired on a Bruker Avance 600 MHz spectrometer operating at a resonance frequency of 600.13 MHz for proton equipped with a 5 mm TCI cryoprobe with a *z*-axis gradient coil. All experiments were acquired at 298 K without sample spinning. ^{1}H chemical shifts were referenced to the residual solvent signals from DMSO-d_6 at δ2.50 and CD_3OD at δ3.30. All experiments were run with samples volumes of 150 μl in 3 mm diameter thin-walled NMR tubes.

2.6. NMR experiments

^{1}H spectra were acquired using the pulse sequence 'zg30' with 4096 transients (64k pts, 3.2 s) and 32 dummy scans. 1D diffusion-edited spectra were acquired with the Bruker pulse sequence 'ledbpgp2s1d' using square gradient pulses to reduce spectral artifacts. The relative gradient field strength and diffusion delay were adjusted to optimize both the quality of solvent suppression and the S/N ratio for each metabolite. The best results were obtained with a 1 ms gradient pulse (at 52.3 G/cm) for both metabolites with an optimum diffusion delay of 200 ms for EASG and 150 ms for MSG. A 0.2 ms gradient recovery delay, a 5 ms eddy current delay, a 6.5 μs pre-scan delay and a 2 s relaxation delay were employed in all experiments. All spectra were acquired with 4096 transients (32k pts, 1.6 s) and 32 dummy scans.

The standard Bruker pulse sequence 'lc1pnf2' was used to acquire the 1D NOESY-presaturation experiment with simultaneous suppression of two solvent signals. The residual solvent signals at δ2.50 for DMSO-d_6 and δ3.30 for H_2O were selected for presaturation in both EASG and MSG samples. Two presaturation pulses were used in this experiment, a 2.4 s pulse during the relaxation delay followed by a 100 ms pulse during mixing time, with an optimized power level of 56 dB for best S/N and solvent signal suppression results. All spectra were acquired using 4096 transients (32k pts, 1.6 s) and 32 dummy scans.

DOSY spectra were acquired using the Bruker pulse program 'ledbpgp2s' using a sine-shaped gradient pulse. The gradient pulse duration used was 1.4 ms while the diffusion delay was optimized to 100 ms. The relative gradient strength was incremented from 5% to 100% sequentially to collect 80 increments with 32 transients each preceded by 8 dummy scans per increment. A 0.35 ms gradient recovery delay, a 5 ms eddy-diffusion delay, a 20 μs pre-scan delay, and a 5 s relaxation delay were also employed in these experiments. DOSY spectra were processed using Bruker Topspin 2.1 employing exponential fitting with a maximum of three components. The presence of at least three components in the sample was indicated by the 1D diffusion-edited experiment as well as LC–MS data (data not shown). Baseline correction was applied to data prior to processing.

For the 1D experiments with EASG, the S/N was calculated using the pair of doublets centered at δ8.34 and noise was measured from δ9.00 to δ9.50 region of the spectrum. For the 1D experiments with MSG the S/N was calculated using the doublet at δ7.92 and noise was measured from δ10.00 to δ10.50.

2.7. Tandem MS

The EASG and MSG were characterized by tandem mass spectrometry on a Thermo Fisher TSQ Quantum Discovery Max mass spectrometer (Thermo Fisher Scientific, Waltham, MA), linked to an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA) and a CTC HTS PAL Autosampler (Leap Technologies, Carrboro, NC). LC–MS analysis of EASG and MSG was performed with a Phenomenex Luna, 5 μm, C18(2), 100 Å, 150 mm × 2.00 mm reverse phase column (Torrance, CA) at a flow rate of 0.3 ml/min. The mobile phase used was 0.1% formic acid in water (solvent-A) and 0.1% formic acid in acetonitrile (solvent-B). Gradient elution was achieved by increasing solvent-B from 0% to 95% over 13 min. Electrospray ionization was employed with the needle potential held at 4.5 kV. MS/MS conditions used were 2 mTorr argon collision gas and 20 eV collision potential. Positive ion mode full scan (50–1200 amu), was conducted with scan time 0.73 s and source collision energy of 10 V. Xcalibur software (version 2.0) was used to acquire all data.

3. Results and discussion

The NMR spectra for the 20 μg and 100 μg samples of EASG dissolved in 150 μl of DMSO-d_6 are shown in Figs. 2 and 3, respectively. The residual solvent and H_2O signals dominate the ^{1}H spectra (Figs. 2A and 3A), obscuring some of the signals from EASG due to the low metabolite concentration. Some signals from the metabolite lie buried under these solvent peaks, making it desirable to use solvent suppression techniques in order to improve the information content of the spectra. As shown in Figs. 2B and 3B, the 1D NOESY-presaturation experiment is effective in suppressing the two main solvent signals. This technique effectively increases the dynamic range of signal detection such that the observed S/N ratios of the metabolite signals, which are well separated from the solvent peaks (e.g. peak at δ8.34), increased by approximately threefold when compared to a standard proton spectrum. This effective improvement in S/N is of special interest as sample quantities are often limited when studying metabolites. However, the signal suppression is non-selective in the 1D NOESY-presaturation experiment, and overlapped metabolite peaks are also suppressed. Selective saturation of the H_2O signal at δ3.30 also suppresses key proton resonance H-11 at δ3.61 and δ3.56 (isomer A and B), the site of glutathione addition to ethacrynic acid, and H-26 at δ3.47. In a structure determination scenario where the identity of EASG was unknown, the correct assignment of H-11 would be critical to the structural characterization of EASG as it represents a new chiral center created during conjugation with glutathione. Suppression of the large solvent signals can also introduce baseline errors that result in integral values inconsistent with the chemical structure for those resonances that are close to the irradiated signal. As shown in Fig. 3B, the proximity of signals H-14b and H-16b (δ2.90, *1.2H*), H-14a and H-16a (δ2.76–2.55, *0.3H*), and H-24 (δ2.27, *1.3H*), to the suppressed residual DMSO-d_6 signal at δ2.50 leads to structurally inconsistent and non-uniform integration (shown in italicized letters) for these peaks in the 1D NOESY-presaturation experiment. Also, ^{13}C satellites of the DMSO-d_6 signal appear in the spectrum (Figs. 2B and 3B) and are overlapped with signals from the metabolite at positions H-14a, and H-16a.

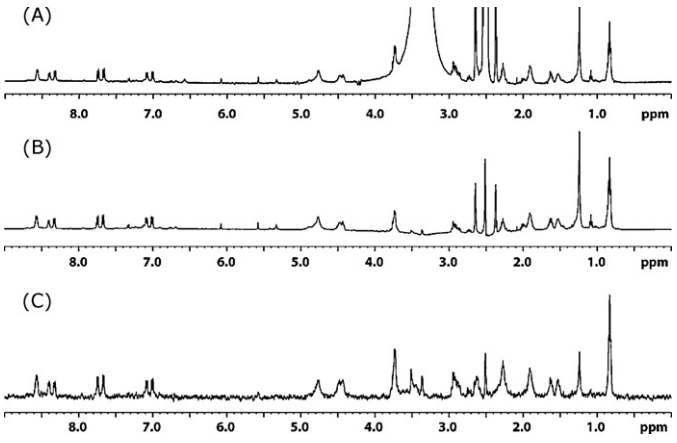

Fig. 2. EASG 20 μg in 150 μl DMSO-d_6. Standard ^{1}H spectrum, S/N = 30 (A), 1D NOESY-presaturation spectrum, S/N = 82 (B), and 1D diffusion-edited ^{1}H spectrum, S/N = 7 (C).

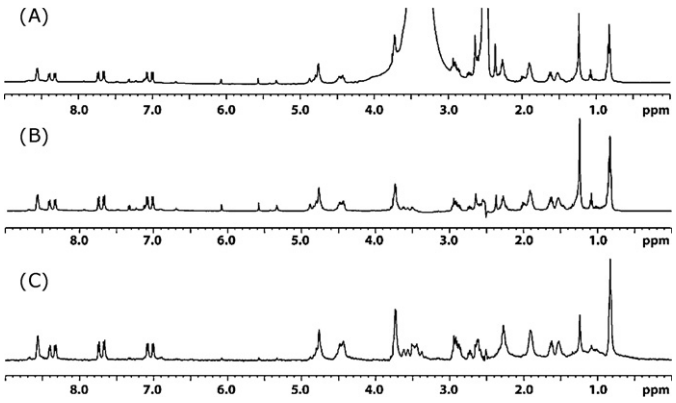

Fig. 3. EASG 100 μg in 150 μl DMSO-d_6. Standard ^{1}H spectrum, S/N = 43 (A), 1D NOESY-presaturation spectrum, S/N = 121 (B), and 1D diffusion-edited ^{1}H spectrum, S/N = 13 (C).

The 1D diffusion-edited ^{1}H NMR spectra of 20 μg and 100 μg samples of EASG in DMSO-d_6 are shown in Figs. 2C and 3C, respectively. Selective suppression of the solvent signals is observed to increase the effective resolution of the peaks of interest close to, and overlapped with the solvent peak. Also, integration of signals H-11 and H-26 (δ3.47–3.61, 2H), H-14b and H-16b (δ2.90, 2H), H-14a and H-16a (δ2.76–2.55, 2H), and H-24 (δ2.27, 2H) were found uniform and consistent with structure in contrast with the 1D NOESY-presaturation experiment. The diffusion-edited spectra were free from baseline distortions and ^{13}C satellites of the solvent. However, due to relaxation losses during the refocusing and diffusion delays employed in the diffusion-edited experiments, signal attenuation is observed for the metabolite signals when compared to the standard 1D ^{1}H spectrum. This can be a significant disadvantage for samples that are available in limited quantities and the loss of signal may require the use of sensitive NMR instrumentation or cryoprobe technologies.

It should be noted that the synthesis of EASG used here introduces a new chiral center (marked with '*' in Fig. 1.) in the molecule resulting in two diastereomers of EASG, consistent with both enzymatic and non-enzymatic conjugation of EA as previously described [13]. These diastereomers were not purified in this study and as a result the ^{1}H NMR spectra of EASG consists of a mixture of diastereomeric signals in the downfield region (δ6.0–9.0), and the appearance of broad and unresolved peaks in the upfield (δ0.0–5.0) region of the spectrum. Additionally, the sample slowly degrades to the parent ethacrynic acid (EA) and free glutathione (GSH) in solution, further complicating spectral assignments. The signals arising from these decomposition products are seen in the NMR spectra

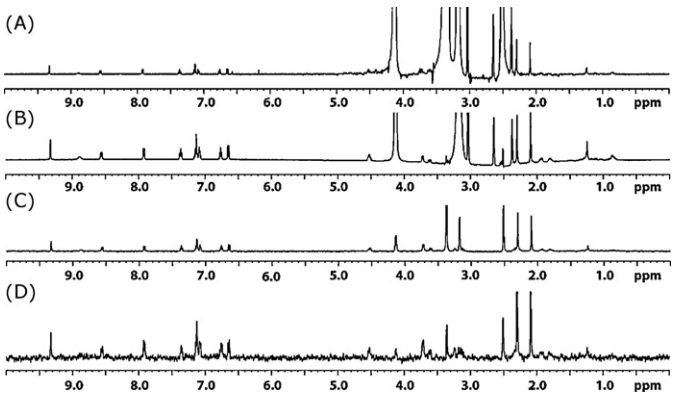

Fig. 4. MSG 20 μg in 150 μl DMSO-d_6 spiked with MeOH. Standard ^{1}H spectrum, S/N = 15 (A), 1D NOESY-presaturation spectrum, S/N = 83 (B), 1D diffusion-edited ^{1}H spectrum, diffusion delay = 150 ms, S/N = 12 (C), and 1D diffusion-edited ^{1}H spectrum, diffusion delay = 200 ms, S/N = 7 (D).

(Figs. 2A–B and 3A–B). As shown in Figs. 2C and 3C, the diffusion-edited experiment is useful in suppressing signals from these small molecular weight impurities and thus simplifies spectral interpretation.

Metabolites are generally purified from biological media in microgram quantities, using reversed-phase chromatography prior to NMR characterization [3,4,17]. Due to the small amounts of material, residual mobile phase solvent signals can reduce the quality of the spectra. To simulate a metabolite isolated using reversed-phase purification, 2 μl of CH_3OH was added to the initial stock of 0.75 mg/ml mefenamic acid glutathione thioester (MSG) in DMSO-d_6 from which the two dilutions were made for analysis. The NMR spectra acquired for 20 μg and 100 μg of MSG in 150 μl of DMSO-d_6 spiked with CH_3OH are shown in Figs. 4 and 5 respectively.

As shown in Figs. 4A and 5A, the standard ^{1}H NMR spectra are dominated by four solvent signals arising from $C\underline{H_3}OH$, H_2O, $CH_3\underline{OH}$, and the residual signal from DMSO-d_6. The 1D NOESY-presaturation experiments (Figs. 4B and 5B) were set-up to suppress the water and DMSO-d_6 signals at δ3.30 and δ2.50 respectively. The $CH_3\underline{OH}$ signal at δ4.13 is also suppressed as a result of saturation exchange with H_2O (Fig. 5B), but the $C\underline{H_3}OH$ signal at δ3.17 is not affected. As seen in Figs. 4B and 5B (by comparison with the corresponding diffusion-edited spectra), H-28 at δ3.25 and H-18a at δ3.13 are overlapped with the solvent signal at δ3.17 and H-26 at δ2.33 is overlapped with the unsuppressed ^{13}C satellite of the residual DMSO-d_6 peak. Additionally, the proximity of signals H-22 (δ3.71, *1.0H*), H-18b (δ3.61, *0.3H*), H-26 and H-8 (δ2.29–2.33, *2.7H*) to the suppressed residual DMSO-d_6 and H_2O signal leads to integration inconsistencies (shown in italicized letters). In comparison, the corresponding diffusion-edited spectra (Figs. 4C–D and 5C–D) show selective suppression of signals from all small molecular weight impurities and thus they are able to resolve metabolite signals, H-28, H-18a and H-26. The integration of signals H-22 (δ3.71, 2H), H-18b (δ3.61, 1H), H-28 (δ3.25, 1H), H-18a (δ 3.13, 1H), H-26 and H-8 (δ2.29–2.33, 5H) was consistent with the proposed structure for MSG and thus the structure could be unequivocally assigned. The observations made in the case of EASG regarding the gains in S/N with respect to signals from the metabolite in the 1D NOESY-presaturation experiment versus the loss observed in S/N in the diffusion-edited spectrum when compared with a standard 1D ^{1}H spectrum, hold true for MSG as well. But, as shown in Figs. 4C–D and 5C–D, by optimizing diffusion-specific parameters such as the diffusion delay, one can find the best compromise between the loss of signal intensity and efficiency of solvent suppression. Alternatively, the gradient strength and gradient pulse duration may be optimized while setting the diffusion delay to the minimum possible value in order to minimize relaxation losses. It may not be necessary in all cases to completely suppress the signals arising from solvents or other impurities as partial suppression of these signals may be sufficient to reduce their intensity while maintaining an appreciable S/N ratio for the signals of interest.

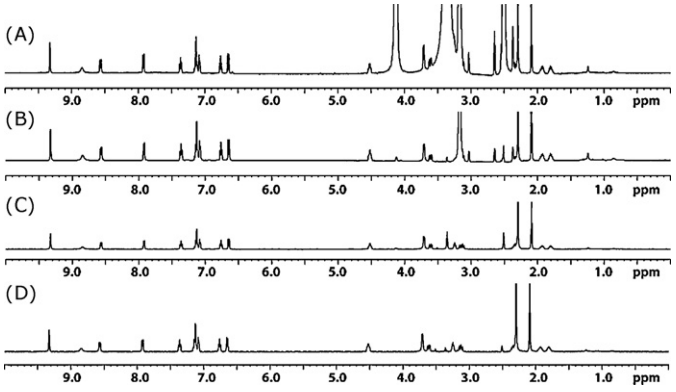

Fig. 5. MSG 100 μg in 150 μl DMSO-d_6 spiked with MeOH. Standard ^{1}H spectrum, S/N = 158 (A), 1D NOESY-presaturation spectrum, S/N = 463 (B), 1D diffusion-edited ^{1}H spectrum, diffusion delay = 150 ms, S/N = 70 (C), and 1D diffusion-edited ^{1}H spectrum, diffusion delay = 200 ms, S/N = 37 (D).

Based on the results from the experiments with EASG and MSG, 1D diffusion-editing proved useful in selectively suppressing signals from solvents and other small molecular weight impurities in complex mixtures containing metabolites. This may be particularly advantageous in the structure determination of metabolites where the paucity of sample available for NMR limits the use of several other NMR techniques. However, care should be taken when using this experiment for the purpose of NMR signal assignment as the data may misrepresent sample composition and presence of impurities that may be otherwise observed in a standard ^{1}H experiment. The signal attenuation observed in the diffusion-edited spectra can be controlled by optimizing a number of parameters as discussed above. Some loss of sensitivity is unavoidable in gradient-based diffusion techniques and these losses need to be balanced with other signal enhancement strategies such as the use of low sample volumes and sensitive instrumentation.

In this study we compared data from a 1D diffusion-edited spectrum with the data obtained from 1D NOESY-presaturation experiment, but other popular solvent suppression techniques such as WATERGATE and excitation sculpting would encounter the same issues as observed in case of 1D NOESY-presaturation, i.e. possible suppression of signals of interest leading to difficulties in spectral analysis and characterization of metabolites.

While 1D diffusion-edited experiments are useful in suppressing signals arising from chemical species that have molecular weights much smaller then the compound of interest, a common problem in characterizing metabolites by NMR post-purification is the presence of co-eluting and/or weakly UV-absorbing endogenous materials. In the case of metabolites isolated from bile, bile acids and fatty acids often end up co-eluting with the metabolites of interest and can complicate the NMR spectra. The signals from these impurities cannot be easily suppressed by 1D diffusion-editing since their molecular weights may be similar to those of the metabolites of interest.

We evaluated the use of DOSY in order to gain 'separation' of the NMR signals of the metabolite from those of the endogenous impurities. To this end, we analyzed bile from a bile duct-cannulated rat that was fed 20 mg of ethacrynic acid (EA). LC–MS demonstrated that the main metabolite in the bile is EASG (data not shown). EASG was purified by reversed-phase chromatography for NMR analysis. The LC–MS and NMR analysis of this purified metabolite suggested

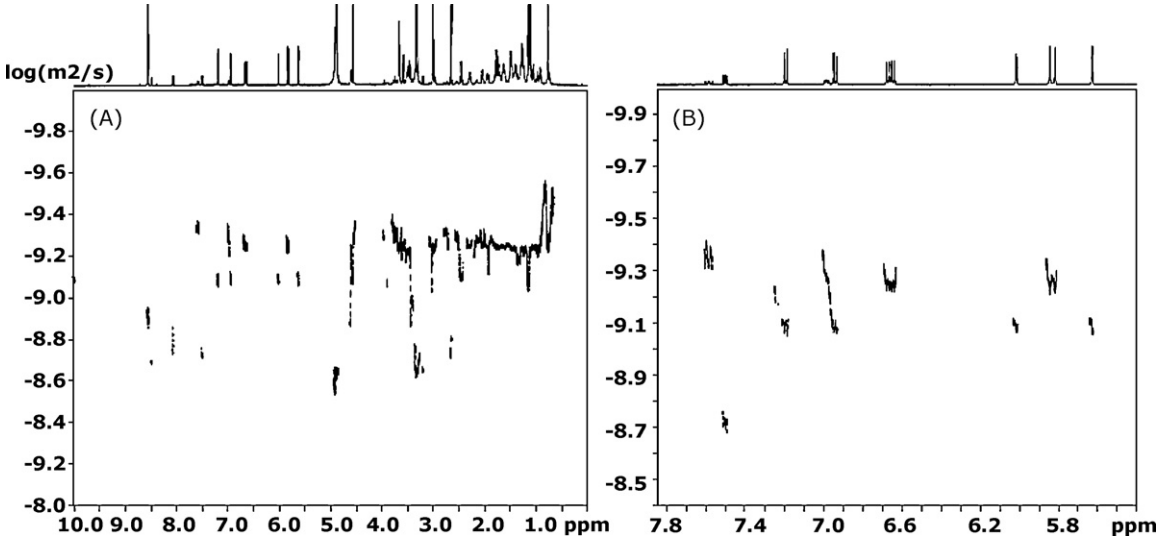

Fig. 6. DOSY spectrum of EASG purified from rat bile in CD_3OD with a ^1H NMR spectrum of the sample projected over the *x*-axis (A), and the downfield region of the DOSY spectrum expanded from δ5.40 to δ7.85 (B).

the presence of EASG, parent drug EA and an unidentified impurity. A DOSY spectrum of this sample is shown in Fig. 6. The NMR peaks in the aromatic region were well resolved and three distinct components could be distinguished in the sample based on the separation of their NMR signals along the diffusion axis (Fig. 6B). These components were identified as EASG (δ7.60, and δ7.00), an uncharacterized bile acid (δ6.65 and δ5.84) and EA (δ7.20, δ6.95, δ6.05 and δ5.65). In the higher field region where spectral overlap was more evident, there was no separation of signals along the diffusion-dimension with the three-component exponential data fitting employed here. It is unclear whether the parent ethacrynic acid observed here was formed as a result of decomposition of EASG, as described above, or was co-purified with the metabolite from bile. These observations suggest a possible use of the DOSY experiment for the determination of the structure of a metabolite of interest in the presence of co-eluting endogenous compounds, provided there is minimal spectral overlap. This experiment can also be used to determine the total number of components present in the NMR sample along with an ability to identify them based on their distinct NMR signals, without the need for laborious chromatography.

4. Conclusions

We have compared data from the 1D NOESY-presaturation and diffusion-edited experiments with standard ^1H NMR using 20 μg and 100 μg samples of mefenamic acid and ethacrynic acid glutathione conjugates. 1D NOESY-presaturation experiments were able to suppress two solvent signals simultaneously while improving the S/N for the metabolite peaks, but, the observed solvent suppression was non-selective and overlapping metabolite signals were also suppressed by using this technique. Baseline errors, solvent ^{13}C satellites, and suppression of signals of interest can lead to errors in the spectrum analysis. In comparison, the 1D diffusion-edited experiments were selective in simultaneously suppressing multiple solvent peaks and other small molecular weight impurities without producing baseline distortions or integration inconsistencies. However, diffusion-edited spectra showed attenuated metabolite signals when compared with a standard ^1H spectrum.

Two-dimensional DOSY was used to analyze the major metabolite isolated from the bile of an ethacrynic acid-fed rat. This experiment was useful in separating the metabolite NMR signals from those of a co-eluting endogenous bile acid and parent drug in the downfield region of the spectrum. In the upfield regions of high spectral overlap, no separation along the diffusion axis was observed. Thus, this experiment provides an easy way to determine the total number of compounds present in the NMR sample without need to run additional analyses, which would further deplete the small amount of material available for structure determination. It also provides a quick read-out of chemical shift information of the metabolite by 'separating' the NMR signals of the metabolite from those of co-eluting endogenous substances, in cases where there is minimal overlap between the same.

Acknowledgements

The authors would like to acknowledge Eric Johnson and Donna Baldisseri of Bruker Biospin Corporation who provided help in optimizing experiments as well as helpful discussions.

References

[1] A.S. Kalgutkar, I. Gardner, R.S. Obach, C.L. Shaffer, E. Callegari, K.R. Henne, A.E. Mutlib, D.K. Dalvie, J.S. Lee, Y. Nakai, J.P. O'Donnell, J. Boer, S.P. Harriman, Curr. Drug Metab. 6 (2005) 161–225.
[2] K. Samuel, W. Yin, R.A. Stearns, Y.S. Tang, A.G. Chaudhary, J.P. Jewell, T. Lanza Jr., L.S. Lin, W.K. Hagmann, D.C. Evans, S. Kumar, J. Mass Spectrom. 38 (2003) 211–221.
[3] Y. Matsuki, J. Dan, K. Fukuhara, T. Ito, T. Nambara, Chem. Pharm. Bull. 36 (1988) 1431–1436.
[4] Q. Chen, G.A. Doss, E.C. Tung, W. Liu, Y.S. Tang, M.P. Braun, V. Didolkar, J.R. Strauss, R.W. Wang, R.A. Stearns, D.C. Evans, T.A. Baillie, W. Tang, Drug Metab. Dispos. 34 (2006) 145–151.
[5] X. Huang, R. Powers, A. Tymiak, R. Espina, V. Roongta, Introduction to NMR and its application in metabolite structure determination, in: D. Zhang, M. Zhu, W.G. Humphreys (Eds.), Drug Metabolism in Drug Design and Development, John Wiley & Sons Inc., Hoboken, 2008, pp. 369–409.
[6] P. Stilbs, Progr. Nucl. Magn. Reson. Spectrosc. 19 (1987) 1–45.
[7] W.S. Price, Annu. Rep. NMR Spectrosc. 38 (1999) 289–354.
[8] P.C.M. van Zijl, C.T.W. Moonen, J. Magn. Reson. 87 (1990) 18–25.
[9] N. Esturau, J.F. Espinosa, J. Org. Chem. 71 (2006) 4103–4110.
[10] R. Giernoth, D. Bankmann, Eur. J. Org. Chem. 2005 (2005) 4529–4532.
[11] S. Bradamante, L. Barenghi, G. Beretta, M. Bonfa, M. Rollini, M. Manzoni, Biotechnol. Bioeng. 80 (2002) 589–593.
[12] C.D. Klaassen, T.J. Fitzgerald, J. Pharmacol. Exp. Ther. 191 (1974) 548–552.
[13] M.L.P.S. van Iersel, M.M.H. van Lipzig, I.M.C.M. Rietjens, J. Vervoort, P.J. van Bladeren, FEBS Lett. 441 (1998) 153–157.
[14] W.-J. Lo, Y.-C. Chiou, Y.-T. Hsu, W.S. Lam, M.-Y. Chang, S.-C. Jao, W.-S. Li, Bioconjug. Chem. 18 (2007) 109–120.
[15] J. Zheng, L. Ma, B. Xin, T. Olah, W.G. Humphreys, M. Zhu, Chem. Res. Toxicol. 20 (2007) 757–766.
[16] M.P. Grillo, L.Z. Benet, Drug Metab. Dispos. 30 (2002) 55–62.
[17] P. Kang, D. Dalvie, E. Smith, S. Zhou, A. Deese, Drug Metab. Dispos. 35 (2007) 1081–1088.