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Short communication

The application of LC–NMR and LC–MS for the separation and rapid structure elucidation of an unknown impurity in 5-aminosalicylic acid

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Abstract

This work has demonstrated the usefulness of combining liquid chromatography–nuclear magnetic resonance spectroscopy (LC–NMR) and liquid chromatography–mass spectrometry (LC–MS) methodologies for a rapid identification of an unknown impurity (**N1**) in the drug 5-aminosalycilic acid. Complementary information obtained from the two methods has revealed plenty of structural information and led to the fast on-line structure determination of **N1** prior to its isolation and purification. The analysis of LC–NMR and LC–MS spectra revealed that **N1** and 5-aminosalycilic acid are structurally closely related compounds. The structure of **N1** was later confirmed by high-resolution NMR spectroscopy of the isolated compound and the atom assignment was made. The approach described here has potential for 5-aminosalycilic acid impurity profiling and monitoring the production process.

Keywords: 5-Aminosalicylic acid; Impurity; Liquid chromatography–nuclear magnetic resonance spectroscopy; Liquid chromatography–mass spectrometry

1. Introduction

To identify and structurally characterize impurities present in bulk drug substances and formulated drug products is one of the primary goals of an analytical laboratory in pharmaceutical industry. The nature of impurities is also very important for formulation stability and possible interactions with excipients. Furthermore, regulatory bodies demand the identification of all impurities above 0.1% level. Complex mixtures, especially those with low amounts of individual components still represent a challenge to analytical chemists working in pharmaceutical research and development. The question still remains whether to apply traditional isolation and purification steps prior to structural elucidation of compounds present in the mixture, or to use an on-line approach involving coupled liquid chromatography–nuclear magnetic resonance spectroscopy (LC–NMR) and liquid chromatography–mass spectrometry (LC–MS) or LC–NMR–MS systems [1–5]. The former approach could be tedious and time demanding. The effectiveness of the latter, however, depends on the mixture complexity, i.e. the number, the amount and nature of compounds present in the sample, especially for LC–NMR analysis which suffers from the low sensitivity [6]. Recently, improvements in cryotechnology, capillary columns and micro-coil probes [7], as well as coupling of the solid-phase extraction [8] have drastically increased the sensitivity of LC–NMR experiments, which in turn made it possible to analyze compounds in the low nanogram quantities. In the last decade LC–NMR and LC–NMR–MS have been widely used to analyze natural products and drug metabolites [6–12], however, drug impurities and their degradation products have less frequently been reported in the literature [13,14].

In our previous papers, we showed that an integrated approach, which combined LC–MS and LC–NMR, provided an efficient methodology for characterization of impurities and degradation products in final drug substances [15,16]. In this study, we further demonstrate the usefulness of LC–MS and LC–NMR for the rapid identification and structural elucidation of an unknown impurity in 5-aminosalycilic acid. 5-Aminosalicylic acid **1**, is a marketed drug (generic name: mesalamine or mesalazine), which is used for the treatment of acute ulcerative colitis and Chron's disease. The drug-related

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impurities and degradation products of **1** have previously been analyzed by micellar electrokinetic capillary chromatography [17,18] and compared to the standard HPLC method described in the United States Pharmacopeia. However, the impurity **N1** has not yet been reported in the literature. The unknown impurity **N1** is formed during the preparation of 5-aminosalicylic acid by the so called sulfanilic acid pathway [19].

2. Experimental

2.1. Chemicals

Acetonitrile and methanol for chromatography (gradient grade, MERCK, Darmstadt, Germany), deuterium oxide (min. 99.9% pure, Cambridge Isotope Laboratories, Andover, USA), DMSO-d_6 (min. 99.9% pure, Cambridge Isotope Laboratories, Andover, USA), water, MilliQ purity (Millipore filters, Billerica, MA, USA), potassium dihydrogen phosphate crystal extra pure (puriss. p.a., Riedel-de Haen, Seelze, Germany), sodium hydrogen phosphate dihydrate crystal extra pure (puriss. p.a., Riedel-de Haen, Seelze, Germany) and sodium salt of octansulphonic acid (MERCK, Darmstadt, Germany) were used. Zorbax SB-C8 collumn with 5 μm particle size (MERCK, Darmstadt, Germany) and nylon filter with 0.45 μm particle size were used (Millipore filters, Billerica, MA, USA).

2.2. Sample preparation

For the LC–MS analysis, the samples were dissolved in a mixture of methanol and water, 1/1 (v/v), in the experiments of the protonated compounds. A system consisting of methanol and D_2O, 1/1 (v/v) was used in the hydrogen/deuterium exchange experiments. The concentration was 0.1 mg/mL. For the LC–NMR analysis the samples were dissolved in D_2O phosphate buffer. The concentration was 0.4 mg/mL.

2.3. Liqiud chromatography

Chromatography was first optimized separately on a 150 mm × 4.6 mm Zorbax SB-C8 column with 3.5 mm particle size using an Agilent Technologies system (Waldbronn, Germany) comprising of Agilent BinPump 1100 Series, DAD 1100 Series detector set at 210 nm. Injection volume was 10 μL and the flow rate was 1.5 mL/min. Elution was isocratic using a mobile phase consisting of 8% methanol, 8% acetonitrile and 84% D_2O buffer solution (10 mM KH_2PO_4 and 10 mM sodium octanesulphonate, pH 2.2).

2.4. LC–MS measurements

An acetonitrile–water system was employed in order to get a chromatographic resolution similar to the one with the phosphate buffer as a component of the mobile phase. The LC–MS system comprised of an Agilent Technologies 1100 system (Waldbronn, Germany) with a quaternary pump, autosampler, column compartment, DAD detector and a Micromass Platform LCZ mass spectrometer (Waters, Milford, USA), operating in Electrospray ionization (ESI), positive ion mode. Each experiment was performed at both low and high in-source collision induced dissociation (CID) parameter.

2.5. LC–NMR measurements

Experiments were performed in the stop flow LC–NMR mode on an Avance DRX500 spectrometer (BrukerBiospin, Rheinstetten, Germany) coupled to a Bruker LC 22 pump, with Bischoff Lambda 1010 UV-detector operating at 220 nm and a BSFU Bruker Stop Flow Unit.

An isocratic composition of 8% methanol, 8% acetonitrile and 84% 0.01 M D_2O phosphate buffer, and 0.001 M sodium salt of octanesulphonic acid was used. The flow rate was 1.2 mL/min. The injection volume was 1 mL. For NMR experiments, an inverse 4 mm detection $^1H/^{13}C$ flow probe (cell volume 120 μL) with z-gradient accessory was used.

One-dimensional NMR spectra were recorded using the WATERGATE and WET pulse sequences for suppression of the acetonitrile, methanol and HOD signals. GARP decoupling module was used to eliminate ^{13}C satellite signals. Spectra were acquired with a 10,000 Hz spectral window and 64 K data points, which gave the digital resolution of 0.6 Hz per point. One hundred to two hundred sixty scans were accumulated to obtain appropriate signal-to-noise ratio.

Two-dimensional narrow region COSY LC–NMR spectra were acquired with the spectral width of 1500 Hz in both dimensions and spectra were transformed into 2 K data points with 512 increments and 64 scans. Data were processed using the unshifted sine square window function. Digital resolution was 1.5 Hz in both dimensions.

2.6. NMR spectroscopy

One- and two-dimensional (^1H, APT, gCOSY, gHSQC and gHMBC) NMR spectra were recorded at ambient temperature on the Avance DRX500 spectrometer using a 5 mm diameter inverse detection probe with z-gradient. The spectra were recorded in DMSO-d_6 with the sample concentration of 20 mg/mL and TMS as the internal standard. The typical spectral conditions for one-dimensional (^1H and APT) and two-dimensional (gCOSY, gHSQC and gHMBC) were used.

3. Results and discussion

For the separation of **1** and its impurity **N1**, experimental conditions were varied until the satisfactory LC profile was obtained. An optimized isocratic methanol–acetonitrile–water separation (see Section 2 for details) was used. The retention times in the LC–DAD chromatogram were 9.964 min and 1.598 min for **1** and **N1**, respectively.

3.1. LC–NMR and LC–MS analyses

After the successful separation, the compound **N1** was sent to the LC–NMR flow probe and ^1H LC–NMR spectra were first recorded using the WATERGATE and WET (Fig. 1a) solvent

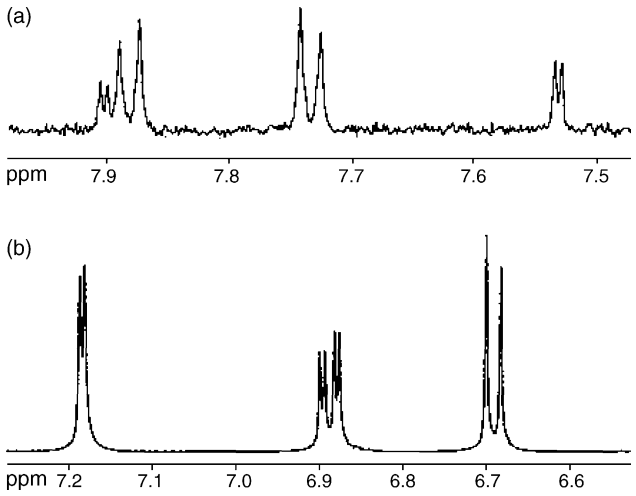

Fig. 1. The aromatic regions of (a) ^{1}H LC–NMR spectrum of **N1** and (b) 5-aminosalycilic acid in DMSO-d_6.

suppression modules. The analysis of the ^{1}H LC–NMR spectrum of **N1** revealed six non-exchangeable protons in the aromatic region 7.5–8.0 ppm (Fig. 1a). A comparison of the ^{1}H LC–NMR spectrum of **N1** and ^{1}H spectrum of **1** recorded in DMSO-d_6 (Fig. 1b) showed that the two compounds are structurally closely related.

A close inspection of the proton–proton coupling constants pointed towards two different sets of protons belonging to the two substituted benzene rings most likely of the biphenyl type. An integration of the proton peak intensities in the ^{1}H LC–NMR spectrum revealed that the first set consists of four protons belonging to one benzene ring and that the second set consists of two protons of the other benzene ring. Furthermore, the value of the coupling constants for the protons in the former set was measured to be 8.5 Hz whereas for the later it amounted to 2.8 Hz. That indicated *para*,*para* substitution in one of the benzene rings whereas the other benzene ring should be tetrasubstituted. These findings were further supported by the analysis of the 2D LC–COSY spectrum whose aromatic part is shown in Fig. 2. The correlation peaks in 2D LC–COSY spectrum were diagnostic for the two spin-systems of the A_2M_2 and AX types in the two benzene rings, respectively. To deduce the complete structure of the impurity LC–MS was performed next.

Prior to LC–MS analysis of the impurity **N1**, we first recorded the mass spectra of the main peak, 5-aminosalicylic acid, in both protonated and deuteriated conditions (Fig. 3a) in order to establish the fragmentation pattern and the number of exchangeable protons. The ESI-MS spectra of **N1** were recorded next (Fig. 3b) and a protonated molecular ion ($[M+H]^+$) at *m*/*z* 310.3 and fragment ions at *m*/*z* 292.3, 158.1 and 154.1 were observed. The in-source CID fragmentation pattern of **N1** was found to be similar to that of **1** (Fig. 3a).

The fragment ion of **N1** at *m*/*z* 292.3 indicated loss of a water molecule as also observed for **1**. The mass fragment at *m*/*z* 154.1 (not assigned in Fig. 3b due to its low intensity) corresponded to the mass of **1** supporting the LC–NMR observation that **N1** and **1** are closely related compounds. That indicated

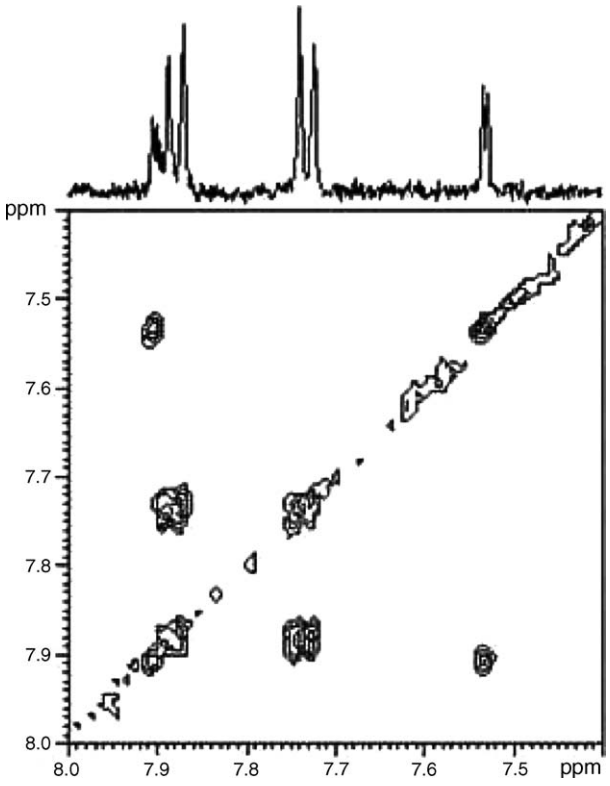

Fig. 2. The part of 2D LC–COSY NMR spectrum of **N1** showing the main correlation peaks.

that 5-aminosalycilic acid moiety is a constitutive part of the impurity **N1**. Furthermore, the isotopic pattern of the molecular ion of **N1**, specifically the intensity of the M + 2 peak afforded a recognition of the sulphur atom. According to the ESI-MS spectrum the product ion at *m*/*z* 158.1 should correspond to the second *para*,*para*-disubstituted benzene ring. A difference in mass indicated the SO_3H group and according to the LC–NMR data it should be in the *para*-position. Next, we carried out LC–MS experiments in methanol/D_2O system. The MS spectra of **1** revealed $[M+D]^+$ ion at *m*/*z* 158 which is four mass units higher than $[M+H]^+$, indicating four exchangeable protons in the molecule being consistent with the structure of **1**. The analysis of MS spectra of **N1** in deuterated mobile phase showed the mass shift of the parent $[M+H]^+$ ion at *m*/*z* 310.3 to $[M+D]^+$ ion at *m*/*z* 315.3. That pointed towards five exchangeble protons in **N1**, one more than in **1**. The fragmentation pattern of deuterated samples was analogous to that in the experiments with protonated samples (Fig. 3).

Hence, if 5-aminosalycilic moiety is present in **N1** as determined by LC–MS, the second phenyl group should be attached at the postion 3 to conform to LC–NMR coupling constant values and 2D correlation peaks data. A complementary information obtained from LC–NMR and LC–MS led us to the structure of **N1** as shown in Fig. 3b.

The value of using an on-line LC–NMR and LC–MS approach for identification and structural characterization of 5-aminosalycilic acid impurities, as demonstrated here, is in the fact that a wealth of information can be obtained rapidly and

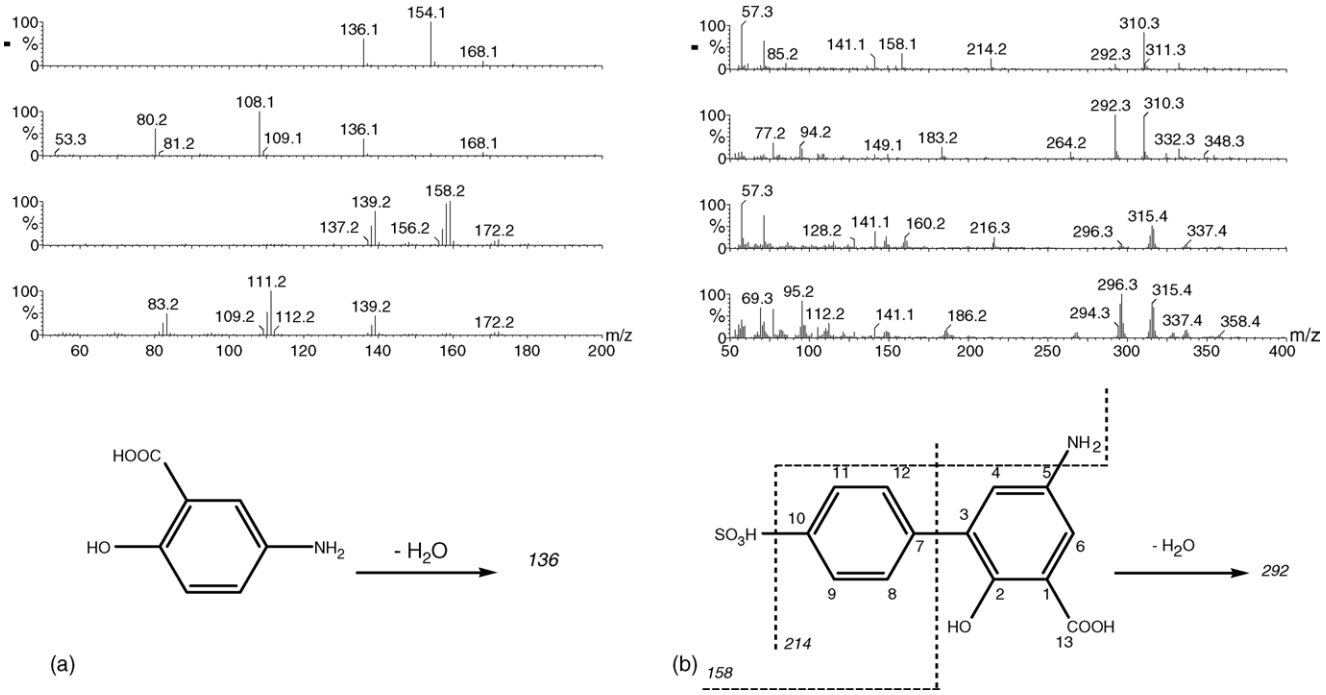

Fig. 3. ESI-MS spectra and fragmentation pattern of (a) 5-aminosalycilic acid (from top to bottom; solvent = MeOH/H_2O, CV = 30 V; solvent = MeOH/H_2O, CV = 55 V; solvent = MeOH/D_2O, CV = 30 V; solvent = MeOH/D_2O, CV = 55 V) and (b) impurity **N1** (from top to bottom; solvent = MeOH/H_2O, CV = 30 V; solvent = MeOH/H_2O, CV = 55 V; solvent = MeOH/D_2O, CV = 30 V; solvent = MeOH/D_2O, CV = 55 V) and the atom numbering.

Table 1
The proton and carbon chemical shifts (ppm) of the 5-aminosalycilic acid impurity **N1**

Atom	δ (ppm)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
^{1}H	–	–	–	7.47	–	7.76	–	7.48	7.71	–	7.71	7.48	–
^{13}C		158.24	130.28	130.51		123.98	136.56	128.89	125.81	147.20	125.81	128.89	172.00

efficiently, prior to isolation and purification which are usually tedious and time demanding steps.

3.2. NMR analysis

In order to confirm the proposed structure of **N1**, isolation and structure elucidation by high-resolution NMR were subsequently performed. Resonance assignments were made by combining one- and two-dimensional homo- and hetero-nuclear NMR experiments of the isolated compound **N1**. The COSY spectra revealed all the expected correlations as observed in LC–COSY, the HSQC spectra yielded an unambiguous assignment for the protonated carbon atoms while correlation peaks in HMBC spectra revealed information about the quaternary carbons and confirmed the assignments of the protonated carbons. The HMBC correlation peaks confirmed the relative position of the substituents in both phenyl rings. The ^{1}H and ^{13}C chemical shifts of **N1** are listed in Table 1.

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