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Combined use of liquid chromatography–nuclear magnetic resonance spectroscopy and liquid chromatography–mass spectrometry for the characterization of an acarbose degradation product

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Abstract

Directly coupled LC–MS and LC–NMR were applied to identify and structurally characterize an acarbose degradation product **A** in acidic media. A comparative analysis of the stop-flow LC–NMR (^{1}H and TOCSY) and LC–MS data provided evidence that **A** is structurally related to acarbose, differing from the parent compound in a number of subunits present in the molecule. Spectral analysis revealed that **A** was the α-glucosidase inhibitor amylostatin XG. Complementary information obtained from the two methods led to the structural elucidation of **A** which was later corroborated by high-resolution NMR spectroscopy of the isolated molecule.
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1. Introduction

Acarbose **1**, a pseudo-tetrasacharide, is an α-glucosidase-inhibitory anti-diabetic drug which reduces postprandial hyperglycemia and hyperinsulinemia [1–5]. Acarbose exerts its activity in the gastrointestinal tract by a reverse inhibition of the enzymatic cleavage of complex carbohydrates to simple absorbable sugars [1,2]. The mechanism of action differs from those of insulin, metformin or sulfonylurea, which are the most frequently used drugs to treat diabetes [1–5]. Acarbose is usually prescribed for type II diabetic patients (non-insulin-dependent diabetes mellitus, NIDDM), that is not well-controlled with sulfonylurea, metformin or insulin. It may be administered as monotherapy and also in combination with insulin to help control blood sugar in type I diabetic patients (insulin-dependent diabetes mellitus, IDDM).

The chemical structure of **1**, shown in Scheme 1, consists of four units, i.e., unsaturated aminocylitol, and three glucopyranose units [6–11].

Acarbose is produced by actinomycetales strains [6,7] and isolated from the fermentation broth. We show here that acarbose decompose in acidic media and a new compound is detected in the LC. This study is focused on the identification and structural elucidation of this degradation product **A**. The main goal is to extract as much structural information as possible using LC–MS and LC–NMR methodologies.

It is now well established and documented that coupling of liquid chromatography with mass spectrometry and/or NMR spectroscopy makes it possible to rapidly obtain detailed structural information on small quantities of substances without the necessity of further purification [12,13].

Prior to LC–MS and LC–NMR analysis the analytical LC method was developed and then adjusted for LC–NMR and LC–MS measurements. The mixture was separately analyzed in the stop-flow LC–NMR and LC–MS modes. The effluents of **A** and acarbose were also analyzed in the LC–MS/MS direct infusion mode. One-dimensional proton and two-dimensional TOCSY sequences were applied with a NOESY type double presaturation module for suppressing HOD and acetonitrile resonances. The structural information collected from LC–MS and LC–NMR analysis led to the full characterization of the degradation product **A**.

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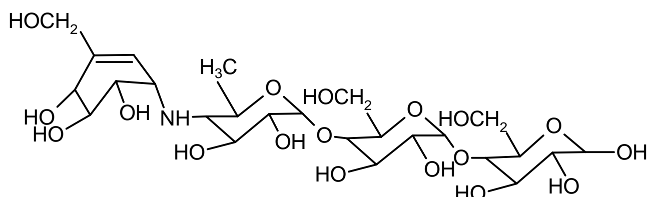

Scheme 1.

2. Experimental

2.1. Reagents and materials

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), acetonitrile for chromatography (gradient grade, MERCK, Darmstadt, Germany), sodium hydroxide 0.1 M solution from Kemika (puriss. p.a., Zagreb, Croatia), deuterium oxide (min. 99.9% pure, Cambridge Isotope Laboratories, Andover, USA), water, Milli Q purity (Millipore filters, Billerica, MA, USA) were used. Nylon filter 0.45 μm particle size was purchased from Millipore (Millipore filters, Billerica, MA, USA) and $–NH_2$ Hibar Lichrospher LC column with 5 μm particle size (MERCK, Darmstadt, Germany).

2.2. Sample preparation

Ten grams of acarbose was dissolved in 100 ml demineralised water and then hydrolyzed with 10 ml 3 M HCl. The solution was stirred continuously at 80 °C. Two aliquots of 20 ml each were neutralized after 30 min and 2 h, respectively, with 1 M NaOH and adjusted to pH 6.3. Both samples were subsequently analyzed by LC.

The concentration of acarbose prepared for LC–MS injection was 10 mg ml^{-1} in all experiments. The concentration of **A** was 0.5% in unsaturated samples and 10% in saturated samples of the acarbose peak area. Compound **A** was concentrated and isolated on the same LC system using the method described below, the only difference being use of a 10 μm particle size $–NH_2$ LC column in the semi-preparative mode.

2.3. LC experiments

Chromatography was performed on a 250 mm × 4 mm $–NH_2$ Hibar Lichrospher LC column with 5 μm particle size in analytical mode and 10 μm particle size in semi-preparative mode, using an Agilent Technologies system 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^{-1}. Elution was isocratic using a mobile phase consisting of 71% acetonitrile and 29% water or D_2O over 30 min. The pH of the mobile phase was adjusted to 6.6 (350 mg sodium hydrogen phosphate dihydrate and 600 mg potassium dihydrogen phosphate diluted in 1000 ml of pure water or deuterium oxide).

2.4. LC–NMR measurements

Experiments were performed in the stop flow LC–NMR mode on an Avance DRX500 spectrometer (Bruker BioSpin, Rheinstteten, Germany), coupled to a Bruker LC 22 pump, with Bischoff Lambda 1010 UV-detector operating at 210 nm and a BSFU Bruker Stop Flow Unit. An isocratic composition of 71% acetonitrile and 29% D_2O at a flow rate of 1.5 ml min^{-1} was used. The injection volume was 25 μl. For NMR experiments, an inverse 4 mm detection $^1H/^{13}C$ flow probe (cell volume 120 μl) with *z*-gradient accessory was used. The hyphenated system was controlled by HyStar software (Bruker BioSpin, Rheinstteten, Germany).

One dimensional NMR spectra were recorded using the double presaturation NOESY pulse sequence (lc1pncwps) with shaped pulses for suppression of the acetonitrile and the water signals. ^{13}C decoupling was applied to eliminate ^{13}C satellites of the solvents. Spectra were acquired with a 7000 Hz spectral window and 64 K data points, giving digital resolution of 0.43 Hz per point. 32–128 scans were accumulated to obtain appropriate signal-to-noise ratio.

Two dimensional phase sensitive TOCSY LC–NMR spectra were acquired with NOESY double presaturation using composite pulses (pulse program: lcmlevf2pcph). Spectra were recorded with a spectral window of 7000 Hz in both dimensions and transferred into 2 K data points with 160 increments and 32–128 scans. Spin lock time was 65 ms. Data were zero-filled in f1 dimension to 512 points and processed using the shifted sine square window function. Digital resolution was 13.67 and 3.42 Hz in f1 and f2 dimensions, respectively.

2.5. LC–MS and LC–MS/MS measurements

Separate LC–MS analyses were made using a Q-TOF Micro mass spectrometer (Micromass, UK) with an electrospray source operating in the positive-ion mode with a Waters HT 2795 LC and a Harvard Pump 11 syringe pump for direct infusion with a flow of 5 μl min^{-1} (for measuring accurate mass with Lock spray option). Capillary voltage was 3000 V and sample cone voltage was 40 V. Source and desolvation temperatures were 80 and 150 °C, respectively. Collision energy was 10 V for MS measurement and 30 V for MS/MS measurement.

The lock-mass reagent was leucine–enkephaline with an exact mass of 556.2771 u. For accurate mass measurement the direct infusion method was used, because the chromatographic method required phosphate buffer and amino columns produced very strong background ionic noise. Half of the scans were generated by the lock-mass reagent. The flow remained unchanged for both lock-mass reagent and analyte at all times. One thousand scans and resolution

better than 5000 were minimally required. MassLynx 4 (Micromass, UK) software was used to calculate molecular formulas of product and precursor ions according to the accurate mass data.

2.6. NMR measurements

All 1D and 2D (^{1}H, APT, gCOSY, gHSQC and gHMBC) NMR spectra were recorded at ambient temperature on the Avance DRX500 spectrometer (Bruker BioSpin, Rheinstteten, Germany), working at 500.13 MHz for ^{1}H and equipped with a 5 mm diameter inverse detection probe with z-gradient.

The sample concentration in D_2O was 20 mg ml^{-1} with TSPD as an internal standard.

1D ^{1}H and APT NMR spectra were obtained with 4000 Hz and 32.000 Hz spectral window, respectively, using 64 K data points. Digital resolution was 0.12 and 0.95 Hz per point, respectively.

2D gCOSY spectra were acquired with a sweep width of 4000 Hz in both dimensions into 2 K data points with 256 increments. Spectra were zero-filled in the f1 dimension to 1 K and processed using an unshifted sine bell window function. Digital resolution was 3.91 Hz per point in both dimensions.

The inverse ^{1}H–^{13}C correlation experiments, gHMQC and gHSQC were recorded at 125.77 MHz using data matrices of 2 K × 256 with 32 scans and processed with a shifted sine bell window function and linear prediction. HMBC spectra were recorded using transfer delay for the evolution of long range C–H couplings of 60 ms with 256 increments into a matrix of 2 K × 256 data points, with a sweep width of 7500 Hz in f2 dimension and 31500 Hz in f1 dimension. Digital resolution was 3.25 Hz per point and 30.70 Hz per point in f2 and f1, respectively.

3. Results and discussion

An LC-UV chromatogram showing the separation of **A** and acarbose is displayed in Fig. 1a. After more than 100 injections, a rapid loss of column efficiency occurred due to the constant stationary phase rinsing. Also, slight shifts in the retention times arose. We therefore used direct infusion in LC–MS/MS experiments.

3.1. LC–MS and LC–NMR

LC–MS and LC–MS/MS analyses of peak **A** (Fig. 1a), additionally analyzed with direct infusion after semi-preparative elution, revealed that **A** lacked one sugar unit compared to acarbose. The mass spectrum exhibits a precursor ion at m/z 484.2. The mass difference of m/z 162 corresponds to the loss of one glucopyranose unit. A consecutive MS/MS cleavage of **A** gave the two additional product ions, m/z 304.1 and m/z 146.1 (Fig. 1b) which were also detected in

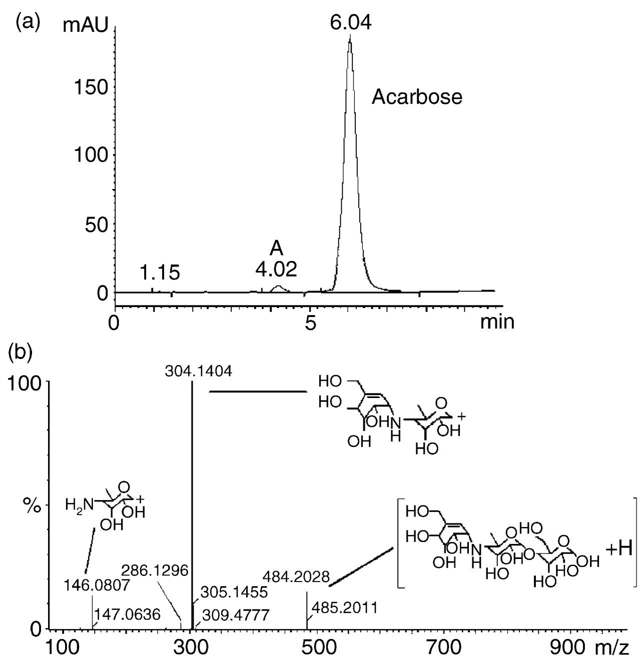

Fig. 1. (a) LC-UV of degradation product **A** and acarbose in acidic media and (b) ESI-MS spectrum and the proposed structures of product and precursor ions of **A**.

the mass spectrum of acarbose. In order to provide further support for one glucopyranose unit cleavage, exact mass measurement was performed on both precursor and product ions of **A**. Two of the most probable calculated molecular formulas are given in Table 1. On the basis of the acarbose structure and its MS fragmentation pattern it is concluded that the correct molecular formulas of the product and precursor ions of **A** are those underlined in Table 1. To confirm the proposed structure of the degradation product as obtained with MS measurement, a subsequent LC–NMR analysis was performed.

After LC-UV separation, compound **A** was sent to the LC–NMR flow cell. The one-dimensional stop-flow ^{1}H LC–NMR spectrum of **A** is shown in Fig. 2, together with the high-resolution ^{1}H spectrum of pure acarbose. The ^{1}H LC–NMR spectrum shows the resonances characteristic of carbohydrates. A comparative analysis of the ^{1}H LC–NMR spectrum of **A** and the ^{1}H spectrum of the pure acarbose

Table 1
Calculated molecular formulas of precursor and product ions of the degradation product **A**

Calculated molecular formulas	Measured mass	Calculated mass	Error (ppm)
$C_4H_{10}N_4O_2$	146.0807	146.0804	2.3
$\underline{C_6H_{12}NO_3}$	146.0807	146.0817	−6.8
$C_{14}H_{18}N_5O_3$	304.1404	304.1410	−1.7
$\underline{C_{13}H_{22}NO_7}$	304.1404	304.1396	2.7
$C_{17}H_{22}N_{15}O_{13}$	484.2025	484.2030	−1.1
$\underline{C_{19}H_{34}NO_{13}}$	484.2025	484.2030	−1.1

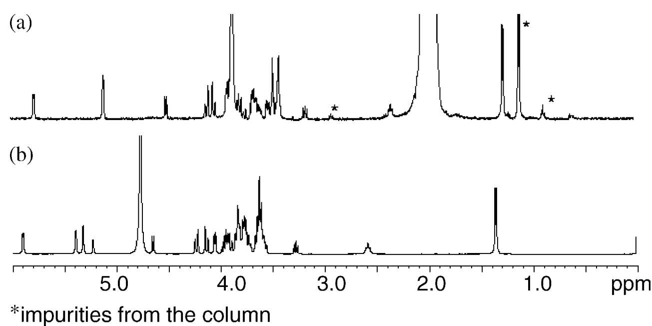

Fig. 2. (a) 500 MHz stop-flow ^{1}H LC–NMR spectrum of **A** and (b) 500 MHz ^{1}H NMR spectrum of acarbose.

(Fig. 2) revealed that **A** was structurally a closely related compound.

According to the integrated proton peak intensities and the absence of some characteristic resonances, it was evident that there was one sugar less in **A** than in acarbose, being consistent with the LC–MS data. The appearance of the two key resonances, i.e. the one belonging to the methyl protons (1.10 ppm) and the other to the olefinic –CH= proton (5.77 ppm) indicated that the structure of **A** contained both aminocylitol and 6-deoxy-D-glucopyranose units. In Fig. 2 it is also clearly seen that one anomeric proton at position 5.4 ppm is missing in the spectrum of **A**, when compared to the spectrum of the parent compound. This resonance was previously assigned to the anomeric proton in one of the glucopyranose units in **1** [6]. The acquisition of the 2D LC-TOCSY sequence (Fig. 3) allowed for further structural characterization of **A**. Although the hydroxyl protons could not be observed in the spectra due to an exchange with deuterium from D_2O, typical peaks of the three structural subunits, each belonging to a different spin-system were identified, i.e., aminocylitol, 6-deoxy-D-glucopyranose and D-glucopyranose (Fig. 4). An expanded view of the LC–NMR TOCSY spectrum in Fig. 3 clearly demonstrates the multistep magnetization transfer between spins belonging to aminocylitol and 6-deoxy-D-glucopyranose units. Only the olefinic and methyl regions are shown in which protons of each sugar moieties can be traced.

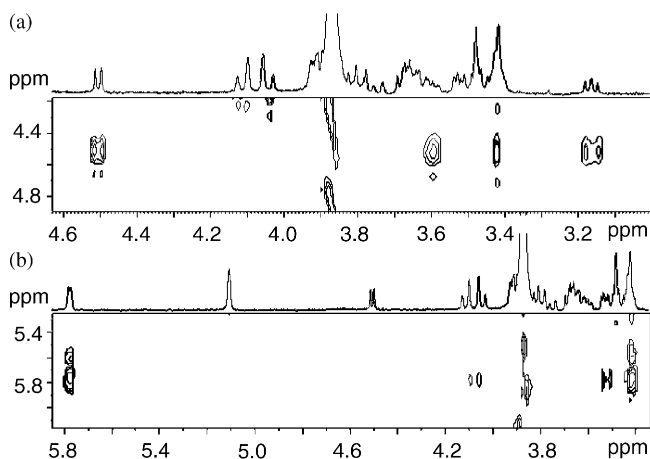

Fig. 3. Expanded views of the LC–NMR TOCSY spectrum of **A** showing correlation peaks of (a) anomeric and (b) olefinic protons.

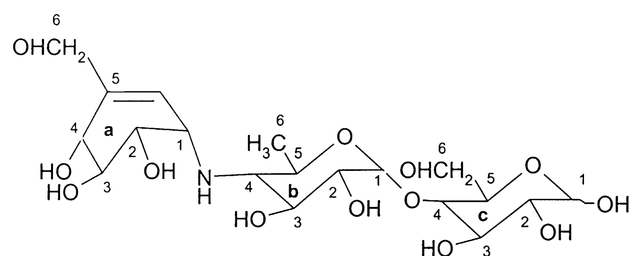

Fig. 4. The structure and atom numbering of **A**.

The data presented above provides substantial evidence that **A** is a compound consisting of three subunits as shown in Fig. 4, produced by simple hydrolytic cleavage of the last sugar unit in **1**. The LC–MS/MS fragmentation pattern (Fig. 1b) is consistent with this. Hence, the advantage of using LC–MS and LC–NMR to analyze acarbose samples over the conventional approach, which includes tedious isolation and purification steps, lies in the fact that sufficient structural information is obtained in a rapid and efficient way, as demonstrated here. This methodology can be exploited

Table 2
^{1}H and ^{13}C chemical shifts (δ/ppm) of the degradation product **A**

Unit	δ (ppm)		
	Atom	^{1}H	^{13}C
a	1	3.53	55.34
	2	3.67	70.14
	3	3.76	72.06
	4	4.04	70.47
	5	–	138.28
	6a	4.22	60.92
	6b	4.12	60.92
	7	5.90	123.11
b	1	5.32	99.10
	2	3.59	72.62
	3	3.59	72.06
	4	2.47	64.30
	5	3.76	68.92
	6	1.34	16.34
c(α)	1	5.23	91.23
	2	3.55	73.92
	3	3.94	72.60
	4	3.61	76.29
	5	3.92	69.32
	6a	3.97	59.95
	6b	3.76	59.95
c(β)	1	4.65	95.11
	2	3.27	73.30
	3	3.74	75.60
	4	3.59	75.60
	5	3.58	76.53
	6a	3.87	60.07
	6b	3.76	60.07

further for acarbose purity profiling and monitoring the production process.

3.2. Off-line NMR and MS

In order to obtain structure confirmation and full atom assignment we have undertaken further steps. The compound **A** in the acarbose sample was isolated and purified by preparative chromatography using the Agilent 1100 system (Agilent Technologies). The subsequent off-line high-resolution NMR analysis, employing homo- and heteronuclear one- and two-dimensional NMR sequences, such as APT, gradient selected COSY, HSQC and HMBC, led to the confirmation and full characterization of **A**. The HMBC correlation peaks involving anomeric atoms such as C-1a, H-4b and H-1b, C-4cα corroborated the relative order of the subunits in the molecule. The proposed structure was found to be consistent with that reported for amylostatin (XG) [14–18], which was also found to exhibit inhibitory activity against some α-glucosidases. However, to the best of our knowledge, a complete atom assignment has not been published yet. The chemical shifts of the compound **A** are given in Table 2.

The off-line MS analysis provided the same structural information about the degradation product, as that obtained with LC–MS. The mass spectra showed precursor and product ions characteristic of **A**.

4. Conclusion

This investigation has demonstrated that directly coupled LC–NMR and LC–MS methods can be used successfully for a rapid characterization of the degradation product in acarbose samples. Combined use of the two methods has provided insight into the structural characteristics of the degradation product. Large amounts of structural information can be obtained prior to impurity isolation, purification and identification by standard methods. This approach can serve as an efficient and reliable tool for assessing the acarbose purity profile and has the potential to be used in monitoring the chemical production process.

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