Journal of Chromatography, 557 (1991) 383–389
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 290

High-performance liquid chromatography–atmospheric pressure ionization mass spectrometry of gymnemic acids

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ABSTRACT

Gymnemic acids (GAs), extracted from the leaves of *Gymnema sylvestre*, are a mixture of triterpene glucuronides possessing various physiological activities. The molecular masses of GA homologues were determined with high-performance liquid chromatography combined with atmospheric pressure ionization mass spectrometry.

A mixture of GAs was chromatographed on an octadecyl silica column eluted with an aqueous methanol solution containing ammonium acetate, and directly introduced into an atmospheric pressure ionization mass spectrometer. Quasimolecular ions of GAs were detected as ammonium adduct ions and/or proton adduct ions. Molecular masses of thirteen different GAs and five compounds not containing glucuronic acid in their molecules were evaluated. Three pairs of geometrical isomers of GAs were found.

INTRODUCTION

Gymnemic acids (GAs) are chemical compounds found in the leaves of the Indian plant *Gymnema sylvestre R. Br. (Asclepiadaceae)*. GAs have been found to inhibit various physiological functions, they affect in particular sweet taste sensing in humans as well as in some other species [1–3]. Furthermore, recent studies have revealed the inhibitory action of GAs against glucose uptake in the small intestine of the rat [4], and glucan synthesis by glucosyltransferase (GTase) from *Streptococcus mutans* [5]. These findings suggest that GAs interact with proteins that take part in sugar recognition.

Gymnemic acids are known to be a mixture of glucuronides of hexahydroxy-olean-12-en(triterpene) and some hydroxyl groups of the structural frame are acylated as shown in Fig. 1. The R groups (R_1–R_5) represent hydrogens (H) or acyl residues

0021-9673/91/$03.50

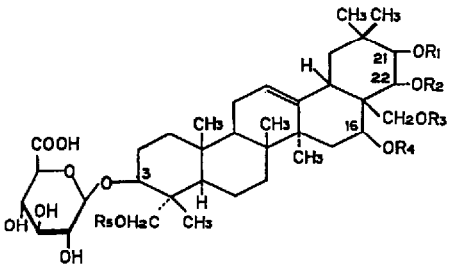

Fig. 1. Presumed molecular structure of gymnemic acid. R_1–R_5 are hydrogen atoms or acyl residues forming ester linkage with triterpene frame.

forming ester linkage with the triterpene framework. Recently, some GA homologues have been isolated, and acetyl, tigloyl and 2-methylbutanoyl residues have been identified as the constituents of R groups by nuclear magnetic resonance (NMR) and mass spectrometric (MS) techniques [6–8]. However, there are the least a few tens of homologues in the leaves, with structures as yet unknown.

In the present study, the GAs were divided into their homologues by molecular mass on an octadecyl silica (ODS) column. The molecular mass and the chemical structure of each GA homologue were investigated by using a combined system of high-performance liquid chromatography (HPLC) and MS.

EXPERIMENTAL

Dried leaves of *Gymnema sylvestre* imported from India were used in this work. Mixtures of GA homologues were extracted according to the method described by Kurihara [2].

The molecular masses of GAs were determined with a Hitachi 655A high-performance liquid chromatograph connected to a Hitachi M-80 mass spectrometer by an interface consisting of a nebulizer and a vaporizer through a PTFE tube (Hitachi, Tokyo, Japan). The vaporized sample and solvent molecules at 300°C were introduced into the ion source of the atmospheric pressure ionization (API) MS system. The drift voltage of spectrometer was set at 160 V.

Chromatography was done on a conventional ODS column (YMC-A312, 150 × 6 mm I.D.; Yamamura, Kyoto, Japan). Mobile phases were prepared as follows: mobile phase A, 0.1 *M* ammonium acetate–methanol (35:65, v/v); mobile phase B, 0.1 *M* ammonium acetate–methanol (10:90, v/v). Isocratic elution was used for 30 min after sample injection, followed by gradient elution from A to B in 10 min. The GA solution (10 mg/ml in the initial mobile phase) was injected via valve equipped with a 10-μl loop, and was eluted at a flow-rate of 1.0 ml/min at room temperature.

All chemicals were purchased from Wako (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan).

RESULTS AND DISCUSSION

Two HPLC chromatograms of GA mixtures on an ODS column are shown in Fig. 2. Chromatogram a was obtained by using acidic (pH 3.2) mobile phase (metha-

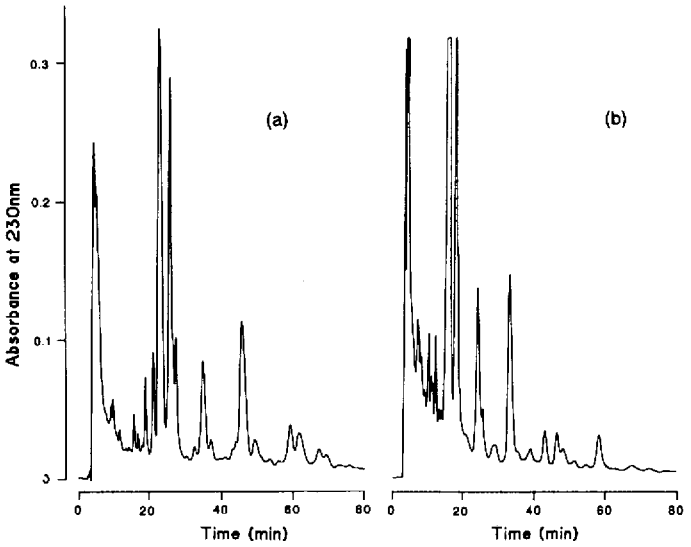

Fig. 2. Chromatograms of a mixture of GAs obtained with (a) the acidic mobile phase (methanol–water–acetic acid; 64:36:0.5 v/v/v, pH 3.2) and (b) the neutral mobile phase (methanol–0.02 *M* phosphate buffer; 60:40 v/v, pH 8.5) In each case, 1 mg of the sample was loaded on an ODS column (YMC A-312, 150 × 6 mm I.D. Temperature, 25°C; detection, UV at 230 nm.

nol–water–acetic acid, 64:36:0.5 v/v/v) and chromatogram b using a neutral (pH 8.5) mobile phase (methanol–0.02 *M* phosphate buffer, 60:40 v/v). In both chromatograms many peaks were observed. As the dissociation of the carboxyl group of glucuronic acid in the GA molecules progressed, the GA molecules became less hydrophobic in the neutral mobile phase than in the acidic mobile phase. In other words, the neutral mobile phase was a rather stronger eluent for GAs than the acidic mobile phase. We also examined the effect of the methanol concentration in the eluent on the retention of GAs. The retention volume of GAs depended on the methanol concentration in the eluent rather than the eluent pH. Therefore, we used a gradient for the methanol concentration in the eluent, as described under Experimental. Some components were much more hydrophobic and eluted with 90% (v/v) methanol, as shown in Fig. 3.

Components in Fig. 2 with retention times less than *ca.* 10 min were considered to be impurities. These were removed by semi-preparative ODS column chromatography before the experiment was performed in order to minimize contamination of the interface from extraneous materials.

Fig. 3 shows a total ion current (TIC) profile over the range *m/z* 400–1000 for the sample solution thus prepared and directly introduced from the column into the APIMS system. As shown, the concentration of methanol in the eluent was increased gradually from 65% to 90% in 10 min after 30 min from the start in order to shorten the analysis time. The addition of ammonium acetate to the eluent was useful to enhance the ionization efficiency of the sample molecule. Under the experimental conditions, quasimolecular ions were observed as ammonium adduct ions $[M + NH_4]^+$ and/or as proton adduct ions $[M + H]^+$.

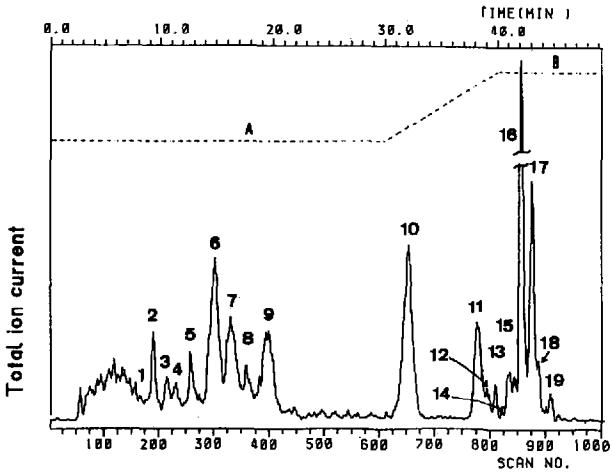

Fig. 3. Total ion current profile of LC–API-MS of GA mixture Total mass range, *m/z* 400–1000; column, ODS (YMC A-312, 150 × 6 mm I.D.); eluents: A = 0.1 *M* ammonium acetate–methanol (35:65, v/v), B = 0.1 *M* ammonium acetate–methanol (10:90 v/v). Gradient elution was begun after 30 min, from 100% A to 100% B in 10 min. Flow-rate, 1.0 ml/min.

The peaks numbered from 1 to 19 in Fig. 3 were selected for the analyses of their mass spectra. Some of these spectra are shown in Fig. 4. Ammonium adduct ions were observed at *m/z* 782 for component 3 and at *m/z* 784 for components 2 and 4: their molecular masses were determined to be 764, 766 and 766, respectively. As the calculated molecular mass of GAs with no acyl residues (R_1–R_5 = H) is 682, the molecular masses of 764 and 766 are consistent with molecular masses of GA homologues containing tigloyl (Tig, *m/z* 82) and 2-methylbutanoyl (MB, *m/z* 84) residues, respectively. The fragment ion at *m/z* 608 in the mass spectrum of component 2 differs from the quasimolecular ion at *m/z* 784 by 176 mass units. So this ion could be attributed to the ammonium adduct ion of the aglycone formed by loss of a glucuronic acid (glcUA) from the ammonium adduct molecular ion at *m/z* 784, which was represented as $[M + NH_4 - glcUA]^+$. The proton adduct ion of the aglycone was also observed at *m/z* 591 in the spectrum of component 2. Furthermore, the peak at *m/z* 471 of component 2 is 120 mass units lower than the fragment ion at *m/z* 591. This ion may be attributed to the fragment ion formed by successive loss of two molecules of water (*m/z* 36) and a 2-methylbutanoyl residue (*m/z* 84) from the proton adduct ion *m/z* 591, $[M + H - glcUA - MB - 2H_2O]^+$.

Similar fragment ions were observed for component 3 at *m/z* 589 and 471. The mass difference of 118 between these two fragment ions, which was two mass units lower than that in the case of component 2, was again consistent with the proposal that the component 3 contained a tigloyl residue. The fragment ions at *m/z* 589 and 471 were thus attributed to $[M + H - glcUA]^+$ and $[M + H - glcUA - tigloyl - 2H_2O]^+$, respectively. For component 4, which is a geometrical isomer of component 2, however, a fragment ion due to the loss of the acyl group was not found, although the fragment ion due to glycoside bond cleavage was clearly apparent at *m/z* 591.

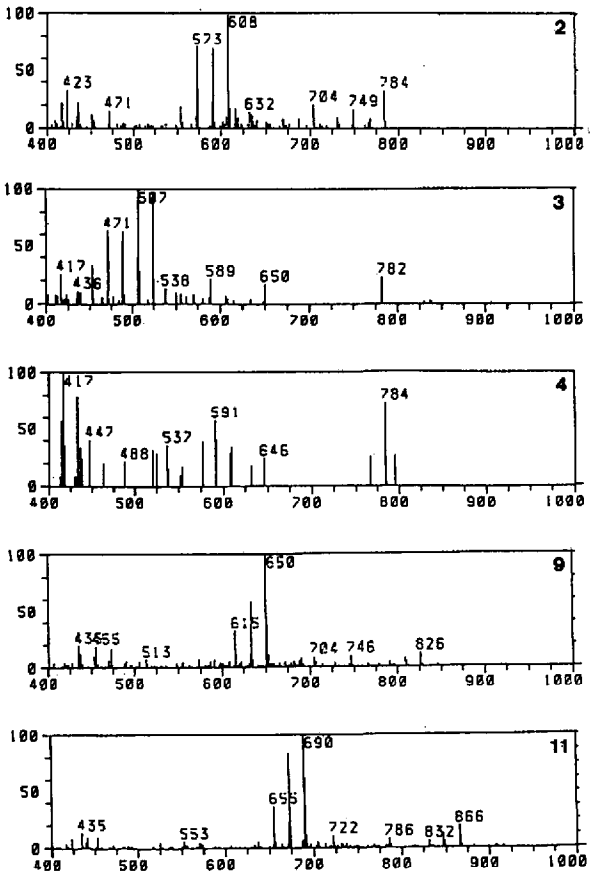

Fig. 4. Typical mass spectra obtained by LC–API-MS. Numbers in the upper right-hand corner of each spectrum correspond to the peak numbers in the total ion current profile in Fig. 3. In each spectrum, the percentage intensity of the ionic current compared with the maximum current in the mass range (m/z) from 400 to 1000 is shown as the ordinate.

Comparing the spectrum of component 9 with that of component 2, both the quasimolecular ion at m/z 826 and the fragment ions at 650, 615, and 513 differ by 42 mass units from those ions of component 2 at m/z 784, 608, 573, and 471, respectively. Therefore, component 9 was consistent with the compound containing an additional acetyl residue to component 2.

The peaks at m/z 866, 690 and 553 in the spectrum of component 11 correspond to those at m/z 784, 608 and 471, respectively, for component 2, increased by 82 mass units. This strongly suggests that component 11 is the homologue formed by further acylation of component 2 with the tigloyl residue. Similar analyses were done for the other fourteen components selected from Fig. 3.

Table I lists the quasimolecular ions, the presumed molecular masses, the fragment ions due to glycosidic bond cleavage and the presumed acyl residues for seventeen components. The data for components 7 and 8 are not listed because their mass spectra showed very complex patterns, indicating that these components were mix-

TABLE I

SUMMARY OF ANALYSIS OF MASS SPECTRA OBTAINED BY LC–API-MS

Component	Quasimolecular ion		Molecular mass	Fragment ion[a]		Presumed acyl residues[b]
	NH_4^+ adduct	H^+ adduct		NH_4^+ adduct	H^+ adduct	
1	782		764			Tig
2	784		766	608	591	MB
3	782		764		589	Tig
4	784		766		591	MB
5		791	790		615	
6[c]	826	(807)	808, (806)	648		MB, (Tig), Ac
9	826		808	650		MB, Ac
10	654		636			
11	866		848	690		MB, Tig
12	864		846		671	Tig, Tig
13	888		870	712		MB, Bz
14			766		573	
15		767	766	608		
16		591	590			
17	638		620			
18		591	590			
19		689	688			

[a] Formed by glycosidic bond cleavage.
[b] Abbreviations: Tig = tigloyl; MB = 2-methylbutanoyl; Ac = acetyl; Bz = benzoyl.
[c] Consist of two components.

tures of two or more compounds. Thirteen components in Table I were identified as GA homologues containing glcUA. Three pairs of isomers were found, whose molecular masses were 764, 766 and 808.

The molecular mass 870 of component 13 was the largest for GA homologues found in the present experiment. The mass difference between component 13 and components 2 and 4 was 104. NMR studies revealed some homologues that showed resonance lines attributable to the benzoyl residue (Bz, *m/z* 104). Therefore, component 13 was identified as the GA homologue derived by further acylation of component 2 or 4 with the benzoyl residue.

The presumed molecular masses of components 14 and 15 (both 766) are the same as those of components 2 and 4. However, these components could not simply be attributed to isomers of components 2 and 4. As shown in Fig. 3, components 14 and 15 were eluted by a higher methanol concentration in the eluent, suggesting that they passess a more hydrophobic structure than components 2 and 4. Therefore, the genins of components 14 and 15 may differ from gymnemagenin. For example, gymnestrogenin, which was proposed by Sinsheimer and Rao [9], is more hydrophobic than gymnemagenin, because gymnestrogenin lacks a hydroxyl group at C-22 of gymnemagenin (Fig. 1).

The molecular masses of components 16 to 19 were smaller than those of the compounds eluted before component 16. Furthermore, the fragment ions due to glycosidic cleavage were not observed in their spectra. It was suggested from these results that they corresponded to aglycones without a sugar moiety in the molecules.

In fact, the proton adduct molecular ion at m/z 591 of component 16 or 18 was the same as that of the fragment ion identified for component 2 or 4.

Present work showed that using LC–MS was quite useful for analysis of such a complex mixture of homologues as gymnemic acids. One of the advantages of using the API interface is that any conventional column can be used without placing a restriction on the choice of flow-rate or mobile phase. In fact, no special design was necessary in carrying out HPLC, except for the addition of ammonium acetate to the mobile phase.

In this study, the molecular masses of thirteen GA homologues and related components were determined. In addition, the acyl residues such as acetyl, tigloyl, 2-methylbutanoyl and benzoyl in GA molecules were identified by analysis of the fragmentation patterns. The GA homologue containing a benzoyl residue was the first one that we identified in the GA mixture. Moreover, we were able to identify three pairs of geometrical isomers in GA mixture. On the basis of the information obtained from the results of LC–API-MS, we are now investigating the complete chemical structures of each GA homologue by in-beam MS and NMR.

ACKNOWLEDGEMENTS

The authors thank Professor Yasutake Hiji for his support in this investigation. They also thank Dr. Shigetake Ganno, Mr. Yoshiaki Kato, and Ms Youko Numajiri, Hitachi Ltd., for their technical assistance with the LC–API-MS measurements and for helpful discussions.

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