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Analysis of Sennosides A and B from Dieter's Tea by HPLC-Diode Array Spectrophotometry and Negative Ion Electrospray Mass Spectrometry

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There is considerable demand in drug testing for a specific and precise analytical method for the identification of sennosides in various food and pharmaceutical preparations. Sennosides are well known as laxative agents (Kadans 1970)and the main active components are diasteroisomers, sennosides A and B (5,5'-bis(B-D-gluco-pyranosyloxy)-9,9',10,10'-tetrahydro-4,4'-dihydroxy-lO,lO'-dioxo(R,R')-[9,9'-bianthracenel-2,2 '-dicarboxylic acid and (5,5'-bis(B-D-glucopyranosyloxy)-9,9',10,10'-tetrahydro-4,4''-dihydroxy-lO,lO'-dioxo(R,S')[9,9'-bi-anthracene]-2,2 '-dicarboxylic acid, respectively)(Figure 1). While thin-layer chromatography (The United States Pharmacopeia 1995) and high performance liquid chromatography using ion pair (Muffat et al. 1986; Sagara et al. 1987) and column switching (Oshima et al. 1991) techniques are commonplace for such analyses, they often encounter sample matrix interference problems which may cause uncertainties in the analytical results. A combination of high performance liquid chromatography and mass spectrometry (LC/MS) will provide unambiguous fingerprint information for chemical structural confirmation. Because the sennoside molecules are polar, thermally labile and not readily accessible to conventional ionization methods, the use of mass spectrometry for sennoside analysis was hampered in the past. Thus, very limited mass spectrometric data is available for underivatized sennosides.

Recent developments in electrospray ionization techniques allow routine analyses of complex biomolecules similar to the sennosides. Stuppner and Sturm (1996) have shown that electrospray mass spectrometry can be used to identify the dianthrones from Cassia angustifolia.

In this article, we report the use of electrospray in the negative mode to obtain mass spectrometric data of sennosides A and B. We also describe LC/MS methods to

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analyze sennosides A and B from dieter's tea extracts.

MATERIALS AND METHODS

Sennosides A and B were obtained from Atomergic Chemetals Corp. Tetraheptyl ammonium bromide was from Fluka Chemika. Ammonium acetate was from EM Science. Acetic acid was from Mallinckrodt. Acetonitrile and methanol were from Burdick & Jackson.

One tea bag (approximately 2.5 gram tea/bag) sample was immersed in 100-ml boiling tap water and allowed to steep for 10 minutes. The tea bag was squeezed lightly and then removed. The tea extract was allowed to cool and was filtered through a 0.45 um filter disc (Gelman Sciences, Ann Arbor, Michigan) before analysis.

An HP 1050 liquid chromatograph equipped with an HP diode array detector (1040M series II), an LC column of Supelcosil LC-18-DB, 25 cm x 4.6 mm ID, particle size 5 um by Supelco, and an autosampler was used. Sennosides were separated with an eluent of 65 % of solution A: 0.06 M of ammonium acetate, pH 5 in water and 35 % of solution B: 0.008 M of tetraheptyl ammonium bromide in acetonitrile. The injection loop was 10 ul and the column flow rate was 1.2 ml per minute. The diode array detector was scanned from 220 nm to 600 nm with a band width of 40 nm.

LC-MS experiments were performed with an HP 1050 liquid chromatograph equipped with a Perkin-Elmer LC-90 UV detector set at 270 nm and an LC column of Phenomenex Ultracarb ODS, 15 cm x 3.2 mm ID, particle size 5 um. The mobile phase was a 50 % (v/v) methanol in water containing 0.1 % acetic acid. The injection loop was 5 ul and the column flow rate was 1.0 ml per minute. A post-column effluent (1:4 split) was introduced to a Finnigan TSQ 700 triple quadrupole mass spectrometer through an electrospray interface at a rate of 200 ul min^{-1}. Nitrogen was used both as the sheath gas at 40-50 psi and the auxiliary gas at 5 psi. An electrospray voltage at 4.5 kV was applied and the capillary temperature was set at 240° C. The mass analyzer was set to monitor negative ions. A mass range of m/z 200 to m/z 950 was scanned every second. For MS/MS analysis, argon was introduced into the collision cell at a pressure of 2 mtorr. The collision cell potential was adjusted from 35 to 60 eV. The MS/MS mass spectra were recorded from m/z 200 to 900 in 1 second.

RESULTS AND DISCUSSION

The two isomers sennosides A and B have distinctly different retention times on the LC-18 column under

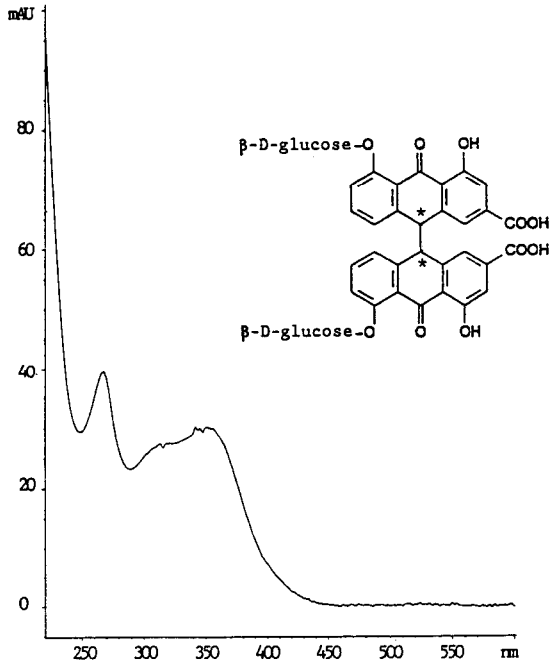

Figure 1. Structure and diode array spectrum of sennoside A(R,R').

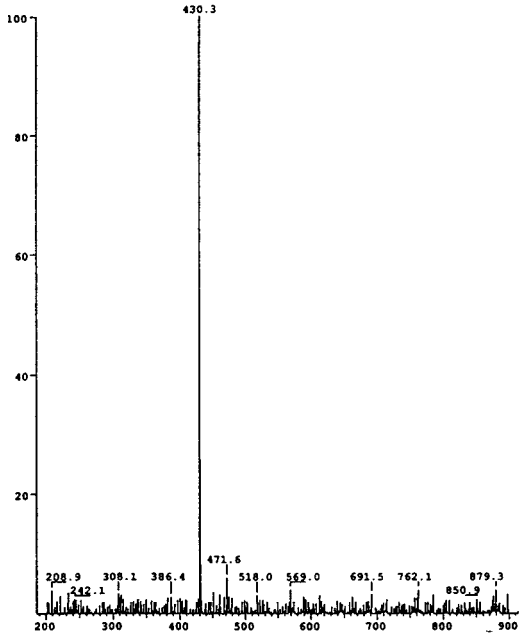

Figure 2. Negative ion electrospray mass Spectrum of sennoside A in 50 % (v/v) aqueous methanol that contained 0.1 % acetic acid.

various eluent compositions. However, to separate sennosides A and B from other components coextracted from the dieter's tea, the addition of a basic ion-pair reagent to the mobile phase is necessary to increase the retention of sennosides A and B. The presence of sennosides A and B can be tentatively identified by 1) comparing the retention times of the chromatographic peaks with those of the sennosides A and B standards and 2) comparing the corresponding diode array spectra for the chromatographic peaks with those of the sennosides A and B standards (Figure 1). This procedure is applicable for screening or product monitoring purposes. When the measured sennosides were suspected to be present at low concentrations (below 20 ppm) or when interferences were severe in some sample matrices, it was difficult to distinguish sennosides from the chromatographic background signals and the determination became uncertain. Consideration was further complicated by the fact that there might be presence of other sennoside constituents (sennosides C, D, E and F) or sennoside derivatives in the sample extracts (Hansel et al. 1992).

Confirmation for the presence of sennosides was made by electrospray ionization (ESI) mass spectrometry in the negative mode. Both compounds did not yield abundant positive ions by electrospray ionization under the described conditions. The mass spectra of standards sennosides A and B are virtually the same and are characterized by an abundant ion at m/z 430 (Figure 2). The ion at m/z 430 can be accounted for as the doubly-charged molecular ion $[M-2H]^{2-}$ which results from the two-proton dissociation of the molecular molecule. Except for the relative response of deprotonated molecular ion, increasing or decreasing the orifice voltage or the capillary temperature could not induce any significant fragmentation. An added advantage of the negative mode electrospray mass spectrometry technique is the selectivity. The majority of the coextracted components, which account for over 85% of the signal in a typical UV chromatogram, were not observed by the mass spectrometer detector at all.

Addition of the ion-pair reagent, tetraheptyl ammonium bromide, to the mobile phase was not needed to aid the chromatographic separation. With the combination of negative ionization and selected ion monitoring, high sensitivity was achieved for sennosides at low levels. This allowed us to develop a simple, quick LC/MS method using single ion monitoring at m/z 430 to analyze sample extracts for sennosides. An ion chromatogram of a tea extract indicating the presence of sennosides A and B (with retention times at 7:20 min and 4:40 min, respectively) is shown in Figure 3. Surprisingly, very

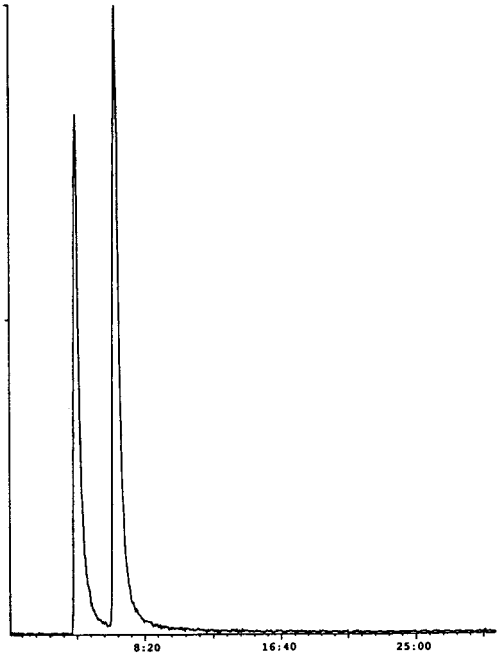

Figure 3. Liquid chromatography electrospray ionization mass spectrometry analysis of sennosides A (7.2 min) and B (4.4 min . Column: LC-18-DB. Mobile phase: 50% (v/v) aqueous methanol that contained 0.1 % acetic acid; split flow rate: 200 ul/min. Orifice votage at 4.5 kV; single ion monitoring at m/z 430.

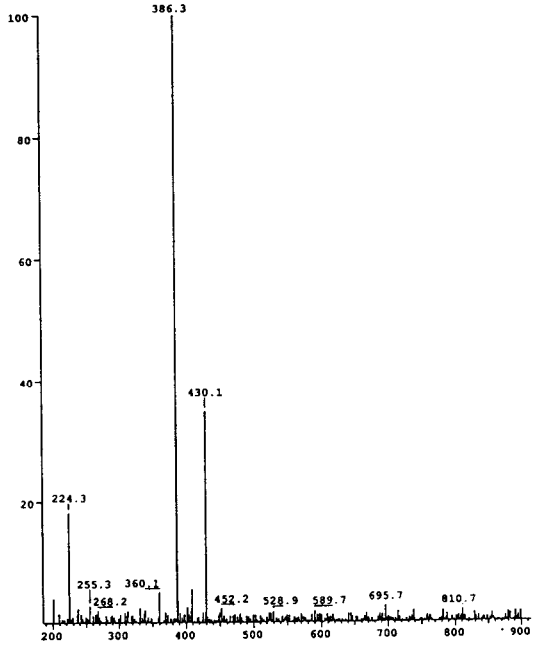

Figure 4. Mass spectrum of sennoside A using "in-source" collision induced dissociation. Source offset votage was set at 20 eV.

little interference was observed under the electrospray conditions used.

An additional mass spectrometric investigation of the ion at m/z 430 was made through "in-source" collision induced dissociation (CID). The source CID offset voltage was set from 10 eV to 80 eV. Again, sennosides A and B showed similar CID spectral pattern. Collision induced dissociation of the anion $[M - 2H]^{2-}$ from the sennosides generated two fragment ions at m/z 224 and 386 (Figure 4). The m/z 386 ion likely resulted from the loss of two carbon dioxides from the doubly-charged molecular ion. The doubly charged fragment at m/z 224 was formed by further dissociation of the RO-glucopyranosyl bond (Figure 5). Complete dissociation of the parent ion occurred at an offset voltage of 30 eV. The ratio of m/z 224 to m/z 386 increased as the offset voltage rose.

These fragmentations were also evidenced in the collision induced dissociation MS/MS experiments. The abundance of the molecular ion was greatest when the collision offset potential was held at 10 eV or below. The intensity of the decarboxylation ion $[M-2H-2CO2]^{2-}$ increased and reached the maximum as the collision offset potential rose to 45 eV. The anion $[M-2H-2CO2-2C6H11O5]^{2-}$ became the dominant ion when the collision offset potential was increased to 60 eV.

Based on the above observation, additional mass spectrometric confirmations of the positive findings of sennosides A and B in preliminary analyses were conducted by monitoring single ions at m/z 224, 386 and 430 using "in-source" CID with an offset voltage of 20 eV. We could then establish an unambiguous proof of structural identification by comparing the retention times and the relative abundance ratios with those of the standards.

Quantification of the sennosides content in tea bag samples was made by HPLC-UV at 270 nm using peak area counts and external standard calibrations from 50 ug/ml to 100 ug/ml. Average recoveries of spiked samples at 10 mg/bag level were 102% (sennoside A) and 95% (sennoside B) with a standard deviation of 2%. The estimated detection limit is 2 mg/bag. The tea bag samples were extracted with boiling water for 10 minutes before analysis. Extending the extraction time up to four hours did not improve recovery. Kazuhiko, et al. reported that good extaction efficiency of sennosides A and B could be obtained using 70% methanol under supersonic irradiation for 30 min (Sagara et al. 1987). We have compared the extraction recoveries experimentally using tap water and 70% methanol as the

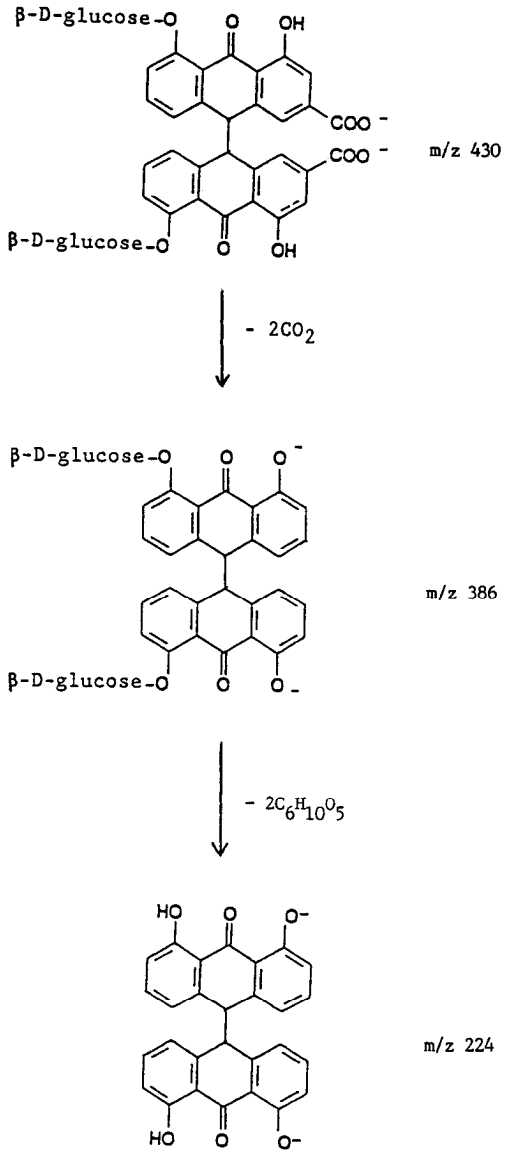

Figure 5. Proposed collision induced dissociation pathways of doubly-charged deprotonated sennoside molecular ion electrospray ionization.

extraction solvent. The results showed that the two procedures produce similar recoveries.

An automated HPLC with a diode array spectrophotometer method has been adopted by our laboratory to screen for sennosides in dietary products. Any suspected positive findings are to be followed by LC/MS confirmation as described above. Although this study was targeted for sennosides A and B determinations, it is believed that the method can be used for analyzing other sennoside compounds including sennosides C, D and F, and their derivatives. We will report these results in the near future.

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