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p-Hydroxybenzoic Acid Alkyl Esters in *Andrographis paniculata* Herbs, Commercial Extracts, and Formulated Products

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Analysis of two commercial extracts of *Andrographis paniculata* using high-performance liquid chromatography (HPLC) with photodiode array absorbance detection showed the presence of several unexpected compounds, which were isolated and identified as methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid by using high-resolution mass spectrometry and nuclear magnetic resonance. Quantitative analysis using HPLC revealed the presence of 0.22% *p*-hydroxybenzoic acid methyl ester (methlyparaben) in one commercial extract, and both 0.11% *p*-hydroxybenzoic acid ethyl ester (ethylparaben) and 0.20% *p*-hydroxybenzoic acid propyl ester (propylparaben) in a second commercial extract of *A. paniculata*. Analyses of additional commercial products of *A. paniculata* in tablet form purchased from Chicago pharmacies also showed the presence of methyl- and ethylparabens. To determine whether these compounds were natural chemical constituents of the plant, pharmacopoeial reference *A. paniculata* plant powder as well as samples of authenticated *A. paniculata* plant materials collected from Indonesia, Hong Kong, and mainland China were obtained and analyzed by HPLC−tandem mass spectrometry (LC-MS-MS). LC-MS-MS analyses confirmed the presence of trace concentrations (<0.0008% w/w) of *p*-hydroxybenzoic acid methyl ester but no *p*-hydroxybenzoic acid ethyl or propyl esters in these plant samples. The limits of detection of the LC-MS-MS assay for these compounds were 5 pg on-column and 5 ppb in the plant material. The levels of these *p*-hydroxybenzoic acid esters measured in the commercial products of *A. paniculata* suggest that they were introduced inadvertently during processing or as artificial additives.

KEYWORDS: ***Andrographis paniculata*; dietary supplement; LC-MS-MS; HPLC-PDA; *p*-hydroxybenzoic acid methyl ester; *p*-hydroxybenzoic acid ethyl ester; *p*-hydroxybenzoic acid propyl ester; methylparaben; ethylparaben; propylparaben**

INTRODUCTION

A medicinal plant used in Chinese and Ayurvedic medicine (*1*−*3*), *Andrographis paniculata* Nees (Acanthaceae) has been used clinically for symptomatic treatment of the common cold and uncomplicated sinusitis, pharyngotonsillitis, pneumonia, and bronchitis (*3*−*5*, *7*). Placebo-controlled, double-blind clinical studies have demonstrated the efficacy of standardized *A. paniculata* extracts (4% andrographolides) for the symptomatic treatment of the common cold (*4*−*7*). *A. paniculata* products are available in the marketplace as tablets for oral administration. Like many other botanical dietary supplements, the production of this product involves the acquisition of source plant materials by cultivation or field collection, processing of the plant materials into a standardized extract, and manufacturing of the standardized extract into suitable dosage forms.

The importance of quality control in each of these steps according to GAP, GLP, and GMP protocols has been reviewed (*8*). During the in-process analysis of potential sources of commercial standardized extracts for the manufacture of *A. paniculata* tablets, the quality control group of Pharmavite, San Fernando, CA, noted the presence of unidentifiable peaks in the high-performance liquid chromatography (HPLC) chromatograms (**Figure 1**) of two candidate commercial extracts. Subsequently, we undertook a study to identify these unknown substances to determine if they occur naturally in authenticated *A. paniculata* plant materials and to determine their concentrations in commercial tablet dosage forms. Therefore, HPLC, liquid chromatography−tandem mass spectrometry (LC-MS-MS), and phytochemical isolation were employed to identify

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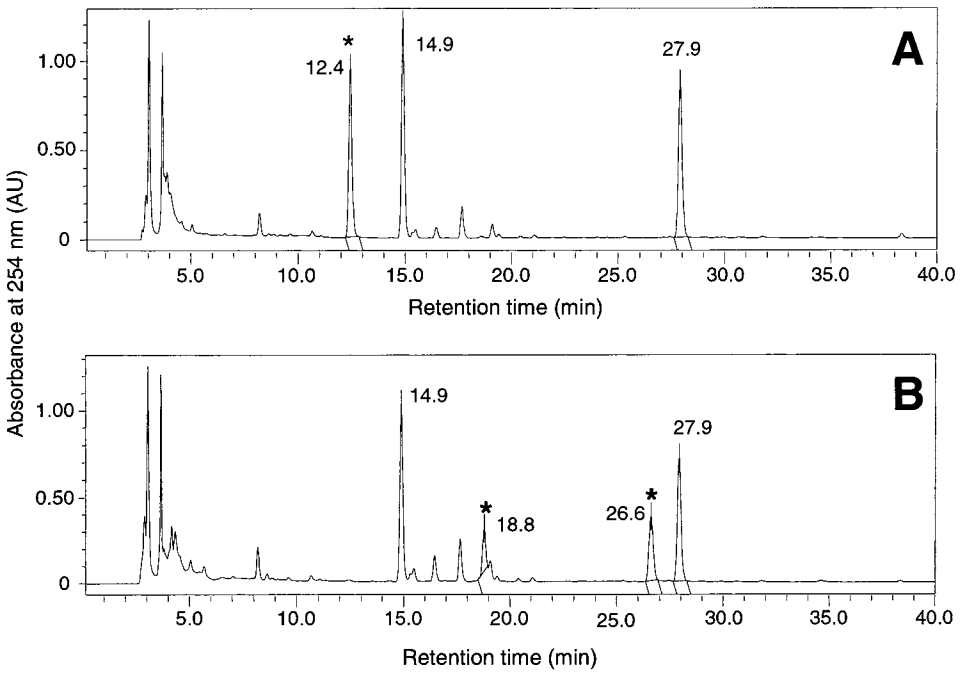

Figure 1. HPLC-PDA chromatograms (only absorbance at 254 nm shown) of commercial *A. paniculata* extracts A (top) and B (bottom). Unidentified peaks eluting at 12.4, 18.8, and 26.6 min are marked with an asterisk (∗).

Table 1. Analyses of *A. paniculata* Products and Specimens for *p*-Hydroxybenzoic Acid Esters

sample		*p*-hydroxybenzoic acid methyl ester (**I**) (methylparaben), % w/w (±SD)	*p*-hydroxybenzoic acid ethyl ester (**II**) (ethylparaben), % w/w (±SD)	*p*-hydroxybenzoic acid propyl ester (**III**) (propylparaben), %, w/w (±SD)
commercial extracts[a,b]	A	0.217 ± 0.004	0.0037 ± 0.0001	ND[c]
	B	0.0040 ± 0.0001	0.112 ± 0.001	0.197 ± 0.004
commercial products[d,e]	C, KanJang, lot 0103001	0.101 ± 0.006	0.00092 ± 0.00003	ND
	D, Vitamin World, lot 47111-02	0.00093 ± 0.00001	0.00182 ± 0.00007	ND
	E, REMDEX	0.137 ± 0.004	0.00176 ± 0.00010	ND
authentic plant samples[b]	F, Beijing	<0.0001	ND	ND
	G, West Java	0.00080 ± 0.00001	ND	ND
	H, Hong Kong	<0.0001	ND	ND
	I, Shanghai	0.00080 ± 0.00001	ND	ND

[a] Commercial extracts provided by Pharmavite. [b] Triplicate samples were prepared and analyzed in duplicate. [c] ND, not detected. [d] Tablets (1 tablet/dose) containing 60–300 mg of standardized *A. paniculata* extract each. [e] Replicate samples were prepared and analyzed in duplicate.

and quantify these unusual compounds in commercial *A. paniculata* products.

EXPERIMENTAL PROCEDURES

Materials. Two commercial *A. paniculata* extracts (A and B, **Table 1**) were provided by Pharmavite (San Fernando, CA). Commercial tablets (C−E, **Table 1**) of *A. paniculata* were purchased at local pharmacies in Chicago, IL. An official Chinese pharmacopoeial reference sample of *A. paniculata* (F; powder, no. 1082-9901) was obtained from the Institute for the Control of Pharmaceutical and Biomedical Products, Ministry of Health (Beijing, China). Authenticated *A. paniculata* plant samples were collected in Sukabumi, West Java, Indonesia (G); New Territory, Hong Kong (H); and Pudong, Shanghai, China (I). Voucher specimens were deposited at the University of Illinois Pharmacognosy Field Station, Downer's Grove, IL. HPLC grade methanol, chloroform, acetonitrile, hexane, ethyl acetate, and *n*-butanol were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized water was produced in-house using a Barnstead water purification system (Newton, MA).

Sample Preparation. Finely pulverized reference authentic plant samples (0.5−1 g each), commercial extracts (0.5−1.0 g each), and tablets (0.3−0.5 g each) were weighed separately into PTFE-capped 20-mL sample vials. An 18-mL aliquot of methanol or 80% methanol in water (for tablets only) was added to each sample, and the mixture was shaken and sonicated at 25−30 °C for 30 min. After cooling, the mixture was filtered through filter paper (Whatman no. 1) into a 250-mL round-bottom flask. The residue was returned to the sample vial and extracted and filtered twice more. Finally, the residue was rinsed three times while on the filter with 15-mL portions of methanol. The combined methanolic extracts were evaporated to dryness in vacuo at 40−45 °C. The residue was redissolved in methanol and transferred to a 10-mL volumetric flask and made up to volume with methanol. The sample solution was centrifuged, and the supernatant (10 μL) was analyzed using HPLC-PDA and HPLC-MS-MS.

Isolation. A sample (60−85 g) of each commercial *A. paniculata* extract (A and B) was sonicated for 60 min with 250 mL of methanol in a 500-mL flask at room temperature and then filtered through filter paper. The residue was returned to the same flask and sonicated with a second 250-mL volume of methanol and filtered. Then the residue was extracted a third time. The combined methanolic extracts were evaporated to dryness under reduced pressure at 40−45 °C. The resulting solid was suspended in water (300 mL) and extracted successively with 200-mL portions (five times each) of hexane, chloroform, ethyl acetate, and *n*-butanol. Because the chloroform and ethyl acetate extracts showed similar HPLC profiles, they were combined and evaporated to dryness under reduced pressure at 40−45

°C. Next, the residue of the combined chloroform and ethyl acetate extracts was fractionated using open column chromatography using a glass column (4 × 60 cm) containing 100 g of silica gel (300−400) mesh. The column was eluted with a step gradient from chloroform to methanol (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 0:100, v/v) at a flow rate of 2−3 mL/min. Each 100-mL fraction was concentrated under reduced pressure and analyzed by HPLC using a Supelco (Bellefonte, PA) Supelcosil LC-18 column (21.2 × 250 mm, 5 mm) with an isocratic mobile phase consisting of 40% acetonitrile in water at 8 mL/min and dectection at 254 nm.

Analysis. NMR spectra were recorded on a Bruker (Rheinstetten, Germany) DPX-300 spectrometer at 29 K using $CDCl_3$ as the solvent and TMS as an internal standard. A frequency of 75.48 MHz was used for ^{13}C NMR, and 300.0 MHz was used for ^{1}H NMR. Exact mass measurements were obtained using a Micromass (Manchester, U.K.) QTOF2 mass spectrometer equipped with negative ion electrospray. Samples for exact mass measurement were infused at 20 μL/min in 50% methanol in water at an ion source temperature of 140 °C.

Column chromatography was carried out on silica gel (200−400 mesh, Selecto Scientific, Suwanee, GA). HPLC-PDA absorbance detection analysis was carried out using a Waters Alliance 2690 HPLC system (Milford, MA) equipped with a 996 photodiode array detector and a Supelco Discovery C_{18} column (4.6 × 250 mm, 5 μm) protected by a Delta-Pak C_{18} guard column at 20 °C. The mobile phase consisted of a 40-min linear gradient from 20 to 50% acetonitrile in water at a flow rate of 1.0 mL/min. The injection volume was 10 μL, and UV spectra were recorded from 200 to 400 nm. Data collection and integration were carried out using Waters Millennium software revision 3.2.

LC-MS-MS was carried out using a Waters Alliance 2690 HPLC system interfaced to a Micromass Quattro II mass triple-quadrupole mass spectrometer equipped with an electrospray ion source operated at 140 °C in negative mode. The HPLC column and solvent system were identical to those used in the HPLC-PDA analysis. The column effluent was split ∼1:5 so that ∼200 μL/min entered the mass spectrometer. Nitrogen was used as both nebulizing gas and drying gas at flow rates of 20 and 450 L/h, respectively. Multiple reaction monitoring (MRM) was used for quantitative analysis using a dwell time of 0.2 s for each of the precursor/product ion pairs. Following the selection of precursor ions by the first quadrupole mass analyzer, collision-induced dissociation (CID) was carried out in the second quadrupole using 1.0×10^{-3} mbar argon at a collision energy of 15−17 eV. Both the first and last quadrupole mass analyzers were operated at unit-mass resolution.

RESULTS AND DISCUSSION

HPLC-PDA chromatograms of the commercial *A. paniculata* extracts A and B showed the presence of unidentified peaks at 12.4 (**Figure 1A**), 18.8, and 26.6 min (**Figure 1B**). Dereplication, which is the process of using HPLC and/or LC-MS to rapidly identify compounds that have been reported to occur in that particular botanical or other organism, failed to identify these peaks as normal constituents reported for this plant. Phytochemical isolation from extract A afforded 15 mg of compound **I** corresponding to the HPLC peak at 12.4 min. Isolation from extract B produced compounds **II** (15 mg) and **III** (5 mg) corresponding to the HPLC peaks at 18.8 and 26.6 min, respectively.

Compound **I** formed a deprotonated molecule at *m/z* 151.0363, which corresponded to an elemental composition of $C_8H_7O_3$ (calculated, 151.0395). The tandem mass spectrum with CID of the $[M - H]^-$ ion of *m/z* 151 is shown in **Figure 2A**. Fragment ions were detected at *m/z* 136.0175 (relative abundance 70%) and 92.0301 (100%) corresponding to the loss of a methyl group from the deprotonated molecule $[M - H - CH_3]^-$ ($C_7H_4O_3$; calculated, 136.0160) and the additional loss of carbon dioxide $[M - H - CH_3 - CO_2]^-$ (C_6H_4O; calculated, 92.0262). These data, together with a comparison of the ^{1}H and ^{13}C NMR spectra of **I** to literature values, confirmed that compound **I** was *p*-hydroxybenzoic acid methyl ester (methylparaben) (*9*).

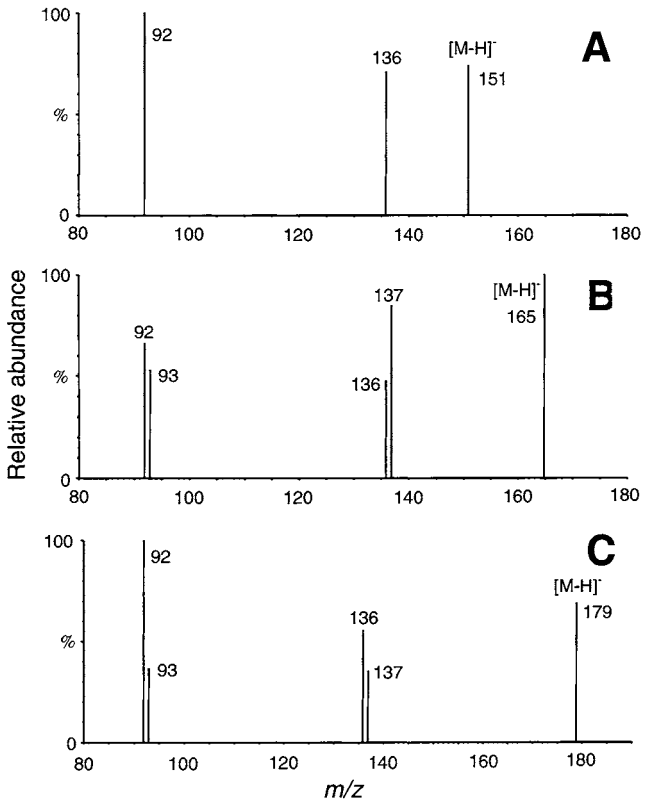

Figure 2. Product ion tandem mass spectra with CID of the deprotonated molecules of (A) compound **I**, (B) compound **II**, and (C) compound **III**.

Compound **II** was shown to have a molecular formula of $C_9H_{10}O_3$ based on the negative ion electrospray exact mass measurement of the deprotonated molecule at *m/z* 165.0541 ($C_9H_9O_3$; calculated, 165.0552). The MS-MS product ion mass spectrum of *m/z* 165 is shown in **Figure 2B**. Four abundant fragment ions were observed at *m/z* 137.0285 (85%) ($C_7H_5O_3$; calculated, 137.0239), 136.0173 (48%) ($C_7H_4O_3$; calculated, 136.0160), 93.0373 (53%) (C_6H_5O; calculated, 93.0340), and 92.0243 (67%) (C_6H_4O; calculated, 92.0262). These ions correspond to $[M - H - C_2H_4]^-$, $[M - H - C_2H_5]^-$, $[M - H - C_2H_4 - CO_2]^-$, and $[M - H - C_2H_5 - CO_2]^-$, respectively. In addition to these mass spectrometric data, a comparison of the ^{1}H and ^{13}C NMR spectra of compound **II** to literature values (*9*) indicated that this compound was *p*-hydroxybenzoic acid ethyl ester (ethylparaben).

The molecular formula of compound **III** was determined to be $C_{10}H_{12}O_3$ on the basis of the exact mass measurement of the deprotonated molecule at *m/z* 179.0708 ($C_{10}H_{11}O_3$; calculated, 179.0708). During MS-MS (see **Figure 2C**), four fragment ions were observed at *m/z* 137.0237 (36%) ($C_7H_5O_3$; calculated, 137.0239), 136.0125 (57%) ($C_7H_4O_3$; calculated, 136.0160), 93.0376 (37%) (C_6H_5O; theoretical, 93.0340), and 92.0243 (100%) (C_6H_4O; calculated, 92.0262), corresponding to $[M - H - C_3H_6]^-$, $[M - H - C_3H_7]^-$, $[M - H - C_3H_6 - CO_2]^-$, and $[M - H - C_3H_7 - CO_2]^-$, respectively. Compound **III** was identified as *p*-hydroxybenzoic acid propyl ester (propylparaben) on the basis of the mass spectrometry data and comparison of its ^{1}H and ^{13}C NMR spectra to those reported in the literature (*9*).

A comprehensive search of the literature revealed that compound **I** has been isolated from at least 20 higher plants

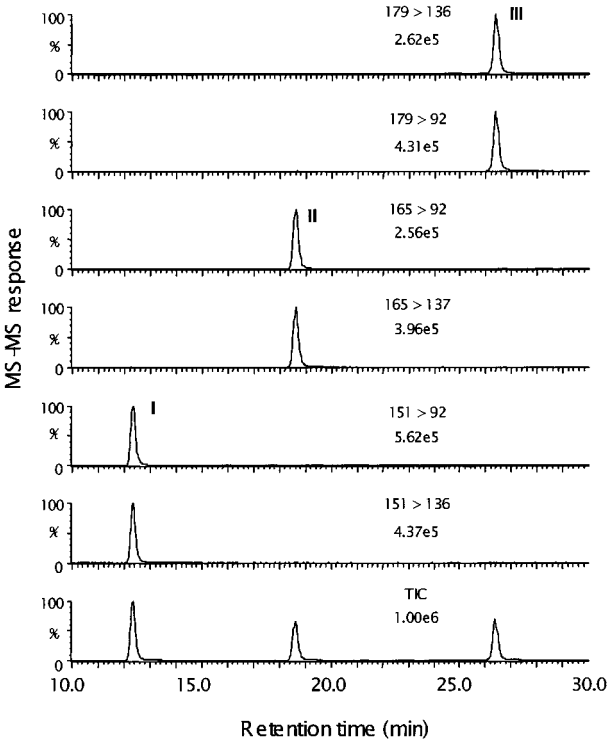

Figure 3. LC-MS-MS MRM chromatograms for a standard mixture of compounds **I**–**III** (10 ng each).

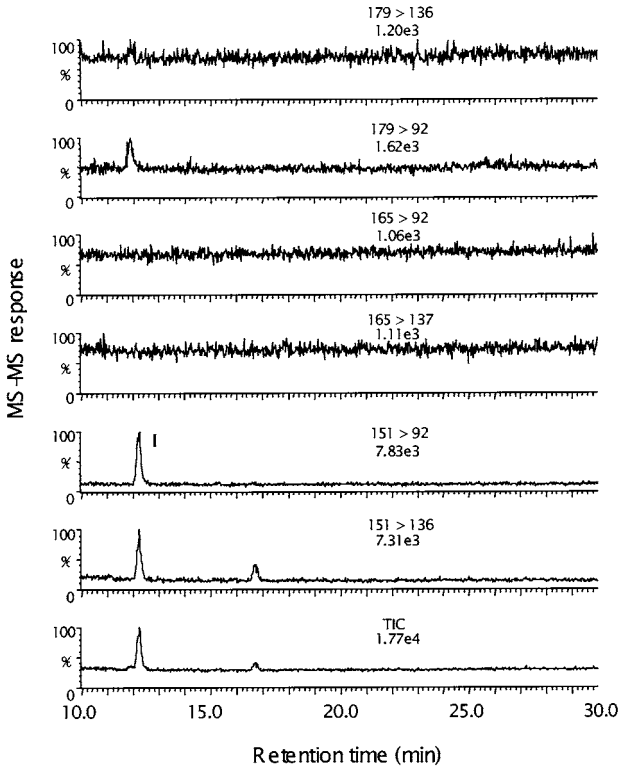

Figure 4. LC-MS-MS MRM chromatogram of a methanolic extract of a Chinese pharmacopoeial reference sample of *A. paniculata* (sample F).

and fungi, including *Lonicera augustifolia* (*10*) and *Guanomyces polytrix* (*11*). Compound **II** has been reported to occur in *Ixeris denticulata f. pinnatipartia* (*12*) and *Dracaena cochinchineses*, which is a traditional Chinese medicine (*13*). Compound **III** has been isolated from *Cannabis sativa* (*14*) and secretions of *Verticillium albo-atrum* (*15*). However, the natural occurrence of **I**, **II**, or **III** in species of the genus *Andrographis* has thus far not been reported. Therefore, reference Chinese pharmacopoeial *A. paniculata* and authenticated plant samples that had been field collected from Indonesia, Hong Kong, and mainland China were analyzed, extracted, and analyzed using LC-MS-MS in order to determine if compounds **I**−**III** are naturally occurring constituents or artificial additives.

On the basis of the tandem mass spectra, precursor/product ions were selected for multiple reaction monitoring (MRM) that was appropriate for selective and sensitive detection during the LC-MS-MS analysis of compounds **I**−**III**. Specifically, MRM ion pairs of *m*/*z* 151 → 136 and 151 → 92 were selected for compound **I**, MRM transitions of *m*/*z* 165 → 137 and 165 → 92 were chosen for compound **II**, and *m*/*z* 179 → 136 and 179 → 92 were used for the detection of compound **III**. These ions were the most abundant in the tandem mass spectra (see **Figure 2**) and represent structural characteristics of each compound. A typical LC-MS-MS MRM chromatogram showing the separation of a mixture of **I**−**III** standards is shown in **Figure 3**. The LC-MS-MS limit of detection for each compound, defined as a signal-to-noise of 5:1, was determined to be 5 pg injected on-column.

Plant samples of *A. paniculata* collected from three different geographical locations and the Chinese pharmacopoeial reference material were extracted and analyzed using LC-MS-MS for compounds **I**−**III**. During the sample preparation and subsequent chromatographic analysis, great care was used to prevent cross-contamination or sample carry-over. The LC-MS-MS chromatograms (**Figure 4**) of the four reference plant materials were identical. Traces of compound **I** but no signals for compounds **II** or **III** were detected in the authenticated plant samples. The identification of compound **I** in these samples was based on the simultaneous appearance of two MRM signals (*m*/*z* 151 → 92 and 151 → 136) at ∼12.2 min during LC-MS-MS, the comparison of the ratio of these two MRM peak areas (1:0.81) with that of standard (1:0.79), and coelution during LC-MS-MS of the unknown with a standard in spiked samples.

Quantitative analyses of compounds **I**−**III** were carried out using HPLC with absorbance detection at 254 nm, and the results are summarized in **Table 1**. The limit of detection of this assay was 1 ng injected on-column. Linear regression analyses of the standard curves for each compound over the range of 2−1000 ng showed correlation coefficients of >0.999. The concentrations of *p*-hydroxybenzoic acid methyl ester (**I**) in the reference plant materials F−I were <0.0001, 0.0008, <0.0001, and 0.0008%, respectively. The levels of compounds **I** and **II** in commercial extract A were 0.217 and 0.0037%, respectively. In commercial extract B, the levels of compounds **I**−**III** were 0.004, 0.112, and 0.197%, respectively. The concentrations of compound **I** in all of the commercial extracts were much higher than in any of the reference plant samples. For example, the level of compound **I** in commercial extract A was 270-fold greater than that in plant sample G or I (**Table 1**).

To determine the presence of compounds **I**−**III** in commercially available formulated products, three brands of *A. paniculata* tablets were obtained from local pharmacies and analyzed using HPLC-PDA and LC-MS-MS. Again, care was exercised to prevent cross-contamination or sample carry-over. As an example, the LC-MS-MS chromatograms of an extract of tablet E are shown in **Figure 5**. Compounds **I** and **II** were detected in all three products, but propylparaben (**III**) was not detected in any of the tablets. The concentration of *p*-hydroxybenzoic acid methyl ester (**I**) in tablet D was similar to the baseline level in some of the authenticated specimens of *A.*

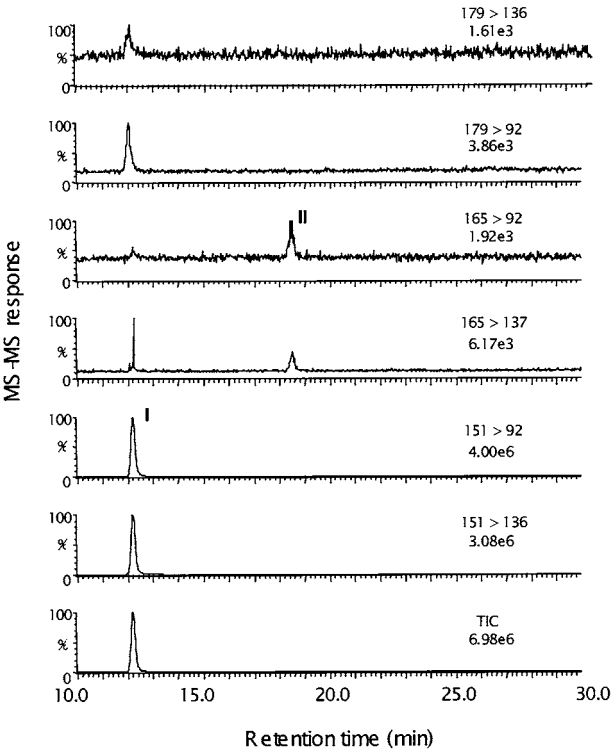

Figure 5. LC-MS-MS MRM chromatogram of a methanolic extract of REMDEX *A. paniculata* tablet.

paniculata, but the level of *p*-hydroxybenzoic acid ethyl ester (**II**) in these tablets was the highest among those tested. The identification of compounds **I** and **II** in these products was based on coelution with standards in spiked samples, the simultaneous appearance of two characteristic MRM signals during LC-MS-MS, and the ratios of the MRM peak areas for unknowns and standards (1:0.79 and 1:0.68, respectively). Together, these data confirm the presence of compounds **I** and **II** in these products.

CONCLUSIONS

To the best of our knowledge, this is the first report of the presence of *p*-hydroxybenzoic acid methyl ester (**I**) in authenticated plant specimens of *A. paniculata*. However, the low concentrations of **I** in this plant do not explain the much higher levels measured in commercial extracts and formulated tablets. The detection of ethyl and propyl esters of *p*-hydroxybenzoic acid (**II** and **III**) in the commercial extracts and the detection of **II** in formulated tablets but not in the authenticated plant samples indicated that these compounds are conceivably additives to the commercial products and not natural products.

An examination of pharmaceutical texts as well as a search of the literature indicates that methyl-, ethyl-, and propylparabens (compounds **I**, **II**, and **III**, respectively) are known preservatives (*16*) frequently found in pharmaceutical injections (*17*) and oral formulations (*19*), processed foods (*18*), fruit juices (*21*), cosmetic products (*20*), and herbal products (*22*). Thus, it is conceivable that the producers of the processed *A. paniculata* extracts added one or more of these *p*-hydroxybenzoic acid esters (parabens) as preservatives. If this is indeed the case, then these products should have been so labeled. Alternatively, this might be a case of contamination or cross-contamination and an example of poor quality control of botanical dietary supplements. In any case, it should be noted that in addition to antimicrobial properties, methylparaben (**I**) has been reported to possess moderate estrogenic (*23*) and smooth muscle relaxant effects (*24*). Therefore, the concentrations of compounds **I**−**III** in other botanical dietary supplements warrant further investigation.

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