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Determination of Allantoin in Dioscorea Rhizoma by High Performance Liquid Chromatography Using Cyano Columns

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Abstract – An easy and reliable HPLC method was developed to determine allantoin in Dioscorea Rhizoma using cyano columns. Qualitative and quantitative analyses of allantoin were performed successfully by cyano columns (YMC-Pack CN column, Zorbax SB-CN column and Discovery® Cyano column). The intraday precision were 0.58 - 3.33% for YMC-Pack CN, 0.41 - 2.20 for Zorbax SB-CN and 0.45 - 1.93% Discovery® Cyano columns, while interday variations were 0.09 - 1.84%, 0.04 - 2.59% and 0.87 - 5.18% for YMC-Pack CN, Zorbax SB-CN and Discovery® Cyano columns. The recoveries of allantoin were in the range at 98.8 - 102.6% (RSD 1.1 - 1.6%) for YMC-Pack CN column, 99.7 - 110.5% (RSD 1.3 - 4.9%) for Zorbax SB-CN column, and 97.2 - 110.1% (RSD 1.8 - 5.7%) for Discovery® Cyano column. The contents of allantoin in four Dioscorea Rhizoma samples were determined by cyano columms and ranged at 4.1-7.1 mg/g dry weight. The present study indicated that HPLC method using cyano column for determining allantoin is a reliable method and this method can be applied to verify allantoin in Dioscorea Rhizoma.
Keywords – Dioscorea Rhizoma, allantoin, HPLC method, cyano column.

Introduction

Dioscorea Rhizoma is a herb which has been widely used as an edible food and as a traditional medicine in the world. It has been used for stimulating the stomach and spleen against poor appetite and has a tonic effect on the lungs and kidneys (Bae, 1999; Frombi *et al*., 2000). Lots of biological activities of Dioscorea Rhizoma have been reported for anti-oxidative (Chang *et al*., 2004), anti-fungal (Sautour *et al*., 2004), anti-mutagenic (Miyazawa *et al*., 1996), hypoglycemic (McAnuff *et al*., 2002), immunomodulaory effect (Choi *et al*., 2004), and it is used as an important ingredient of dietary supplements and cosmetics in pharmaceutical industry now days.

Many phytochemical studies revealed that purine derivatives, saponins, starches, mucilage are present as main constituents in Dioscorea Rhizoma, and allantoin, one of purine derivatives, is well-known biologically active compound in *Dioscorea* species (Fu *et al*., 2006; Fu *et al*., 2005; Zhang *et al*., 2004).

Allantoin is a common constituent of plants such as legumes and coffee (Matsumoto *et al*., 1977; Mazzafera *et al*., 1999). The role of allantoin has been suggested that it is present as a nitrogen storage form in plants or as a product in the detoxification process of ammonia in plant tissues (Siegfried *et al*., 1975), and it has been demonstrated that *Dioscorea* species contain higher level of allantoin content than any other plants (Fu *et al*., 2006). Therefore, allantoin could be a good standard substance for the quality control of Dioscorea Rhizoma because of their pharmacological activities and abundance in *Dioscorea* spp. So far, there have been many reports to determine allantoin in biofluid with HPLC, LC-MS/MS, GC-MS (Berthemy *et al*., 1999; Czauderna *et al*., 2000; George *et al*., 2006; Shingfield *et al*., 1998; Terzuoli *et al*., 1995), and a capilliary electrophresis method has been published for measuring allantoin content in Dioscorea Rhizoma (Zhang *et al*., 2004). But these techniques require somewhat complex process including time and labor consuming sample preparation and adjusting buffer condition. The present study describes a simple and reliable HPLC method using cyano column in order to determine allantoin in Dioscorea Rhizoma.

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Experimental

Instrumentation and chromatographic condition – HPLC analysis was performed by Gilson HPLC system

(Gilson, USA) equipped with two pumps (321 pump), UV/Vis detector (UV/VIS-151) and autosampler (234 autoinjector). Data acquisition and analysis were carried out by Gilson Unipoint software. The columns used in this study were YMC-Pack CN (250 × 4.6 mm ID, 5 μm, YMC, Japan), Zorbax SB-CN (250 × 4.6 mm ID, 5 μm, Agilent, USA), Discovery® Cyano (250 × 4.6 mm ID, 5 μm, Supelco, USA), J'sphere ODS H80 (250 × 4.6 mm ID, 5 μm, YMC, Japan), YMC-Pack Pro C18 RS (250 × 4.6 mm ID, 5 μm, YMC, USA) and Inertsil ODS-3 (250 × 4.6 mm ID, 5 μm, GL Science, USA). The chromatographic conditions were used as follows; UV wavelength: 215 nm; mobile phase: MeCN/H_2O (2 : 98); flow rate: 0.2 mL/min in 1 - 5 min, 0.5 mL/min in 5 - 15 min, 0.2 mL/min in 15 - 20 min. Injection volumes of all samples were 20 μL. Water and acetonitril used for HPLC analysis were HPLC grade and purchased from Fisher Scientific Korea Ltd. (Seoul, Korea).

Plant materials – Dioscorea Rhizoma samples were collected from different regions in Korea, and identified by Prof. Gwang Jin Chang, the Korea National Agricultural College. All samples were freeze-dried and pulverized to give fine powder. The samples were stored in the freezer at –60 °C until being used.

Sample preparation – This study was carried out according to the method established by Fu *et al*. (2006) with modification. Two grams of freeze-dried powder were suspended in 5 mL of distilled water. After addition of ten times amount of 95% ethanol, the mixture was extracted by ultrasonication for 30 min at room temperature, and stored at 4 °C for overnight. The supernatant evaporated under dryness. Ten mL of distilled water was added and ultrasonicated for 15 min, and one mL was taken and centrifuged at 4 °C at 7700 g for 15 min. The solution was diluted ten times with distilled water and subjected to HPLC to determine the content of allantoin. Authentic samples of allantoin was dissolved in distilled water (1 mg/mL) and diluted to desired concentration in the range of 5 - 125 μg/mL and kept at 4 °C until being used. Allantoin and allantoic acid were purchased from Sigma (St. Louis, USA). All samples of allanton, allantoic acid and Dioscorea Rhizoma extracts were filtered through 0.22 μm millipore filter before injection.

Calibration – Sequential standard solutions of allantoin (5.0 μg/mL, 12.5 μg/mL, 50.0 μg/mL and 125.0 μg/mL) were tested to obtain the linearity of calibration curve. Each standard solution was injected five times consecutively. The calibration curves were achieved by plotting the ratio of peak area versus concentration (μg/mL). The linearity was obtained by linear regression analysis calculated by the least square regression. The limit of detection (LOD) was calculated as signal-to-noise (S/N) ratio of 3, while the limit of quantification (LOQ) was defined as S/N ratio of 10 for each column.

Precision and accuracy – The precision of this HPLC method was obtained by intra- and inter-day variations. The intraday precision was evaluated by analyzing the results of five consecutive injections of sequential standard solutions of allantoin (5 - 125 μg/mL) as described above, while inter-day variation was performed by five consecutive injections of sequential standard solutions of allantoin on different five days. The precisions were expressed by the calculation of relative standard deviations [RSD (%) = (SD/mean) × 100%]. The accuracy of the HPLC method was achieved by the performing recovery test. Two concentration levels (10 and 20 μg/mL) of allantoin were spiked directly to the methanol extract of Dioscorea Rhizoma samples and analyzed as described above. The recoveries were calculated as: Recovery (%) = $(C_1 - C_0) \times 100 / AC$, where C_0 and C_1 are the measurements before and after addition of allantoin standard, respectively, and AC is the amount of added allantoin.

Results and Discussion

Column selection – Allantoin showed little or no retention, due to the extreme polar nature, in the conventional reversed-phase C18 column. This phenomenon resulted in difficulties to separate allantoin from other polar constituents. Thus, many researchers determined allantoin and other purine derivatives with derivatization method. But this technique had disadvantage of time and labor consuming in derivatization process. Therefore, a simple, selective and accurate analytical method was needed to separate of allantoin in Dioscorea Rhizoma. Six HPLC columns were tested in order to select the optimal separation condition. Three of them were cyano columns, and the rest of them were reversed-phase C18 columns. Good retentions of allantoin were obtained with cyano columns. The retention times of allantoin were in the range of 9.8 - 10.8 min in three cyano columns, and three conventional reversed-phase C18 columns showed shorter retention times (5 - 7 min). When they were applied to plant samples, allantoin in all Dioscorea Rhizoma samples was successfully separated from other polar impurites in cyano columns (Fig. 1 - 3), but not in C18 columns (data not shown). These results indicated that cyano columns were more effective than C18 columns to determine allantoin.

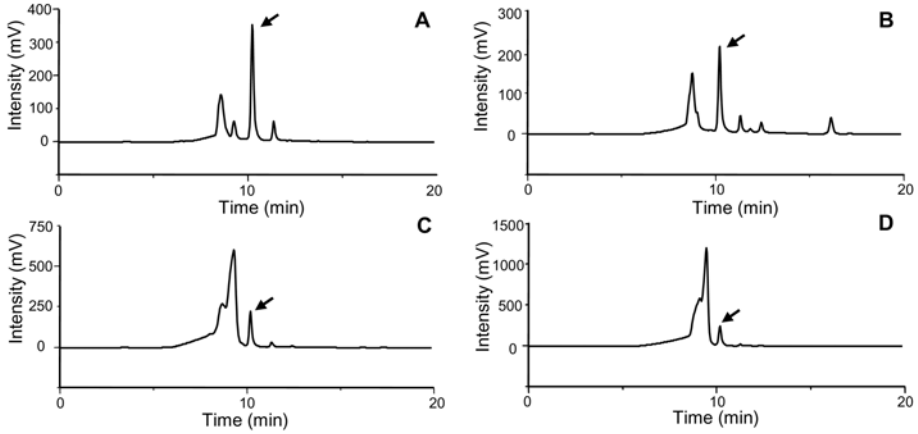

Fig. 1. HPLC chromatograms of Dioscorea Rhizoma samples on YMC-Pack CN column. Samples were (A) *D. opposita* Thunb. (Ham-An), (B) *D. opposita* Thunb. (Chung-Ju), (C) *D. batatas* Decne. (Jin-Ju) and (D) *D. japonica* Thunb. (Jin-Ju).

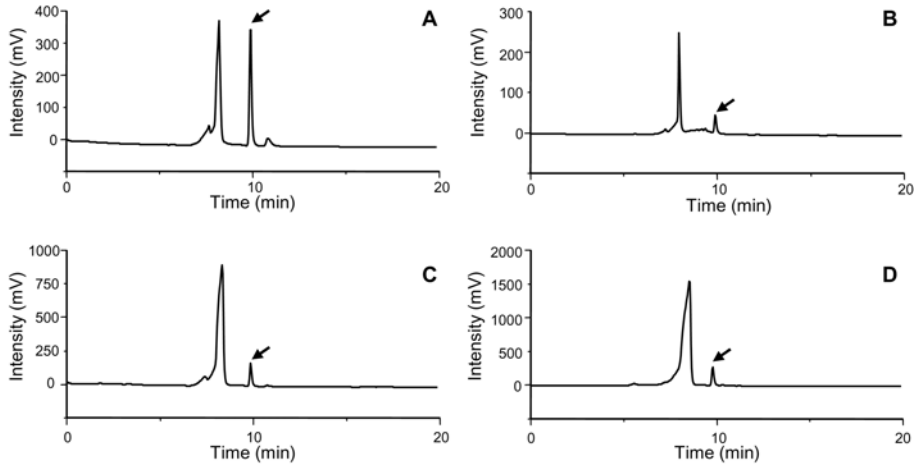

Fig. 2. HPLC chromatograms of Dioscorea Rhizoma samples on Zorbax SB-CN column. Samples were (A) *D. opposita* Thunb. (Ham-An), (B) *D. opposita* Thunb. (Chung-Ju), (C) *D. batatas* Decne. (Jin-Ju) and (D) *D. japonica* Thunb. (Jin-Ju).

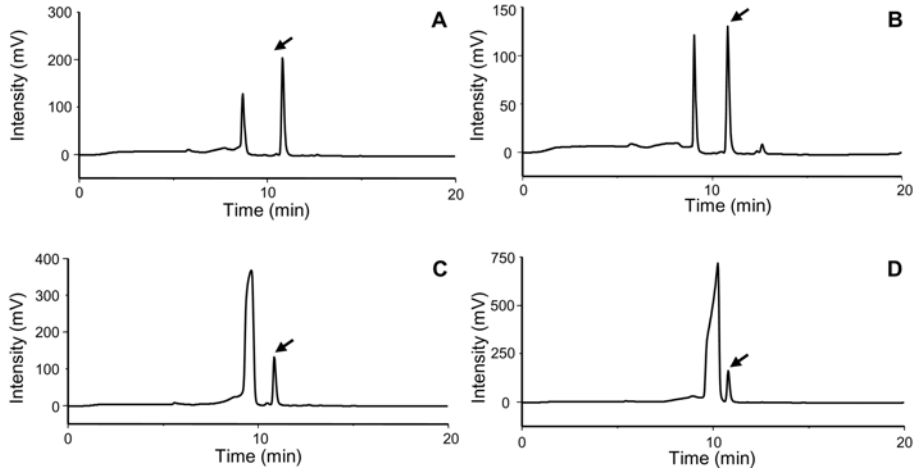

Fig. 3. HPLC chromatograms of Dioscorea Rhizoma samples on Supelco® Cyano column. Samples were (A) *D. opposita* Thunb. (Ham-An), (B) *D. opposita* Thunb. (Chung-Ju), (C) *D. batatas* Decne. (Jin-Ju) and (D) *D. japonica* Thunb. (Jin-Ju).

Table 1. Results of intra- and Interday variations

Columns	TC[a]	EC[b]	RSD[c]	A[d]
Intraday (n = 5)				
YMC-Pack CN	5.0	4.91	3.33	98.2
	12.5	12.41	1.23	99.3
	50.0	50.28	1.00	100.6
	125.0	124.90	0.58	99.9
Zorbax SB-CN	5.0	5.1	2.20	102.7
	12.5	12.5	1.49	99.8
	50.0	49.8	0.90	99.7
	125.0	125.1	0.41	100.1
Discovery® Cyano	5.0	5.20	1.93	103.9
	12.5	12.39	0.61	99.1
	50.0	49.85	0.80	99.7
	125.0	125.06	0.45	100.1
Interday (n = 5)				
YMC-Pack CN	5.0	4.82	1.84	96.3
	12.5	12.36	1.34	98.8
	50.0	50.53	0.67	101.1
	125.0	124.8	0.09	99.8
Zorbax SB-CN	5.0	5.2	2.59	103.7
	12.5	12.3	1.33	98.3
	50.0	50.0	0.26	100.0
	125.0	125.1	0.04	100.1
Discovery® Cyano	5.0	5.2	5.18	109.9
	12.5	12.5	0.65	99.4
	50.0	49.4	0.94	101.6
	125.0	125.7	0.87	98.5

[a]Theoletical concentration of allantoin (μg/mL); [b]Experimental concentration of allantoin (μg/mL); [c]Relative standard deviation = SD/mean × 100 (%); [d]Accuracy (%) = AC/EC × 100 (%)

Calibration – Good linearity was achieved in the range from 5.0 to 125.0 μg/mL for allantoin with cyano columns. The regression equation was $y = 25078x + 17710$, where y is the peak area, and x (μg /mL) is the amount of allantoin, and correlation coefficient was 0.9999 for YMC-Pack CN column. Zorbax SB-CN and Discovery® Cyano column showed regression equations as $y = 22481x + 10502$ and $y = 24981x + 32479$, and correlation coefficients were 1.0 and 0.9999, respectively. The LOD and LOQ in this method were 1.5 - 2.0 μg/mL and 5.0 μg/mL, respectively.

Precision and accuracy – The intraday precision were 0.58 - 3.33% for YMC-Pack CN, 0.41-2.20 for Zorbax SB-CN and 0.45-1.93% Discovery® Cyano column, while interday variations were 0.09-1.84%, 0.04-2.59% and 0.87-5.18% for YMC-Pack CN, Zorbax SB-CN and Discovery® Cyano column (Table 1). The recoveries of allantoin were in the range at 98.8 - 102.6% (RSD 1.1 - 1.6%) for YMC-Pack CN column, 99.7 - 110.5% (RSD 1.3 - 4.9%) for Zorbax SB-CN column, and 97.2 - 110.1% (RSD 1.8 - 5.7%) for Discovery® Cyano column (Table 2). These results demonstrated that analysis of allantoin using cyano column is a reliable method.

Table 2. Recovery of allantoin standard added to Dioscorea Rhizoma samples (n = 5)

Columns	Samples (Origin)	AC[a]	EC[b]	R[c]	RSD[d]
YMC-Pack CN	*D. opposita* (Ham-An)	10.00	10.15	101.5	1.81
		20.00	19.88	99.4	2.55
	D. opposita (Chung-Ju)	10.00	10.26	102.6	1.91
		20.00	20.28	101.4	1.32
	D. batatas (Jin-Ju)	10.00	9.91	99.1	1.71
		20.00	19.98	99.9	1.25
	D. japonica (Jin-Ju)	10.00	9.88	98.8	1.05
		20.00	19.82	99.1	2.60
Zorbax SB-CN	*D. opposite* (Ham-An)	10.00	10.13	101.3	1.32
		20.00	20.68	103.4	2.22
	D. opposita (Chung-Ju)	10.00	10.28	102.8	2.45
		20.00	20.14	100.7	1.22
	D. batatas (Jin-Ju)	10.00	11.05	110.5	4.03
		20.00	21.76	108.8	1.85
	D. japonica (Jin-Ju)	10.00	9.97	99.7	4.88
		20.00	20.04	100.2	1.62
Discovery® Cyano	*D. opposite* (Ham-An)	10.00	9.96	99.6	1.80
		20.00	19.32	96.6	2.08
	D. opposita (Chung-Ju)	10.00	10.16	101.6	5.71
		20.00	22.02	110.1	2.27
	D. batatas (Jin-Ju)	10.00	9.72	97.2	3.55
		20.00	19.92	99.6	2.13
	D. japonica (Jin-Ju)	10.00	10.53	105.3	3.87
		20.00	20.26	101.3	1.85

[a]Amount of added allantoin (μg/mL); [b]Experimental concentration of allantoin (μg/mL); [c]Recovery = $(C_1 - C_0) \times 100 / AC$ (%), where C_0 and C_1 are the measurements before and after addition of allantoin standard; [d]Relative standard deviation = SD / mean × 100 (%)

Allantoin and allantoic acid determination – It has been reported that allantoin is converted to allantoic acid by mild alkaline hydrolysis and then allantoic acid is converted to urea and glyoxylic acid by acid hydrolysis (Fu *et al*., 2006). Furthermore, the allantoin changed to its more stable form, allantoic acid, during storage time, which causes errors to determine allantoin contents in Dioscorea Rhizoma. In order to confirm whether or not allantoic acid affect allantoin determination, standard samples of allantoin and allantoic acid were subjected to three cyano columns and three reversed-phase C18 columns. The conventional C18 columns could not

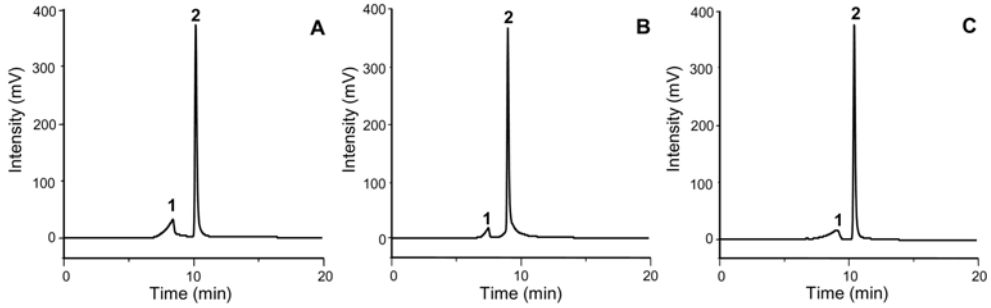

Fig. 4. HPLC Chromatograms of standard mixture (**1**: allantoic acid, **2**: allantoin). Columns: (A) YMC-Pack CN; (B) Zorbax SB-CN; (C) Supelco® Cyano.

Table 3. Amounts of allantoin in Dioscorea Rhizoma samples (n = 5)

Columns	Samples (Origin)	Allantoin contents (mg/g dry wt.)
YMC-Pack CN	*D. opposita* (Ham-An)	6.9 ± 0.13
	D. opposita (Chung-Ju)	4.1 ± 0.07
	D. batatas (Jin-Ju)	6.2 ± 0.10
	D. japonica (Jin-Ju)	4.5 ± 0.07
Zorbax SB-CN	*D. opposita* (Ham-An)	7.0 ± 0.10
	D. opposita (Chung-Ju)	4.0 ± 0.09
	D. batatas (Jin-Ju)	6.4 ± 0.07
	D. japonica (Jin-Ju)	4.5 ± 0.09
Discovery® Cyano	*D. opposita* (Ham-An)	7.1 ± 0.12
	D. opposita (Chung-Ju)	4.1 ± 0.06
	D. batatas (Jin-Ju)	6.4 ± 0.04
	D. japonica (Jin-Ju)	4.4 ± 0.03

separate allantoin and allantoic acid (data not shown), but three cyano columns separate perfectly these two compounds (Fig. 4). The retention times of allantoic acid and allantoin were 8.4 and 10.3 min for YMC-Pack CN column, 7.5 and 9.8 min for Zorbax SB-CN column, 9.1 and 10.8 min for Discovery® Cyano column. These results indicated that allantoic acid did not interfere with determination of allantoin in this method.

Quantitative analysis of allantoin in Dioscorea Rhizoma – Quantitative analysis of allantoin in Dioscorea Rhizoma was performed by aforementioned HPLC method. Dioscorea Rhizoma samples were collected or purchased from the different regions in Korea, and the amounts of allantoin in four Dioscorea Rhizoma samples were ranged from 4.1 to 7.1 mg/g dry weight (Table 3).

Conclusion

Dioscorea Rhizoma has been used for numerous pharmaceutical preparations in Korea. The present study showed a simple, precise and accurate method for the determination of allantoin, and this method could be applied to determine allantoin in Dioscorea Rhizoma and various pharmaceutical preparations possessing Dioscorea Rhizoma.

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