Analysis method of Crude drug extract by HPLC

Plant materials

Aerial parts and roots of Crude drug extact for eg. A. racemosus and A. curillus were collected in four different seasons e.g. April, June, August and October 2008, from six different altitudes such as Mandal, cultivated (2000 m), Mandal, wild (2200 m), Kasturivihar, cultivated (2500 m), Pokhari, wild (2200 m), Khirshu, wild (1800 m) and Ukhimatha, wild (1500 m) of Uttarakhand, India.

Chemicals and reagent

Acetonitrile (HPLC grade), Deionised water, Sarsasapogenin was isolated from A. officinalis which was characterized previously and was derivatized to acetyl sarsasapogenin.

Preparation of standard

250 mg shatavarin-IV (sarsasapogenin triglycoside) was refluxed with 50 ml 2N HCl-MeOH (1:1) on water bath for 5 h, neutralized with Ag_2O, filtered. Filtrate was concentrated in vacuo, diluted with water and extracted with ethylacetate to afford the aglycone (sarsasapogenin) structure of which was confirmed by Co-TLC, mp and IR with standard [mp 200˚C, IR 3400 (OH) 2925 (C-H) 1150 (CO)]. Acetyl derivative of sarsasapogenin was prepared by the reaction of sarsasapogenin and acetic anhydride in equimolar ratio in the presence of pyridine, the completion of reaction was monitored by TLC. The product was purified (as it had slight impurity of the parent) by radial chromatography (chromatotron) containing 2 mm thick Silica gel, TLC standard grade Silica gel G with fluorescent indicator as the stationary phase and hexane: ethylacetate (9:1) as the mobile phase. Sarsasapogenin acetate was crystallized in hexane mp 144-145˚C, IR (cm-) 2929, 2850, 1739, 1460 and 1262; [IR (cm-) 2937, 2919, 2862, 1737, 1467, 1448 and 1227 Lit.]

Preparation of samples

All dried plant materials were extracted through Soxhlet in methanol. The -OH group of sarsasapogenin is converted into -OCOCH3 by the reaction of dried extracts (5 g) with acetic anhydride (0.025 ml) and pyridine (0.201 ml) for six hours at 40°C and the crude extract was dried by rotary evaporator. 10 mg dry acetylated extract was dissolved in 10 ml extraction solvent to get 1 mg/ml solution, filtered through 0.25 µm syringe filter and injected to Waters HPLC system.

RP-HPLC conditions

HPLC analysis was carried out on injection valve with a 20 µl loop, a 486 UV variable wavelength detector (set at 210 nm) sensitivity was 0.001, 5 µm C8 (150 × 3.9 mm i.d.) column. The analytes were eluted isocratically at a flow rate of 0.5 ml/min. Chromatograms were generated on software. The HPLC instrument was operated at room temperature (23 ± 2°C). Each diluted extract 20 µl was injected in to the HPLC three times and the average peak area was reported and used for quantification.

Calibration

The standard solutions of acetyl sarsasapogenin containing 1 to 100 µg/ml were prepared and injected in triplicate into the HPLC reverse phase column, calibration curves were constructed and their linear ranges determined. Calibration curves were plotted by the peak area against concentration of each analytes. The linearity was evaluated by linear regression analysis. The calibration curve was in good linear correlation with correlation coefficient 0.9997. The limit of detection (LOD), defined as the lowest detectable amount of analyte was calculated by using the formula, LOD = (b+3σb)/a where a is the slope of the calibration curve, b is intercept and σb is the standard deviation associated with the intercept.

Validation

Standard stock solution (100 µg/ml) was diluted with extraction solvent (water: acetonitrile) to give different concentrations (1, 5, 10, 15, 20 ppm). Intra day (injecting each concentration three times within 24 h) and inter day analysis (injecting each concentration three times over 3 days with each injection separated by at least 24 h) were run to check reproducibility. The standard deviation (SD) and relative standard deviation (RSD) were calculated shown in Table 1. Recovery test was used to evaluate the accuracy of the method. Accurate amounts of acetylsarsasapogenin was added to 10 mg MeOH extract of Asparagus species and then analysed as samples. The percentage recoveries were determined by the equation [(C3-C2)/C1] ×100 where C1 represents the amounts of standard spiked, C2 represents the original amount of marker in extracts and C3 represents the total (observed) amount of marker in extracts. RSD was determined by the formula, % RSD = (SD/mean) × 100.

Table 1. Linearity study of acetyl sarsasapogenin.

	Calibration curve equation	Slope	Intercept	R^2	LOD µg	RT (RSD %)	PA (RSD %)
	Y=23092x+40362	23092	40362	0.9997			
Intra day	Y=23722x+42344	23722	42344	0.9997	2.026	0.3984	1.5071
	Y=23456x+44492	23456	44492	0.9997			
	Y=24315x+34548	24315	34548	0.9998			
Inter day	Y=24317x+42365	24317	42365	0.9995	1.995	0.9821	0.8761
	Y=23649x+34923	23649	34923	0.9997			

RT=Retention time, PA= Peak area and LOD= Limit of detection.

Table 2. Concentration (in %) of sarsasapogenin in A. racemosus and A. curillus

Samples	Collection (April)	Collection (June)	Collection (August)	IV Collection (October)
ArMD (Roots, Cultivated)	1.33	0.62	0.21	0.61
ArMD (Leaves, Cultivated)	0.31	0.36	0.35	0.22
Ar KV (Roots, Cultivated)	1.62	0.19	0.93	1.43
Ar KV (Leaves, Cultivated)	0.54	0.58	0.31	0.42
ArNP (Roots, Wild)	2.96	0.32	0.47	0.44
ArNP (Leaves, Wild)	1.48	2.55	0.67	0.1
ArUM (Roots, Wild)	1.83	2.11	0.37	0.5
ArUM (Leaves, Wild)	1.87	0.84	0.57	0.36

AcKS (Roots, Wild)	1.53	0.39	0.42	1.27
AcKS (Leaves, Wild)	0.56	0.85	0.83	0.98
AcNP (Roots, Wild)	2.55	3.72	0.57	1.3
AcNP (Leaves, Wild)	0.91	0.21	0.84	0.83
AcMD (Roots, Wild)	2.06	0.39	0.68	3.7
AcMD (Leaves, Wild)	0.51	0.38	0.68	0.84

RESULTS AND DISCUSSION

Chromatographic behaviour of acetylated sapogenin of Asparagus species in extracts was analyzed by HPLC-UV on C8 stationary phase using water: acetonitrile in 30: 70(v/v) ratios as mobile phase in isocratic mode. The purpose of derivatization of sarsasapogenin to acetyl sarsasapogenin was to overcome the detection problem. Several preliminary experiments were conducted to develop RP-HPLC method for the separation of derivatized sapogenin in Asparagus species by adjusting water, acetonitrile and λmax to obtain better resolution and detection. Chromatograms were achieved within 15 min for standard and 30 min for Asparagus roots and leaves extracts by use of mobile phase water: acetonitrile (30:70 v/v) at 210 nm. Identification was confirmed by comparison of retention time and quantification by linear regression equation (y=mx+c). Derivatized sarsasapogenin was eluted at 5.2 min. Data obtained on the concentration of bioactive is shown in Table 2. The chromatograms of standard, A. racemosus and A. curillus are shown in Figure 2.

The LOD obtained for analyte was 2.02 ppm for intra day and 1.99 ppm for inter day analysis. RP-HPLC method was validated by determining relative standard deviation (RSD) of inter and intra day analysis. The RSD value for retention time and peak areas were 0.398 and 1.50 and 0.982 and 0.876 for intra day and inter day analysis, respectively (Table 1). The recovery test was used to evaluate the accuracy of the developed method. The average recovery % is greater than 98% and concentration of acetyl sarsasapogenin was in the range of 0.1 to 3.72% in Asparagus species (Table 1). The concentration of sarsasapogenin varies from site to site and season to season. In the samples studied, roots had higher ercentage of bioactive than leaves. Maximum concentration was found in A. curillus (wild) rhizomes collected from Nagnath-Pokhari (2200 m). Highest concentrations of sarsasapogenin in most of the Asparagus samples were found in those samples which are collected from Mandal (2000 to 2200 m) in June, which seems to be appropriate altitude for cultivation and proper harvesting period is June.

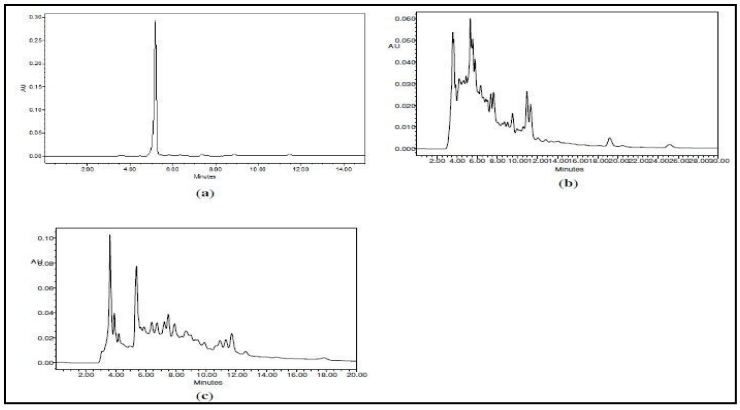

Figure 1. Chromatograms of (a) Standard, (b) A. racemosus and (c) A. curillus root extract.