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Taxol content of various *Taxus* species in Hungary[1]

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Abstract

The anticancer drug taxol was separated and quantitatively determined in bark and foliage of different *Taxus* species by high-performance liquid chromatography to prove the presence of taxol in Hungarian *Taxus* species. The measurements were carried out with photodiode array detection using a porous graphitized carbon column, and a water:dioxan 54:46 v/v eluent. Taxol was established as being present in measurable amounts in each Hungarian *Taxus* species. According to the results bark was richer in taxol than foliage. It could also be observed that the older the bark or foliage, the more taxol it contained. The validation process proved that the method is reliable and can be used for the separation and quantitative determination of taxol in both the bark and foliage of *Taxus* species grown in Hungary.

Keywords: Taxol content; Hungarian *Taxus* species; Porous graphitized carbon column

1. Introduction

Taxol is a highly functionalized taxane diterpene amide, which occurs as a minor component in various species of *Taxus*. In recent years taxol has become one of the most important natural products. Isolation of taxol can cause problems; for example, to obtain taxol from bark (the bark of *Taxus brevifolia*) is the main source of taxol nowadays), a large number of trees must be felled. Taxol was first isolated in 1971 by Wall and his collaborators, and was shown to possess antileukemic and tumor inhibitory properties. It was the first compound with a taxane ring which was shown to have such activity [1–3]. It is effective against a variety of cancers, such as resistant ovarian cancer and breast cancer. Taxol has been shown to promote tubulin polymerization and stabilize microtubules against depolymerization. It promotes microtubule assembly, and microtubules complexed with taxol appear to be stable against cold or calcium disassembly [4,5]. Taxol prevents cell division without affecting DNA, RNA, or protein syntheses. In spite of its prominent properties, progress on developing taxol as a drug was slow, mainly because of the difficulty of isolating it from the trees of the *Taxus* species, and because of its lipophilicity, which made formulation difficult [6–9].

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Many efforts have been devoted to the development of chromatographic methods suitable for the separation and quantitative determination of taxol in the extracts of *Taxus* species. Due to its low separation power thin layer chromatography has not been frequently used [10]. High-performance liquid chromatography (HPLC) has been extensively used for the determination of taxol using mainly C_{18} [11] and phenyl-bonded silica [12,13] supports. Porous graphitized carbon (PGC) is a non-polar adsorbent which can be used in both the normal and reversed-phase modes and is stable across the whole pH range from 0–14. It possesses a rigid, planar surface, which is capable of dispersion and charge-transfer interactions [14,15]. The planar surface of the PGC allows special stereoselectivity [16]. The retention on a carbon phase seems to be determined by how much contact is possible between a solute and the carbon surface [17,18].

Validation is a prerequisite of any reliable chromatographic analysis [19]. Many chromatographic parameters have been proposed for inclusion in the validation process, such as linearity of the calibration curve [20], sensitivity and selectivity of the detection of the solute to be analyzed, inter-day and intra-day reproducibility [21], instrument precision [22], etc.

The aim of this study was to determine the taxol content of various *Taxus* species in Hungary and to validate the HPLC method used for the separation and quantitative determination of taxol in the foliage and bark of various *Taxus* species.

2. Experimental

Measurements were carried out on a PGC column (Shandon Hypercarb 100 × 4.6 mm i.d., particle size 7 μm; Shandon Scientific Ltd., Cheshire, UK). The HPLC equipment consisted of an ISCO model 2350 HPLC pump and an ISCO model 2360 gradient programmer (Isco Inc., Lincoln), a Rheodyne injector with a 20 μl sample loop (Rheodyne Inc., Cotati), a Waters 991 photodiode array detector (Waters, Milford, Ma), an NEC Power Mate SX/16 computer (NEC Technologies, Inc., Boxborough, USA) and a Waters 5200 printer plotter. The detection wavelength range was 225–399 nm; the calibration curve and qualitative evaluation were carried out at 228 nm, the absorption maximum of taxol. The flow rate was 0.8 ml min^{-1}. The peak purity test of taxol was carried out on each sample at various wavelengths. The measurements were carried out in reversed phase separation mode with isocratic elution, where the eluent was a mixture of 1,4-dioxan and water (46:54 v/v). The experiments were carried out at room temperature (22–24°C). Each solvent was of HPLC-grade purity. A taxol standard (from *Taxus brevifolia*, min. purity 95%) was purchased from Sigma Chemie GmbH (Deisenhofen, Germany) and was used without further purification. Each determination was run in quadruplicate.

The name and source of *Taxus* species under investigation are listed in Table 1 and Table 2. Sample preparation was carried out by mixing a ground sample (400 mg) with methanol (30 ml) and stirring for 1 h. The extract was filtered and methylene chloride (50 ml) and water (80 ml) were then added to the sample in a separatory funnel. The methanol–water phase was extracted with three volumes of methylene chloride (50 ml each) and these extracts were evaporated to apparent dryness using a rotary evaporator. The sample was redissolved in 8 ml of methylene chloride and dried again under an N_2 atmosphere. For the HPLC analysis the sample was redissolved in 200 μl of methanol.

Linear regression analysis was used to determine the relationship between the taxol peak area (y) and the concentration of taxol injected (x):

$$y = a + bx \quad (1)$$

Data for the linear regression were obtained by measuring standards of five different concentrations of taxol between 0.5 and 0.005 μg μl^{-1} and four parallel determinations were made at each concentration. The recovery of the extraction method was determined by adding taxol standards to the samples of bark and foliage and by carrying out the determination of the taxol content as described above. The inter-day and intra-day reproducibility of the method were elucidated

Table 1
Taxol content in the foliage of various *Taxus* species grown in Hungary

Taxus species	Age (years)	Source	Taxol content (mg g^{-1})	
			Mean	RSD (%)
Taxus hunevelliata	1	I[a]	0.032	5.24
Taxus × *media*	1	I	0.036	5.88
Taxus cuspidata	1	II[b]	0.037	4.86
Taxus baccata "a" sign male clone	1	I	0.027	4.57
Taxus baccata "a" sign male clone	2	I	0.041	5.04
Taxus b. "Aurea"	1	I	0.040	4.59
Taxus canadensis	1	II	0.095	4.21
Taxus b. "Aurea"	1	II	0.053	5.33
Taxus × *media "Hicksii"*	1	I	0.056	4.42
Taxus b. "Fastigiata"	1	II	0.027	5.39
Taxus b. "Dovastoniana"	1	II	0.071	4.48
Taxus baccata basic species	1	III[c]	0.146	3.77
Taxus b. "Aurea"	1	IV[d]	0.025	4.76
Taxus b. "Fastigiata"	1	V[e]	0.100	5.20
Toreya californica (male)	1	III	0.027	4.16
Toreya californica (female) "e" sign clone	1	III	0.031	4.51
Taxus baccata (female)	1	III	0.029	5.03
Taxus b. "Elegentissima"	1	II	0.029	4.57

[a] I = Horticultural University, Central Plantation, Budapest
[b] II = Horticultural University, Arboretum
[c] III = Erdötelek, Hungary
[d] IV = Szombathely, Hungary
[e] V = Tahi, Hungary

by injecting the same extract four times on three consecutive days and calculating the mean and standrd deviation of the measurements. The differences between the mean value and the standard deviation was calculated by the "*t*" and "*F*" probes respectively.

3. Results and discussion

Taxol has been well separated from the impurities in the extracts of both bark and foliage for each *Taxus* species grown in Hungary, proving the good separation capacity of the PGC column (Fig. 1). Each sample contained taxol; however, the taxol content varied considerably. The peak purity test indicated that the taxol peak is not entirely homogeneous. This result can be explained by the supposition that other taxol derivatives (taxoteres) present in the extracts co-elute with taxol and are detected by the peak purity test differed slightly according to the wavelength used, indicating the different absorption capacities of taxomers. The foliage extracts contained in each case more impurities than the bark extracts, indicating that taxol can be purified more easily from the bark than from the foliage of *Taxus* species. However, it has to be borne in mind that foliage represents a renewable source of taxol whereas the use of bark is limited by the number of trees. The taxol contents of the samples calculated from the calibration curve, and taking into consideration the results of peak purity tests, are compiled in Tables 1 (foliage) and 2 (bark). Foliage generally

Table 2
Taxol content in the bark of various *Taxus* species grown in Hungary. For sources of I–V see Table 1

Taxus species	Age (year)	Source	Taxol content (mg g^{-1})	
			Mean	RSD (%)
Taxus brevifolia	1	I	0.048	5.25
Taxus b. "Overeyndenri"	1	I	0.079	4.60
Taxus b. "Fastigiata" "Aurea"	1	V	0.037	4.74
Taxus b. "Repanda"	1	V	0.048	3.33
Taxus b. "Overeyndenri"	1	V	0.024	3.92
Taxus b. "Overeyndenri"	2	V	0.039	4.29
Taxus baccata "b" sign clone	1	III	0.040	4.71
Taxus media "Hicksii"	1	I	0.031	4.37
Taxus b. "Fastigiata" "Aurea"	1	VI[a]	0.023	3.96
Taxus b. "Semperaurea"	1	VI	0.049	5.09
Taxus b. "Adpressa"	1	VI	0.047	4.87
Taxus b. "Aurea"	1	IV	0.056	3.79
Taxus baccata "c" sign clone	1	I	0.029	4.61
Taxus b. "Overeyndenri"	1	V	0.044	3.71
Chepatotaxus "Harringt."	1	III	0.023	4.09
Taxus b. "Lutea" (female)	1	III	0.179	3.61
Taxus baccata (male)	1	I	0.061	5.62
Taxus baccata "d" sign clone	1	III	0.068	5.18
Taxus baccata "e" sign clone	1	III	0.093	4.55

[a] VI = Aggostyán, Hungary.

contained less taxol than the bark, indicating that the bark is a richer source of taxol than the foliage. The foliage of *Taxus b. baccata* basic species contained the highest amount of taxol, followed by *Taxus b. "Fastigiata" 5* and Taxus *canadensis*. The lowest quantity of taxol was found in *Taxus baccata* "a" sign male clone, *Taxus b. "Fastigiata" 2*, *Taxus b. "Aurea" 3* and *Toreya californica* (male). The bark of *Taxus b. Lutea* (female) contained the highest amount of taxol followed by *Taxus baccata* "e" sign clone. *Taxus b. "Fastigiata Aurea" 4* and *Chepatotaxus "Harringt"* contained the lowest amount. It is interesting to note that the older sample of both foliage and bark contained noticeably more taxol, proving that taxol production is higher in the older *Taxus* species. However, the taxol content of *Taxus* species grown in Hungary is markedly lower than that of tropical species.

The calibration curve showed a linear relationship between the peak area (y) and the concentration of taxol (x):

$$Y = 4.40 \times 10^{-2} + (1.70 \pm 0.03) \times 10^{-5}x \qquad (1)$$

indicating that the HPLC method can be successfully used for the quantitative determination of taxol in various *Taxus* species. The recovery of taxol varied between 83–88% depending on the accompanying matrix in the extracts; however, no significant differences were observed between the recoveries from the bark and foliage. This result suggests thjat the majority of taxol can be extracted from both the bark and the foliage without using complicated extraction procedures such as microwave or Soxhlet extraction. No significant differences were found between the mean values and the standard deviation of the inter-day

Fig. 1. Chromatogram of foliage extract of *Taxus cuspidata* on PGC column. Detection wavelength, 228 nm; eluent, water: dioxan 54: 46 v/v; flow rate, 0.8 ml min^{-1}.

and intra-day reproducibility of the method (data not shown), indicating again the suitability of the HPLC procedure for the separation and quantitative determination of taxol.

It can be concluded from the data that the anticancer drug taxol was proved to be present in measurable amounts in Hungarian *Taxus* species. According to the results bark generally contained a higher amount of taxol than foliage and the older the bark or foliage, the more taxol it contained. Investigations are under way to study the distribution of taxol in the various plant tissues and its dependence on the vegetation period.

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