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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ALKALOID SPECTRUM IN THE ROOTS AND CAPSULES OF THE SPECIES AND HYBRIDS OF *PAPAVER* SECTION *OXYTONA*

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SUMMARY

A reversed-phase high-performance liquid chromatographic method was developed for the simultaneous quantitation of the alkaloids of *Papaver* species in section *Oxytona*: salutaridine, thebaine, oripavine, alpinigenine, isothebaine and orientalidine. The concentrations of the alkaloids were compared in roots and capsules of the species *P. bracteatum, P. orientale* and *P. pseudo-orientale* and their interspecific hybrids. The alkaloid spectrum was similar for the two parts of each plant, and significant correlations were found between the concentration of each alkaloid in the roots and in the capsules. The importance of the results for the screening of plant populations and for breeding purposes is discussed.

INTRODUCTION

The species of the section *Oxytona, Papaver bracteatum, P. orientale* and *P. pseudo-orientale,* have aroused increased interest due to the pharmacological properties of some of their alkaloids, particularly thebaine, found in *P. bracteatum*[1,2]. This alkaloid is the natural precursor of codeine and can be converted also into other drugs[3]. Various chromatographic systems for the separation and determination of *Papaver* alkaloids have been described[4–12]. However, none is adequate for the simultaneous quantitation of the main alkaloids of the *Oxytona* species, namely, isothebaine, alpinigenine, oripavine, orientalidine and thebaine (Fig. 1). Such a method is needed for studies of the interspecific hybrids expected to have the alkaloid spectrum of both parents.

Most chemical studies with *Papaver* species have employed capsules for the alkaloid analysis, and other plant parts were investigated only in a few cases[13]. So far, no comparative and systematic screening of the main alkaloids present in the capsules and roots of the three species of the section *Oxytona* and their hybrids has been reported. Studies of the thebaine content in the roots and capsules of *P. brac-*

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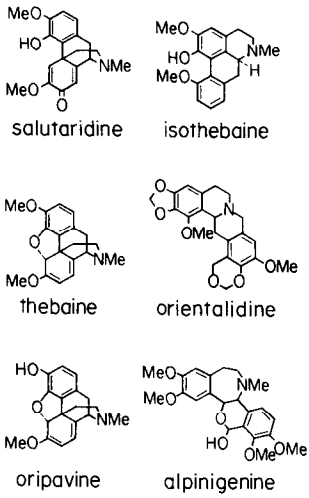

Fig. 1. Structures of the major alkaloids of *Papaver* species of section *Oxytona*. Me = methyl.

teatum plants from different origins have been reviewed[14]. The thebaine and oripavine concentrations in roots and capsules of *P. bracteatum, P. orientale* and their F_1 hybrid were compared[15], and the alkaloid content in various parts of *P. orientale* and *P. pseudo-orientale* was studied[16,17].

In the present study an improved high-performance liquid chromatographic (HPLC) procedure adapted to distinguish between the predominant alkaloids of the species of the section *Oxytona* was developed. This method was further used for the analysis and comparison of the alkaloid spectrum in the roots and capsules of *P. bracteatum, P. orientale, P. pseudo-orientale* and their interspecific hybrids.

EXPERIMENTAL

Plant material

The plant populations used were: a breeding line of *P. bracteatum* (PB) selected for early flowering and high thebaine, from an original accession from Iran (P.I. 381442); a *P. orientale* (PO) accession (P.I. 376815) from Iran and two accessions of *P. pseudo-orientale* (PPO) one from Iran (P.I. 375952) and the other from the U.S.S.R. (P.I. 372772). For each accession the identification of the species was confirmed by determination of the chromosome number (PB, $2n = 14$; PO, $2n = 28$; PPO, $2n = 42$) and morphological characters. From each species, F_1 and F_2 hybrids, five to ten plants were examined. The capsules and roots of each plant were collected and analysed separately. For a detailed description of the growing conditions and the interspecific crosses see Milo *et al.*[18].

Extraction of the alkaloids

A modified method of Fairbairn and Helliwell[11] was used for the extraction. Exactly 250 mg of dried powder plant material were extracted with 20 ml of 5% aqueous acetic acid at room temperature for about 18 h. The solution was filtered

and 7 ml of hexane were added. The aqueous fraction was made alkaline with ammonium hydroxide, and extracted with 3 × 15 ml chloroform–2-propanol (3:1). The pooled extracts were evaporated to dryness under reduced pressure. The residue was dissolved in 2.5 ml of methanol and filtered through a RC 55, 0.45-μm membrane filter (Schleicher & Schull, Dassel, F.R.G.).

High-performance liquid chromatography

A Tracor liquid chromatograph was used and included a Model 951 pump unit, a 980 A solvent programmer, a 970 A detector and a computing integrator Model CI-10B (LDC/Milton Roy). The reversed-phase HPLC was carried out on a LiChrosorb Superspher® RP-18 column (Merck, particle size 4 μm, 125 mm × 4 mm I.D.). Separation of the alkaloids was accomplished at 25°C by the mobile phase 5% 2-propanol, 40% acetonitrile, 55% water with 1% ammonium carbonate. The solvent flow-rate was 1 ml/min and detection was at 280 nm. All the solvents used (methanol, 2-propanol, acetonitrile, water) were of HPLC grade. Thin-layer chromatography (TLC) was performed as described previously[12]. For alkaloid quantitation, a linear correlation ($r = 0.999$) was observed between the sample concentration and the HPLC-integrated peak area, in the range of 0.01–1.0 mg/ml for each alkaloid.

Reference compounds

Samples of the various purified alkaloids were generous gifts from Dr. L. A. Anderson (School of Pharmacy, University of London, U.K.) and professor D. Lavie (The Weizman Institute of Science, Israel). The identity of the alkaloids was confirmed by co-chromatography with references in both TLC and HPLC.

RESULTS AND DISCUSSION

Two reversed-phase HPLC procedures for the alkaloid separation were evaluated. The first method used a 250 mm × 4 mm RP-18 column with a gradient solvent programme: A, 60% water with 1% ammonium carbonate, 30% acetonitrile and 10% methanol; B, 80% acetonitrile and 20% methanol. The programme started with solvent A for 20 min, and then 5% of solvent B per min were added; under these conditions, good separation with the following retention times was obtained: oripavine, 19 min; alpinigenine, 22 min; isothebaine, 27 min and thebaine, 37 min.

In order to avoid the use of the gradient programme and to shorten the analysis time, a second procedure was elaborated as described in the Experimental section. The chromatogram of the standard alkaloids is shown in Fig. 2. Most of the previously reported HPLC methods were developed for the analysis of the opium alkaloids[4–7].

We present herewith a rapid and sensitive method which is well suited for quantitative studies and for genetic analysis of *Papaver* alkaloids in interspecific hybrids in the section *Oxytona*. In some of the chromatograms, other unidentified peaks were obtained, indicating that this method might be appropriate for the separation of additional alkaloids. The accuracy of the assay was evaluated from six replications of a mixture (0.1 mg/ml) of the three major alkaloids and from four replicated samples of a bulk plant material from PO. In the mixture, the average and standard

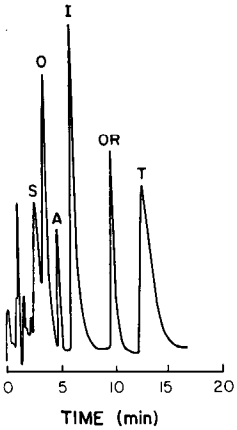

Fig. 2. Chromatogram of an artificial mixture of purified alkaloids. Column: LiChrosorb C_{18} (RP) Superspher 4 μm. Eluent: 2-propanol–acetontrile–water (5:40:55) with 1% ammonium carbonate; flow-rate, 1 ml/min. Dectection: 280 nm. Salutaridine = S; oripavine = O; alpinigenine = A; isothebaine = I; orientalidine = OR; thebaine = T.

deviation ($\cdot 10^3$) of the integrated areas were: oripavine, 144.7 ± 5.2; isothebaine, 236.9 ± 25.2 and thebaine, 224.4 ± 21.2. In the plant sample, the average and standard deviation of the concentrations of these alkaloids were: 0.097 ± 0.009, 0.019 ± 0.007 and 0.065 ± 0.01 respectively.

It was found that the alkaloid spectra of the roots and capsules were similar in each plant for each species (Fig. 3) and hybrid (Fig. 4) examined. However, quantitative differences in the various alkaloids were observed between the two parts of each plant. The chemical spectra of the three species are consistent with those reported in other studies[13,19]. Significant correlations ($p = 0.05$) were found between the contents of each major alkaloid in the capsules and in the roots for the entire plant population. The correlation coefficients were 0.65, 0.60 and 0.82 for isothebaine, oripavine and thebaine, respectively. The concentrations of the major alkaloids isothebaine, thebaine and oripavine in the different parents and hybrids are shown in Table I. The distribution of the different alkaloids in the roots and capsules followed different patterns in the three species and hybrids.

In PB only thebaine was found; its content in the capsules was significantly much higher than in the roots, 2.15 and 0.92%, respectively. This feature was found also in plants from the population of F_2 (PB × PPO) having thebaine as the predominant alkaloid. In PO the total alkaloid concentration was low, oripavine and thebaine being the major alkaloids. No significant differences between roots and capsules were detected for these compounds. Isothebaine was the major alkaloid in PPO and its content in the capsules was higher than in the roots. However, the accession from Iran (PPO 31) showed an higher content (0.72% in the capsules) and a larger variation compared with the second accession (PPO 82) from the U.S.S.R.

In the F_1 hybrid (PB × PO) only oripavine and thebaine were present; in some plants an additional unidentified peak was observed, see Fig. 2. The oripavine content in capsules (0.51%) and roots (0.49%) was much higher in this hybrid than in the PO parent (0.09%); on the other hand, the thebaine concentration was much lower

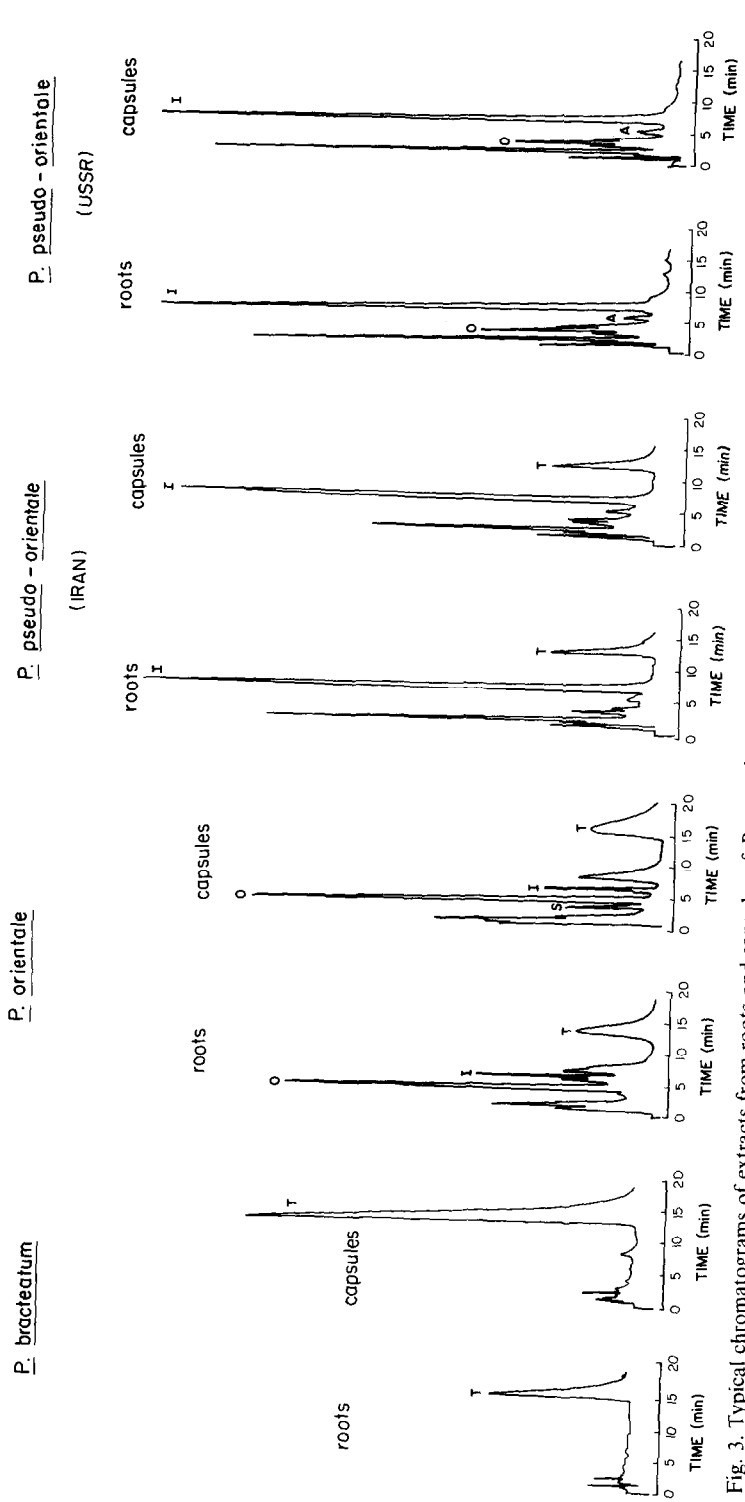

Fig. 3. Typical chromatograms of extracts from roots and capsules of *Papaver bracteatum*, *P. orientale* and *P. pseudo-orientale*. Other details as in Fig. 2.

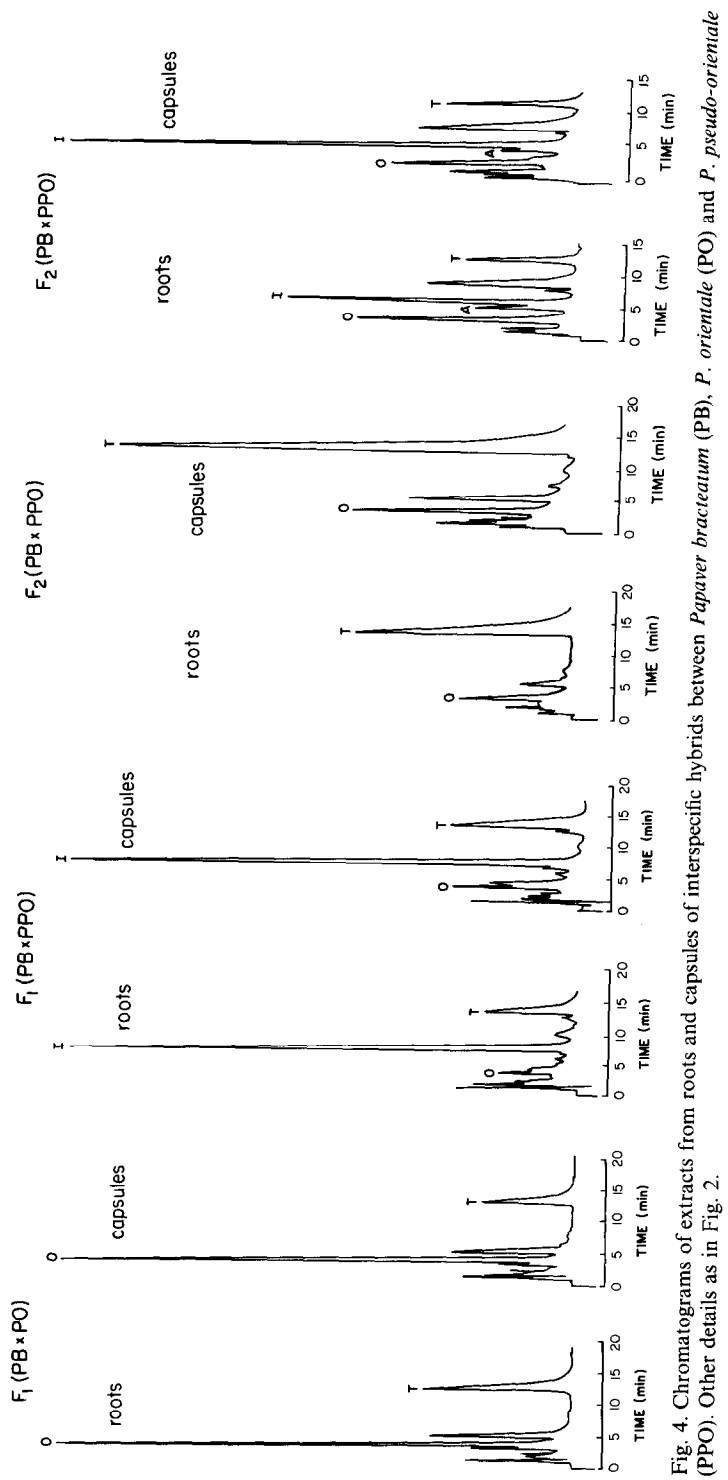

Fig. 4. Chromatograms of extracts from roots and capsules of interspecific hybrids between *Papaver bracteatum* (PB), *P. orientale* (PO) and *P. pseudo-orientale* (PPO). Other details as in Fig. 2.

TABLE I

CONTENT OF THE MAJOR ALKALOIDS (% DRY WEIGHT) IN CAPSULES AND ROOTS OF *PAPAVER BRACTEATUM* (PB), *P. ORIENTALE* (PO), *P. PSEUDO-ORIENTALE* (PPO) AND THEIR HYBRIDS

Species or hybrid	*Isothebaine*		*Thebaine*		*Oripavine*	
	Capsules	*Roots*	*Capsules*	*Roots*	*Capsules*	*Roots*
PB			2.15* (1.24–3.75)	0.92 (0.45–1.23)		
PO	0.03 (tr–0.09)	0.001 (tr–0.003)	0.06 (0.02–0.13)	0.07 (tr–0.16)	0.09 (0.02–0.13)	0.09 (0.08–0.09)
PPO 31	0.72 (0.08–1.38)	0.41 (0.003–0.65)	0.007 (tr–0.02)	0.004 (tr–0.01)	0.03 (0.004–0.04)	0.03 (0.01–0.05)
PPO 82	0.33 (0.27–0.41)	0.20 (0.17–0.24)	0.04 (tr–0.08)	0.04 (tr–0.07)	0.05 (tr–0.1)	0.06 (0.03–0.09)
F_1						
PB × PO			0.07 (0.09–0.11)	0.14 (0.08–0.23)	0.51 (0.30–0.70)	0.49 (0.26–0.78)
PB × PPO_{31}	0.11 (0.01–0.21)	0.16 (0.08–0.27)	0.07 (0.06–0.08)	0.07 (0.05–0.11)	0.04 (0.02–0.05)	0.02 (0.01–0.05)
F_2						
PB × PPO_{31}	0.16 (0.06–0.20)	0.09 (0.07–0.12)	0.26 (0.07–0.59)	0.15 (0.06–0.24)	0.07 (tr–0.13)	0.08 (0.01–0.18)

* The values are means from 5–10 plants; under each value, the range is given in parentheses. tr = Traces, <0.003% of dry weight.

than that of PB. Similar patterns have been reported[15]; however, the low alkaloid content of PO in our study might result from differences in growing conditions or in the origin of the plant populatons.

The alkaloid spectrum of the F_1 hybrid (PB × PPO 31) was similar to that of the PPO 31 parent. No significant differences between capsules and roots were obtained. When compared with the parents, the oripavine concentration of this hybrid (0.04%) was similar to PPO, its isothebine content was reduced and thebaine (0.07%) was higher than in PPO but much lower than in PB.

A wide range of alkaloid spectra was observed in the plants of the segregating generation F_2 (PB × PPO); however, the similarity between roots and capsules of each plant remained.

The constancy of the alkaloid spectrum in the roots and capsules, and the significant correlations found between the content of each alkaloid in these parts, show that chemical screening could be conducted on either capsules or roots. The use of capsules, however, requires a long growth period of the plants, until flowering and capsule maturation. Indeed, the species of the section *Oxytona* require cold for flowering or are biannual[14,20]; moreover, the plant biomass obtained from the capsules in much lower than that available from the roots. So far, chemical analysis has been performed mainly on capsules[13]. The findings of the present work and the

previous considerations show that the roots can be exploited to advantage in the chemical screening of these species and hybrids in breeding and genetic studies of the alkaloids' accumulation. Using roots for the chemical analysis therefore enhances the selection process for a desired alkaloid spectrum by reducing the growth period of the plants and the generation time.

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