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Short communication

Determination of alkaloids from the colchicine family by reversed-phase high-performance liquid chromatography

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Abstract

A reversed-phase HPLC gradient method is described to analyze some alkaloids from genus *colchicum*, with particular reference to the thio series members; at the same time the reliability of the results has been assessed, by calculating the main validation parameters. Moreover a short outline of the UV spectral features of the involved molecules explains the criteria adopted to investigate the presence of some minor related substances in the samples to be analyzed. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Alkaloids; Thiocolchicine; Thiocolchicoside

1. Introduction

The chemistry of the alkaloids from genus *colchicum* has been extensively reviewed in the last forty years [1,2], but they still are the object of further investigations, owing to their unique structural features and their broad biological activity. In this context a collaboration agreement with Indena[1] led us to consider the analytical implications of a project aimed at realizing on a pilot plant scale a conversion process thiocolchicine→thiocolchicoside — the thio prefix designating a class of compounds where the 10-methoxy group in the C tropolone ring is substituted by a thio methyl. Actually, whereas several papers have been published on HPLC analysis of colchicine in vegetable [3], as well as in biological materials [4,5] no report, even in very recent articles [6], deals with the separation of the thio derivatives. This prompted us to search for an HPLC method suitable to monitor the aforesaid conversion reaction.

The general structures of the colchicum alkaloids are outlined below (Fig. 1), while the molecules of our concern are listed in Table 1, as a function of the related substituting groups.

Referring to the thiocolchicine structure outlined above, the 3 position on the A ring appears to be of particular interest, since the molecule resulting from replacing the ethereal methyl with a glucosidic moiety, the so called thiocolchicoside, has a marked physiological activity as a skeletal muscle relaxant drug (Muscoril)®. Considering the 7 position on the C ring, besides the *N*-acetyl, also its lower *N*-formyl homologous is usually present as a minor component in the natural extracts. The reaction intermediate 3 (*O*) demethyl TCN and the formyl–acetyl pairs, both

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[1]Indena spa — Via Don Minzoni 6, Settala (Mi), Italy 20090 — is an international company active in the field of plant extracts.

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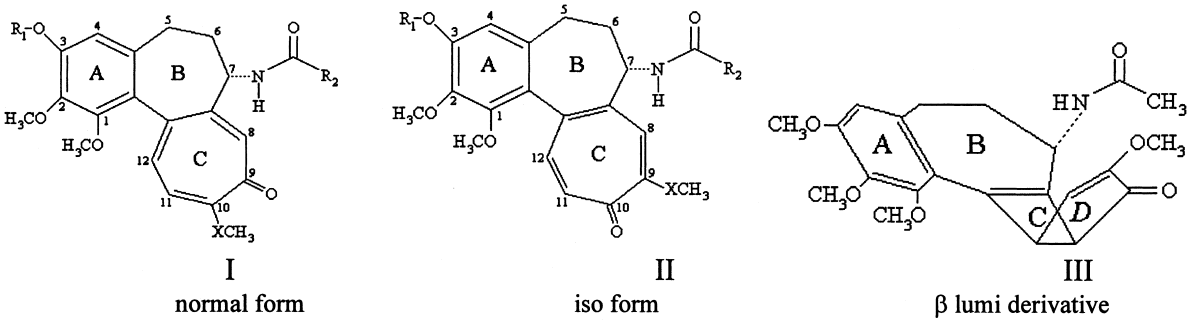

Fig. 1. General structures of Colchicum alkaloids.

for TCN and TCD structures, were all involved into the reaction mixture to be analyzed.

2. Experimental

2.1. Material and reference substances

Solvents (ACN, MEOH, THF) were of HPLC grade, the salt (KH_2PO4) and the phosphoric acid used for the mobile phase preparation were of RP grade (C. Erba RP): water was purified by a Milli Q system. Alkaloids references and standards were kindly supplied from Indena.

Warning: these substances should be handled with the greatest care because of their marked toxicity.

2.2. Chromatographic operating conditions

The analysis were run on a HP 1090 liquid chromatograph equipped with a DR5 gradient pump, an automatic injection system, an UV Diode Array Detector (3D version) and an analytical Leochem work station interfaced with a Vectra (Pentium 166) Hewlett Packard PC. The column was a Hypersil C_{18}, BDS, 5 μm, 100×4.6 mm, supplied by CPS.[2] The mobile phase was composed of: ACN (solvent A), solution KH_2PO_4 5 g/l adjusted to pH 4.5 with phosphoric acid (solvent B), THF (solvent C), delivered accordingly to a linear gradient profile (solvents ratio as v/v): 0–7 min. A:B 0.5:93.5→4:90; 7–20 min. A:B→12:82; 20–26 min. A:B 12:82; 26.1–30 min. A:B→0.5: 93.5, C being held constant at 6%. The flow-rate was set at 1 ml/min., the injection volume at 7 μl, the oven temperature at 35°C and the detector wave length at 380 nm.

Table 1

Compounds	R_1	R_2	X
Colchicine	CH_3	CH_3	O
Colchicoside	glucosyl	CH_3	O
Thiocolchicine (TCN)	CH_3	CH_3	S
N-formyl, *N*-deacetyl TCN	CH_3	H	S
3 *O*-demethyl TCN	H	CH_3	S
Thiocolchicoside (TCD)	glucosyl	CH_3	S
N-formyl, *N*-deacetyl TCD	glucosyl	H	S

2.3. Standard solution and samples preparation

About 10 mg of TCN, 3 *O*-demethyl-TCN and TCD standard, weighed on an analytical balance, were dissolved in MeOH to a final volume of 100 ml. A weighed amount (about 5 g) of a mass reaction sample was suspended in MeOH to a final volume of 100 ml and filtered on paper.

The standard and the samples were kept in the dark in a refrigerator.

The quantification was performed using an external standard.

3. Results and discussion

3.1. Analytical conditions

Even though the literature reports examples of isocratic as well as gradient elution, the large polari-

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ty spread between the species to be analyzed, ranging from unhydroxylate to polyol structures, led us to prefer the latter technique. Different compositions have been suggested for the mobile phase, encompassing ion-pair as well acetate or phosphate buffers, in a pH range from 5.5 to 7.6. However, considering the chemical structure of our alkaloids, we discarded the IP option for a suitably buffered system, since a functional group susceptible of ion pairing is present only in the 3 *O*-demethyl TCN. A salt concentration range from 3 to 7 g/l was considered, but 5 g/l proved to be the most suitable condition, affording both the highest efficiency and a better peak shape. The symmetry factors, calculated on the standards, are shown in Table 2.

Besides, we set the pH value at 4.5, according to the acidity level of our sample — a mixture of pseudo rather than true alkaloids, owing to the masking of the aminic nitrogen by acylation at 7. In any case to ensure a thorough suppression of the phenol ionization, a pH value was selected far lower than the pK_a of 3 demethyl TCN, supposedly ranging between 9.6 and 10, in analogy with the *o*/*m* diphenol mono methyl ethers [7], assumed as model molecules. With regard to the organic buffer modifiers, a ternary system was developed (ACN–THF–phosphate), which proved to be the best approach for satisfactory separation of the analytes present in the mass reaction, including the acetyl–formyl pairs, both for TCN and TCD derivatives, in spite of their very similar polarity. In turn THF alone showed good resolution capability, but the ternary system was equally preferred because of a better transmittancy value in the UV region we were interested in. In contrast, the ACN–buffer or ACN–MeOH–buffer systems, partly derived from literature [3,4], gave no separation between the aforesaid critical couples.

Table 2

Compounds	Symmetry*		
	3 g/l	5 g/l	7 g/l
Thiocolchicoside (TCD)	1.10	1.07	1.09
3 *O*-demethyl TCN	1.07	1.05	1.06
Thiocolchicine (TCN)	1.04	1.01	1.14

* calculated as the square root of the peak moments ratio

The column types already used in previous works, i.e. C_{18} packaging, was maintained and the best results could be achieved by an Hypersil BDS 5 μm, 10 cm length, that succeeded in reducing peak tailing, possibly because of an improved silica deactivation process. Referring to the other operating parameters, the temperature of the oven column did not seem to affect the separations and was therefore held at a default value of 35°C, the flow-rate having been set at 1 ml/min as an acceptable compromise between resolution and analysis length.

The analysis of a mixture of references is shown in Fig. 2, while the profile of a mass reaction sample is reported in Fig. 3

In order to select the best detection conditions, the UV spectra of our references were individually recorded during the elution (cf. Fig. 4); their maxima belong to two distinct UV regions, at 245–260 and 350–380 nm (with a rather less intense absorption) to be both ascribed to the tropolonic chromophore system [8]. Referring to the latter band a bathochromic shift (350→370–380 nm) can be pointed out from the oxygenated to the thio series, whereas an hypsochromic effect is shown by the lumi thiocolchicines — typical photochemical tropolone ring rearrangement products, ensuing in a shortening of the conjugation versus the colchicine series (267←353 nm). Consequently the choice of a suitable set of wavelengths allowed us to check for the possible presence of the oxo impurities (our mass reaction consisting in principle only of thio derivatives) and also for the undue formation of artefacts like the lumi[3] derivatives. Actually, since no meaningful absorbance could be detected at the expected RT neither at 267 nor at 350 nm, we concluded that these byproducts were absent in our sample. Moreover, checking for the purity of the three main peaks of our conversion process, through a spectral test provided by the detection system (similarity curve technique) since the purity-factor values were all above 999, we could exclude the presence of any other hidden substance.

[3]Two diastereoisomeric pairs could be assumed (β and γ) hardly distinguishable on a mere UV spectral basis, their chirality ensuing from the trans/cis orientation of the 7 acetamido group versus the D ring [9,10].

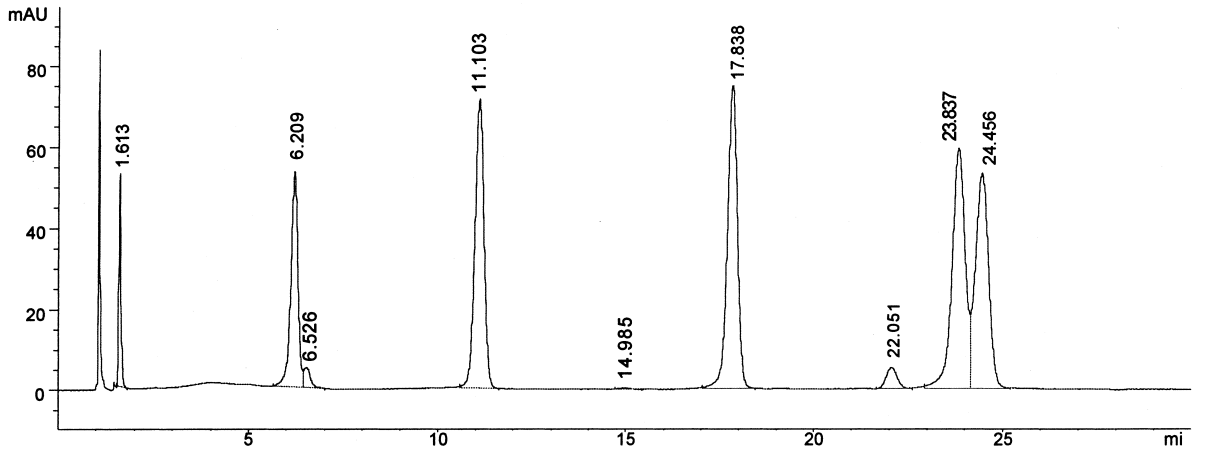

Fig. 2. Analysis of a mixture of references at 380 nm: RT 1.61 colchicoside; RT 6.21 Thiocolchicoside (TCD); RT 6.53 *N*-deacetyl, *N*-formyl TCD; RT 11.1 Colchicine; RT 17.84 3 *O*-demethyl TCD; RT 22.05 β Lumi-colchicine; RT 23.84 Thiocolchicine (TCN); RT 24.46 *N*-deacetyl, *N*-formyl TCN.

While working at 267 and 350 nm proved useful for qualitative purposes, the optimum λ for the quantification was set at 380 nm, where the fairly high ε values shown by the involved molecules secure a suitable analytical response.

3.2. Linearity

The detector response has proved linear in the following range: TCD from 0.91 to 910 μg/ml; 3 *O*-demethyl TCD from 0.96 to 960 μg/ml; TCN from 0.88 to 880 μg/ml.

(Nine data points — replicated three times — have been considered for each analyte).

The related linearity plots meet the following equations:

TCD: $y = 1.001972x - 0.425481$ ($R^2 = 0.999999$);

3 *O*-demethyl TCD: $y = 0.993745x - 0.197689$ ($R^2 = 1.000000$)

TCN: $y = 0.994094x - 0.538666$ ($R^2 = 0.999996$)

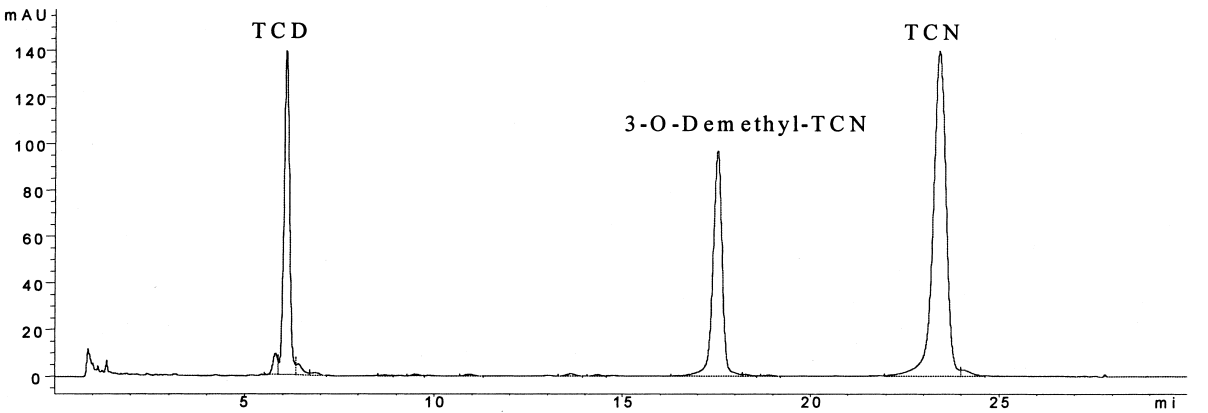

Fig. 3. Profile of a mass reaction in the conversion thiocolchicine→thiocolchicoside.

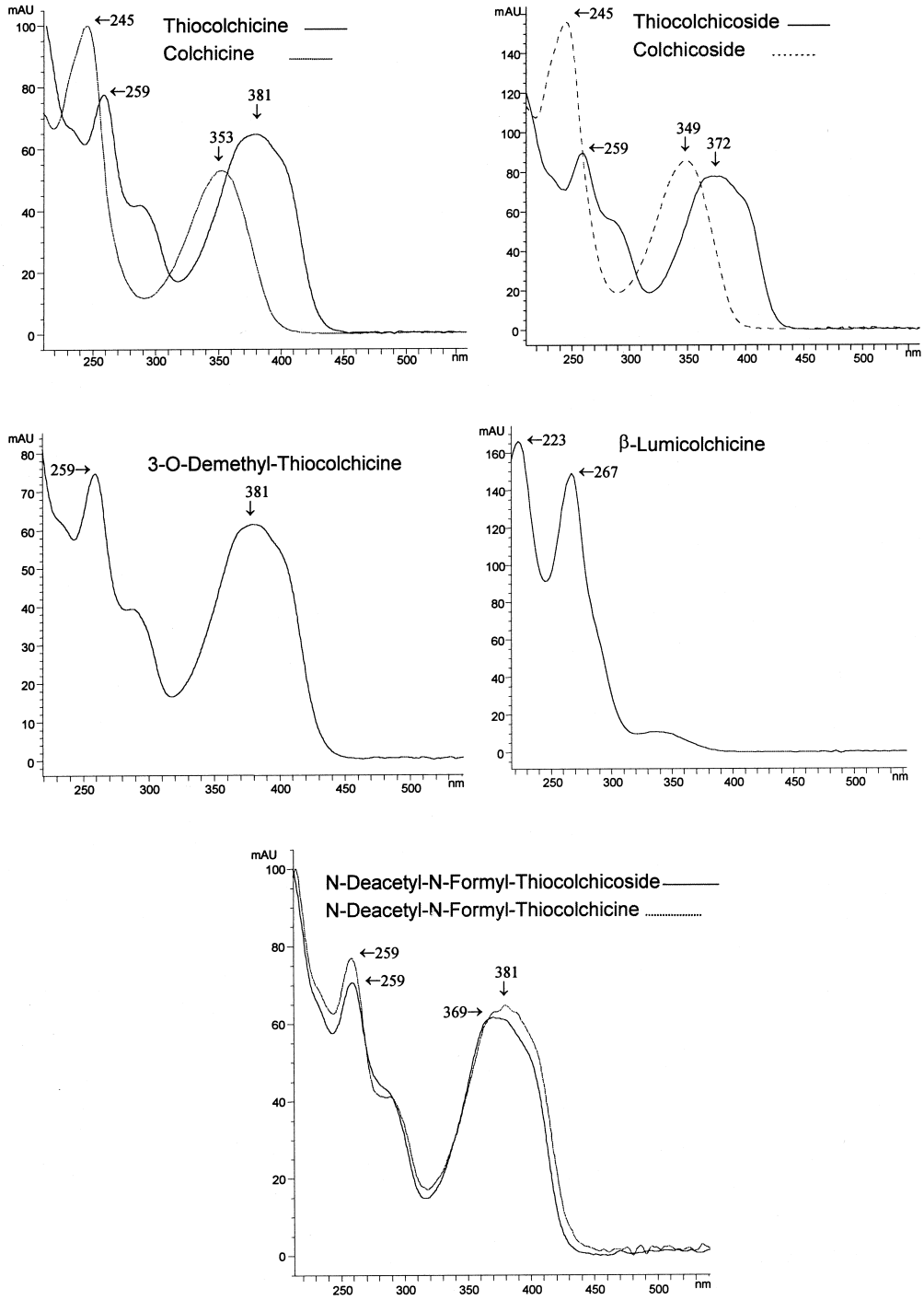

Fig. 4. UV spectra of some colchicum alkaloids in the oxo and thio series.

3.3. Precision

Operating on a sample of the conversion process — five replications, each being injected twice — the precision % is: ±0.63 for TCD; ±0.68 for demethyl-TCN and ±0.61 for TCN ($P = 0.05$)

4. Conclusions

An effective reversed-phase HPLC method has been developed to analyze an alkaloidic mixture containing some thiocolchicine derivatives never previously investigated by HPLC. By a suitable selection of chromatographic parameters a satisfactory resolution has been obtained, especially referring to some pairs of homologues alkaloids, requesting a careful solvent selectivity modulation, because of their close polarity pattern. Some further information about the possible presence of the oxo compounds and of a photochemical rearrangement products has been acquired on the basis of the UV spectral features of the alkaloids being analyzed. The purity of the peaks detected on the mass reaction has also been assessed on a spectral basis.

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