Journal of Chromatography, 188 (1980) 280–284

CHROM. 12,335

Note

High-performance liquid chromatographic separation of colchicine and its phenolic and N-desacetylated derivatives

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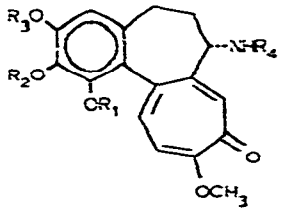
(Received August 20th, 1979)

Colchicine (I), a traditional drug used to relieve the symptoms of gout[1], has been recognized for some time as an anti-tumor agent[2]. Work has lately focused on the antineoplastic properties of several derivatives of colchicine, which are reported to be potentially less toxic and of equal or greater potency than the parent compound[3]. N-Desacetylcolchicine (II) is currently undergoing preclinical trials[4], and N-methyl-N-desacetylcolchicine (Demecolcine, III), as well as the phenolic derivatives, 3-demethylcolchicine (IV) and 2-demethylcolchicine (V), show promise for further pharmacological evaluation[5,6]. Additionally, the phenols, IV and V, are known mammalian[7] and microbial[8] metabolites of colchicine. Demecolcine (III) is also a potential internal standard for the determination of I and its known metabolites in biological samples.



I: $R_1, R_2, R_3 = CH_3$; $R_4 = COCH_3$

II: $R_1, R_2, R_3 = CH_3$; $R_4 = H$

III: $R_1, R_2, R_3 = CH_3$; $R_4 = CH_3$

IV: $R_1, R_2 = CH_3$; $R_3 = H$; $R_4 = COCH_3$

V: $R_1, R_3 = CH_3$; $R_2 = H$; $R_4 = COCH_3$

VI: $R_2, R_3 = CH_3$; $R_1 = H$; $R_4 = COCH_3$

Few analytical methods have been reported for colchicine and its derivatives. Thin-layer chromatography has been successfully used to separate some colchicine derivatives[8,9] and recently high-performance liquid chromatography (HPLC) has been applied to analyze colchicine (I)[10,11] and 3-demethylcolchicine (IV)[12]. No chromato-

graphic system has previously been described that resolves all the compounds I–VI. The present method enables a rapid identification of colchicine derivatives in biological extracts and is currently being applied to the evaluation of colchicine transformations in microbial fermentations.

MATERIALS AND METHODS

Colchicine (I) was purchased from Aldrich (Milwaukee, Wisc., U.S.A.). Samples of colchicine derivatives II–VI were kindly provided by Dr. J. A. R. Mead and Dr. A. Brossi of the National Cancer Institute (Bethesda, Md., U.S.A.). The phenols (IV and V) were additionally prepared according to the Udenfriend oxidation system as modified by Schonharting *et al.*[13]. Colchicine and its derivatives were observed as single, homogeneous spots by silica gel and alumina thin-layer chromatography and R_F values obtained agreed with reported results for development with chloroform–ethanol (96:4) on alumina[8] for compounds IV and V, and with chloroform–acetone–diethylamine (7:2:1) for compounds I and III on silica gel[9]. Electron-impact (EI) mass spectra for I–VI were in agreement with published data[14–16].

Organic solvents were chromatographic or pesticide quality obtained from MCB Manufacturing Chemists (Cincinnati, Ohio, U.S.A.). Water was deionized and double distilled in glass. All other reagents were certified A.C.S. grade. Mobile phases were prepared by filtering solvents through glass fiber pads (Whatman GF/F glass fiber paper), mixing and degassing by sonication prior to use. HPLC columns (μBondapak C_{18}, particle size = 10 μm; 30 cm × 4 mm I.D.) were obtained from Waters Assoc. (Milford, Mass., U.S.A.).

Standard solutions

For initial HPLC work, solutions of 0.5 mg/ml of I and *ca.* 0.3–0.5 mg/ml of II–VI were prepared in the HPLC mobile phase. All solutions were protected from light.

HPLC system

A Tracor 950 pump attached to a Rheodyne 20-μl loop injector and a Tracor 970A variable-wavelength detector were used. Detection was at 350 nm using a sensitivity of 0.32 a.u.f.s. for 20 μl of the standard solutions. A mobile phase flow-rate of 2.0 ml/min was employed.

RESULTS AND DISCUSSION

Various closely related isocratic mixtures were evaluated for satisfactory resolution of the phenolic colchicines IV and V in order to obtain a potentially successful mobile phase. After this initial evaluation, the resolution of the closely related desacetyl derivatives, II and III, was accomplished by modifying the pH of the phosphate buffer in the mobile phase. If final resolutions were not adequate, variations were systematically made in mobile phase compositions.

The successful separation of compounds I–VI is shown in Fig. 1. The μBondapak C_{18} column provided nearly complete resolution of 3-demethylcolchicine (IV) from 2-demethylcolchicine (V) with most of the buffer systems employing acetonitrile

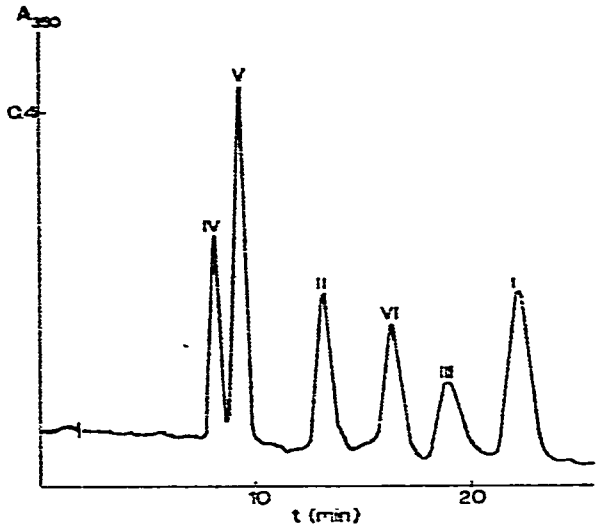


Fig. 1. HPLC separation of colchicine (I), N-desacetylcolchicine (II), N-methyl-N-desacetylcolchicine (III), 3-demethylcolchicine (IV), 2-demethylcolchicine (V) and 1-demethylcolchicine (VI) (*ca.* 1–2 μg each) on a 10-μm μBondapak C_{18} column, 30 cm × 4 mm I.D., eluted at 2 ml/min with acetonitrile–methanol–phosphate buffer, pH 6.0 (16:5:79). UV detector set at 350 nm, 0.08 a.u.f.s. Sample size, 20 μl. Pressure, 1430 p.s.i.g.

or acetonitrile–methanol as an organic modifier (see Table I). Peak shape as well as resolution for these compounds improved using systems containing 5% methanol in addition to acetonitrile as the organic modifier. However, the N-desacetylcolchicine co-chromatographed with colchicine in systems containing alkaline buffer (Table I, mobile phase A), and a successful separation was only achieved when compounds II and III were ionized using pH 6 buffer in the mobile phase. The fraction of acetonitrile

TABLE I

RETENTION TIMES OF COLCHICINE AND ITS DERIVATIVES ON A μBONDAPAK C_{18} COLUMN WITH VARIOUS ACETONITRILE–METHANOL–PHOSPHATE BUFFER MOBILE PHASES

The flow-rate is constant at 2 ml/min; pressure 1390 p.s.i.g. Mobile phases: *A* = acetonitrile–methanol–phosphate buffer, pH 7.6 (μ = 0.05) (17:5:78); *B* = acetonitrile–methanol–phosphate buffer, pH 6.0 (μ = 0.05) (17:5:78); *C* = acetonitrile–methanol–phosphate buffer, pH 6.0) (μ = 0.05) (15:5:79); *D* = acetonitrile–methanol–phosphate buffer, pH 6.0 (μ = 0.05) (15:5:80).

Compound	*Retention time (min) on mobile phase*			
	A	*B*	*C*	*D*
I	15.8*	17.7	24.4	30.7
II	15.8*	11.2	14.0	17.1
III	23.1	15.8	20.3	23.5*
IV	6.6	6.9	8.7	10.9
V	7.4	7.7	10.1	12.9
VI	11.5	13.2	18.0	22.2*

* Unresolved compound.

in the acidic buffer system was critical to the relative retention of the unionized (I, IV–VI) to ionized (II, III) compounds, as shown in Table I (mobile phases B–D). For example, a decrease of 2% in the acetonitrile fraction (going from mobile phase B to D) increased the retention time of colchicine (I) from 17.7 to 30.7 min while the retention time of ionized N-desacetylcolchicine (II) increased from 11.2 to 17.1 min. Likewise, N-methyl-N-desacetylcolchicine (III) and 1-demethylcolchicine (VI) were successfully resolved in mobile phase B but unresolved in mobile phase D due to their relative mobilities as ionized and unionized species, respectively.

The most satisfactory separation was obtained with methanol–acetonitrile–phosphate buffer, pH 6.0, (0.038 *M* KH_2PO_4 + 0.005 *M* K_2HPO_4, $\mu = 0.05$) (16:5:79), which is listed as mobile phase C in Table I. This HPLC system was employed to evaluate the biotransformation products in a chloroform extract of an *S. spectabilius* culture which had previously been incubated with colchicine. The resulting chromatogram (Fig. 2) clearly shows the presence of the 3- and 2-demethylcolchicines in the extract. The described HPLC system (Fig. 1) is currently being used to develop quantitative methods for the determination of compounds I–VI in biological systems. Results from these investigations will be the subject of a future report.

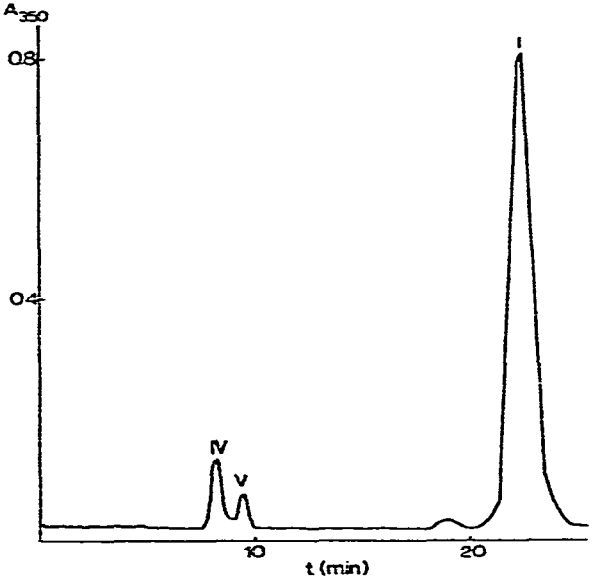


Fig. 2. HPLC separation of 3-demethylcolchicine (IV) and 2-demethylcolchicine (V) in a chloroform extract from an *S. spectabilius* incubation with colchicine (I). The dried extract was redissolved in mobile phase. Same chromatographic conditions as Fig. 1 except 1.28 a.u.f.s. Sample size, 10 μl. Pressure, 1430 p.s.i.g.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service Grant CA 24171. The authors are grateful to Dr. J. A. R. Mead and Dr. A. Brossi of the National Cancer Institute for their generous samples of the colchicine derivatives.

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