Journal of Chromatography, 219 (1981) 321–324
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,149

Note

High-performance liquid chromatographic determination of N,N-dimethylcolchiceinamide and its metabolites, N-methylcolchiceinamide and colchiceinamide, in microbial culture

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(Received June 30th, 1981)

Although colchicine (I) has been used for centuries in the treatment of gout, recent interest in colchicine derivatives stems from their potential use as antineoplastic agents[1]. There is ample evidence to indicate that certain colchicine analogues, particularly colchiceinamide derivatives such as II–IV (see Fig. 1), exhibit higher therapeutic indicies, and hold more promise for clinical application[2,3].

We are currently examining the use of micro-organisms to prepare colchicine derivatives metabolically and to study their metabolic alteration[4]. Studies involving the microbial transformations of N-methylcolchiceinamide (II) and N,N-dimethylcolchiceinamide (III) have necessitated the development of new analytical procedures for these substrates and their metabolites. We chose high-performance liquid chromatography (HPLC) for its demonstrated utility in the rapid analysis of microbial extracts and delineation of metabolic pathways[5]. The devised method may also be useful in other pharmaceutical, pharmacokinetic, and clinical studies of colchiceinamide derivatives used as antineoplastic agents.

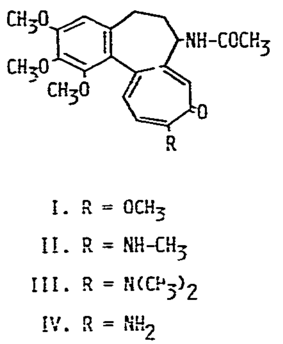
EXPERIMENTAL

Reagents

All solvents and reagents were analytical grade or better. Solvents for chromatography were chromatographic grade (LiChrosorb; MCB, Cincinatti, OH, U.S.A.). Water was deionized and double distilled in glass. Mobile phases were prepared by the filtration of individual solvents through glass fiber pads (GF/F grade; Whatman, Clifton, NJ, U.S.A.), mixing, and degassing prior to use.

Standard compounds

Colchicine (I) and N-methylcolchiceinamide (II) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Colchiceinamide (IV) was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Compounds I, II, and IV gave spectral and physical data consistent with literature reports[4]. N,N-Dimethylcolchiceinamide (III) was synthesized from colchicine according to the method of Hartwell *et al.*[6], and recrystallized from light petroleum (b.p. 60–68°C). This compound gave the follow-



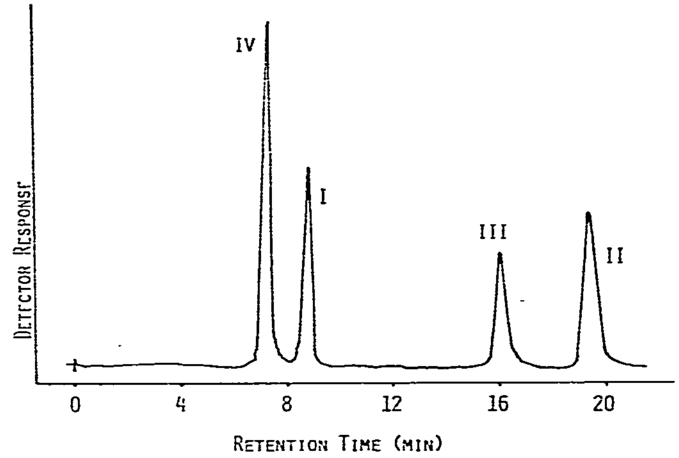


Fig. 1. Structures of colchiceine (I) and the colchiceinamide derivatives N-methylcolchiceinamide (II), N,N-dimethylcolchiceinamide (III) and colchiceinamide (IV).

Fig. 2. HPLC separation of colchicine (I) and the colchiceinamide derivatives colchiceinamide (IV), N-methylcolchiceinamide (II), and N,N-dimethylcolchiceinamide (III). Chromatographic conditions are described in the Experimental section.

ing analytical data: m.p. 138–143°C, re-solidifies, and re-melts at 173–174°C [rept.[6] 145°C (foams), 203–205°C (melts); and[7] 174–176°C]; proton magnetic resonance spectrum ($CDCl_3$) ppm (multiplicity, assignment); 1.97 (s, 3H, $COCH_3$), 2.0–2.6 (m, 5H, C-5, C-6, C-7), 3.15 (s, 6H, $N(CH_3)_2$, 3.60, 3.85 and 3.93 (3s, 9H, C-1–, C-2– and C-3–OCH_3), 6.45 and 6.58 (m, 2H, C-4 and C-11), 7.20 and 7.33 (m, 2H, C-2 and C-8), identical with reported spectrum[8]; mass spectrum, m/e (% relative abundance), 412 (100), 398 (39), 384 (12), 369 (11), 357 (16), 341 (12), 325 (18), 310 (15), 298 (10), consistent with analogous spectra[9]. All of the standard compounds were homogeneous as determined by thin-layer chromatography and HPLC.

Chromatographic system

A Tracor Model 950 pump and 970A variable-wavelength detector (Tracor Industries, Austin, TX, U.S.A.) with a 20-µl loop injector (Rheodyne, Berkeley, CA, U.S.A.) were employed for all HPLC analyses. Detection was at 370 nm, which represents a maximal compromise for all four compounds analyzed. An HP Model 3380A reporting integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.) at an input sensitivity of 0.1 v/a.u. and a slope sensitivity of 1 mV/min was used for peak area measurements and chromatographic recording. The column used was a 5-µm Ultrasphere-ODS (Beckman), 25 × 0.46 cm I.D. The mobile phase consisted of 0.1 *M* triethylamine hydrochloride in phosphate buffer (0.02 *M*, final pH adjusted to 2.2)–acetonitrile–methanol (715:200:85).

Extraction and analysis

Spiked samples were analyzed using a single extraction step. A stock solution was prepared consisting of 1 mg each of colchiceinamide (IV), N-methylcolchiceinamide (II), and N,N-dimethylcolchiceinamide (III), in 25 ml of chloroform.

Portions of 0.625, 1.25, 2.50, 3.75, and 5.00 ml were pipeted into duplicate 125 × 16 mm I.D. silylated glass extraction tubes, and the solvent was removed under a gentle nitrogen stream. The residue was taken up in 1 ml of *Streptomyces griseus* culture (NRRL B-599) grown according to a two-stage fermentation procedure[4]. Samples were alkalinized with 1.0 ml of saturated aqueous sodium bicarbonate solution, and extracted with 2.0 ml of chloroform containing 100 μg of colchicine (I) as the internal standard. The mixture was agitated for 20 min at 18 rpm on an Alignot mixer Model 4651 (Ames Co., Elkhart, IN, U.S.A.) and centrifuged. A 1-ml portion of the chloroform layer was taken to dryness under a nitrogen stream, reconstituted in 1.0 ml of acetonitrile–methanol (1:1), filtered, and subjected to HPLC analysis.

The resultant peak areas were used to plot standard curves for II–IV as peak area ratio (standard compound/colchicine) *vs.* μg of standard compound per ml of culture. Typical standard curves were as follows: colchiceinamide, slope = 0.018, y-intercept = 0.03, r = 0.999; N-methylcolchiceinamide, slope = 0.13, y-intercept = 0.02, r = 0.998; N,N-dimethylcolchiceinamide, slope = 0.090, y-intercept = 0.01, r = 0.999.

RESULTS AND DISCUSSION

Fig. 2 illustrates the chromatographic separation of colchiceinamide (IV), the internal standard, colchicine (I), N-methylcolchiceinamide (II), and N,N-dimethylcolchiceinamide (III). Several modifications of the previously reported methods for the separation of colchicine derivatives[10] were necessitated by the observation that the N-methyl derivative, II, had the same retention time as the N,N-dimethyl derivative, III. In addition, colchiceinamide (IV) exhibited no separation from the desired internal standard, colchicine (I).

The first modification involved the use of a 5-μm, instead of a 10-μm column, thus increasing the resolution of II and III. Second, the addition of triethylamine hydrochloride to the mobile phase[11] was found to improve the peak shape. We found a linear increase in the number of theoretical plates with reference to the peaks for II and III as the concentration of triethylamine was increased from zero to 0.8%, due entirely to improved peak shape with no alteration of retention time. Our results indicate that compounds II–IV are not protonated at the pH of the mobile phase (pH 2.2) (see below). It appears that triethylamine, in its protonated form at this pH, functions to improve peak shape by a "competing base" mechanism[11].

The use of an acidic pH (2.2) allowed for the resolution of colchiceinamide (IV) and colchicine (I). Colchicine is the only readily available compound of similar chemical structure to II–IV which would not be a potential metabolite; hence, it was chosen as an appropriate internal standard. Colchicine and colchiceinamide exhibit the same retention time in a chromatographic system reported earlier[10] for analyzing colchicine metabolites and related derivatives. However, Fig. 3 illustrates that a drastic decrease in k' values occurs only for the colchiceinamide derivatives (II–IV) as the pH is decreased below 3.0, presumably due to the protonation of the relatively non-basic vinylagous amide of the tropolone ring. Colchicine, on the other hand, exhibits little pH effect, as would be expected. Thus, baseline resolution of I and IV was accomplished at pH 2.2, as indicated in Fig. 2.

A method has been developed for the simple extraction and rapid analysis of

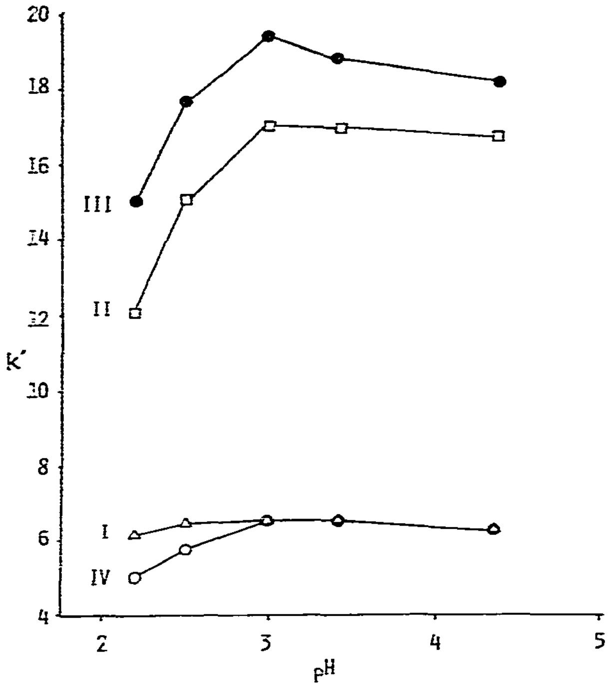


Fig. 3. Variation in capacity factor (k') as a function of pH for colchiceinamide (IV) (○), colchicine (I) (△), N-methylcolchiceinamide (II) (□), and N,N-dimethylcolchiceinamide (III) (●). Chromatographic conditions are described in the Experimental section.

colchiceinamide derivatives in microbial cultures. The procedure has been applied to growing cultures of *Streptomyces griseus* to follow the sequential N-dealkylation of III to II to IV (results to be published elsewhere). The method may also have application in pharmaceutical and clinical analyses of these important antineoplastic agents.

ACKNOWLEDGEMENTS

This research was supported by PHS Grant CA24172 from the National Cancer Institute. J.T.H. also acknowledges partial financial support from NCI.

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