Journal of Chromatography, 315 (1984) 233–241
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,188

SEPARATION OF COLCHICINE AND RELATED HYDROLYSIS AND PHOTODECOMPOSITION PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, USING COPPER ION COMPLEXATION

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(Received August 20th, 1984)

SUMMARY

The high-performance liquid chromatographic separation of colchicine and its hydrolysis and photodecomposition products has been investigated. Separation of colchicine, N-desacetyl-colchicine, β- and γ-lumicolchicine can be accomplished by modification of existing methods; however, under these conditions the tropolone derivatives, colchiceine and trimethylcolchicinic acid, do not elute. Based on the observed complexation of tropolone with metal ions, copper(II) ions were incorporated in the eluent enabling the direct detection and quantitation of the tropolone derivatives. Separation of colchicine, isocolchicine and their respective derivatives has been optimised for both phosphate buffer and aq. copper solutions.

INTRODUCTION

Although the therapeutic use of colchicine (I, Fig. 1) for the treatment of gout is well established[1], it is the biochemical action of this drug which has received the

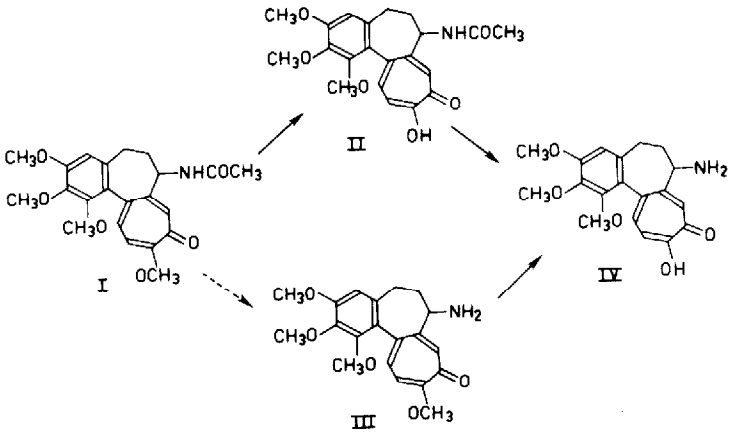

Fig. 1. Hydrolytic degradation pattern of colchicine (I).

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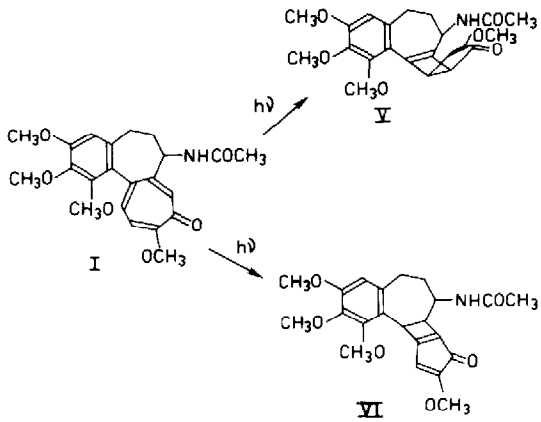


Fig. 2. Photodecomposition products of colchicine.

most attention in recent years[2,3]. Colchicine has been shown to bind to tubulin, an ubiquitous protein in eukaryotic cells, inhibiting the formation of microtubules[4]. This action has generated considerable interest in the use of colchicine as a model for development of anti-tumour agents[5,6].

The separation of colchicine and colchiciside in Colchicum seeds (*Colchicum autumnale* L.) using reversed-phase high-performance liquid chromatography (HPLC) was examined by Forni and Massarani[7] and Petitjean *et al.*[8] In order to investigate the metabolism of colchicine by bacteria, Davis and Klein[9] subsequently developed methods for the separation of the three demethylated phenolic isomers of colchicine and N-desacetylcolchicine (III, Fig. 1). The biosynthesis of colchiceine (II, Fig. 1) by microbes has been examined by derivitisation of colchiceine to ethylcolchicinate and ethylisocolchicinate before chromatographing[10].

Colchicine in solution is not stable; it has been shown to undergo quantitative photodecomposition to form β- and γ-lumicolchicine (V and VI, respectively, Fig. 2) with smaller amounts of the dimer, α-lumicolchicine being formed[11]. Depending on the pH of the solution, colchicine may also undergo hydrolysis of the methoxytropone moiety to form colchiceine (II, Fig. 1) with subsequent amide hydrolysis to form trimethylcolchicinic acid (IV, Fig. 1)[12]. As the methoxytropone is more labile to hydrolysis, the intermediate N-desacetylcolchicine (III, Fig. 1), formed from the alternative hydrolytic route, is not obtained as a major product[12]. Isocolchicine (VII), which contains an isomeric methoxytropone moiety to colchicine and is inactive as a microtubule inhibitor, has also been shown to undergo similar photodecomposition[13] and hydrolytic reactions to colchicine.

The separation of colchicine, N-desacetylcolchicine and a number of other related derivatives has been reported[10,14]. However, as colchiceine failed to elute as the free tropolone, it could only be quantitated as ethoxytropones[10].

As part of our investigations into the interaction of colchicine with tubulin, it was essential to develop a method to identify and quantitate not only the parent drug but also the possible decomposition products which may be formed in solution. Investigation of the chromatographic behaviour of the decomposition products of colchicine has enabled the separation of the free tropolone derivatives (II and IV, Fig.

1) and the major photodecomposition products (V and VI, Fig. 2) using a novel HPLC method which utilises complexation with the copper(II) ion.

EXPERIMENTAL

Chemicals and reagents

Colchicine (I), trimethylcolchicinic acid (IV), β- and γ-lumicolchicine (V, VI) were obtained from Sigma (St. Louis, MO, U.S.A.). Colchiceine (II) was prepared by the method of Chapman *et al.*[12]. Supplementary quantities of trimethylcolchicinic acid were prepared by the hydrolysis of colchicine according to the method of Wilson and Friedkin[13]. N-Desacetylcolchicine (III) and N-desacetylisocolchicine (VIII) were prepared by treating trimethylcolchicinic acid with diazomethane and separated by column chromatography[13]. All synthesised compounds were characterised by thin-layer chromatography (TLC), mass spectrometry and proton nuclear magnetic resonance spectroscopy, their spectra being in agreement with published data. Methanol and acetonitrile were HPLC grade (Waters Assoc., MA, U.S.A.). Copper sulphate, potassium dihydrogen phosphate, sulphuric acid, phosphoric acid and dimethyl sulphoxide were of analytical grade (Ajax Chemicals, N.S.W., Australia). Water was distilled in glass and then purified with a Milli-Q Reagent System (Millipore, Bedford, MA, U.S.A.)

HPLC system

The HPLC apparatus consisted of a Waters Model 6000A solvent delivery system, with a Model 480 variable-wavelength detector and a Model 710B (WISP) automatic sample processor (Waters Assoc.). Separation was achieved using a reversed-phase C_{18} radial compression cartridge (Radpak B, spherical 10 μm) housed in an RCM-100 radial compression module.

Solvent systems used in this study were either 0.025 *M* potassium dihydrogen phosphate adjusted to pH 2.5–5.5 with orthophosphoric acid, or an aqueous solution of copper sulphate (0–1%) adjusted to a range of pH values between pH 2.5 and 4.0 with 2.5 *M* sulphuric acid. The organic phase was a mixture of methanol–acetonitrile (25:75). All reported data were obtained using a 35% organic phase. All solvents were filtered and degassed under vacuum prior to use. Solvent flow-rate was set at 3.0 ml/min and separation performed at ambient temperature (23°C). The eluant was monitored at 350 nm.

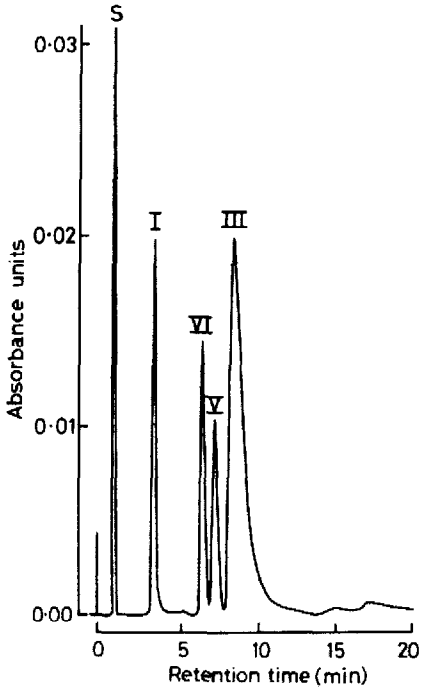
Standard solutions (1.0 mg/ml) of I–VIII were prepared in either dimethylsulphoxide or methanol and protected from light. A composite solution of the colchicine derivatives (I–VI) was prepared in methanol from stock solutions by dilution to a final concentration of 17.5 μg/ml of I, 35.0 μg/ml of II and III and 70.0 μg/ml of IV, V and VI. A composite solution of isocolchicine (VII) and its hydrolytic derivatives (II, III and VIII) was prepared with final concentrations of 17.0, 34.0, 136.0 and 136.0 μg/ml, respectively. For establishing chromatographic conditions for separation, 20 μl of the composite solutions were injected, followed by individual injections of each derivative at the same concentration. Detection limits for each compound were obtained by appropriate dilution or reduction in the injection volume of composite and individual solutions and are based on three separate determinations.

RESULTS AND DISCUSSION

Initial investigation of the conditions required for the separation of colchicine (I) and its decomposition products (II–VI) using methanol–phosphate buffer (35:65) at pH 5.5 resulted in separation of I, V and VII; however, III failed to elute after 60 min. Substitution of acetonitrile for methanol as the organic phase resulted in elution of I, III, V and VI, with partial co-elution of V and VI. Isocolchicine (VII) and N-desacetylisocolchicine (VIII) could also be adequately separated using this system. The use of a mixed organic phase of methanol–acetonitrile (25:75) was found to be optimal for separation and elution time for I, III, V and VI and also for VII and VIII (Figs. 3 and 4).

However, under these conditions colchiceine (II) failed to elute. This problem had been previously encountered by Klein and Davis[9,10], who synthesised derivatives of colchiceine. As the hydroxytropone ring of colchiceine is tautomeric, alkylation results in the formation of both ethylcolchicinate and ethylisocolchicinate.

The failure of colchiceine to elute was thought to be due to the hydroxytropone moiety, as trimethylcolchicinic acid (IV) also failed to elute under these conditions. As hydroxytropone behaves as a vinylogous acid, displaying constant behaviour sim-



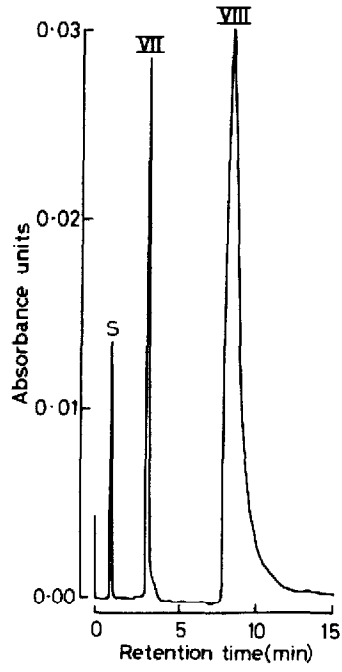


Fig. 3. HPLC separation of colchicine (I), N-desacetylcolchicine (III), β-lumicolchicine (V) and γ-lumicolchicine (VI) on a 10-μm Radpak B, C_{18} reversed-phase radial compression column, eluted at 3.0 ml/min with the solvent acetonitrile–methanol–phosphate buffer (0.025 *M*), pH 5.6 (26.25:8.75:65). Detector wavelength, 350 nm; 0.05 a.u.f.s.

Fig. 4. HPLC separation of isocolchicine (VII) and N-desacetylcolchicine (VIII). Conditions as for Fig. 3

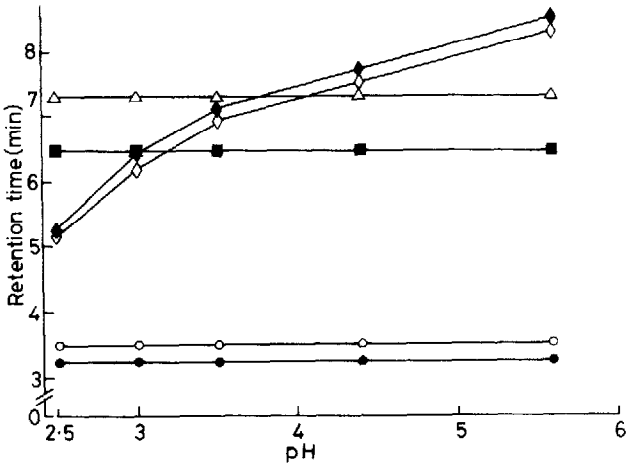


Fig. 5. Plots of retention time of colchicine derivatives as a function of pH. Conditions as for Fig. 3, except that the pH of phosphate buffer was adjusted to required value with phosphoric acid. Colchicine (I), ○; isocolchicine (VII), ●; N-desacetylcolchicine (III), ◇; N-desacetylisocolchicine (VIII), ◆; β-luminocolchicine (V), △; γ-lumicolchicine (VI), ■.

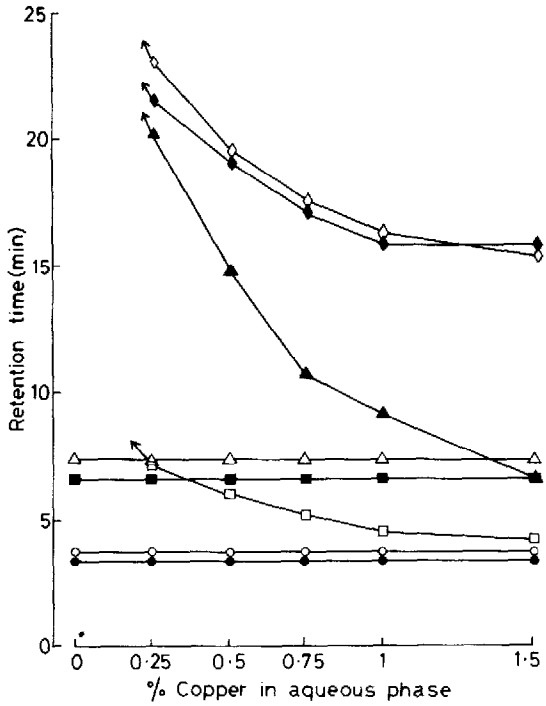


Fig. 6. Plots of retention time of colchicine derivatives as a function of copper ion concentration in the aqueous phase. Column conditions as specified in Fig. 3. Solvent, acetonitrile–methanol–aq. copper sulphate (26.25:8.75:65). Variation of the percentage copper sulphate was achieved by appropriate dilution of a stock 5% copper sulphate solution with distilled water. Colchiceine (II), □; trimethylcolchicinic acid (IV), ▲ and all other symbols are as shown in Fig. 5. Arrow indicates that at 0% copper sulphate, compounds fail to elute.

ilar to carboxylic acid, reduction in eluent pH was examined. Reduction of pH from 5.5 to 2.5, the pH range where ionisation of the hydroxytropone is minimal, demonstrated that this phenomenon is not pH dependent as both II and IV failed to elute. For both desacetyl derivatives (III and VIII) elution was however observed to be pH dependent, probably due to the presence of the free amino group, while for the acetylated derivatives I, V, VI and VII elution was pH independent (Fig. 5).

It appeared to be preferable to investigate further separation methods rather than to synthesise derivatives of colchiceine (II) and trimethylcolchicinic acid (IV), with the attendant needs to standardise the reaction(s) and separate the four derivatives. The chemistry of the hydroxytropone system has been extensively studied and is the subject of a number of reviews[15,16]. Hubler[17] originally described the fact that colchiceine can complex with metal ions to form stable complexes. The copper–colchiceine complex was subsequently isolated and characterised by Zeisel[18]. The order of stability of eight metal complexes formed with tropolone (hydroxytropone), calculated from stability constants, has been demonstrated to be Cu(II) $\gg$ Be > Pb > Zn, Ni > Co(II) > Mg > Ca[19,20].

Based on these studies, investigation of the incorporation of metal ions into the eluent to complex the tropolone moieties of II and IV was undertaken. Metal ions giving both neutral (Cu^{2+} and Pb^{2+}) and ionised (Zn^{2+}) metal–tropolone com-

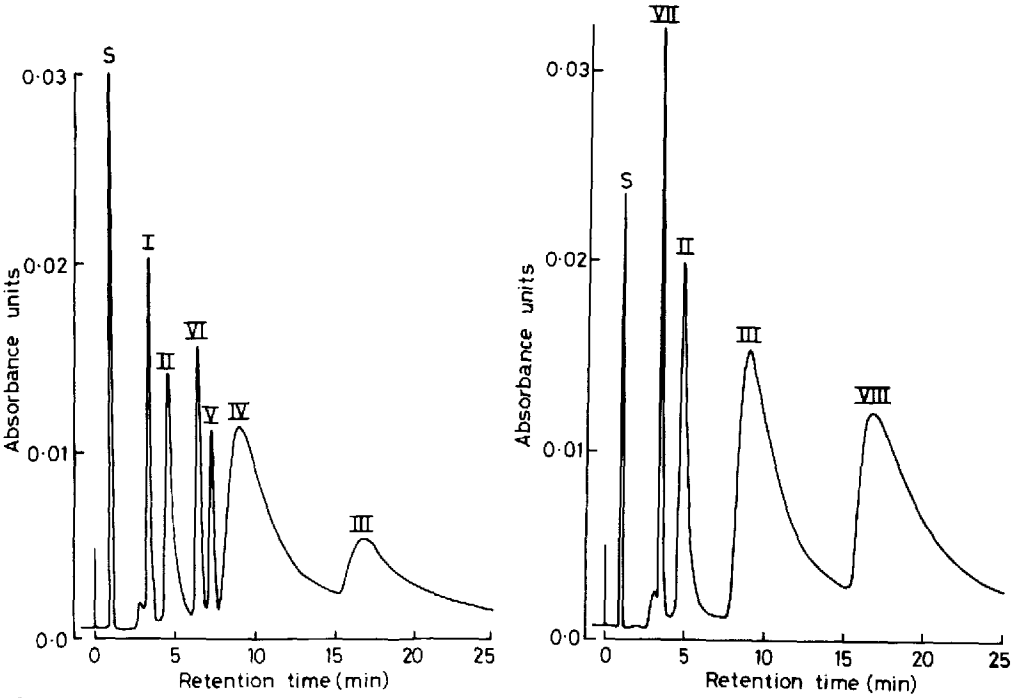


Fig. 7. HPLC separation of colchicine derivatives. Column conditions were as specified in Fig. 3. The compounds were eluted using acetonitrile–methanol–1% copper sulphate solution at pH 4.0 (26.25:8.75:65) at a flow-rate of 3.0 ml/min, detector wavelength, 350 nm; 0.005 a.u.f.s. Colchicine (I), colchiceine (II), β-lumicolchicine (V), γ-lumicolchicine (VI), trimethylcolchicinic acid (IV) and N-desacetylcolchicine (III).

Fig. 8. HPLC separation of isocolchicine hydrolysis products. Conditions as specified in Fig. 7. Isocolchicine (VII), colchiceine (II), trimethylcolchicinic acid (III) and N-desacetylcolchicine (VIII).

plexes were examined[16]. The use of 1% $PbSO_4$ and 1% $ZnSO_4$ in 35% organic phase [comprising methanol–acetonitrile (25:75)] in distilled water did not permit elution of either II or IV. However, the incorporation of 1% $CuSO_4$ allowed elution of both these compounds. This probably reflects a difference in the rates of formation and stability of the complexes under the conditions of analysis.

The presence of buffer was found to be undesirable due to resultant precipitation of $CuPO_4$ during solvent preparation. Decreasing the percentage of $CuSO_4$ from 1.50 to 0.25% resulted in an increase in both retention time and peak width of II and IV (Fig. 6), the effect being more profound with IV. Subsequent analysis of the N-desacetyl derivatives, III and VIII, demonstrated a dependency on Cu^{2+} concentration which was analogous to that of II (Fig. 6).

These results indicate that III and VIII also interact with Cu^{2+} possibly via the free amino group and either the keto group of III or the oxygen of the methoxy group of VIII. The peak shapes of compounds complexed in this manner are significantly broader than that of II (Figs. 7 and 8). When the hydroxy and the amino groups are protected (as for I, V, VI and VII), the elution characteristics do not show any dependency on Cu^{2+} concentration. Elution of the complex was pH dependent for both the tropolone (II, IV) and the amino derivatives (III, VIII), with reduced retention times and improved peak shapes at lower pH (Fig. 9). For routine analysis, pH 3.0–4.0 was considered to be satisfactory in view of the reduced stability of re-

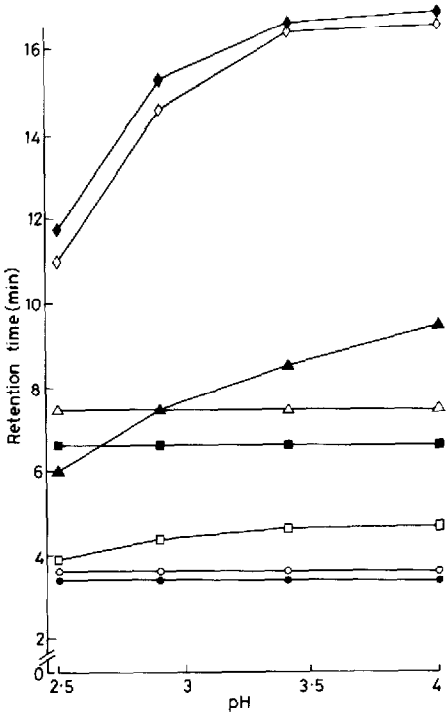


Fig. 9. Plot of retention time of colchicine derivative *versus* variation in pH for eluent containing copper ions. Column conditions were as specified in Fig. 3. The compounds were eluted at 3.0 ml/min in acetonitrile–methanol–1% copper sulphate solution (26.25:8.75:65). pH was varied by the addition of 2.5 *M* H_2SO_4. Symbols are as specified in Fig. 6.

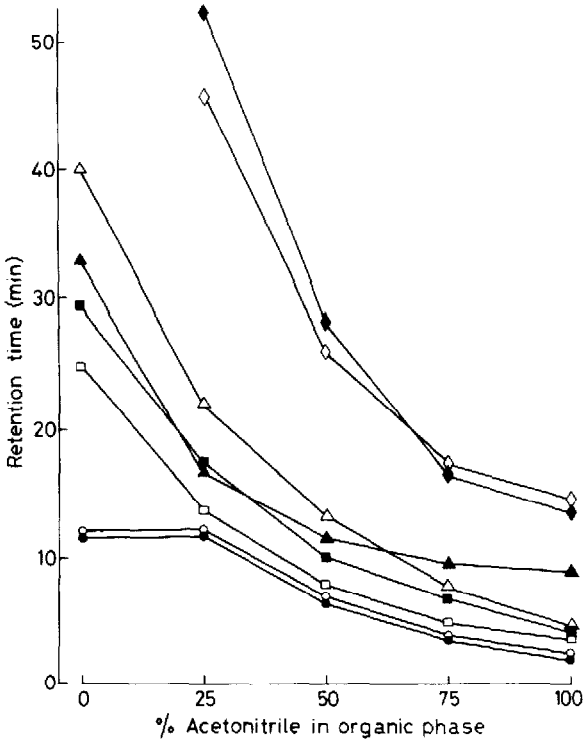


Fig. 10. Plot of retention time of colchicine derivatives as a function of the percentage of acetonitrile in the organic phase. Column conditions were as specified in Fig. 3. The compounds were eluted at 3.0 ml/min in variable mixtures of acetonitrile and methanol comprising total organic phase of 35%. The aqueous phase, comprising the residual volume, was a 1% copper sulphate solution at pH 4.0. Symbols are as specified in Fig. 6.

versed-phase columns under strongly acidic conditions (pH < 2.5).

As shown in Fig. 10, the use of a 35% organic phase with increasing ratios of methanol to acetonitrile in a 1% aq. $CuSO_4$ solution resulted in increasing retention times. The optimum ratio of methanol–acetonitrile for analysis was 25:75. The N-desacetyl derivatives, III and VIII, failed to elute in 120 min when 100% methanol was used as the organic phase.

The detection limits observed were I, 2 ng; II, 4 ng; III, 8 ng; IV, 8 ng; V, 20 ng; VI, 20 ng; VII, 2 ng and VIII, 8 ng for the standard solutions when the detection limit was defined as signal-to-noise ratio of 5 to 1 for the system 35% organic phase [methanol–acetonitrile (25:75)] in 1% aq. $CuSO_4$ at pH 4.0.

CONCLUSIONS

Investigations of methods for the separation of colchicine and related compounds by HPLC have previously been focussed on methods aimed at specific objectives, for example the analysis of Colchicum seeds or the identification of metabolites in mammals or microbes. No study has been undertaken to identify and quantitate the degradation products formed by colchicine in solution. To achieve this

objective, a novel HPLC method which enables direct analysis of tropolones has been developed utilising Cu^{2+} in the eluent. This method avoids the chemical derivatisation and multiple peak quantitation previously involved in tropolone analysis. This method will enable examination of the rates of decomposition of colchicine in solution by either hydrolysis or photolytic mechanisms and aid in the investigation of the interaction of colchicine and related compounds with tubulin.

ACKNOWLEDGEMENTS

This work was supported by Australian Wool Board Grant Number L/9/921. The mass spectra and nuclear magnetic resonance spectra were kindly provided by Mr. Bruce Tattam, Pharmacy Department, Sydney University, Sydney, Australia.

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