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DETERMINATION OF THE ANTIMITOTIC AGENTS N-DESACETYLCOLCHICINE, DEMECOLCINE AND COLCHICINE IN SERUM AND URINE

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SUMMARY

In an effort to characterize the pharmacokinetic behavior of the antimitotic agent N-desacetylcolchicine a selective, sensitive high-performance liquid chromatographic method was developed for the determination of N-desacetylcolchicine, demecolcine and colchicine in serum or urine. To 0.5 ml of serum or 0.1 ml of urine diluted to 0.5 ml were added 50 μl demecolcine (2 μg/ml) which serves as the internal standard. The sample was extracted using a C_2 reversed-phase solid extraction column N-Desacetyl-colchicine, colchicine and the internal standard were eluted from the column with methanol. The combined eluates were evaporated to dryness and the residue was reconstituted with water. The reconstituted sample was injected into a C_{18} reversed-phase column and eluted using a mobile phase consisting of 0.1 *M* potassium dihydrogenphosphate, 5 m*M* 1-pentanesulfonic acid in methanol and acetonitrile with a final pH of 6.0, at a flow rate of 1.5 ml/min. N-Desacetylcolchicine, colchicine and the internal standard were detected using a variable-wavelength ultraviolet detector at 254 nm. The limit of detection was 0.4 ng/ml for desacetylcolchicine and 4.0 ng/ml for colchicine. The method is linear over a concentration range of 1.0–200 ng/ml. The method has been shown to be a rapid, reliable method to monitor N-desacetylcolchicine levels in clinical trials in cancer patients.

INTRODUCTION

There is continuing interest in the development of antimitotic agents as therapeutic alternatives in the treatment of neoplastic disease. Several derivatives of colchicine have been investigated with N-desacetylcolchicine (DAC), formerly referred to as trimethylcolchicinic acid methyl ether *d*-tartrate or TMCA, showing the highest therapeutic index among sixteen colchicine deriv-

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atives tested in an experimental mouse tumor system [1]. It has been postulated that desacetylated derivatives of colchicine act by inhibition of spindle formation resulting in mitotic arrest. Phase II clinical trials have shown that DAC demonstrates activity against Hodgkin's lymphoma, chronic granulocytic leukemia and melanoma. Toxicities were reported to primarily consist of leukopenia, thrombocytopenia, alopecia, dermatitis, stomatitis, diarrhea and nausea [2–4]. It is difficult to predict toxicity with this drug as a function of dose since the maximum tolerated dose and the pharmacokinetics of DAC have not been reported and occurrence of toxic effects is poorly correlated with the total drug dose [3].

Methods used to determine colchicine concentrations in biological fluids have been previously reported using thin-layer chromatographic (TLC), radioimmunoassay and high-performance liquid chromatographic (HPLC) methods [5–8]. Davis and Klein [9] developed an analytical methodology which separated the N-desacetylated derivative of colchicine using HPLC. By modifying the proportion of acetonitrile, methanol and potassium phosphate in the mobile phase, they reported that the retention times of DAC and colchicine were greater than 10 and 20 min, respectively, using a reversed-phase C_{18} analytical column. The method, however, did not include procedures on sample preparation and the reported sensitivity of the method was only 0.3 mg/ml.

In view of the potential toxicity of DAC, low doses of the order of 4–6 mg/m^2 are currently being used in clinical investigations which will result in relatively low steady-state serum DAC levels. The investigation of the pharmacokinetics of DAC in patients necessitated the development of an assay that was highly selective with a limit of detection less than 5 ng/ml.

The procedure described in this report is a selective HPLC method which will allow quantitation of DAC and colchicine in plasma, serum or urine when administered in therapeutic doses.

EXPERIMENTAL

Materials and reagents

Desacetylcolchicine, colchicine, demecolcine (internal standard) and 1-pentanesulfonic acid sodium salt were obtained from Sigma (St. Louis, MO, U.S.A.) and were used without further purification. Potassium dihydrogenphosphate was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.), potassium hydroxide from Matheson Coleman & Bell (Norwood, OH, U.S.A.) and HPLC-grade methanol and acetonitrile from EM Science (Cherry Hill, NJ, U.S.A.). All chemicals and reagents were certified ACS grade. Solid reversed-phase extraction columns (Bond Elut C_2, 100 mg, 1 ml) were purchased from Analytichem International (Harbor City, CA, U.S.A.). Pooled normal human serum was obtained from Pantex (Santa Monica, CA, U.S.A.). Double-distilled, deionized water was used for the preparation of all reagent solutions.

Instrumentation

The HPLC system was manufactured by Shimadzu (Kyoto, Japan) and consisted of two Model LC-6A solvent delivery pumps, a Model DIL-6A autosampler injector, a Model SCL-6A system controller, a Model SPD-6A variable-wavelength UV detector and a Model C-R5A data processor. Samples (50 μl) were injected by an autosampler into a guard column (C_{18}, 5 μm, 1.5 cm$\times$4.6 mm I.D.) followed by an analytical column (Microsorb C_{18}, 5 μm, 15 cm$\times$4.6 mm I.D.) (Rainin, Emeryville, CA, U.S.A.). The absorbance of the effluent was monitored using a variable-wavelength detector at 254 nm for plasma or 350 nm for urine samples.

Mobile phase

Samples were eluted isocratically using a mobile phase consisting of 0.1 *M* potassium dihydrogenphosphate buffer containing 5 m*M* of 1-pentanesulfonic acid–methanol–acetonitrile (60:26.6:13.4, v/v), adjusted to pH 6.0 using 0.1 *M* potassium hydroxide. The solution was filtered through a Magna Nylon 66 membrane filter (Micron Sep, Honeoye Falls, NY, U.S.A.) and deaerated using helium.

Preparation of standard solutions

Standard solutions of DAC and colchicine were prepared by dissolving each in distilled water to obtain concentrations of 1.0 and 10.0 μg/ml. Pooled normal human serum was spiked with the standard solutions of DAC and colchicine to achieve final concentrations of 1, 5, 25, 50, 100 and 200 ng/ml. All spiked serum samples were stored at -70°C prior to use.

Sample preparation

To 0.5 ml of serum or 0.1 ml of urine diluted to 0.5 ml were added 50 μl of demecolcine internal standard solution (2 μg/ml). The serum or urine samples containing the internal standard were passed through a C_2 reversed-phase solid extraction column which was preconditioned by passing through one cartridge volume of methanol and two cartridge volumes of water. The columns were washed by passing through 1.0 ml of distilled water. DAC, colchicine and the internal standard were eluted from the column using 3$\times$0.35 ml of methanol. The combined methanol eluate was collected and evaporated to dryness in a water bath at 40–50°C under a stream of dry, filtered air. The residue was reconstituted in 150 μl of water, and 50 μl were injected into the HPLC system.

A standard curve of peak-height ratio of DAC or colchicine to internal standard versus DAC or colchicine concentration, respectively, was determined using least-squares linear regression. Solid-phase extraction efficiency was determined by comparing the drug concentrations before and after passage through the extraction column. Assay reproducibility was determined at five

different concentrations measured on six consecutive days. Between-run and within-run precision were determined at two DAC concentrations.

RESULTS AND DISCUSSION

The structures of DAC, colchicine and demecolcine are shown in Fig. 1. The ultraviolet absorbance spectra for DAC and colchicine at a concentration of 10 ng/ml in water are shown in Fig. 2. Absorbance maxima for DAC occur at 204.2, 241.4 and 350 nm, and for colchicine at 245.2 and 350 nm. Considering both the sensitivity and selectivity of DAC and colchicine, 254 nm was selected as the analytical wavelength for monitoring both compounds. Following injection into a precolumn, the sample was eluted isocratically with the mobile phase for 10 min. This was followed by a 2-min washout using 80% acetonitrile and an additional washout for 12 min with the mobile phase prior to the next injection. A representative chromatogram for a plasma sample containing 100 ng/ml each of DAC and colchicine is shown in Fig. 3. The retention times for DAC, demecolcine and colchicine were 6.85, 8.20 and 9.41 min, respectively. The total run-time, including a pre-injection washout, required about 26 min. Between-run column washout consumed the majority of the time required for a single assay. The washout was necessary to assure complete removal of re-

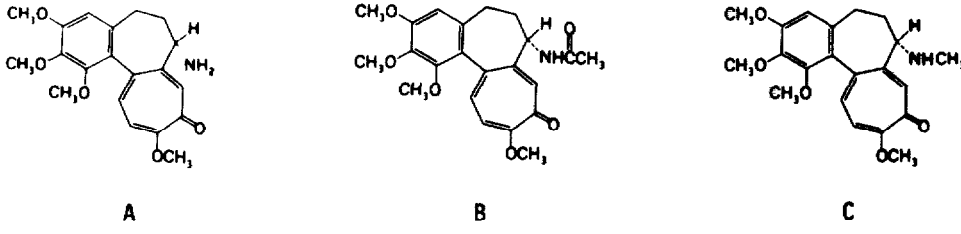

Fig. 1. Structures of desacetylcolchicine (A), colchicine (B) and demecolcine (C).

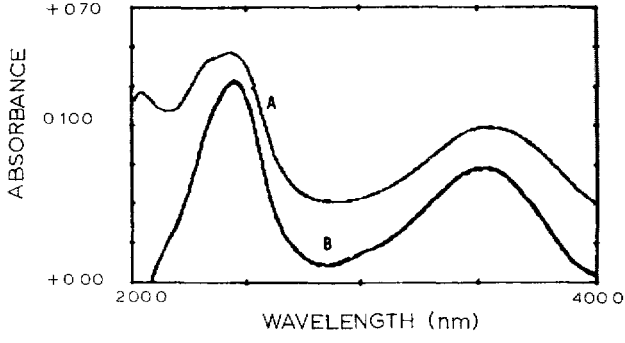

Fig. 2. Ultraviolet absorbance spectra of desacetylcolchicine (A) and colchicine (B) at a concentration of 10 ng/ml in water.

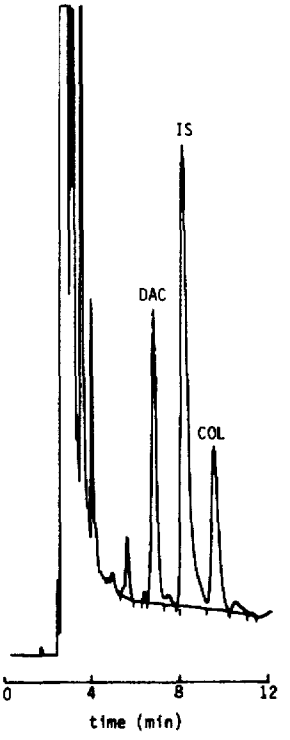

Fig. 3. HPLC profile of DAC and colchicine (COL) at a concentration of 100 ng/ml in pooled human serum. IS = internal standard.

sidual interfering substances on the column. In addition, because of the relatively high solute content in the mobile phase, thorough washing of the column with 80% acetonitrile after each injection was required. Under the chromatographic conditions described above, peaks for DAC, colchicine and demecolcine (internal standard) were resolved completely and separated from interference peaks caused by matrix effects. The minimum amount which could be detected was 0.2 ng for DAC and 1.0 ng for colchicine. In urine samples and several patient serum samples, an interference peak located adjacent to the DAC peak could not be completely resolved (Fig. 4A). In these cases, an analytical wavelength of 350 nm was used which eliminated the interference peaks as shown in Fig. 4B. The peak-height ratio of DAC and colchicine to the internal standard was linear over the concentration range 1.0–200 ng/ml at both 254 and 350 nm.

Assay variability at five concentrations on six separate days is shown in Table I. The standard curve was linear from 1 to 200 ng/ml DAC with a slope of 101.882 ± 3.70 and a mean correlation coefficient of 0.9987. Between-run and within-run precision were determined at two different concentrations. The

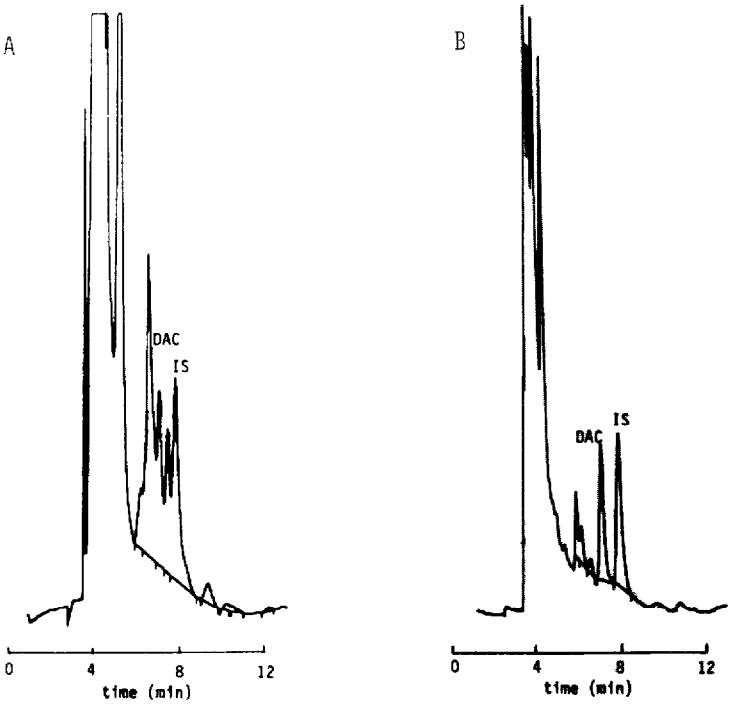

Fig. 4. HPLC profile of DAC from a urine sample monitored at 254 nm (A) and 350 nm (B). IS = internal standard.

TABLE I

DAY-TO-DAY VARIABILITY OF THE DAC ASSAY AT FIVE DIFFERENT CONCENTRATIONS MEASURED ON SIX SEPARATE DAYS

Actual concentration (ng/ml)	Measured concentration (mean ± S.E.M.) (ng/ml)
1.0	1.6 ± 0.67
5.0	5 3 ± 0.82
25.0	23.9 ± 1.88
50.0	53.7 ± 2.00
100.0	98.3 ± 1.14
200.0	199 6 ± 0.86

Standard curve (six separate days)
Slope (mean ± S.E.M.) 101.882 ± 3.70
y-Intercept (mean) −1.190

TABLE II

DETERMINATION OF BETWEEN-RUN PRECISION FOR THE ANALYSIS OF DESACETYLCOLCHICINE

Assay No.	Concentration (ng/ml)	
	5 ng/ml	50 ng/ml
1	5.82	49.76
2	5.30	50.99
3	4 57	51.22
4	4.15	51.33
5	4.32	49.85
Mean ± S.E.M.	4.83 ± 0.32	50.63 ± 0.34

TABLE III

WITHIN-RUN PRECISION FOR THE DETERMINATION OF DESACETYLCOLCHICINE

Assay No.	Concentration (ng/ml)	
	40 ng/ml	100 ng/ml
1	38.15	104.76
2	36.64	106.69
3	36.71	108.58
4	39.19	113.15
5	35 11	109.23
Mean ± S.E.M	37.16 ± 0.70	108.48 ± 1 40

TABLE IV

RECOVERY OF DESACETYLCOLCHICINE AND COLCHICINE FOLLOWING EXTRACTION USING C_2 SOLID-PHASE EXTRACTION COLUMN

Sample concentration (ng/ml)	Mean recovery (%)	
	DAC	Colchicine
200	98.4	100.0
150	97.3	102.9
100	93.8	97.6
50	100.8	86.0
10	105.6	103.2
Mean	99.2 ± 1.96	97.9 ± 3.16

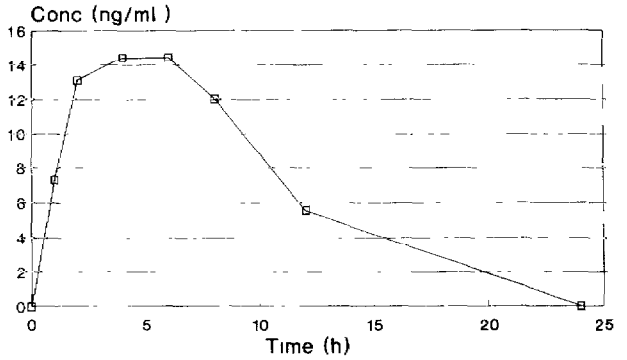

Fig 5 Plasma DAC concentration–time profile in a patient administered an oral dose of 11.2 mg of DAC

between-run precision was determined at 5 and 50 ng/ml and is shown in Table II. The mean value ($\pm$S.E.M.) of five assays for 5 ng/ml sample was 4.83 ± 0.32 and 50.63 ± 0.34 for the 50 ng/ml samples. The relatively high S.E.M. (0.32) for the 5 ng/ml sample can be attributed to assay variability and was not unexpected at extremely low plasma concentrations. The within-run precision was determined at DAC concentrations of 40 and 100 ng/ml and are shown in Table III. Five repeated assays for the 40-and 100-mg samples resulted in mean values of 37.16 ± 0.70 and 108.48 ± 1.40 ng/ml, respectively.

Mean extraction efficiency using C_2 solid extraction columns at five different DAC and colchicine sample concentrations from 10 to 200 ng/ml ranged from 93.8 to 105.6% (mean $99.2 \pm 1.96\%$) for DAC, and 86.0 to 103.2% (mean $97.9 \pm 3.16\%$) for colchicine (Table IV). Solid extraction proved to be more efficient and cleaner than solvent extraction using dichloromethane [7,8].

Dose ranging studies and an investigation of the pharmacokinetics of DAC in cancer patients are currently being conducted. Fig. 5. shows a typical plasma concentration–time profile for DAC in a patient following oral administration of 11.2 mg of desacetylcolchicine. The analytical method is a simple, selective, sensitive and reproducible HPLC assay for the quantitative determination of DAC and colchicine in plasma and urine.

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