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Note

Colchicine quantitation by high-performance liquid chromatography in human plasma and urine

M. LHERMITTE*

Laboratoire de Toxicologie, Faculté de Pharmacie de Lille, 59045 Lille Cedex and Unité des Protéines U 16, Inserm, Place de Verdun, 59045 Lille Cedex (France)*

J.L. BERNIER

Unité des Protéines U 16, Inserm, Place de Verdun, 59045 Lille Cedex (France)

D. MATHIEU

Unité de Réanimation Respiratoire et Médicale, Hôpital Calmette, 59045 Lille Cedex (France)

M. MATHIEU-NOLF

Centre Anti-Poisons, Hôpital Calmette, 59045 Lille Cedex (France)

F. ERB

Laboratoire de Toxicologie, Faculté de Pharmacie de Lille, 59045 Lille Cedex (France)

and

P. ROUSSEL

Unité des Protéines U 16, Inserm, Place de Verdun, 59045 Lille Cedex (France)

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The clinical features of colchicine poisoning have been well documented over the past 40 years [1—4]. Most reported cases were associated with the

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ingestion of doses of colchicine of 50 mg or more. Other reports have described severe toxic side-effects and some fatalities following doses of 5–15 mg.

Colchicine is given under medical supervision for the treatment of malignancy or gout [5–7]. In a few instances the toxicity was attributed retrospectively to poor renal function, but in others the possibility of individual sensitivity to colchicine has been raised [5].

Despite the awareness of the low therapeutic index of colchicine, little has been done, until recently, to study its pharmacokinetics, largely due to the lack of a suitable method for the estimation of colchicine in body fluids.

Colchicine has been determined using colorimetric methods [8, 9], which were insufficiently sensitive for use in humans taking therapeutic doses. Bourdon and Galliot [10] developed a fluorimetric method, and colchicine has also been determined in body fluids by gallium chelate formation [11]. The pharmacokinetic parameters of colchicine, the half-life and the volume of distribution, have been determined in human volunteers by a radioisotope dilution technique [12] and later by radioimmunoassay [13–15].

More recently, Jarvie et al. [16] and Caplan et al. [17] have described a high-performance liquid chromatographic (HPLC) method for the estimation of colchicine in a poisoned patient.

A method that is sufficiently rapid and accurate at the relatively high levels found in cases of colchicine overdose has been developed in our laboratory, in order to provide some prognostic guidance to the clinicians with such patients. This method could assist in gaining an appreciation of the therapy used after an intoxication. Preliminary results obtained from a single case are reported.

EXPERIMENTAL

Chemicals and reagents

Colchicine was a gift from Laboratories Houde I.S.H., (Paris, France) and used directly. Morpholinopropylcolchicamide was used as internal standard

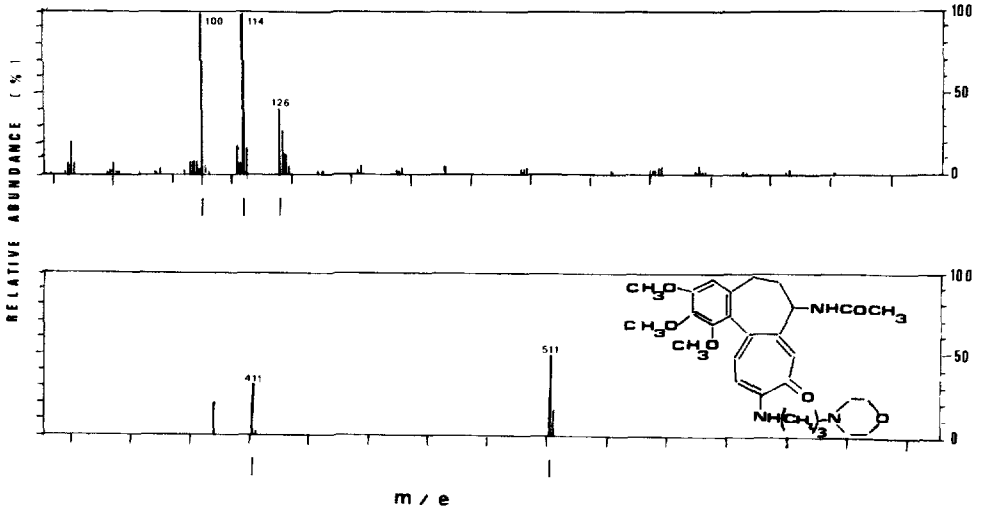

Fig. 1. Electron-impact mass spectrum of internal standard (morpholinopropylcolchicamide).

(Fig. 1). It was synthesized by refluxing 1 g of colchicine (2.5 m*M*) with 0.36 g of N-(3-aminopropyl)morpholine (2.5 m*M*) in 50 ml of pyridine for 4 h. The solution was concentrated to 20 ml in vacuo and cooled to give the expected amide, which can be crystallized from ethanol: 1.1 g (86%); m.p.: 145°C; M^+ (electron-impact) = 511; IR: 1670 cm^{-1} ($\nu_{C=O}$ amide), 2800—2820 cm^{-1} (ν_{CH_2} morpholinoalkyl). *Acetonitrile was HPLC grade (Carlo Erba, Italy)*; all other chemicals and solvents were reagent grade. Distilled water was purified by passing it through a reverse-osmosis four-filter system (Millipore, Bedford, MA, U.S.A.).

Mass spectrometric analysis

The structure of the internal standard was assessed using a mass spectrometer—computer system (Riber-mag) with a 70-eV electron-impact ionization source.

Chromatographic conditions and instrumentation

A Varian (Palo Alto, CA, U.S.A.) Model 5000 liquid chromatograph equipped with a Valco sample injection valve and UV variable-wavelength detector (Varichrom) was used for the analysis. Chromatography was performed on a 300 × 4.0 mm I.D. stainless-steel Micropak MCH 10-μm column (Varian), with a precolumn (40 × 4 mm I.D.) that contained the same phase. The mobile phase was actonitrile—water (50:50) at a flow-rate of 2.0 ml/min and a pressure of ca. 140 bar. The separation was performed at ambient temperature. Colchicine and internal standard were detected at a wavelength of 245 nm.

Standard solutions

Standard stock solutions of colchicine and internal standard (morpholinopropylcolchicamide) were prepared in methanol at a concentration of 100 μg/ml. These were refrigerated at 4°C and found to be stable for several weeks in the dark.

The internal standard stock solution (100 μg/ml) was diluted 1:100 with methanol, and 100 μl of this solution were used for internal standardization (100 ng).

Plasma standards (calibration standards) were prepared at concentrations of 5, 10, 20, 25, 50, 75 and 100 ng/ml. The 100 ng/ml standard was prepared by adding 50 μl of the colchicine stock solution (100 μg/ml) to 50 ml of drug-free human plasma. The other standards were then prepared by stepwise dilutions with drug-free plasma. These calibration standards were stored deep-frozen (—20°C) in small portions until needed for analysis.

Urine standards (calibration standards) were prepared at concentrations of 0.25, 0.5, 1, 2.5, 3.75 and 5 ng/ml. The 5 ng/ml standard was prepared by adding 50 μl of the colchicine stock solution (100 μg/ml) to 50 ml of drug-free human urine to yield a concentration of 100 ng/ml. This solution was diluted 1:20 with drug-free urine to yield a concentration of 5 ng/ml. The other standards were prepared by stepwise dilutions with drug-free urine. Calibration standards are lower in urine than in plasma, because the extraction procedure is carried out with 20 ml of urine. The urine calibration standards were stored deep-frozen (—20°C) in small portions until needed for analysis.

Plasma extraction procedure

Calibration curve. To 1.0 ml of each solution of plasma standard in a 50-ml centrifuge tube containing 100 ng of internal standard (morpholinopropylcolchicamide) were added 1.0 ml of 8 *M* ammonium hydroxide and 15 ml of dichloromethane. The tube was mechanically shaken for 10 min and then centrifuged at 850 *g* for 5 min. The aqueous phase (upper) was transferred to another tube and re-extracted in a similar manner. The two organic phases were mixed and ethanol (10.0 ml) was added. The tube was vortexed for 5 min and then centrifuged at 850 *g* for 5 min. The supernatant was evaporated to dryness under nitrogen at 50°C. The residue was redissolved in 100 μl of mobile phase (acetonitrile—water, 50:50), and 50 μl were injected into the liquid chromatograph.

Samples. A suitable volume of plasma (up to 1 ml) was combined with 100 μl of internal standard (100 ng of morpholinopropylcolchicamide), 1 ml of 8 *M* ammonium hydroxide and 15 ml of dichloromethane. This mixture was further treated as described for the calibration curve.

Urine extraction procedure

Calibration curve. To 20 ml of each solution of urine standard in a 50-ml centrifuge tube containing 100 ng of internal standard were added 5 ml of 8 *M* ammonium hydroxide and 15 ml of dichloromethane, and extraction proceeded as described for plasma. The upper aqueous phase was re-extracted twice with 15 ml of dichloromethane. The organic phases were transferred to a tube containing anhydrous sodium sulphate and filtered through Whatman 1 paper into a clean tube. The solvent was evaporated to dryness under nitrogen at 50°C. The residue was redissolved in 1 ml of distilled water and passed through a Sep-Pak C_{18} cartridge (Waters). The cartridge was eluted with 5 ml of acetonitrile—water (50:50). The solution was evaporated to dryness under nitrogen at 50°C. The final residue was dissolved in 100 μl of mobile phase (acetonitrile—water, 50:50), and 50 μl were injected into the liquid chromatograph.

Samples. Urine samples of 20 ml were further treated as described for the calibration curve.

Quantitation

Calibration standards covering the anticipated concentration range (5—100 ng/ml) in methanol, plasma and urine were processed. Peak area ratios of colchicine to the internal standard were measured, and the calibration was obtained from linear regression of the peak area ratio against concentration. This line was then used to calculate the concentration of the drug in the unknown samples.

Recovery

Extracts from urine and from plasma, prepared as described above, were compared with a direct assay of standards in methanolic solution. These relative recoveries were determined for two different concentrations. The absolute recoveries were also determined for these two different concentrations from extracts of urine and plasma, treated using the procedure described

above, except that the internal standard was omitted. All extraction sample residues were reconstituted in 100 μl of the solution of internal standard (1 μg/ml) in mobile phase (acetonitrile—water, 50:50). In this recovery analysis, morpholinopropylcolchicamide served as external standard.

Interference

Interferences from endogenous material and from drugs commonly used in therapeutic treatment or often found in poisoned patients were researched (Table I). Drugs were tested at concentrations of 500 ng/ml.

TABLE I

DRUGS TESTED FOR POSSIBLE INTERFERENCE IN THE HPLC ASSAY OF COLCHICINE

Vinbarbital	Meprobamate
Amobarbital	Prazepam
Secobarbital	Chlordiazepoxide
Phenobarbital	Chlorazepam
Barbital	Medazepam
Butalbital	Diazepam
Butobarbital	Lorazepam
Thiopental	Clonazepam
Caffeine	Nitrazepam
Theophylline	Quinidine

Human toxicokinetic studies

The procedure was used to analyse the in vivo disposition of colchicine in a poisoned patient, found after absorption of 31 mg of drug. Blood was collected frequently into heparinized tubes over a period of 12 h after the admission. The plasma was separated, frozen and stored in the dark at −20°C until analysed. During the period of admission, 300 ml of urine were collected.

RESULTS AND DISCUSSION

Fig. 2 shows the separation and quantitation of colchicine in human plasma and urine using morpholinopropylcolchicamide as internal standard. In the chromatograms which were obtained after extraction of 1.0 ml of blank plasma or 20 ml of blank urine, no additional peaks that could interfere with the determination of colchicine and internal standard are present. Fig. 2A represents a chromatogram of a blank plasma. Fig. 2B is a chromatogram obtained after extraction of 1.0 ml plasma containing 5 ng/ml colchicine. Fig. 2C is a chromatogram obtained after extraction of 20.0 ml of urine containing 0.5 ng/ml colchicine. Drug and internal standard are well separated with retention times of 3.5 and 4.7 min, respectively.

The introduction of a N-(3-aminopropyl)morpholine chain makes the molecule more hydrophilic and thus modifies its retention time in the column with an acetonitrile—water mobile phase. Nevertheless, the polycyclic moiety of colchicine remains unchanged, involving only a slight difference of separation which permits a good comparison between the peaks of colchicine and its internal standard.

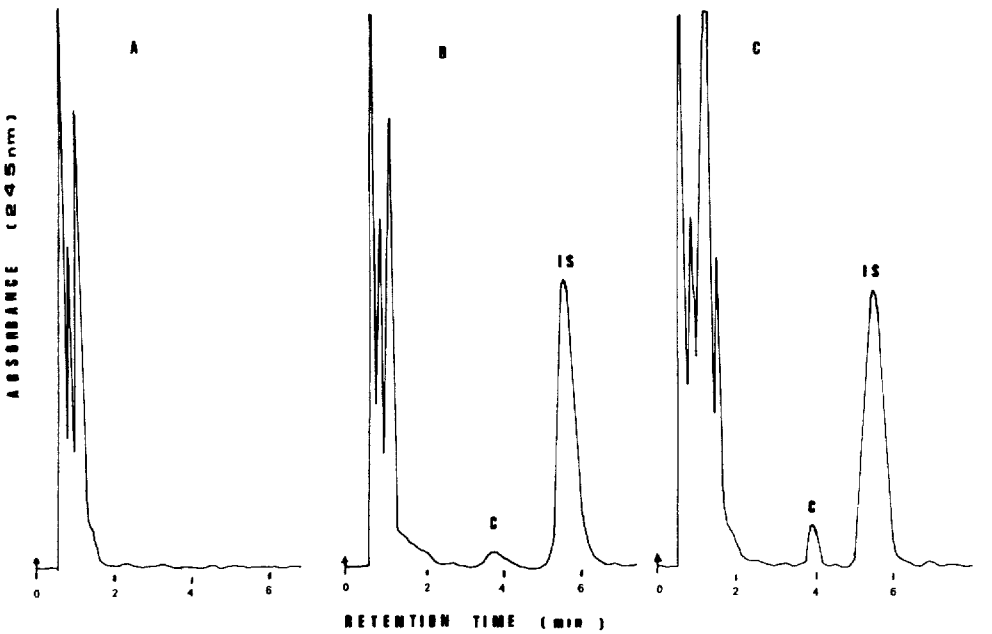

Fig. 2. HPLC profiles of (A) extracted serum blank, (B) human plasma (1 ml) containing 5 ng/ml colchicine and 100 ng/ml internal standard, (C) human urine (20 ml) containing 0.5 ng/ml colchicine and 100 ng/20 ml internal standard. Peaks: C = colchicine; IS = internal standard.

The calibration curves were obtained using methanolic solution of standards, human plasma or human urine spiked with 5—100 ng/ml colchicine and 100 ng/ml internal standard. There was a good correlation between the amount of colchicine added to the human plasma and urine and the amount detected in the samples of both 1.0 ml of plasma and 20.0 ml of urine. The linear regression equations of data are shown in Table II. Calibration curves in plasma and urine showed good linearity between peak area ratios and concentrations from 5 to 100 ng/ml, and the present method is able to detect 5 ng/ml colchicine. The intra- and inter-assay precision data for colchicine in both plasma and urine are summarized in Table III. There was little variation in colchicine determination with coefficients of variation below 9%.

Analytical relative and absolute recoveries of colchicine both in plasma and urine were determined at two concentrations (10 and 50 ng/ml) and are reported in Table IV. For absolute recovery, morpholinopropylcolchicamide was used as external standard.

TABLE II

LINEAR REGRESSION EQUATIONS FOR COLCHICINE

Methanolic solution	$y = 0.0190x - 0.0153$	($r = 0.9937$)
Plasma extraction	$y = 0.0176x - 0.0035$	($r = 0.9984$)
Urine extraction	$y = 0.0193x + 0.0041$	($r = 0.9985$)

y = peak area ratio colchicine to internal standard; x = colchicine concentration.

TABLE III

INTRA- AND INTER-ASSAY COEFFICIENT OF VARIATION FOR COLCHICINE

Sample	Concentration (ng/ml)	Within-run C.V. (%) (n^* = 15)	Day-to-day C.V. (%) (n = 16)
Plasma	10	8.73	7.70
	50	2.09	1.80
Urine	10	4.34	6.00
	50	4.30	6.00

*n = Number of determinations.

TABLE IV

RELATIVE AND ABSOLUTE RECOVERIES OF COLCHICINE

Sample	Concentration (ng/ml)	Relative recovery (%)	Absolute recovery (%)
Plasma	10	94 ± 4	78 ± 2
	50	98 ± 3	90 ± 1
Urine	10	94 ± 8	86 ± 3
	50	98 ± 3	90 ± 2

The assay was shown to be selective, without interference from endogenous material and from other drugs commonly used in therapeutic treatment or often found in poisoned patients (except for quinidine, which eluted at the same retention time as colchicine) (Table I).

The HPLC procedure described herein has been used for the assay of human plasma samples obtained from one poisoned subject. After ingestion of 31 mg of colchicine, the colchicine plasma levels were 720 ng/ml at 20 min, 212 ng/ml at 125 min, 132 ng/ml at 305 min and 120 ng/ml at 605 min. In these assays, only 100 μl of plasma sample were used. The level of colchicine found in the urine of the same patient was 5 μg/l.

In summary, this HPLC assay shows good reproducibility, sensitivity and selectivity. It has the advantage of being a relatively convenient, rapid and simple method. It was developed in response to a clinical problem and can provide an indication of the plasma colchicine concentration in as little as 10 min and an accurate answer within 1 h, once standards have been processed. This method was applied to the analysis of samples from poisoned patients and will be easily applicable to toxicokinetic studies in humans.

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