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Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713597273>

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To cite this Article Samanidou, V. F. , Sarantis, G. A. and Papadoyannis, I. N.(2006) 'Development and Validation of a Rapid HPLC Method for the Direct Determination of Colchicine in Pharmaceuticals and Biological Fluids', *Journal of Liquid Chromatography & Related Technologies*, 29: 1, 1 – 13

To link to this Article: DOI: 10.1080/10826070500357755

URL: <http://dx.doi.org/10.1080/10826070500357755>

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Development and Validation of a Rapid HPLC Method for the Direct Determination of Colchicine in Pharmaceuticals and Biological Fluids

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Abstract: A reversed-phase high performance liquid chromatographic (RP-HPLC) method is developed and validated for the determination of the colchicine, an anti-gout alkaloid. The analytical column, an Inertsil ODS-2, 5 μm , 250 \times 4 mm, was operated at ambient temperature. Isocratic elution with A: 48% $\text{CH}_3\text{COONH}_4$ 0.05 M and B: 52% CH_3OH , was used at a flow rate of 1.0 mL/min. Inlet pressure was 270 kg/cm². UV detection was performed at 245 nm.

The Limit of Detection was 0.5 ng, per 20 μL injection volume, while linearity held up to 2.5 ng/ μL . Codeine was used as Internal Standard at a concentration level of 10 ng/ μL . The statistical evaluation of the method was performed in terms of intra-day ($n = 8$) and inter-day calibration ($n = 8$) and was found to be satisfactory, with high accuracy and precision results.

The method was successfully applied to commercial pharmaceuticals and biological fluids. Blood serum samples, after deproteinization with acetonitrile, yielded high recovery rates from 97.2 to 104.6%, while direct analysis of urine after a 10-fold dilution provided recovery rates ranging from 89.5 to 104.0%.

Keywords: Colchicine, Pharmaceuticals, Biological fluids, Serum, Urine, HPLC

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INTRODUCTION

Colchicine (N-5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[a]heptalen-7-yl acetamide), the alkaloid of meadow saffron is isolated from various species of *Colchicum* of Liliaceae family, mainly from *Colchicum autumnale* L.^[1] It has been used in the treatment of acute flares of gout, pseudogout, and acute gouty arthritis, specifically relieving the pain of acute attacks.^[2,3] Additionally colchicine is a relatively potent inhibitor of mitosis, and it has been investigated as an antineoplastic agent for the treatment of amyloidosis in Familial Mediterranean Fever and scleroderma.^[4]

The mode of action of colchicine in gout is unknown. It is thought to decrease lactic acid production by the leukocytes and, thereby, decrease uric acid crystals deposition causing severe pain, redness, and swelling of the affected joints.^[5]

However, despite of its therapeutic properties there are several reports of colchicine toxicity in literature mostly attributed to suicidal overdose. It is widely accepted that a dose of 0.5 mg/kg can cause gastrointestinal problems, while a dose over 0.8 mg/kg usually results in cardiogenic shock. The mechanism whereby colchicine exerts its toxic effects can be related to the drug's cytotoxic effects on the labile cell population by means of disturbing the replication process in such tissues.^[2,6] Colchicine is primarily deacetylated by the liver, although as much as 30% of it may be excreted unchanged by the kidney.^[7]

Various analytical techniques have been used for the determination of colchicine and several analytical methods can be found in the literature for the analysis of biological matrices, as well as pharmaceuticals, dietary supplements, plant material tissues, and milk samples from the intoxicated animals. Most of the reported methods involve RP-HPLC with UV detection and isocratic or gradient elution.^[3,6-15] High performance liquid chromatography coupled to mass spectrometry was also applied for the determination of colchicine at ppb levels in human biofluids (urine, serum, plasma, and whole blood), as well as in post-mortem blood and other tissues and fluids, where colchicine was detected by selected-ion monitoring mode.^[2,16,17]

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS-MS) has been used for the evaluation of commercial Ginkgo and Echinacea dietary supplements for colchicine.^[18]

Other chromatographic techniques used for colchicine determination include Gas-liquid chromatography,^[19] as well as GC/MS in a case of Colchicine poisoning report of a fatal case.^[20]

TLC methods have been reported for the quantitative determination of the alkaloid in several matrices such as plant material, tissues, and milk samples from the intoxicated animals, pharmaceuticals, and vegetal extracts.^[21-24]

Other analytical techniques for colchicine determination include: Indirect atomic absorption spectroscopy by means of complexation with copper, which was proposed for the determination of colchicine in biological samples,^[25]

fluorescence analysis by hydrolysis and complexation with lanthanum^[26] or calcium ion,^[27] spectrophotometry involving the oxidation of colchicine by iron (III) chloride and subsequent complexation of iron (II) with 1,10-phenanthroline forming a red coloured complex having maximum absorbance at 510 nm.^[28]

Electroanalytical techniques used for the determination of colchicine include adsorptive stripping voltammetry based on the controlled adsorptive accumulation of the drug at the hanging mercury drop electrode, followed by voltammetric determination of the surface species,^[29] adsorptive voltammetry on a carbon paste electrode,^[30] and differential pulse polarography at a glassy electrode and a hanging mercury electrode.^[31,32]

Radioimmunoassays^[33–35] and optical-fibre sensors for the determination of colchicine have also been introduced for colchicine analysis.^[36–38]

Sample preparation techniques used for colchicine determination include SPE in serum and urine,^[8] LLE with dichloromethane in post-mortem fluids or tissue homogenates,^[7] the use of Extrelut columns with dichloromethane in blood and bile,^[3,6,11] and extraction with ethyl acetate in placental blood from patients using herbal medicines, after removing red blood cells and proteins by centrifugation and filtration.^[12]

HPLC-DAD was used in heart blood, urine, gastric contents, and bile analysis, in a case report of colchicine poisoning. Colchicine was eluted in 13.1 min. Gradient elution was performed at 30°C and detection at 233 nm. Sample preparation involved extraction with dichloromethane.^[15]

The alkaline extraction of biological fluids (urine, serum, plasma, and whole blood) with a mixture of diethyl ether and methylene chloride 7:4 in post-mortem blood, and methylene chloride in other tissues and fluids was also reported.^[16]

Supercritical fluid extraction with carbon dioxide was reported only for colchicine isolation from plant materials such as seeds of *Colchicum autumnale*.^[14,39]

In the present paper an accurate, precise, and rapid analytical method was developed and validated for the direct HPLC determination of colchicine in pharmaceuticals and biological fluids: blood, serum, and urine. The present method can be applied both to toxicological diagnosis and to therapeutic monitoring of colchicine.

EXPERIMENTAL

Instrumentation and Chromatography

A Shimadzu (Kyoto, Japan) LC-10AD pump was used to deliver the mobile phase to the analytical column, Inertsil ODS-2, 5 μm , 250 \times 4 mm (MZ Analytical, Mainz, Germany). Sample injection was performed via a Rheodyne 7125 injection valve (Rheodyne, Cotati California, U.S.A) with a

20 μL loop. Detection was achieved by an SSI 500 UV-vis detector (SSI, State College, PA, U.S.A.) at a wavelength of 245 nm and a sensitivity setting of 0.002 AUFS. Data acquisition was performed using software designed for chromatography, developed by Professor P. Nikitas (Laboratory of Physical Chemistry, Chemistry Department of University of Thessaloniki). A glass vacuum filtration apparatus obtained from Alltech Associates was employed for the filtration of the ammonium acetate solution, using 0.2 μm membrane filters (Schleicher and Schuell, Dassel, Germany). Degassing of solvents was achieved by helium sparging before use. Dissolution of compounds was enhanced by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany). A Glass-col, Terre Haute, IN 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pretreatment. All evaporations were performed with a 9-port Reacti-VapTM evaporator, Pierce, Model 18780 (Rockford, IL, USA). The UV spectrum of colchicine for selecting the working wavelength of detection was taken using a Varian DMS 100S UV-vis double-beam spectrophotometer (Varian, Inc. Corporate Headquarters, Palo Alto, CA, USA).

The mobile phase was $\text{CH}_3\text{COONH}_4$ 0.05 M- CH_3OH 48 : 52 v/v. Inlet pressure observed at a flow rate of 1.0 mL/min was 270 kg/cm². The injection volume was 20 μL . Codeine was used as internal standard at a concentration of 10 ng/ μL .

Samples, Chemicals, and Reagents

Colchicine (95% HPLC) was supplied by Sigma (Sigma-Aldrich Chemie BV, The Netherlands). Codeine was from Merck (Darmstadt, Germany). Methanol and acetonitrile of HPLC grade were purchased from Carlo Erba (Rodano, Italy). Ammonium acetate of analytical grade was supplied by Riedel-de-Haen (Seelze, Germany). Ultrapure water provided by a Milli-Q[®] purification system (Millipore, Bedford, MA, USA) was used throughout the study.

In Greece, colchicine is commercially available only as 1 mg tablets. Colchicine[®] Houdé 1 mg tablets are manufactured by Usiphar (Compiègne, France).

Serum samples were kindly provided by the Blood Donation Unity of a State Hospital, while urine samples were provided by healthy volunteers.

Stock standard solutions (100.0 ng/ μL) were prepared in water. Working aqueous standards were prepared by appropriate dilution at 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, and 2.5 ng/ μL . These solutions when kept refrigerated were found to be stable for at least 3 months.

An aqueous solution of codeine at a concentration of 10 ng/ μL was selected as the most suitable internal standard.

Validation of the Method

Method validation was performed in terms of linearity, repeatability, intermediate precision, accuracy, and sensitivity.

The linearity response was assessed in the range of 0.05–2.5 ng/ μ L. Method validation regarding repeatability was achieved by replicate injections of standard solutions at low, medium, and high concentration levels (0.2, 1.0, and 2.0 ng/ μ L (microliter)), where peak areas were measured versus peak area of the internal standard. Statistical evaluation revealed relative standard deviations at different values for eight injections. Intermediate precision study was conducted during routine operation of the system over a period of eight consecutive days. In both validation processes, accuracy was determined by replicate analysis and it was expressed as relative error of measurement.

Samples of biological fluids spiked at three concentration levels (0.2, 1.0, and 2.0 ng/ μ L (microlite) for urine and 1.0, 2.0, and 2.5 ng/ μ L (microliter) for serum) were used for estimating accuracy and precision in terms of recovery. Between-day assay covered a period of eight days, while within-day repeatability was checked by eight replicates of spiked biological samples.

The sensitivity of the developed method was checked in terms of limits of detection (LOD) and quantitation (LOQ). The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal to-noise ratio. This was taken as the lowest concentration in the calibration range.

Sample Preparation

Pharmaceuticals

Ten commercial tablets (labeled concentration 1 mg/tablet) were weighed to determine the mean weight. Then, they were finely powdered in a porcelain mortar and an accurately weighed portion of the pooled sample equivalent to the antibiotic content of one tablet: 0.0746 g, was quantitatively transferred to a 100 mL volumetric flask, dissolved, and diluted to volume with water. After filtration, further dilution provided solutions containing the following concentrations 0.2, 0.5, and 1.0 ng/ μ L. Eight replicate injections were performed for each solution. All working solutions contained the internal standard.

Biological Fluids: Blood Serum and Urine

To an aliquot of 100 μ L of pooled blood serum, proteins were precipitated using 500 μ L of acetonitrile. After centrifugation for 15 min at 3500 rpm,

the supernatant was evaporated to dryness at 45°C under a gentle nitrogen stream. The dry residue was reconstituted with 100 μL of methanol.

Pooled urine samples were directly analyzed after a 10-fold dilution and filtration.

Calibration curves were constructed using spiked serum and urine samples at concentration levels in the range from 0.05 to 2.5 $\text{ng}/\mu\text{L}$.

RESULTS AND DISCUSSION

Chromatography

A typical chromatogram obtained using the developed method conditions is illustrated in Fig. 1A. Retention times revealed were 5.0 min. for colchicine and 3.8 min for codeine. Resolution factor between colchicine and the internal standard was 2.

Method Validation

Linearity and Sensitivity

Calibration curves were obtained by least-squares linear regression analysis of the peak areas ratio of colchicine to internal standard versus colchicine absolute amount. The method was linear up to 2.5 $\text{ng}/\mu\text{L}$, with a correlation coefficient of 0.993.

The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three and it was found to be 0.4 ng. The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the signal-to-noise ratio and it was found to be 1.0 ng.

Table 1 summarizes all calibration and sensitivity data.

Precision and Accuracy

The accuracy and precision of the method based on within-day repeatability was performed, by replicate injections ($n = 8$) of three standard solutions, covering different concentration levels: low, medium, and high, where peak areas were measured, in comparison to the peak area of the internal standard. The reproducibility (between-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of eight consecutive days. Statistical evaluation revealed relative standard deviations, at different values. Table 2 summarizes the results of the method validation regarding accuracy, within-day, and

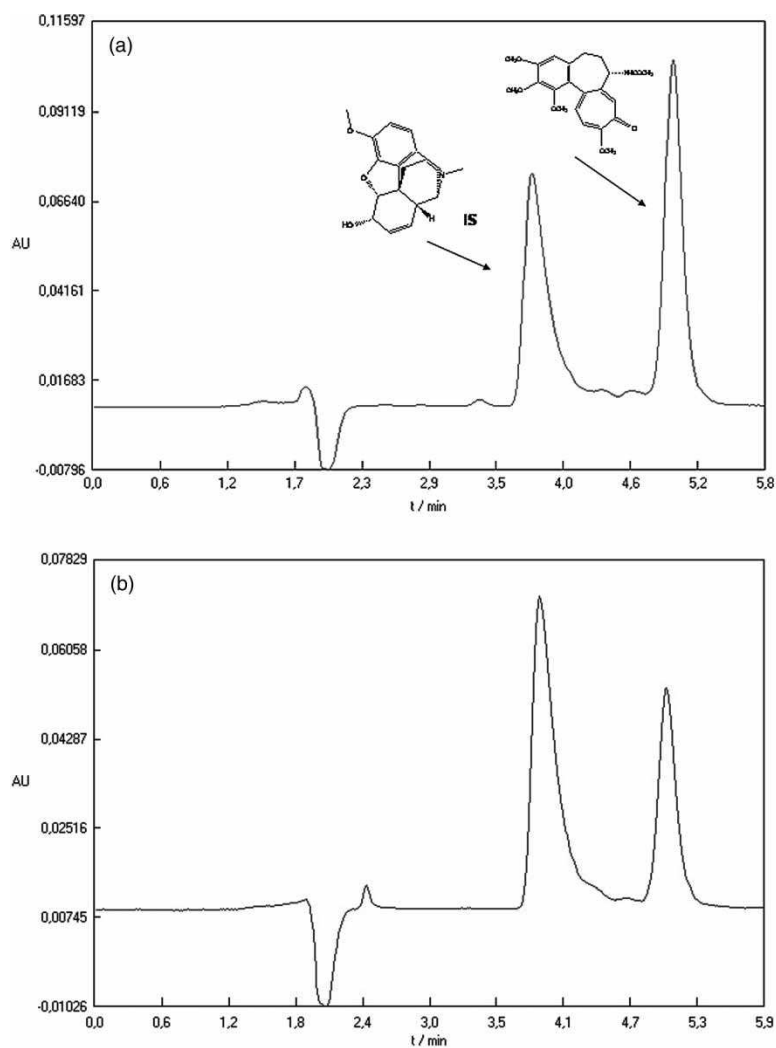


Figure 1. (a) Chromatogram of colchicine (5.0 min) in the presence of codeine as Internal Standard (3.8 min). (b) Chromatogram of colchicine (5.0 min) determination in pharmaceutical tablets in the presence of Codeine (3.8 min).

between-day precision assays. The measured concentrations had RSD values <7%, with relative error (inaccuracy) in the range of -9.2 to 8.1%.

Accuracy was expressed as relative error calculated by the equation:

$$\text{Relative error (\%)} = \frac{[\text{Mean determined value} - \text{theoretical(added amount)}]}{\text{theoretical}} \times 100$$

Table 1. Calibration data for simultaneous determination of colchicine in standard solutions, blood serum and urine. Peak area ratio measurement with 10 ng/ μ L codeine as internal standard

Samples	Slope	Intercept	R	LOD (ng)	LOQ (ng)	Upper limit (ng/ μ L)
Standard solutions	0.024709 \pm 0.00053	0.005974 \pm 0.010664	0.9993			
Blood serum	0.034471 \pm 0.001311	-0.046405 \pm 0.044464	0.9985	0.4	1	2.5
Urine	0.03244 \pm 0.000782	0.054922 \pm 0.017581	0.9994			

Application to Pharmaceuticals and Biological Fluids

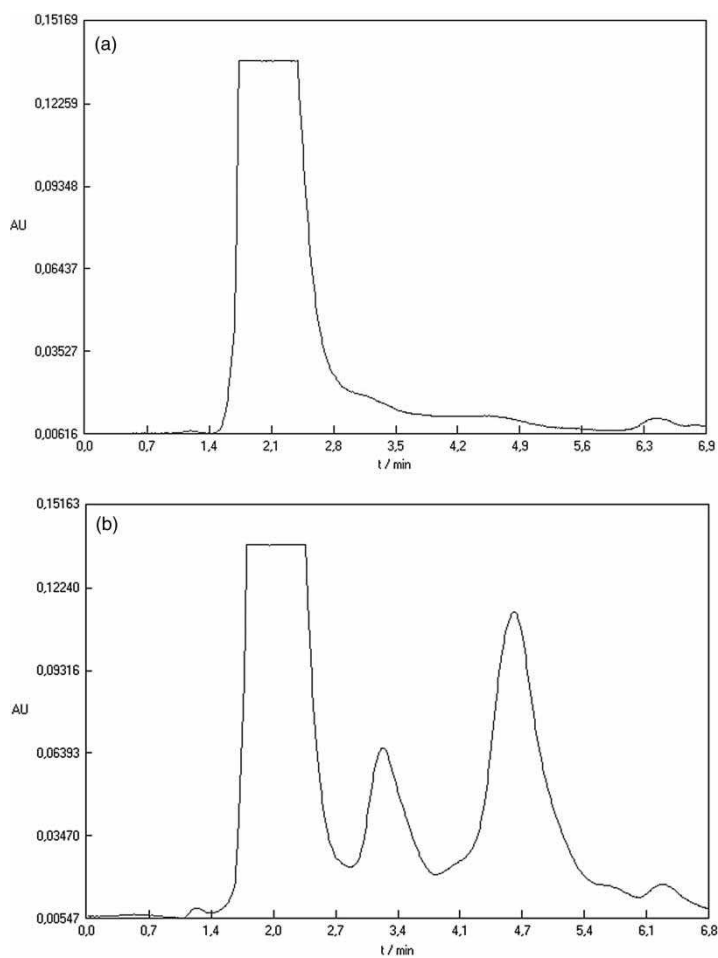
The developed method was subsequently applied to pharmaceuticals and biological fluids. Results of colchicine determination in tablets are presented in Table 3. Satisfactory accuracy and precision is observed. A typical

Table 2. Within-day and between-day precision and accuracy study for colchicine in standard solutions, blood serum and urine samples

Added (ng)	Within-day n = 8			Between-day n = 8		
	Found (ng) \pm SD	RSD	RE%	Found (ng) \pm SD	RSD	RE (%)
Standard solutions						
4	3.63 \pm 0.18	4.9	-9.2	3.90 \pm 0.27	6.9	-2.5
20	20.07 \pm 0.04	2.4	+0.4	20.98 \pm 0.60	2.8	+4.9
40	42.04 \pm 0.736	1.8	+5.1	43.23 \pm 1.94	4.5	+8.1
	Found (ng) \pm SD	RSD	Recovery%	Found (ng) \pm SD	RSD	Recovery%
Blood serum						
20	18.52 \pm 0.52	2.8	92.6	19.45 \pm 0.96	5.0	97.2
40	43.46 \pm 1.45	3.3	108.6	41.86 \pm 0.44	3.3	104.6
50	48.52 \pm 1.19	2.4	97.0	49.29 \pm 0.44	0.9	98.6
Urine						
4	3.53 \pm 0.25	7.1	88.2	3.58 \pm 0.28	7.8	89.5
20	20.85 \pm 0.64	3.1	104.2	20.81 \pm 0.08	0.4	104.0
40	39.50 \pm 0.84	2.1	98.8	39.60 \pm 0.12	0.3	99.0

Table 3. Colchicine determination in pharmaceuticals

Added ng	Measured ng \pm SD	RSD	RE (%)	Colchicine mg/tablet
4	3.98 \pm 0.09	2.2	-0.5	0.995 ^a \pm 0.022
10	10.90 \pm 0.13	1.2	+10.9	1.09 ^a \pm 0.01
20	22.37 \pm 0.13	0.6	+11.8	1.12 ^a \pm 0.01
Labeled amount 1 mg/tablet				1.08 ^b \pm 0.01

^aMean values from eight measurements.^bMean value of 3 \times 8 measurements.**Figure 2.** (a) Blank chromatogram of serum sample. (b) Chromatogram of colchicine (4.7 min) determination in spiked serum sample in the presence of codeine (3.4 min).

chromatogram is illustrated in Fig. 1B, while chromatograms of blank and spiked samples of blood serum and urine are presented in Fig. 2A, Fig. 2B, and Fig. 3A and 3B, respectively. No endogenous interference is noticed in biological matrices.

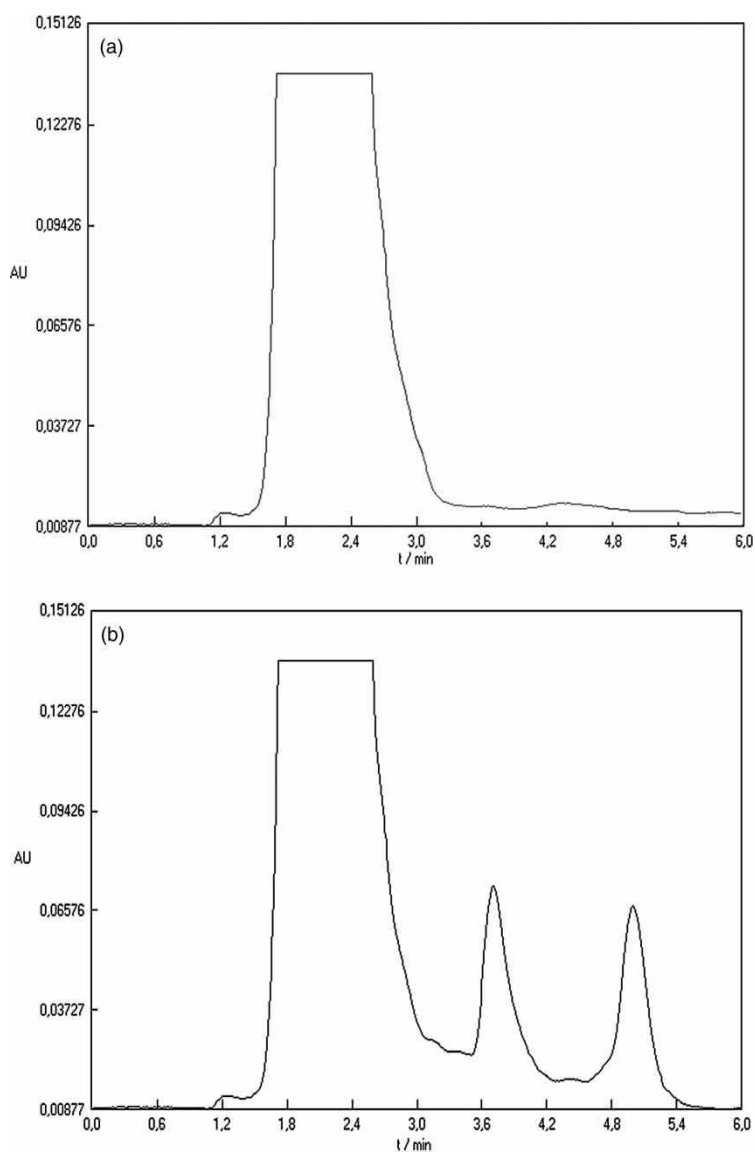


Figure 3. (a) Blank chromatogram of urine sample. (b) Chromatogram of colchicine (5.0 min) determination in spiked urine sample in the presence of codeine (3.8 min).

The method was also validated using spiked samples of biological fluids. The results are summarized in Tables 1 and 2. High recovery rates are obtained, ranging from 97.2 to 104.6% for blood serum samples and from 89.5 to 104.0% for urine samples. No sample preparation is needed except for deproteinization for serum samples and a ten-fold dilution and filtration for urine samples. The assay procedures are simple with satisfactory precision and accuracy (RSD <8%).

CONCLUSIONS

The method described herein is a simple, validated assay that can be readily used for the determination of colchicine. The assay procedures are simple with satisfactory precision and accuracy. RSD values obtained were lower than 8%.

High percentage recoveries of colchicine from biological fluids were noticed without endogenous interference. By avoiding an extraction technique, sample losses are eliminated and time of sample analysis is reduced, since sample preparation steps are minimized. The method can be applied both to toxicological diagnosis and to the therapeutic monitoring of colchicine.

ACKNOWLEDGMENTS

The authors would like to thank Professor P. Nikitas (Laboratory of Physical Chemistry, Chemistry Department of University of Thessaloniki) for kindly providing the chromatographic software for data acquisition.

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Received March 22, 2005

Accepted April 29, 2005

Manuscript 6632