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DETERMINATION OF NOSCAPINE IN PLASMA BY LIQUID CHROMATOGRAPHY

MARGARETA JOHANSSON*

ACO Läkemedel AB, Box 3026, S-171 03 Solna 3 (Sweden)

STAFFAN EKSBORG

Karolinska Pharmacy, Box 60024, S-104 01 Stockholm 60 (Sweden)

and

ASTRID ARBIN

ACO Läkemedel AB, Box 3026, S-171 03 Solna 3 (Sweden)

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SUMMARY

A liquid chromatographic method has been developed for the determination of noscapine in plasma. Noscapine and the internal standard, papaverine, were extracted into methylene chloride by column extraction. The separation was performed on a straight-phase liquid chromatographic system using a mobile phase of hexane—methanol—chloroform—diethylamine. A high detection selectivity was obtained by UV detection at 310 nm. The precision of the method was 3.8% (standard deviation) at a level of 89 ng/ml and 9.5% (standard deviation) at 5.9 ng/ml. The selectivity of the analytical method was evaluated by comparing analytical results after isolation of extracts of plasma samples on reversed- and straight-phase liquid chromatographic systems.

INTRODUCTION

Noscapine is a widely used antitussive agent. Plasma and urine levels of noscapine have been determined fluorimetrically [1]. Even though the selectivity of the fluorimetric method was increased by the modifications suggested by Nayak et al. [2], the sensitivity was not sufficient for pharmaco-

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kinetic studies. The present paper gives a liquid chromatographic method for the determination of plasma levels of noscapine suitable for this purpose. Noscapine and the internal standard, papaverine, are extracted from plasma samples by column extraction using methylene chloride—butanol (19:1) as organic phase. The crude extract is evaporated and partly purified before injection into a straight-phase liquid chromatographic column. A high detection selectivity and sensitivity have been obtained by photometric detection of the eluate at 310 nm. Using 2.00 ml of plasma for the analysis, levels of 5 ng/ml can be determined with a precision better than 10% S.D.

The metabolism of noscapine has not been established in detail [3—5]. To confirm that there were no interferences from metabolites, the selectivity of the present method has been evaluated by comparing analytical results from extracts of plasma samples after isolation on reversed- and straight-phase liquid chromatographic systems.

Depending on the pH, the lactone ring on the noscapine molecule can be either opened (noscapine acid) or closed (noscapine), and the equilibrium between these two forms (Fig. 1) has been studied in detail. The apparent first-order rate constants for the process have been determined as well as the equilibrium constants. In buffer solutions the relative amounts of noscapine/noscapine acid are close to unity at physiological pH and increase with decreasing pH. However, in blood and plasma samples no transformation of noscapine into noscapine acid occurred.

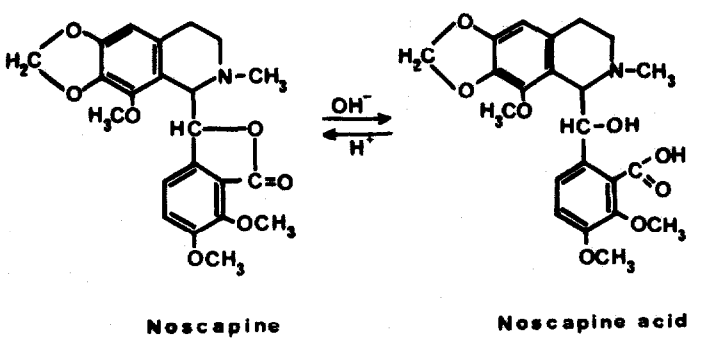

Fig. 1. The equilibrium process between noscapine and noscapine acid.

EXPERIMENTAL

Apparatus

The pump was an LDC 711 solvent delivery system. The chromatographic detector was an LDC Spectromonitor III having a 10.0 mm pathlength and a cell volume of 8 μl. Unless otherwise stated the absorbance of the eluate was measured at 310 nm. A Rheodyne Model 71-20 injection valve with a sample loop of 100 μl or a Waters Model U6K Universal injector was used.

The straight-phase column (250 mm × 4 mm I.D.) was a Hibar® LiChrosorb Si 60, 5 μm (E. Merck, Darmstadt, G.F.R.). The reversed-phase column (150 mm × 4.6 mm I.D.) was home-packed with Spherisorb S 5 ODS (Phase Separations, Queensferry, Great Britain).

The extractions were performed on glass columns (20 mm I.D.) packed with Extrelut® (E. Merck). The Extrelut was purified in a large column (1000 mm × 90 mm I.D.) by ethanol and dried at 100°C.

Chemicals

Noscapine (Bios Courtelier, Brussels, Belgium) and papaverine (E. Merck) were used as bases. 1-Pentanesulfonic acid sodium (Eastman-Kodak, Rochester, NY, U.S.A.) and human albumin (Behring Institut, Behringwerke AG, Marburg, G.F.R.) were used as received. Toluene, methanol and *n*-hexane were of LiChrosolv quality (E. Merck); all other chemicals were of analytical grade and used without further purification.

Mobile phase

The mobile phase for the straight-phase system consisted of hexane—methanol—chloroform—diethylamine (86.5:10.1:3.4:0.034, v/v), which was freshly prepared every day. For the reversed-phase system the mobile phase was composed of 0.005 *M* pentanesulfonic acid in a mixture of methanol—water—acetic acid—triethylamine (40:53:6:1, v/v). The flow-rate was 1.0 ml/min for both systems.

Determination of partition coefficients

The partition experiments were performed in centrifuge tubes by mechanical shaking at 25 ± 1°C (equilibration time 15 min), using equal volumes of organic and aqueous phases. Noscapine and papaverine were initially dissolved in the organic phases (previously equilibrated with the aqueous phase). The concentration of the drugs in the organic phase was determined by straight-phase liquid chromatography after evaporation of an aliquot of the organic phase to dryness with nitrogen and redissolving the residue in the same volume of the mobile phase. The concentration in the aqueous phase was calculated as the difference between the initial concentration in the organic phase and the concentration in the organic phase found at the equilibrium stage.

Determination of apparent first-order rate constants for hydrolysis and lactonization in buffer solutions

The hydrolysis of noscapine was studied at 25.0 ± 0.1°C by mixing 2.00 ml of noscapine (78 μg/ml) in 0.1 *M* phosphoric acid and 20.0 ml of the appropriate buffer (μ = 1.0). The decrease of noscapine and the increase of noscapine acid were monitored by reversed-phase liquid chromatography with photometric detection at 280 nm until the equilibrium stage was reached. Before injection into the liquid chromatograph the buffer solutions were mixed with an equal volume of mobile phase.

The lactonization was studied as described above by mixing 4.00 ml of noscapine acid (39 μg/ml) in 0.1 *M* sodium hydroxide and 20.0 ml of the appropriate buffer (μ = 0.1).

Determination of the degree of hydrolysis and lactonization in blood and 5% (w/v) albumin solution

The degrees of hydrolysis and lactonization were studied at 37.0 ± 0.1°C in

100 ml of blood (containing sodium heparin), 100 ml of 5% (w/v) human serum albumin or 100 ml of phosphate buffer pH 7.4 (μ = 1.0) by adding 14.9 μg of noscapine in 40 μl of 0.1 *M* phosphoric acid or 14.9 μg of noscapine acid in 400 μl of 0.1 *M* sodium hydroxide, respectively.

The total amount of noscapine and noscapine acid was determined by transformation of noscapine acid to noscapine by mixing the sample (2.00 ml) with 1.00 ml of 0.4 *M* phosphoric acid, giving a pH of 2.6, and leaving the solution overnight at room temperature. The solution was analysed according to the analytical method referring to the concentration of noscapine. The amount of noscapine was determined according to the analytical method; however, toluene–butanol (19:1) was used in the initial extraction, since the extraction of noscapine acid was negligible (< 5%) into this organic phase. The amount of noscapine acid was calculated as the difference between the total amount of noscapine and noscapine acid and the amount of noscapine alone.

Analytical method

To the plasma sample (2.00 ml) were added 240 ng of papaverine (internal standard) and 5.0 ml of phosphate buffer pH 4.0 (μ = 1.0). The mixture was poured into the column (packed with 4.0 g of silica) and was allowed to soak for 10 min. Noscapine and papaverine were eluted with 20 ml of methylene chloride–butanol (19:1). The eluate was evaporated to dryness under nitrogen flow at 50°C. The residue was dissolved in 1.0 ml of 0.1 *M* phosphoric acid, extracted with 1 ml of heptane (discarded), neutralized with 1 ml of phosphate buffer pH 6 (μ = 1.0) and extracted with 2.0 ml of toluene–butanol (19:1). After centrifugation the toluene phase was evaporated to dryness with nitrogen flow at 50°C. The residue from the extraction procedure was redissolved in 200 μl of the mobile phase and 50–100 μl were injected into the chromatographic column.

A calibration curve was obtained by analysing five standard samples containing 2.00 ml of blank plasma, 240 ng of internal standard and spiked with noscapine in the concentration range 6–400 ng/ml.

RESULTS AND DISCUSSION

The following symbols are used:

$[\ ]_{org}$, $[\ ]$ = concentrations of ions and molecules in organic and aqueous phase, respectively;

C_{org}, C_{aq} = total concentrations in organic and aqueous phase, respectively;

$k'_{HA} = \frac{a_H + [A]}{[HA^+]}$ = apparent acid dissociation constant of HA^+;

$k_d = \frac{[A]_{org}}{[A]}$ = partition coefficient of A;

$D_A = \frac{C_{A\ org}}{C_{A\ aq}}$ = partition ratio of A.

Extraction

The partition coefficients of noscapine and papaverine were studied using heptane, methylene chloride—butanol (19:1) and toluene—butanol (19:1) as the organic phases and phosphate buffers as the aqueous phases. Constants were evaluated graphically from the following equation by means of the linear plot of D_A^{-1} versus a_{H^+} (ref. 6)

$$\frac{1}{D_A} = \frac{1}{k_d} + \frac{a_{H^+}}{k_d \cdot k'_{HA}}$$

The products $k_d \cdot k'_{HA}$ were calculated from the slopes (Table I). In the analytical method methylene chloride—butanol (19:1) was preferred as initial extraction solvent because of its good extraction properties even at low pH and its high volatility, facilitating subsequent evaporation. Quantitative extraction (> 99%) of noscapine and papaverine into methylene chloride—butanol (19:1) was possible from aqueous buffer solutions pH > 3.8 using equal phase volumes. Extraction of plasma (diluted three times with phosphate buffer, giving a pH of 4.7) with methylene chloride—butanol (19:1), however, produced protein precipitation which reduced the amount of organic phase available for transfer. This fact may explain the low extraction yield (about 80%) obtained in batch extraction experiments from diluted plasma samples.

TABLE I

PARTITION COEFFICIENTS FOR NOSCAPINE AND PAPAVERINE

Aqueous phase: phosphate buffer, μ = 0.1. Temperature: 25°C.

Organic phase	$-\log k_d \cdot k'_{HA}$ ± S.E.	
	Noscapine*	Papaverine**
Methylene chloride—butanol (19:1)	1.43 ± 0.08	1.82 ± 0.04
Toluene—butanol (19:1)	3.02 ± 0.02	3.56 ± 0.02
Heptane	5.24 ± 0.01	5.58 ± 0.01

*pk'_{HA} = 6.8 (ref. 8).
**pk'_{HA} = 6.4 (ref. 11).

To improve the reproducibility and also the extraction yield, column extraction has been used in the present method for quantitative transfer of noscapine and papaverine from buffered plasma (pH 4.7) into the organic phase. The drugs are eluted within the first 15 ml of the organic extractant passing through the column.

After the initial extraction and evaporation of the organic phase, noscapine and papaverine were redissolved in phosphoric acid. Coextracted lipophilic compounds were removed by extraction with heptane. By extraction of the drug and internal standard into toluene—butanol (19:1) the amount of hydrophilic impurities was reduced to a large extent.

Liquid chromatography

Noscapine and papaverine were easily separated on both straight- and reversed-phase systems. The capacity factor (k') for the straight-phase system was 2.8 for noscapine and 3.8 for papaverine, and for the reversed-phase system 3.0 and 3.9, respectively. The straight-phase column had about 9100 theoretical plates and the reversed-phase column about 1200. Moreover, the molar absorptivity of noscapine in the mobile phase suitable for the straight-phase system was about 30% higher than in the mobile phase used in the reversed-phase system. Calculations based on equations given in ref. 7 showed that the straight-phase system should give about three times higher sensitivity than the reversed-phase system (Figs. 2 and 3), and hence the straight-phase system was preferred in the analytical method.

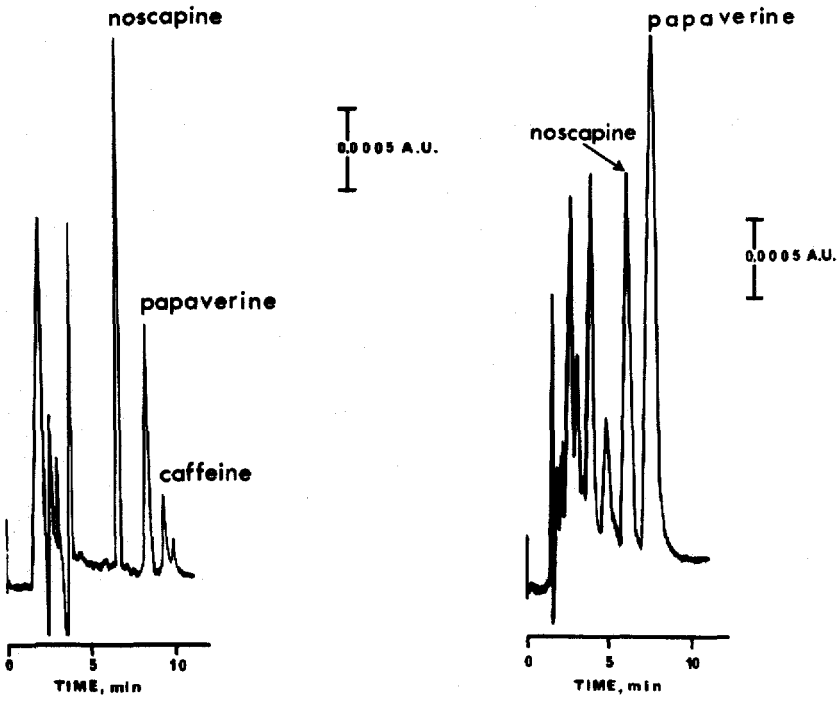

Fig. 2. Straight-phase liquid chromatogram of a plasma sample containing 197 ng/ml noscapine and 240 ng of papaverine. The noscapine peak corresponds to 49 ng.

Fig. 3. Reversed-phase liquid chromatogram from the same plasma sample that is shown in Fig. 2. The noscapine peak corresponds to 98 ng.

When the straight-phase system was used for separation of plasma extracts, the capacity factor (k') for caffeine, which eluted behind papaverine, decreased continuously. To avoid interference from the caffeine peak it was necessary to regenerate the column after about 150 injections of plasma extracts, by running about 100 ml of water through the chromatographic system. By this procedure the retention time for caffeine returned to its original value. With

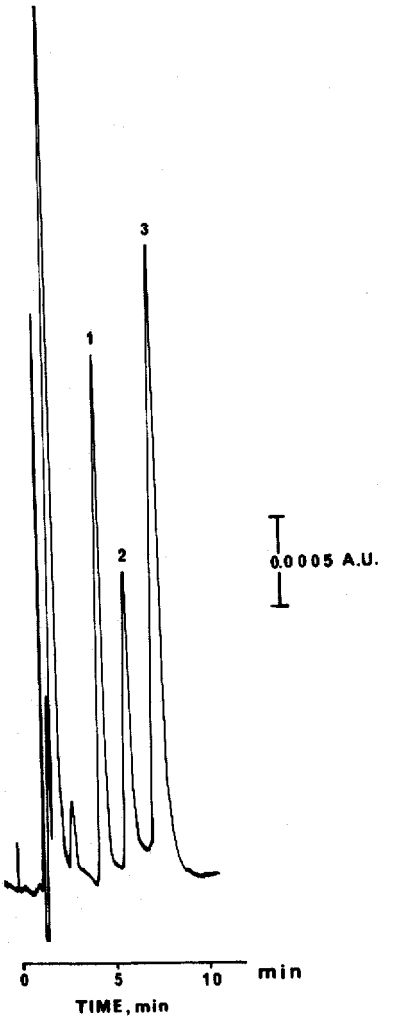

Fig. 4. Reversed-phase liquid chromatogram with chromatographic detection at 280 nm. Injected amounts: 186 ng of noscapine acid (peak 1), 149 ng of noscapine (peak 2), and 119 ng of papaverine (peak 3).

this handling the columns have been used for at least 1500 injections without any signs of deterioration.

Simultaneous determination of noscapine acid, noscapine and papaverine was possible only on the reversed-phase system (Fig. 4). This system was therefore used in the studies of hydrolysis and lactonization of noscapine and noscapine acid, respectively, in buffer solutions.

Internal standard

Papaverine is a suitable internal standard since it eluted just after noscapine in the chromatographic systems used. Papaverine has, like noscapine, a high UV absorbance at 310 nm which enables a high detection selectivity. The extraction properties of papaverine and noscapine are also very similar (Table I).

Hydrolysis and lactonization in aqueous solution

A pH-dependent equilibrium between noscapine and noscapine acid in aqueous solutions (Fig. 1) has previously been described [8]. The apparent first-order rate constants (k) for hydrolysis of noscapine and lactonization of noscapine acid in buffer solutions are given in Table II. The relative amounts of noscapine/noscapine acid at the equilibrium stage at different pH are given in Fig. 5. The data obtained with the selective analytical procedure used in the present paper, with concomitant determination of noscapine and noscapine acid, were of the same magnitude that Pawelczyk and Zajac [8] found by the less-selective photometric technique.

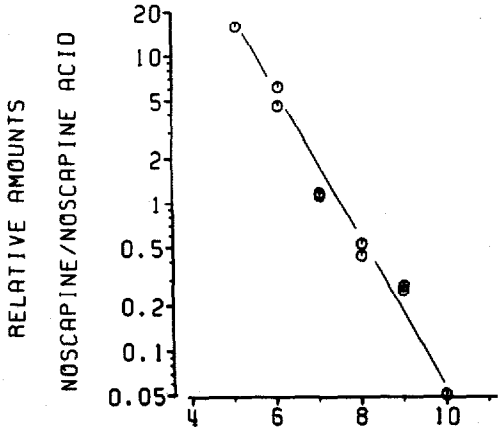

Fig. 5. The relative amounts of noscapine/noscapine acid at equilibrium in buffer solutions at different pH. Temperature 25.0 ± 0.1°C.

TABLE II

APPARENT FIRST-ORDER RATE CONSTANTS (k) FOR THE EQUILIBRATED HYDROLYSIS OF NOSCAPINE AND THE LACTONIZATION OF NOSCAPINE ACID IN BUFFER SOLUTIONS (μ = 1.0) AT DIFFERENT pH

Temperature: 25.0 ± 0.1°C.

pH	$-(k \pm$ S.E.$) \cdot 10^2$ (h^{-1})	
	Hydrolysis	Lactonization
2.0	—	156 ± 7
2.6	—	61 ± 2
3.0	—	29.8 ± 0.3
4.0	—	8.8 ± 0.1
5.0	—	9.5 ± 0.5
6.0	8.5 ± 0.3	8.0 ± 0.3
7.0	13.1 ± 0.3	11.9 ± 0.1
8.0	16.5 ± 0.6	17.3 ± 0.7
9.0	18 ± 1	16.3 ± 0.8
10.0	23.0 ± 0.4	—

Hydrolysis and lactonization in blood and 5% (w/v) albumin solution

The results from the study of the equilibrium between noscapine and noscapine acid in buffer solution indicate that both forms may exist in blood and plasma samples. However, noscapine in blood or 5% (w/v) albumin solution was not transformed into noscapine acid within 24 h. Addition of noscapine acid to blood and 5% (w/v) albumin solution gave an almost complete transformation of noscapine acid to noscapine within 24 h (Fig. 6). The difference in equilibrium stage between buffer, blood and albumin solution may depend on the strong binding of noscapine to proteins. Plasma samples from three patients collected 0–6 h after the intake of 150 mg of noscapine were analyzed for the total amount of noscapine and noscapine acid as well as for noscapine only. The mean value of the quotients (noscapine + noscapine acid)/noscapine was 1.01 with a standard deviation of 0.102 (n = 24). Hence, it can be concluded that it is unlikely that noscapine acid is a metabolite of noscapine in man.

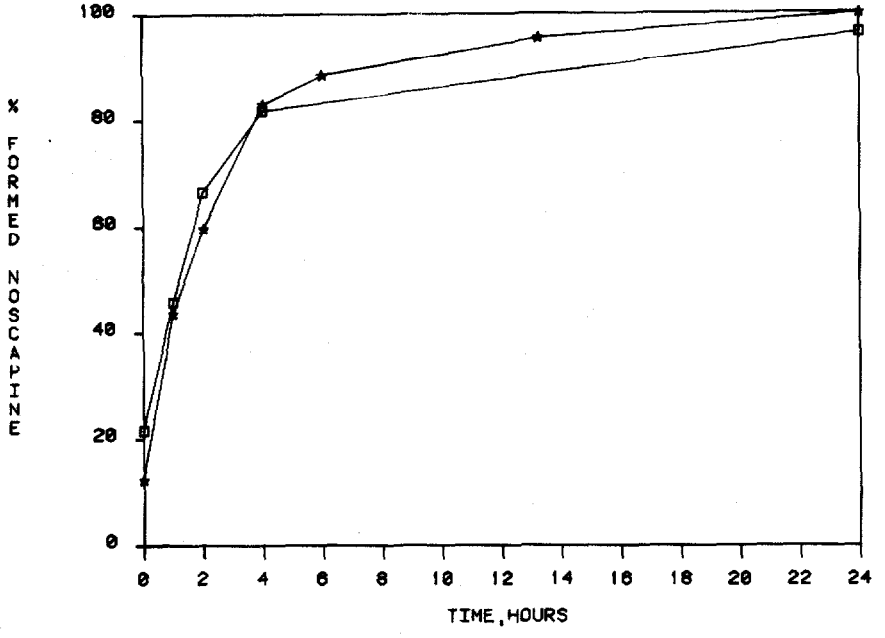

Fig. 6. Transformation of noscapine acid to noscapine in blood (□) and in 5% (w/v) albumin solution (*).

Selectivity of the analytical method

A high detection selectivity towards endogenous compounds and also towards noscapine acid was obtained in the present method using a detector measuring at 310 nm (Fig. 7) (cf. ref. 9). The selectivity of the proposed analytical method against other possible metabolites than noscapine acid was evaluated by comparing the analytical results of noscapine from plasma extracts with reversed- and straight-phase systems. The analytical results from the reversed-phase system/straight-phase system were plotted against the analytical results from the straight-phase system (Fig. 8) [10]. The plot (n =

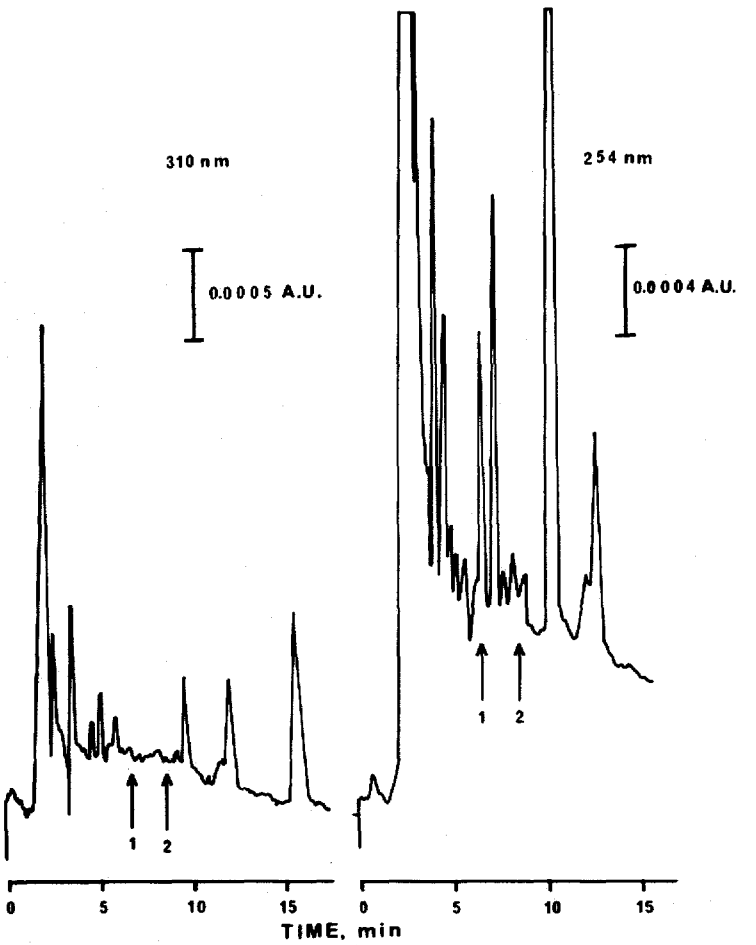

Fig. 7. Detection selectivity. Blank plasma samples were analyzed according to the proposed analytical method with two detectors coupled in series and detection wavelengths of 310 nm and 254 nm, respectively. 1 and 2 indicate the retention times of noscapine and papaverine, respectively.

TABLE III

PRECISION OF THE TWO LIQUID CHROMATOGRAPHIC SYSTEMS AT DIFFERENT LEVELS

Drug level (ng/ml)	Standard deviation (%)	
	Straight-phase	Reversed-phase
89	3.8	6.6
18	7.3	9.3
5.9	9.5	—*

*Signal-to-noise ratio < 2.

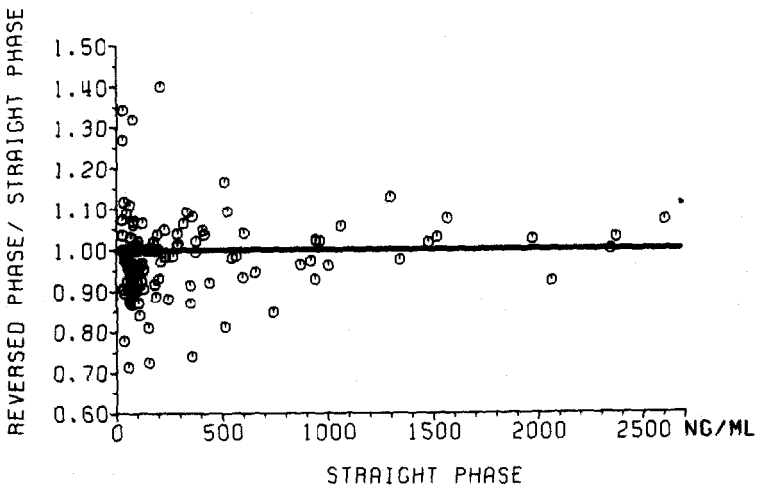

Fig. 8. Comparison of plasma levels of noscapine from the analytical results of the reversed-phase and straight-phase systems.

111) gives a mean value of 0.99 and a standard deviation of 10.6% for plasma levels > 25 ng/ml, i.e. essentially the same results were obtained by the two methods, which implies no codetermination of metabolites in any of the methods.

Recovery, precision and sensitivity

Quantitation was made by construction of a calibration curve by plotting the peak height ratios of noscapine/papaverine against the concentration of noscapine. The linear calibration curve had a correlation coefficient of 0.9999, a slope of $(1.015 \pm 0.001) \cdot 10^{-2}$ and an intercept of 0.02 ± 0.03.

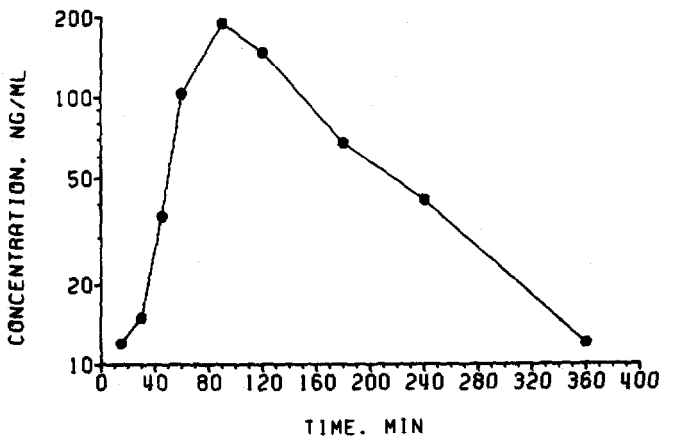

Fig. 9. Plasma levels of noscapine after an oral dose of 150 mg of noscapine.

The absolute recovery from blank plasma samples spiked with 92 ng/ml noscapine, obtained by using aliquots throughout the method, was 81% compared with direct injection of noscapine dissolved in mobile phase.

The precision of the proposed methods is presented in Table III. Using 2.00 ml of plasma for the analysis, the lower limit for determination was 5 ng/ml for the straight-phase system and 15 ng/ml for the reversed-phase system.

Application to biological samples

A chromatogram from 2.00 ml of plasma containing 197 ng/ml noscapine and 240 ng of papaverine is given in Fig. 2. The same plasma sample chromatographed with the reversed-phase liquid chromatographic system is shown in Fig. 3. The plasma levels after an oral dose of 150 mg of noscapine are presented in Fig. 9.

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