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DETERMINATION OF NOSCAPINE AND ITS METABOLITES IN PLASMA BY COUPLED-COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

Noscapine, narcotoline and cotarnine were quantified in deproteinized plasma samples by using a coupled-column liquid chromatographic system. The drug and the metabolites were first separated into two groups on a short polar precolumn (–CN) with an acidic mobile phase, containing a low content of acetonitrile. The metabolites were transferred to a hydrophobic analytical column (C_{18}) and separated with a mobile phase containing a counter ion and a co-ion in an acidic buffer with an high acetonitrile content. Noscapine was transferred to another hydrophobic analytical column (C_{18}) with a mobile phase containing a counter ion in an acidic buffer with an high acetonitrile content. Ultraviolet detection at 310 nm was used for all three compounds. The limits of quantitation were 9 ng/ml for noscapine, 13 ng/ml for cotarnine and 20 ng/ml for narcotoline. The within-day precisions were better than 6% (relative standard deviation), and the absolute recoveries were above 82%.

INTRODUCTION

Noscapine is a centrally active antitussive agent. The metabolism of the drug is not known in detail, but a few demethylated metabolites have been identified, *e.g.*, narcotoline[1], cotarnine, hydrocotarnine and meconine[2,3]. Plasma levels of noscapine have previously been determined using normal phase[4] or reversed-phase liquid chromatography[4–6]. The metabolites have been identified only in urine[2,3] and in rat liver microsomes[7].

In recent years the use of coupled columns for the analysis of drugs in biological material has increased rapidly[8,9]. The technique increases the sample throughput by minimizing the sample clean-up and by permitting automation[10]. Furthermore, it is an alternative to gradient elution for the determination of compounds with different polarities, *e.g.*, a drug and its metabolites[11,12].

Coupled-column systems, based on ion-pairing principles, have been evaluated

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for noscapine, narcotoline and cotarnine[13,14]. In this study the most suitable of the previous systems was applied to the quantitation of noscapine and its metabolites in plasma. The aims were to make the sample-work up as simple as possible, to develop a method with high reliability and to achieve the necessary selectivity and sensitivity.

EXPERIMENTAL

Chemicals

Noscapine base was obtained from Macfarlan Smith (Edinburgh, U.K.), cotarnine chloride dihydrate from EGA-Chemie (Albuch, F.R.G.) and narcotoline from Diosynth-Apeldorn (Oss, The Netherlands). The sodium salt of dodecyl sulphate (DDS) was obtained from Eastman Kodak (Rochester, NY, U.S.A.) and N,N-dimethyl-N-octylamine (DMOA) from ICN Pharmaceuticals (Plainview, NY, U.S.A.). The perchloric acid (suprapure quality) and the buffer substances (analytical quality) were obtained from Merck (Darmstadt, F.R.G.). The water in the precolumn mobile phase and the acetonitrile was of HPLC quality and obtained from Merck. The water used in the analytical column mobile phases was purified in a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

Apparatus

The liquid chromatographic system consisted of a Kontron Tracer MCS, 670 valve switching unit, with a Tracer timer 210 (Kontron, Zürich, Switzerland). The pumps were LKB 2150 (LKB, Bromma, Sweden) and Waters M-6000A (Waters Assoc., Milford, MA, U.S.A.). The samples were injected with a Waters WISP 710 B automatic injector. Noscapine was detected at 310 nm with a LDC/Milton Roy SM 4000 (Laboratory Data Control, Riviera Beach, FL, U.S.A.), narcotoline and cotarnine at 310 nm with a Spectroflow 783 (Kratos Analytical, Ramsey, NJ, U.S.A.). The output signal from the UV detectors was connected to two Shimadzu C-R3A-integrators (Shimadzu, Kyoto, Japan).

Liquid chromatographic system

The chromatographic system is shown in Fig. 1. The precolumn was a Brownlee Spheri-5 CN (30 mm × 4.6 mm, 5 μm; Brownlee Labs., Santa Clara, CA, U.S.A.) with a mobile phase containing 6–10% (v/v) of acetonitrile in a phosphate buffer (pH 2; ionic strength, $I = 0.05$). The analytical column for the metabolites was a laboratory-packed Nucleosil C_{18} (100 mm × 4.6 mm, 3 μm, Macherey-Nagel, Düren, F.R.G.) with a mobile phase containing 2 m*M* DDS–0.1 m*M* DMOA in a 35% (v/v) solution of acetonitrile in phosphate buffer (pH 2, $I = 0.05$). The analytical column for noscapine was a Spherisorb ODS-2 (100 mm × 4.6 mm, 3 μm; Phase Separations, Queensferry, U.K.) with a mobile phase containing 3 m*M* DDS in a 40% (v/v) solution of acetonitrile in phosphate buffer (pH 2, $I = 0.05$). An enrichment column for noscapine was inserted in the backflush mode in a switching valve between the precolumn and the analytical column. The enrichment column (20 mm × 3.8 mm) was laboratory-packed by the slurry technique with Spherisorb ODS-2, 3 μm. The flow-rate was always 1 ml/min. A single-column system was used for evaluation of the absolute recoveries of the protein precipitation. This column was a Spherisorb Ph (250 mm × 4.6 mm, 5 μm) with a mobile phase containing 32.5% (v/v) acetonitrile in phosphate buffer (pH 2, $I = 0.05$). The eluate was monitored at 310 nm.

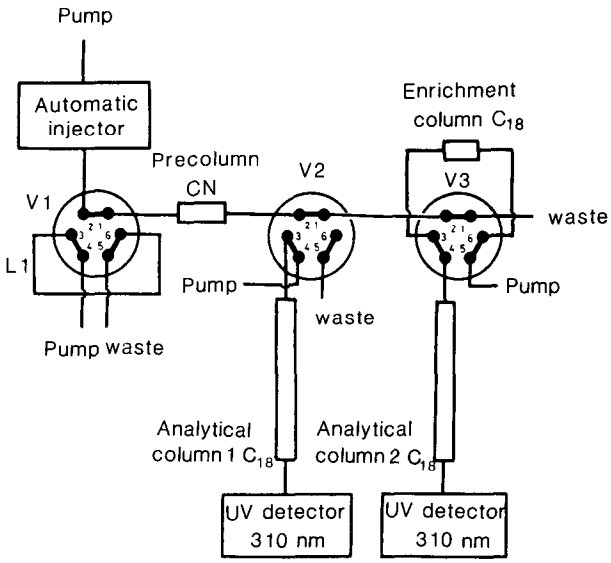

Fig. 1. Scheme of the chromatographic system. Chromatographic conditions: precolumn, Brownlee CN (5 μm, 30 mm × 4.6 mm); analytical column 1, Nucleosil C_{18} (3 μm, 100 mm × 4.6 mm); analytical column 2, Spherisorb ODS-2 (3 μm, 100 mm × 4.6 mm); enrichment column, Spherisorb ODS-2 (3 μm, 20 mm × 3.8 mm); precolumn eluent, 6–10% (v/v) acetonitrile in phosphate buffer (pH 2); analytical column 1 eluent, 2 m*M* DDS–0.1 m*M* DMOA in 35% (v/v) acetonitrile in phosphate buffer (pH 2); analytical column 2 eluent, 3 m*M* DDS in 40% (v/v) of acetonitrile in phosphate buffer (pH 2); detection wavelength, 310 nm; injection volume, 500 μl.

Column switching

Noscapine and the metabolites were first separated into two fractions on the precolumn. The metabolites were transferred to analytical column 1 for further separation. Noscapine was enriched on a short (C_{18}) column before being backflushed to analytical column 2. Between injections, the precolumn was cleaned by creating a step gradient of acetonitrile. A mixture of 50% (v/v) acetonitrile in phosphate buffer (pH 2, $I = 0.05$) was pumped into the loop (1.6 ml) through valve 1 with the pump in the Kontron unit. By switching the valve, the gradient was created. The timetable of the switching events is given as Table I.

Sample preparation

Plasma (1.000 ml) was mixed carefully with acetonitrile (0.125 ml) and perchloric acid (0.250 ml). The samples were centrifuged at 5000 rpm for 10 min. Then, the supernatant (0.800 ml) was adjusted to about pH 3, using 0.380 ml of a solution containing 1 *M* trisodium citrate and 5 *M* sodium hydroxide (1:4). A 500-μl sample was injected into the precolumn.

Mass spectrometry

Mass spectra were recorded in the electron impact (EI) (70 eV) or chemical ionization (CI) (ammonia) mode, using a Shimadzu QP 1000 mass spectrometer, equipped with a direct inlet probe. The probe temperature was programmed from

TABLE I

SWITCHING EVENTS

Time	*Valve*	*Event*
0		Plasma sample is injected
0.9 A*	V_2 on	Precolumn and analytical column 1 are connected in series and narcotoline and cotarnine are enriched on the top of analytical column 1
B**	V_2 off	Precolumn and analytical column 1 are disconnected. Elution of the metabolites starts
0.9 C***	V_3 on	Precolumn and enrichment column are connected in series and noscapine is enriched
D§	V_3 off	Enrichment column and analytical column are connected
D§ +1.0	Kontron pump on	Loop is filled with 50% acetonitrile in acidic buffer
D§ +1.6	Kontron pump off	
D§ +2.1	V_1 on	Precolumn is purified with acetonitrile in acidic buffer
D§ +3.6	V_1 off	Regeneration of the precolumn is completed

* A = Time for the start of the metabolite fraction, *e.g.*, 2.0 min.
** B = Time for the end of the metabolite fraction, *e.g.*, 3.5 min.
*** C = Time for the start of the noscapine fraction, *e.g.*, 6.0 min.
§ D = Time for the end of the noscapine fraction, *e.g.*, 8.5 min.

room temperature to 250°C at a rate of 40°C/min. The mass was scanned from *m/z* 40 to 450 (EI) and from *m/z* 100 to 450 (CI) with a cycle time of 4 s. Since the instrument has no direct facilities for reading the ion-source pressure, the conditions for chemical ionization were adjusted by setting the ratio of *m/z* 18:*m/z* 35 to approximatively 3:1. The presence of N-demethylated noscapine in the collected fraction was monitored by selected ion monitoring (SIM) of the ions *m/z* 191, 193 and 206 for the EI mode, and *m/z* 195, 204, 206 and 212 for CI mode. The time for collecting the N-demethylated fraction from the liquid chromatographic system was decided by UV monitoring at 310 nm. Four fractions, each containing about 180 ng, were collected and pooled. Then the pH was adjusted to about 8, using sodium hydroxide, and the pooled fraction was extracted for 15 min with 1.0 ml of 1% heptafluorobutanol in dichloromethane. After centrifugation, the organic phase was extracted with two portions of 2 ml 0.01 *M* sodium hydroxide. Finally, the organic phase was evaporated, and the residue was redissolved in 10 μl dichloromethane. A 3-μl sample was evaporated at the direct probe.

RESULTS AND DISCUSSION

Sample preparation

In aqueous solution, there is a pH-dependent equilibrium between noscapine lactone and noscapine acid[15]. At low pH, the lactone form prevails, and at an high pH the acid form. At pH 7.4, the two forms will exist in equal amounts. However, in blood and in 5% (w/v) albumin solution no measurable amount of noscapine was

TABLE II

ABSOLUTE RECOVERY OF NOSCAPINE, NARCOTOLINE AND COTARNINE AFTER ADDITION OF VARYING VOLUMES OF ACETONITRILE TO PLASMA BEFORE PROTEIN PRECIPITATION

Work-up procedure: 1.00 ml plasma was mixed with varying volumes of acetonitrile and 0.250 ml perchloric acid (11.4 *M*).

Added volume of acetonitrile (ml)	*Absolute recovery (%)*		
	Noscapine	*Narcotoline*	*Cotarnine*
0.050	81	63	79
0.075	80	74	85
0.100	81	76	84
0.125	90	91	92
0.150	89	88	91
0.200	76	83	72

converted into noscapine acid[4]. The reason for this is probably a strong protein binding of the lactone.

Isolation of noscapine from plasma has previously been performed by liquid–liquid extraction, both in tubes and on a solid phase[4]. By using a coupled-column system, a direct injection technique can be substituted for the manual isolation procedure. In order to minimize deterioration of the precolumn, the plasma proteins were precipitated before injection. Precipitation with 1 *M* perchloric acid (1.00 ml for 1.00 ml plasma) gave only *ca.* 50% recovery. Addition of acetonitrile to plasma before precipitation with perchloric acid increased the recoveries (Table II), until a maximum was reached at 0.125 ml. Dilution of perchloric acid before precipitation decreased the absolute recoveries (Table III). The use of 11.4 *M* perchloric acid for precipitation made pH adjustment of the supernatant necessary in order to avoid degradation of the analytes. At pH 3 the compounds were stable for at least 24 h. An increase to almost quantitative recoveries (> 95%) was obtained by diluting the plasma in phosphate buffer (pH 7.4) before precipitation (Table IV). However, with this high dilution a large injection volume was necessary in order to exceed the low

TABLE III

ABSOLUTE RECOVERY OF NOSCAPINE, NARCOTOLINE AND COTARNINE USING VARYING CONCENTRATIONS OF PERCHLORIC ACID

Work-up procedure: 1.00 ml plasma was mixed with 0.125 ml acetonitrile and 0.250 ml perchloric acid.

Concentration of perchloric acid (M)	*Absolute recovery (%)*		
	Noscapine	*Narcotoline*	*Cotarnine*
1.4	57	82	79
2.8	61	91	83
5.7	76	98	89
11.4	88	99	90

TABLE IV

ABSOLUTE RECOVERY OF NOSCAPINE, NARCOTOLINE AND COTARNINE AFTER DILUTION OF PLASMA BEFORE PRECIPITATION

Work-up procedure: plasma was diluted to 1.00 ml in phosphate buffer, pH 7.4 (I = 0.2) and mixed with 0.125 ml acetonitrile and 0.200 ml perchloric acid (11.4 M).

Volume plasma (ml)	*Absolute recovery (%)*		
	Noscapine	*Narcotoline*	*Cotarnine*
0.25	95	99	97
0.50	87	95	93
0.75	84	92	95
1.00	74	84	87

minimum determinable concentration. As a compromise, 0.125 ml acetonitrile and 0.250 ml 11.4 M perchloric acid were added to undiluted plasma and 500-μl sample was injected into the precolumn.

The reliability of the protein precipitation was checked by addition of albumin (3 mg/ml) and orosomucoid (2 mg/ml) to plasma. This did not affect the absolute recoveries of the analytes. Because lipophilic compounds, *e.g.*, 1 mg/ml cholesterol or 10 mg/ml oleic acid did not dissolve in plasma, the influence of plasma lipophilicity on absolute recoveries was checked by using plasma from fasted or fed persons. No differences in the absolute recoveries of the analytes were obtained.

Chromatography

The chromatography of noscapine and the metabolites has previously been studied in different coupled-column systems, based on the heart-cut technique[14]. Noscapine and the metabolites were separated into two fractions on a polar precolumn (–CN). Each fraction was then transferred to an analytical column for final separation. Transfer of the precolumn eluate produced system peaks in the analytical columns[14]. The system peaks are formed as a consequence of disturbed equilibria on the analytical column. Each deviating mobile phase component may give rise to a system peak, which contains the mobile phase component in deficiency or excess.

The system peaks were used to obtain high peak performance of the analytes on the analytical column, in order to improve the sensitivity. The precolumn eluent giving the best results with respect to separation, efficiency and production of system peaks had a low content of acetonitrile in an acidic buffer. By using an analytical column eluent containing an high content of acetonitrile and DDS (1 mM), a compressed noscapine peak was obtained when eluted together with an increasing gradient of the system peak[14]. However, when noscapine is co-eluted with the system peak, the selectivity for structurally related compounds, *e.g.*, metabolites is decreased[14]. By increasing the acetonitrile content to 40% (v/v) and the DDS concentration to 3 mM, the system peak and the noscapine peak were separated. This improved the separation of noscapine from the unidentified metabolite (Fig. 2). Furthermore, the peak performance of noscapine was unaffected, *i.e.*, neither band broadening nor peak compression was obtained.

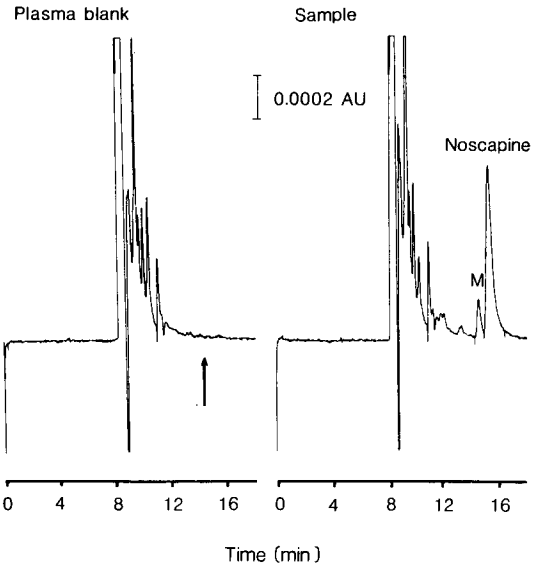

Fig. 2. Plasma blank and sample chromatogram. The plasma sample was taken after an oral dose of 200 mg noscapine. The noscapine concentration corresponds to 242 ng/ml plasma. Chromatographic conditions as in Fig. 1. The arrow indicates the retention time for noscapine. M = Unidentified metabolite.

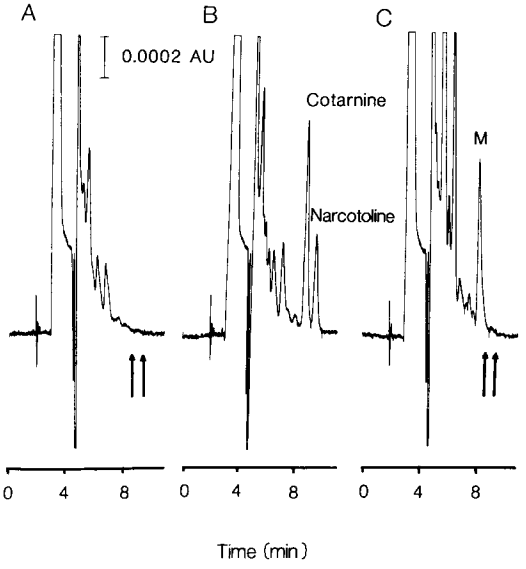

Fig. 3. Chromatogram of narcotoline and cotarnine. (A) Plasma blank; (B) plasma spiked with 99 ng/ml cotarnine and 102 ng/ml narcotoline; (C) plasma after an oral dose of 300 mg noscapine. Chromatographic conditions as in Fig. 1. The arrows indicate the retention times for cotarnine and narcotoline. M = Unidentified metabolite.

For the metabolites an eluent containing an high concentration of acetonitrile, DDS and DMOA was used. Even in this case, the system peak produced contained an excess of DDS[14]. The peak performance of the analytes was unaffected and no efforts were made to elute the metabolites together with the system peak for fear of lowering the selectivity. A good resolution of cotarnine, narcotoline and endogenous compounds in deproteinized plasma was obtained by using 2 m*M* DDS–0.1 m*M* DMOA in a solution of 35% (v/v) acetonitrile in an acidic buffer (Fig. 3). Another unidentified metabolite appeared (Fig. 3C) in plasma samples collected after an oral dose of the drug.

Tentative mass spectrometric identification of N-demethylated noscapine

The retention time of the unidentified metabolite on the noscapine column coincides with that of N-demethylated noscapine. The EI mass spectrum of N-demethylated noscapine produced an intense ion at *m/z* 206, orginating from the isoquinoline part. An ion at *m/z* 191 was probably produced by the loss of a methyl group from the ion at *m/z* 206. Furthermore, the ion at *m/z* 193 originated from the isobenzofuranone part. The LC eluate produced signals at *m/z* 191, 193 and 206 in the SIM analysis. The relationship of the relative intensities between the fragments in the eluate corresponded to the relative intensities of the reference compound. The abundance of the molecular ion at *m/z* 399 was very low and could not be registered, by EI or CI. CI with ammonia produced fragments at *m/z* 204 and 206 from the isoquinoline part and at *m/z* 195 and 212 (most likely from the isobenzofuranone part). The fragment at *m/z* 212 may be due to the addition of ammonia to the isobenzofuranone structure (*m/z* 196). The relative intensities of the fragments at *m/z* 195, 206, 212, but not *m/z* 204, in the eluate were in accordance with the intensities of the reference compound. A mass spectrum of the collected fraction could not be obtained by EI or CI, probably because the concentration of the metabolite was too low owing to losses during the work-up procedure. However, the data obtained indicate that the metabolite is N-demethylated noscapine. As pointed out earlier, the eluate from the column used for narcotoline and cotarnine also showed an unidentified metabolite (Fig. 3). In accordance with the above discussion, this peak may correspond to N-demethylated narcotoline or cotarnine. Further support for this is the retention behaviour of dextromethorphan and its N- and O-demethylated metabolites in a liquid chromatographic system[8] similar to the one used here for noscapine and its metabolites. Since no reference compounds for the O-demethylated metabolites are available, mass spectrometric identification is difficult. Furthermore, the main part of the O-demethylated metabolites probably exists in the form of conjugates.

Detection

An high detection selectivity and sensitivity is required when low levels of a drug must be determined in biological samples. Unfortunately, noscapine has no functional groups useful for derivatization. Oxidation of noscapine by bromine generated on-line has been tried, but the detection limit is too high (>20 ng)[16]. Oxidation with a coulometric detector (ESA, Coulochem Model 5100A) requires an high potential. The signal continuously increases up to the highest level tested, 1.2 V. At this high potential the background current is too high. The lowest detection limit of noscapine was obtained with UV detection at 211 nm, which was *ca.* 15 times better

TABLE V

DAY-TO-DAY VARIATION

R.S.D. = Relative standard deviation.

Compound	*Added (ng/ml)*	*R.S.D. (%)*	*Interval (days)*
Noscapine	15	9.5	9
	250	5.5	15
Narcotoline	26	15.6	10
	263	6.4	15
Cotarnine	14	17.6	12
	271	4.5	15

than that at 310 nm. The more selective fluorescence detection with a xenon lamp at 316/427 nm (Perkin-Elmer LS-4 fluorescence spectrometer) gave a four times lower signal-to-noise ratio than UV at 310 nm. Fluorescence detection with a deuterium lamp at 211/370 nm (Kratos 980 fluorescence detector) gave twice the signal-to-noise ratio of UV at 310 nm. However, when the main part of this study was performed only an UV detector was available. UV detection at 211 nm resulted in too much endogenous interference when 3 m*M* DDS was used. By increasing the DDS concentration, some interference disappeared, but another complication appeared —the resolution of noscapine and the unidentified metabolite was too low. Another way to decrease the endogenous interference was to increase the detection wavelength. Thus detection at 310 nm was used in the routine determinations. Injection of 2.00 ml of the supernatant instead of 0.5 ml gave only a small increase of the noscapine peak width from the precolumn. However, the amount of endogenous interference in the eluate from the analytical column increased considerably, limiting the injection volume to 0.5 ml of the supernatant.

Quantitation and stability of the system

The method has been used in an automated fashion for routine determinations. The samples were worked up manually (protein precipitation and buffering) and

TABLE VI

RELATIVE STANDARD DEVIATIONS AND ABSOLUTE RECOVERIES (%) OF NOSCAPINE, COTARNINE AND NARCOTOLINE

Compound	*Added (ng/ml)*	*R.S.D. (%)*	*Absolute recovery (%)*	*n*
Noscapine	9.3	5.9	84	7
	15	6.0	89	9
	250	1.2	86	7
Cotarnine	13	4.5	82	8
	241	1.5	88	9
Narcotoline	20	4.4	92	8
	263	2.5	95	9

injected automatically overnight. Before start-up, the retention times for the precolumn were checked daily, and the actual column-switching times were entered in the Tracer timer. The analyte peaks were integrated and evaluated by comparison with a standard curve, constructed from standards prepared like the samples. A calibration solution was injected as every tenth sample to ensure the stability of the system. The day-to-day variation is shown in Table V. The compounds were stable for more than 8 months at –20°C.

The absolute recoveries and the within-day precisions are shown in Table VI. The absolute recoveries were determined by comparison with direct injections into the precolumn of the compounds dissolved in a mixture containing phosphate buffer (pH 7.4), perchloric acid and acetonitrile (1.00 ml + 0.250 ml + 0.125 ml), adjusted to a pH of *ca.* 3. Plasma levels down to 9 ng/ml for noscapine, 13 ng/ml for cotarnine and 20 ng/ml for narcotoline were determined.

In general, 50–60 samples were analyzed overnight. The precolumn was used for about 3–4 days, corresponding to the injection of 45–60 ml of deproteinized plasma before the peaks became deformed and the resolution decreased. After change to a new precolumn, the retention time of the noscapine peak decreased by *ca.* 2–3 min after the first 5–7 plasma injections. Therefore, the time for the column switching event was determined after pretreatment of the column with 6–8 plasma injections. Between each day the retention time of noscapine on the precolumn decreased slightly. This was compensated by decreasing the acetonitrile content of the precolumn eluent by 1.0% for each day the precolumn was used.

CONCLUSION

Coupled-column liquid chromatography was used for quantitation of noscapine and its metabolites. Prior to injection on a short precolumn the plasma proteins were precipitated with acetonitrile and perchloric acid. Noscapine and the polar metabolites were separated into two groups on the precolumn and each group was transferred to an analytical column for further separation. The precolumn was used for about 45–60 ml of deproteinized plasma before the peaks deteriorated and the resolution decreased.

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