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# DETERMINATION OF ERGOT ALKALOIDS IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

PER OLOF EDLUND

*Department of Pharmacology, Central Research and Control Laboratory of the National Corporation of Swedish Pharmacies, Box 3045, S-171 03 Solna (Sweden)*

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## SUMMARY

Liquid chromatographic methods for the determination of ergotamine and methylergometrine in plasma have been developed. The samples are extracted with an organic solvent at pH 9.0, cleaned by extractions and finally injected on an ODS-Hypersil reversed-phase column with acetonitrile-ammonium carbonate buffer as the mobile phase. The polarity of solvents used for extraction and the mobile phase are varied with the compounds of interest. Ergocristine is used as internal standard for ergotamine, and methysergide for the determination of methylergometrine. The stability of samples and standard solutions for calibration are discussed. Conditions for high selectivity and sensitivity of detection are given. Concentrations down to 100 pg/ml of plasma can be detected with a 3-ml sample.

## INTRODUCTION

Ergotamine is still the mainstay in the treatment of migraine, and methylergometrine is used in obstetrics as a uterus-contracting agent. The therapeutic doses of these drugs are rather low (0.2–2 mg) and plasma concentrations after a single dose are in the range of ng/ml to pg/ml.

Determination of the total radioactivity after administration of tritium-labeled ergot alkaloids has been used in pharmacokinetic investigations [1, 2]. Several radioimmunoassay (RIA) methods for the determination of ergot alkaloids [3–6] and lysergic acid diethylamide (LSD) [7, 8] have been described. RIA methods using lysergic acid antisera for determination of ergopeptides are not selective or sensitive enough to be used in pharmacokinetic investigations. More selective and sensitive methods have been developed by Shran et al. [6] who used antisera elicited against conjugates of human serum albumin and ergopeptide linked via the indole nitrogen of the alkaloid.

Fluorimetric [9] and thin-layer chromatographic methods [10] are not sensitive enough for analysis of ergot alkaloids in plasma samples. However, sensitive and selective methods for the determination of LSD by liquid chromatography with fluorescence detection have been used in forensic work [8, 11]. Several liquid chromatographic systems have been developed for separating ergot alkaloids [12–19] but no applications for plasma have been published to our knowledge. This report describes methods suitable for the determination of ergotamine and methylergotamine in plasma. The described methods can be used for other derivatives of lysergic acid after minor modifications.

## EXPERIMENTAL

Ergotamine tartrate was obtained from WHO Collaborating Centre for Chemical Reference Substances (Solna, Sweden). Ergotaminine, ergocristine, methylergometrine hydrogen maleate, methysergide hydrogen maleate and dihydroergotamine methane sulphonate were kindly supplied by Sandoz (Basle, Switzerland). Ethyl acetate, cyclohexane, butanol and acetonitrile were of LiChrosolv® quality (Merck, Darmstadt, G.F.R.). Test tubes (50 ml) with glass stoppers were used for extraction and 10-ml conical tubes for evaporation of organic extracts. All test tubes were silanized with 2% Drifilm (Pierce, Rockford, IL, U.S.A.) in cyclohexane followed by three washes of methanol.

### *Preparation of calibration solutions*

A stock solution of ergotamine tartrate and ergotaminine was prepared so that 40% of the total alkaloid content consisted of ergotaminine. Ethanol (96%) purged with nitrogen was used as solvent. The stock solution was further diluted with deoxygenated ethanol together with ergocristine to obtain seven solutions in the range of 0–75 ng/ml ergotamine and 100 ng/ml ergocristine.

Methylergometrine solutions with methysergide as internal standard were prepared in a similar manner; 0.1 ml of these solutions were used for calibration. The solutions were found to be stable for 60 days at –50°C.

### *Instruments*

A liquid chromatographic pump (Waters M-6000 A) with a U6K injector was connected to a 250 × 4.6 mm I.D. stainless steel column packed with ODS-Hypersil, 5 µm particles (Chrompack, Middelburg, The Netherlands). Columns were packed with a slurry of particles in dichloromethane followed by methanol. The mobile phase consisted of acetonitrile–0.01 M ammonium carbonate in glass-distilled water. Thirty per cent acetonitrile was used for the separation of methylergometrine and methysergide; 50% acetonitrile was used to separate ergotamine, ergocristine and ergotaminine.

The outlet of the column was connected to a Schoeffel FS 970 fluorescence detector for liquid chromatography. The monochromator at the excitation side was set at 328 nm, combined with a band-pass filter of 320 nm to exclude higher orders of diffraction. A 389-nm cut-off filter was used at the emission side, and the time constant was set to 4 sec.

### *Extraction procedure*

Plasma or serum (3 ml), 0.1 ml of internal standard solution and 3 ml of 1 M ammonia-hydrochloric acid buffer (pH 9.0) are extracted by 20 ml of organic solvent during 15 min, followed by centrifugation. The organic phase is transferred by pasteur pipette to a new tube. The organic phase is extracted with 2 ml of 0.05 M sulphuric acid during 10 min. The organic phase is aspirated off, and 2 ml of ammonia-hydrochloric acid buffer (pH 9.2) are added to the aqueous phase to yield pH 9.0. The aqueous phase is extracted by a fresh 10-ml portion of organic phase, transferred to a 10-ml tube and evaporated with a stream of nitrogen at 50°C. Then 0.2 ml of mobile phase from the liquid chromatograph is added, followed by mixing and agitation in an ultrasonic bath for 5 min. An aliquot of 50–150  $\mu$ l is injected into the liquid chromatograph. The organic solvent used for extraction consisted of cyclohexane-1-butanol (9:1) for the extraction of ergotamine, and ethyl acetate for the extraction of methylergometrine. The concentrations of ergotamine, ergotaminine and methylergometrine are evaluated by using a calibration graph established from seven standard samples. The standard samples were prepared from standard solutions described above and 3 ml of blank Seronorm® (Nyegaard & Co., Oslo, Norway).

## RESULTS AND DISCUSSION

### *Extraction*

Ergotamine is an amphoteric compound with  $pK_{a_1} = 6.25$  [20] and  $pK_{a_2} \approx 11$ . Kleimola [4] determined distribution ratios for ergotamine with different organic solvents and used benzene with 5% isoamyl alcohol for extraction in an RIA method for ergot alkaloids. Hooper et al. [9] also used benzene for extraction of biological samples in a fluorimetric assay of ergotamine. In general it is desirable to use a solvent with as low a polarity as possible to reduce co-extraction of the matrix components, but which still gives quantitative extraction. We preferred to use cyclohexane-1-butanol (9:1) prior to benzene due to the toxic properties of benzene. Cyclohexane-butanol gave pure extracts with a high absolute recovery. A more polar solvent was needed for extraction of methylergometrine. Ethyl acetate was found to give clean extracts and high absolute recovery (Table I). Re-extraction to acidic aqueous phase was used to increase the selectivity of the extraction and to concentrate the sample.

### *Chromatography*

Separation of ergot alkaloids in fermentation media has been performed with LiChrosorb NH<sub>2</sub> [15]. Szepesy et al. [16] used silica columns for group separation and contaminants. Stereo- and structural isomers were separated on reversed-phase packings. Reversed-phase columns have been widely used for the separation of mixtures and isomers of ergot alkaloids [17, 19], and gradient systems are used for the determination of degradation products [13, 14].

Two modes of operation are possible for the chromatographic separation of bases on reversed-phase columns. The components can be chromatographed as free bases at alkaline pH or as cations in an ion-pair complex with a suitable counter-ion. The ion-pair mode of operation is advantageous with respect to

TABLE I  
ABSOLUTE RECOVERY AND PRECISION

	<i>n</i>	Amount (ng) added to 3 ml of Seronorm	Absolute recovery (%)	Relative peak height versus internal standard (C.V. %)	Detection limit in 3-ml sample (pg/ml)
Methylergometrine	6	1.50	53 ± 4	10	100
Methysergide	6	10	50 ± 2		
Ergotamine	6	7.89	79 ± 7	2.5	100
	6	1.58	92 ± 6	3.2	
Ergotaminine	6	5.27	91 ± 7	6.6	200
	6	1.07	99 ± 6	7.7	
Ergocristine	12	10	84 ± 8		

column stability since silica is susceptible to hydrolysis at alkaline pH. However, a much better selectivity for the separation of epimers of the ergot alkaloids is obtained when the free bases are chromatographed (Table II). Large differences in capacity factors are obtained for lysergic acid—isolysergic acid epimers. Wurst et al. [15] suggested that this depends on a difference in the possibility of intramolecular hydrogen bonding. Epimers with an intramolecular hydrogen bond are much more lipophilic than epimers which lack such a bond. ODS-Hypersil columns with acetonitrile—aqueous ammonium carbonate mixtures as mobile phase provided very efficient and selective separation systems suitable for determination of ergot alkaloids in plasma.

We have used the same column for several months and hundreds of analyses without any sign of deterioration despite the high pH (9.0) used.

TABLE II  
CAPACITY FACTORS FOR DIFFERENT MOBILE PHASES

	Mobile phase 1*	Mobile phase 2**
Ergotamine	6.4	2.3
Ergotaminine	7.7	8.5
Ergocristine	12.6	4.9

\*Mobile phase 1 = 400 ml of acetonitrile, 1.9 g of sodium heptanesulphonate (0.05 M), 10 ml of glacial acetic acid and glass-distilled water to make one litre.

\*\*Mobile phase 2 = 500 ml of acetonitrile diluted with 0.01 M ammonium carbonate to make one litre.

### *Detection*

The combination of solvent extractions, high separation efficiency of the chromatographic system, intense fluorescence of the lysergic acid derivatives and selectivity of detection, makes it possible to measure concentrations down to 100 pg/ml with a 3-ml plasma sample. The detection limit in plasma samples

is restricted by the volume of sample available and the noise of the fluorimetric detector. Due to the efficiency of the chromatographic separation it is possible to optimize the conditions of fluorimetric detection for maximum sensitivity rather than selectivity. High sensitivity is gained by maximum ratio of output of photons to the photomultiplier, versus background fluorescence. Detectors equipped with filters give higher output of light than detectors with monochromators but the choice of wavelength is more restricted. The instrument used in this work was equipped with a diffraction-grating monochromator for excitation. A cut-off filter was used on the emission side to exclude scattered light and to minimize loss of light emitted. The intense fluorescence of the ergot alkaloids is diminished if the 9:10 double bond is hydrogenated. With the chromatographic system used for ergotamine the detection limit for dihydroergotamine was determined to be 1 ng at excitation wavelength 280 nm, without any filter on the emission side.

### *Stability of samples and standards*

Ergotamine undergoes spontaneous epimerization at C-8 to ergotaminine, epimerization at C-2 in acidic solutions to form "aci-compounds". Addition of water to the 9:10 double bond can occur, catalyzed by UV light. Furthermore, the indole is susceptible to oxidation by oxygen. The stability of ergotamine tartrate in aqueous solution has been studied by Krieglård and Kisbye [21]. Heat treatment at pH 3.6 was used to obtain epimerization equilibrium at C-8 in solutions for injection. Hartman et al. [22] studied the stability of liquid hydrated ergot alkaloid preparations as a function of solvent polarity. Stable solutions were obtained with water-alcohol mixtures with a dielectric constant between 30 and 45. The information about the stability of solutions used for injections can not be extrapolated to the concentration range of standard solutions used for calibration. The rate of oxidation might be dependent on concentration, and solutions used for calibration differs by four to five orders of magnitude from solutions used for injection. The epimerization reactions will be without importance if the solutions are stored at a low enough temperature ( $-50^{\circ}\text{C}$ ), but oxidation might occur due to the low energy of activation for oxidation [22].

Another possibility to obtain solutions that are stable with respect to epimer composition is to prepare solutions with equilibrium concentrations of epimers.

We have used both approaches for the preparation of ergotamine/ergotaminine standard solutions. The ergotaminine concentration should be 40% of the total [21] to obtain equilibrium. Ergocristinine was not added since it is not readily available, and it would prolong the chromatography time due to its high elution volume.

Standard solutions prepared with nitrogen-purged ethanol can be stored at  $-50^{\circ}\text{C}$  for at least 60 days without degradation. Serum standards were prepared from Seronorm and aqueous solutions of ergotamine and ergotaminine and stored at  $-50^{\circ}\text{C}$ . The stability was followed by repeated analyses during the time of storage. It was found that the samples could be stored for at least 60 days without appreciable degradation (Table III). Prolonged storage of samples is not recommended until a thorough evaluation of long-term stability has

TABLE III  
STABILITY OF SPIKED SERUM SAMPLES

Time of storage at -50°C (days)	n	Concentration found (ng/ml)		Calculated concentration (ng/ml)	
		Ergotamine	Ergotaminine	Ergotamine	Ergotaminine
0	1	38.8	24.4	39.6	26.2
24	1	31.2	20.7	39.6	26.2
44	1	31.4	20.5	39.6	26.2
129	1	38.8	27.5	39.6	26.2
144	1	40.5	28.2	39.6	26.2

been performed. A standard solution containing only ergotamine was extracted according to the "extraction procedure" to check if any ergotaminine was formed during work-up of the samples. No measurable concentration of ergotaminine was found. No ergocristinine was observed in chromatograms after work-up of samples if it was not present from the beginning.

The above-described method has been used in our laboratory for one year for the analysis of ergotamine, ergotaminine and methylergometrine in plasma with excellent results. Absolute recoveries and coefficients of variation are presented in Table I.

Plasma concentration profiles of ergot alkaloids after intravenous administra-

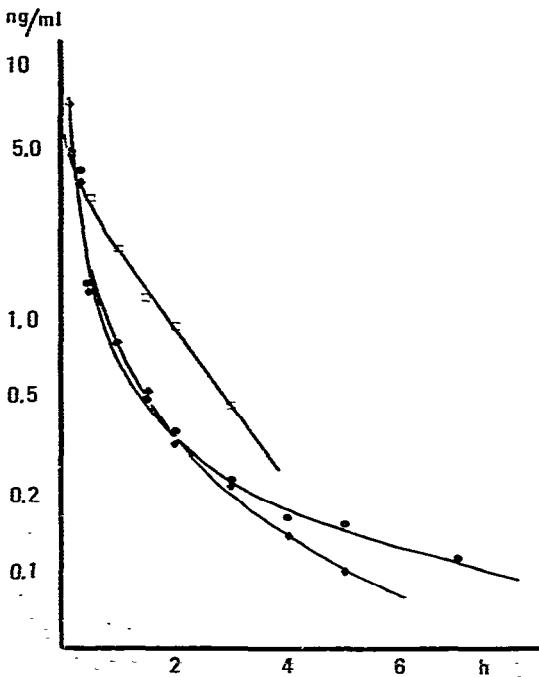


Fig. 1. Plasma concentration profiles from a patient after a 250- $\mu$ g dose of ergotamine tartrate (containing 133  $\mu$ g ergotamine base and 89  $\mu$ g ergotaminine base); ergotamine (●), ergotaminine (○). Plasma concentration profile from a patient after a 200- $\mu$ g intravenous dose of methylergometrine maleate (□).

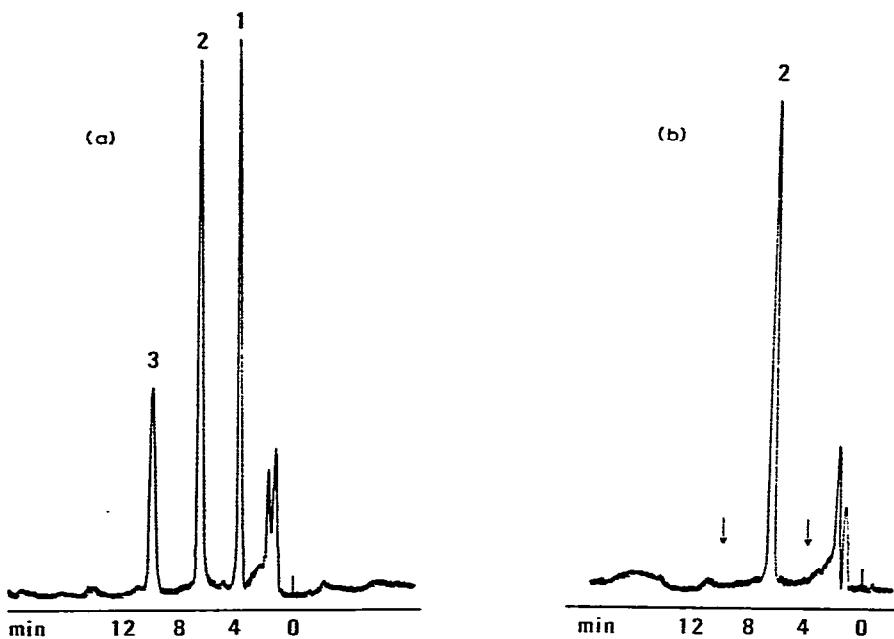


Fig. 2. (a) Chromatogram from a 3-ml plasma sample containing 3.2 ng of ergotamine (1), 2.1 ng of ergotaminine (3) and 10 ng of ergocristine (2). (b) The pertinent blank plasma containing 10 ng of ergocristine (2). Injection volume: 90  $\mu$ l of a total 200  $\mu$ l. Mobile phase 2; flow-rate = 1.5 ml/min.

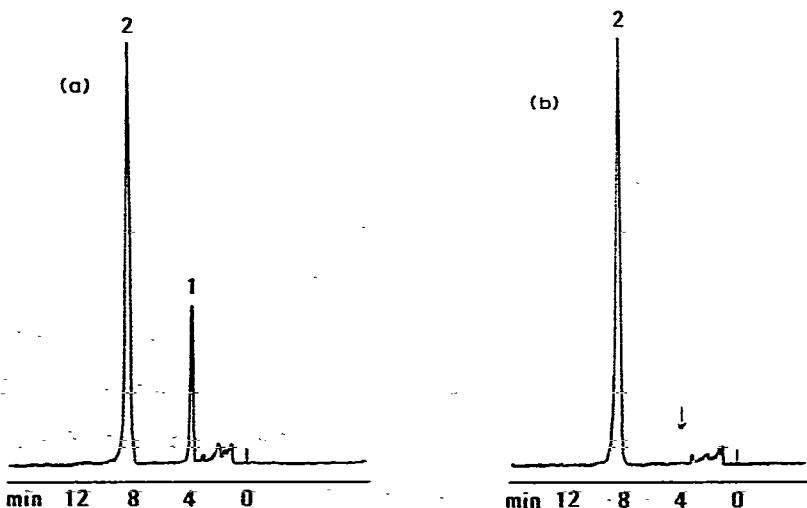


Fig. 3. (a) Chromatogram from a 3-ml plasma sample containing 2.3 ng of methylergometrine (1) and 9.0 ng of methysergide (2). (b) The pertinent blank plasma containing 9 ng of methysergide (2). Injection volume: 90  $\mu$ l of a total 200  $\mu$ l. Mobile phase was 300 ml of acetonitrile diluted with 0.01 M ammonium carbonate to one litre; flow-rate = 1.5 ml/min.

tion are shown in Fig. 1. Chromatograms obtained with 3-ml plasma samples are presented in Figs. 2 and 3 and with a 3-ml Seronorm blank in Fig. 4.

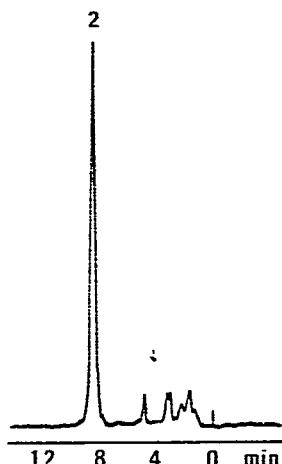


Fig. 4. Chromatogram obtained with a 3-ml Seronorm blank containing 9 ng of methysergide. Conditions as in Fig. 3.

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