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Note

Reversed-phase liquid chromatography for the determination of galanthamine and its metabolites in human plasma and urine

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Galanthamine (I, Fig. 1) is a tertiary amine alkaloid that belongs to the phenanthrene group. It is frequently used in clinical practice in connection with its anticholinesterase activity in both the central and peripheral systems [1–5]. This drug is available for clinical use as Nivalin® (Pharmachim, Sofia, Bulgaria) and Galanthamine (Medexport, Moscow, U.S.S.R.).

Recently in this laboratory a thorough investigation of the pharmacokinetics and metabolism of galanthamine was performed. A reversed-phase liquid chromatographic (RPLC) method for the analysis of galanthamine and its metabolites in blood plasma and urine was developed.

Claessens et al. [6] reported a high-performance liquid chromatographic method for the determination of galanthamine in blood plasma, urine and bile, using normal-phase chromatography on 3-μm silicagel. The application of *n*-hexane–dichloromethane–ethanolamine (500:500:0.25, v/v/v) as eluent gave rapid results, with a detection limit of ca. 5 ng/ml. However, the coefficient of variation at this level is relatively high (37.8%), and the use of normal-phase chromatography causes some inconvenience.

The present reversed-phase method is more suitable and, moreover, the two major metabolites, epigalanthamine (II) and galanthaminone (III) (Fig. 1), are also conveniently quantified. They were identified by means of mass spectra after their thin-layer chromatographic separation from biological in vivo experiments.

Since the substances in question contain a basic nitrogen atom, a greater retention and peak tailing during chromatographic separation was observed. This effect is due to silanol sites remained unchanged during the modification of the silica

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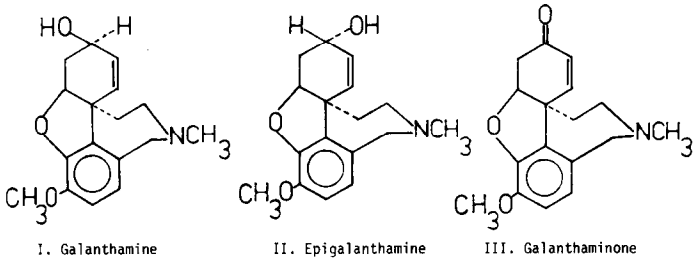

Fig. 1. Structures of compounds I, II and III.

gel surface with alkylamines. This unfavourable effect was overcome by adding dibutylamine to the mobile phase [7,8].

EXPERIMENTAL

Drugs and reagents

Galanthamine was obtained from Pharmachim. Codeine (an internal standard) was of pharmacopoeial purity. Epigalanthamine and galanthaminone (standard substances) were obtained from the Research Chemical Pharmaceutical Institute (Sofia, Bulgaria). Methanol and chloroform, p.a. grade, were used after redistillation. Dibutylamine (Fluka, Buchs, Switzerland), phosphoric acid (85%), trichloroacetic acid, sodium sulphate and sodium hydroxide (Merck, Darmstadt, F.R.G.) were used without further purification.

Apparatus

The experiments were performed on a liquid chromatograph, Perkin-Elmer Series 1/1, with a fixed-wavelength (280 nm) detector (Perkin-Elmer LC-15). Detection was at 0.008 a.u.f.s. A Rheodyne valve 7010 with an injection loop of 100 μl was used.

The pH value of the non-aqueous mobile phase was measured potentiometrically by means of a digital pH meter, Radiometer PHM 84, with a combined glass/reference electrode, Radiometer GK 2401C. The calibration of the cell was performed according to ref. 9.

RPLC conditions

A 125×4 mm I.D. Hibar-LiChrosorb RP-8 5-μm column (Merck) was used. The methanol–water (40:60) mobile phase modified with $5 \cdot 10^{-3}$ *M* dibutylamine at pH 7 (adjusted with 85% phosphoric acid) and a flow-rate of 1.2 ml/min were chosen. The presence of dibutylamine significantly improved the chromatographic behaviour of the substances investigated. The mobile phase was prepared by mixing 400 ml of methanol, 0.85 ml of dibutylamine and 0.2 ml of 85% phosphoric acid and making to 1000 ml with distilled water. The mixture was degassed before use. All chromatographic investigations were performed at room temperature.

Plasma and urine standards

A stock solution was prepared by dissolving 12.82 mg of galanthamine hydrobromide, 10.0 mg of epigalanthamine and 10.0 mg galanthaminone in 100.0 ml of distilled water, to yield the equivalent of 100 μg/ml galanthamine base and the other two substances. This solution was diluted with blank human plasma or urine to make standard solutions with final concentrations of 0.1–10 μg/ml.

Internal standard solution

Codeine base (11.11 mg) was dissolved in 100.0 ml of freshly distilled chloroform. The concentration of the solution obtained was 111.1 μg/ml. It was diluted with chloroform to prepare the internal standard solution of 5.6 μg/ml codeine.

Sample preparation

For 2.00 ml of blood plasma, 2.0 ml of 20% trichloroacetic acid was used as a precipitating agent. The mixture was shaken for 1 min on a vortex mixer. After centrifugation at 2000 *g* for 10 min, 3.00 ml of the supernatant were transferred in another test-tube, and 0.5 ml of 5 *M* sodium hydroxide were added to bring the pH of the supernatant to 11. Then 5.00 ml of chloroform, containing 5.6 μg/ml codeine, were added. The tube was rotated on a vortex mixer for 30 s and centrifuged at 2000 *g* for 5 min. A 4.5-ml aliquot of the clear solution was filtered through sodium sulphate. The filter was washed with 1.0 ml of chloroform. The extracts were evaporated at 62–65 °C. The dry residue was dissolved in 0.2 ml of mobile phase, and 100 μl were injected into the chromatograph. The same procedure was applied for the preparation of urine samples, but without the precipitation step.

Calibration curves

Standard solutions containing 0.1–10 μg/ml galanthamine, epigalanthamine and galanthaminone and 5.6 μg/ml codeine were prepared in plasma and urine. Calibration curves were constructed by plotting the peak-height ratios of the substances and internal standard against the drug concentration in each sample. The slope of the standard curves was calculated by a least-squares procedure with a high correlation coefficient ($r=0.998$; $n=16$).

RESULTS AND DISCUSSION

Precision and detection limits

The precision of the method was assessed by repeated analysis of plasma and urine samples containing various concentrations of galanthamine, epigalanthamine and galanthaminone. The plasma and urine levels were calculated from the calibration curves. The results are shown in Table I, which shows that the relative standard deviations are ca. 3% for the three substances.

The detection limits were found to be 0.05 μg/ml in plasma and urine. They were determined by extrapolating a plot of peak heights versus standard concentrations to a signal-to-noise ratio of 3. The noise was determined from the standard deviation of several measurements of the response of blank plasma (urine) measured at the retention times of the substances.

TABLE I

PRECISION OF THE PROPOSED METHOD

C.V. = coefficient of variation; $n = 5$.

Compound	Concentration added (μg/ml)	Plasma		Urine	
		Found (mean ± S.D.) (μg/ml)	C.V. (%)	Found (mean ± S.D.) (μg/ml)	C.V. (%)
Galanthamine	0.5	0.49 ± 0.012	4.66	0.51 ± 0.011	4.52
	1.0	1.01 ± 0.010	4.05	1.01 ± 0.010	4.05
	2.0	1.99 ± 0.010	1.79	2.01 ± 0.010	1.80
	4.0	4.02 ± 0.018	1.46	4.01 ± 0.010	1.34
	6.0	5.99 ± 0.017	1.01	5.99 ± 0.018	1.08
Epigalanthamine	0.5	0.51 ± 0.011	4.42	0.49 ± 0.012	4.66
	1.0	0.98 ± 0.014	4.40	0.99 ± 0.011	4.12
	2.0	2.01 ± 0.014	1.84	2.01 ± 0.014	1.84
	4.0	4.02 ± 0.018	1.46	4.01 ± 0.010	1.34
	6.0	6.01 ± 0.018	1.05	6.01 ± 0.018	1.05
Galanthaminone	0.5	0.49 ± 0.013	4.75	0.49 ± 0.012	4.66
	1.0	1.01 ± 0.012	4.12	1.01 ± 0.012	4.12
	2.0	2.01 ± 0.010	1.75	1.99 ± 0.010	1.79
	4.0	4.01 ± 0.014	1.44	3.99 ± 0.010	1.34
	6.0	5.99 ± 0.016	0.94	6.01 ± 0.016	0.94

Recovery and application of the method

The recovery was checked by means of in vitro experiments in which a known amount (1 μg/ml) of each substance had undergone the whole procedure. Then the peak heights obtained after extraction were compared against those obtained when the same amounts of galanthamine, epigalanthamine and galanthaminone from aqueous stock solutions were chromatographed. The results from ten experiments showed a recovery of 97.5 ± 2.6%. Interference from other substances was not observed but further research in this field is envisaged.

The method proposed was used for pharmacokinetic investigations of galanthamine. The substance was administered orally and subcutaneously to ten healthy volunteers and ten patients. The drug was administered at a dose of 10 mg of Nivalin (tablets and injections).

Fig. 2b shows a chromatogram of a human plasma extract, taken 180 min after the oral administration of a tablet of Nivalin. This shows that the substances under investigation are well resolved from the endogenous peaks. Besides the plasma components (see Fig. 1a), the peaks of galanthamine, epigalanthamine and codeine can be seen. Quantitative data are obtained from the chromatogram by peak-height analysis: the concentrations are 3.24 μg/ml galanthamine and 0.74 μg/ml epigalanthamine.

Fig. 3 shows a typical chromatogram of human urine extract, taken 12 h after subcutaneous administration of the drug. The peaks of galanthamine, epigalanthamine, galanthaminone, internal standard and urine components can be seen in the chromatogram. The concentrations determined are 0.98 μg/ml galanthamine, 2.54 μg/ml epigalanthamine and 0.66 μg/ml galanthaminone.

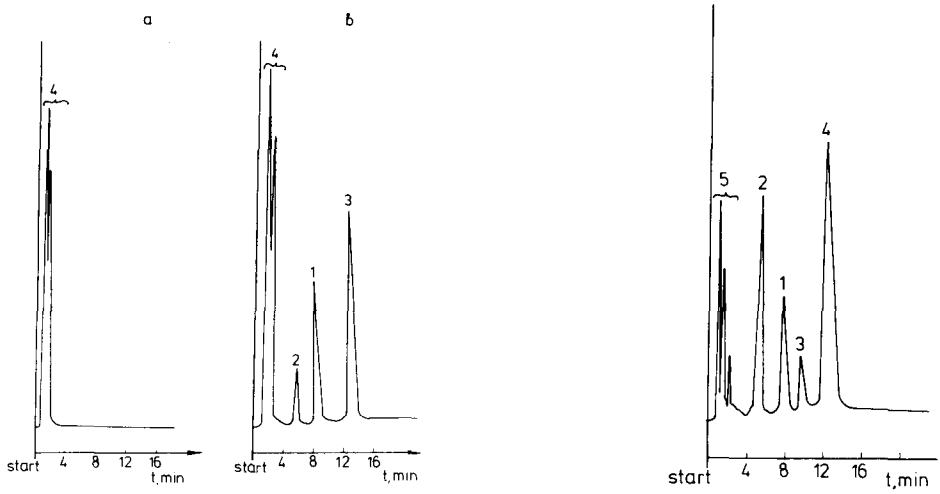

Fig. 2. Chromatograms of human plasma extracts. (a) Galanthamine-free plasma without internal standard: peaks marked 4 are plasma components; (b) chromatogram obtained 180 min after oral administration of a tablet of Nivalin. Peaks: 1 = galanthamine (3.24 μg/ml); 2 = epigalanthamine (0.74 μg/ml); 3 = codeine (internal standard, 5.6 μg/ml); 4 = plasma components.

Fig. 3. Chromatogram of human urine extract obtained 12 h after subcutaneous application of Nivalin. Peaks: 1 = galanthamine (0.98 μg/ml); 2 = epigalanthamine (2.54 μg/ml); 3 = galanthaminone (0.66 μg/ml); 4 = codeine (5.6 μg/ml); 5 = urine components.

CONCLUSION

The present RPLC method for the determination of galanthamine and its metabolites in human plasma and urine has sufficient sensitivity, selectivity and reliability for the measurement of the drug, and can be used for pharmacokinetic and metabolism studies.

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