Journal of Chromatography, 275 (1983) 345—353
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1676

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GALANTHAMINE, A LONG-ACTING ANTICHOLINESTERASE DRUG, IN SERUM, URINE AND BILE

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(First received December 12th, 1982; revised manuscript received February 9th, 1983)

SUMMARY

The anticholinesterase drug galanthamine is obtained from alkalinized serum by repeated liquid—liquid extraction. The resulting extract is approximately 100 times concentrated with respect to the original sample. Quantitative determination of galanthamine is performed with normal-phase liquid chromatography using a mixture of dichloromethane—*n*-hexane and ethanolamine as an eluent. Phenacetin is used as internal standard. The absorption of the column effluent is monitored at 235 nm. No endogenous sources of interference have been observed. A galanthamine serum level of 5 ng/ml is found as the minimum detectable concentration; the coefficient of variation at this level is 37.8% (n = 4). For the assay of galanthamine in the concentration range 10—100 ng/ml, standard deviations vary between 18.9 and 2.5% (n = 32).

INTRODUCTION

The alkaloid galanthamine hydrobromide is a tertiary amine belonging to

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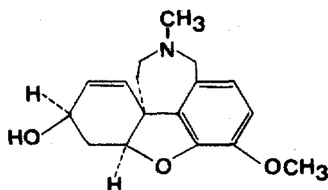

Fig. 1. Structural formula of galanthamine.

the phenanthrene group (see Fig. 1). Since the compound is an anticholinesterase drug, its pharmacological actions are both central and peripheral.

Galanthamine was isolated in 1952 by Proskurnina and Yakovleva [1] from the bulbs of the common snowdrop, *Galanthus woronowi* (Amaryllidaceae family) and in 1956 by Paskow and Iwanova-Bubewa from the bulbs of *Galanthus nivalis* (see ref. 2). It is available for clinical use as Nivalin (Pharmachim, Sophia, Bulgaria) and as Galanthamine (Medexport, Moscow, U.S.S.R.). Until now, galanthamine has mainly been in clinical use for the reversal of the neuromuscular blockade caused by various curare-like agents [2—5].

Antagonism of the respiratory depressant effect of opiates such as morphine, pethidine, dextromoramide, etc., in both animals and man has also been reported [6—9]. Furthermore, galanthamine has been used to reverse the central anticholinergic syndrome caused by scopolamine [10, 11] and certain central effects of droperidol and diazepam [12].

This broad variety of central effects of galanthamine and especially the finding that, in rabbits, the compound reversed opiate-induced respiratory depression, but not the concomitant analgesia [9], makes galanthamine a useful pharmacological tool in the search for better antagonists in anaesthesia.

In further (clinical-)pharmacological studies, concentration—effect relationships will be highly important; therefore, a sensitive and reproducible method for the determination of galanthamine in biological materials has to be developed.

Some work, mostly in the field of thin-layer chromatography, has been done on the assay of galanthamine [13—17]. High-performance liquid chromatographic (HPLC) determination of alkaloids in general has been studied by several authors. Wu and Wittick [18] and Rasmussen et al. [19] describe methods for the separation of alkaloids, using reversed-phase chromatography. Verpoorte and Baerheim Svendsen [20—22] and Rasmussen et al. [19] report the application of normal-phase chromatography on silica. Also the use of polar bonded phases had been reported [23]. To obtain low detection limits and short analysis times, particle sizes of 5 and 3 μm were selected and the capacity factors (k') of the components of interest were kept below 6.

The development of the method was started using reversed-phase chromatography on octadecyl phases. In spite of the available tools of this technique we failed to develop a method with the required sensitivity. The application of normal-phase chromatography on 3 μm silica gel and a mixture of *n*-hexane—dichloromethane—ethanolamine (500:500:0.25, v/v) as eluent gave good results in terms of detection limit and speed of analysis. The necessary pretreatment of the serum samples was started with extraction columns; however, even extensive experimental work gave no satisfactory results. We found

a repeated liquid—liquid extraction method very selective, but unfortunately it is more time-consuming.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade (Merck, Darmstadt, G.F.R.). Aqueous solutions were made in double-distilled water.

Dichloromethane and *n*-hexane were both of HPLC grade (Merck) and purified prior to use by filtration through a 0.45-μm HVLP membrane (Millipore Corporation, Bedford, MA, U.S.A.).

Galanthamine HBr and the standard phenacetin were obtained from Koch-Light Labs. (Colnbrook, Great Britain) and Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands), respectively.

Apparatus

The experiments were performed on a liquid chromatograph consisting of a reciprocating pump (Model 100-A, Beckman Instruments Inc., Berkeley, CA, U.S.A.) and a variable-wavelength UV detector (type Uvidec 100-III, Jasco, Tokyo, Japan) operating at 235 nm and 2.5×10^{-3} a.u.f.s. The pump delivered a constant flow of 1 ml/min. Injections of 10 μl were made with an injection valve equipped with a 20-μl loop (Model CV-6-UHPa-N60, Valco, Houston, TX, U.S.A.). The reversed-phase columns, 15 $\times$ 0.46 cm, using Polygosil 60-C_{18} 5 μm as packing material (Macherey-Nagel, Düren, G.F.R.) were home-made. The columns were constructed of stainless steel tubing (SS-316, Handy and Harman, Norristown, PA, U.S.A.) 15 cm $\times$ 6.4 mm O.D. $\times$ 4.6 mm I.D.

The normal-phase columns, 10 $\times$ 0.46 cm, were pre-packed with CP^{tm} Micro Spher Si 3 μm manufactured by Chrompack (Middelburg, The Netherlands).

To suppress the influence of fluctuations of temperature, tubing and column were suitably isolated. Detector signals were registered with a Yew recorder (Yokogawa Electric Works, Tokyo, Japan).

Galanthamine serum standards

Prepare a stock solution by dissolving 25.64 mg of galanthamine HBr in 200 ml of water. This solution is equivalent to 10^5 μg of galanthamine base per l. Dilute this solution with blank human serum to make serum standards with final galanthamine concentrations of 5, 10, 20, 40, 50, 70, and 100 ng/ml. Serum standard amounts of approximately 5 ml are frozen. There is no degradation of galanthamine after six months of storage at -20°C.

Sample preparation

Pipet 2.0 ml of serum into a 10-ml glass test tube and add 2.0 ml of a 20% (w/v) aqueous solution of trichloroacetic acid as protein-precipitating agent. The tube is then rotated on a Vortex mixer for 10 sec, allowed to stand at room temperature for 5 min, and centrifuged at 1600 *g* for 10 min.

A 3.0-ml aliquot of the supernatant is transferred in a 25-ml glass test tube

with stopper, and alkalinized by adding 0.6 ml of 4 *M* sodium hydroxide and 2.0 ml of buffer (1 *M* ammonia solution titrated with 2 *M* hydrochloric acid to pH 9.0). Extraction of galanthamine base is carried out twice with 5 ml of dichloromethane (DCM) by Vortex mixing for 2 min and centrifuging at 1600 *g* for 5 min. DCM extracts are transferred to a clean 25-ml glass test tube with stopper and back-extracted twice with 5 ml of 0.05 *M* sulphuric acid by Vortex mixing for 2 min and centrifuging at 1600 *g* for 5 min. Sulphuric acid extracts with galanthamine are transferred to a clean 25-ml glass test tube with stopper and alkalinized by adding 0.4 ml of 4 *M* sodium hydroxide, and 2.0 ml of buffer pH 9.0, prepared as described above. Subsequently galanthamine base is extracted twice with 5 ml of DCM by Vortex mixing for 2 min and centrifuging at 1600 *g* for 5 min. The DCM phase with galanthamine base, obtained after each extraction, is transferred to a 10-ml conical test tube, containing 0.5 ml of phenacetin internal standard solution in DCM (12.5 μg/l). The organic phase is evaporated under a gentle stream of nitrogen at 50°C in a heating bath. The inside wall of the test tube is washed with 0.5—1 ml of DCM, and the DCM is then evaporated to dryness as mentioned above. Each extract is dissolved in 25 μl of mobile phase by Vortex mixing for 30 sec. Then a 10-μl aliquot is injected immediately into the chromatograph.

Chromatographic procedures

The silica column was loaded with ethanolamine by pumping an approximately 0.1% solution of ethanolamine in *n*-hexane—dichloromethane (1:1,

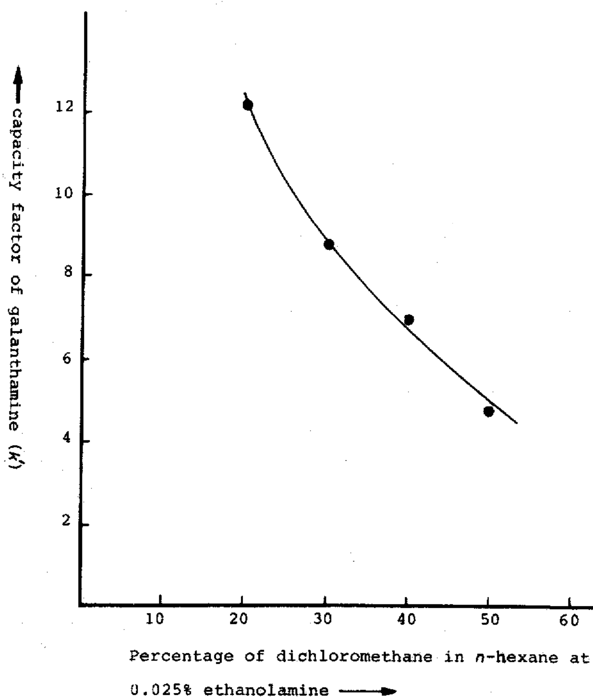

Fig. 2. Influence on the capacity factor (k') of galanthamine by various percentages of dichloromethane in the eluent mixture consisting of dichloromethane, *n*-hexane and a constant amount of 0.025% ethanolamine.

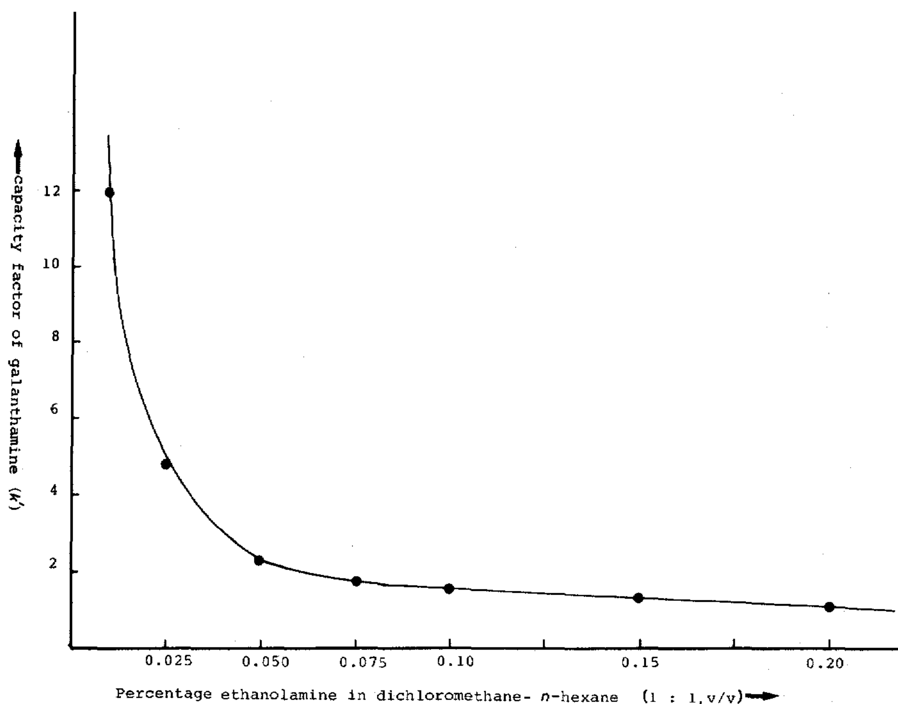

Fig. 3. Influence on the capacity factor (k') of galanthamine by various amounts of ethanolamine in the eluent mixture consisting of n-hexane and dichloromethane (1:1, v/v).

v/v) for about 1 h. Then the eluent, dichloromethane—n-hexane—ethanolamine (500:500:0.25, v/v), was pumped through the system until equilibrium; preferably this was done overnight. The capacity ratio (k') of galanthamine can be controlled by the content of dichloromethane and ethanolamine as is shown in Figs. 2 and 3, respectively. In practice the control of k' by the ethanolamine content is more easy.

The HPLC equipment and conditions described above permit the detector to operate at 235 nm and 2.5×10^{-3} a.u.f.s. Galanthamine has an absorption maximum at 288 nm, and a molar extinction coefficient of 2500 $l\ mol^{-1}\ cm^{-1}$. At shorter wavelengths the extinction coefficient increases at 235 nm to 7500 $l\ mol^{-1}\ cm^{-1}$, providing a more sensitive measurement. Application of lower wavelengths is limited by the quality and absorption of the eluent. In practice, no problems were encountered working at 235 nm.

High demands are put on the quality of the eluent because of the abovementioned facts, but also to prevent the appearance of interfering peaks in the chromatograms.

Quantitation

The peak height ratio of galanthamine base to the internal standard phenacetin is used for quantitation. The peak height ratios for the serum standards are plotted against concentration to obtain a standard calibration curve.

RESULTS

Typical chromatograms for serum samples containing 0 and 5 ng/ml galanthamine are presented in Fig. 4a and b, respectively; phenacetin is added as internal standard.

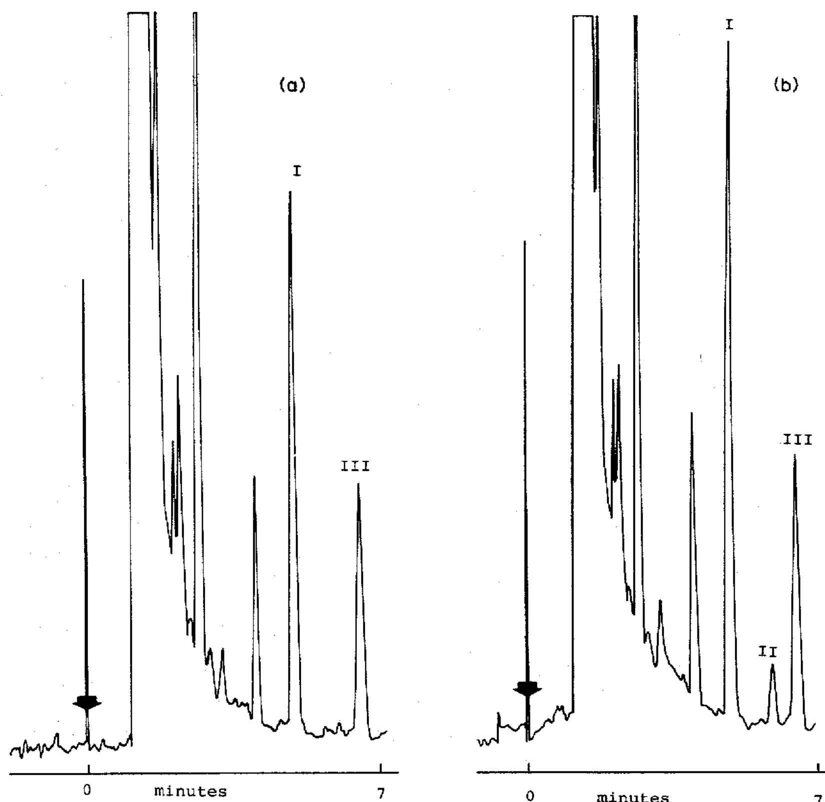

Fig. 4. Chromatograms of serum samples containing 0 ng/ml galanthamine (a) and 5 ng/ml galanthamine (b). Conditions: flow-rate 1 ml/min; eluent *n*-hexane—dichloromethane—ethanolamine (500:500:0.25, v/v); 10 µl injection; UV detection at 235 nm, 2.5×10^{-3} a.u.f.s. I = pollution of dichloromethane, II = galanthamine, III = phenacetin.

Calibration curve

The galanthamine/phenacetin ratios, calculated after determination of the serum standards 10, 20, 40, 50, 70, and 100 ng/ml, are used for constructing a calibration curve. The correlation coefficient is calculated by a least-squares procedure and is equal to 0.9996 (n = 32).

Precision and detection limit

The precision of the assay method was assessed by repeated analysis, on different days, of serum samples containing various concentrations of galanthamine. Galanthamine serum levels were calculated by means of a calibration curve. The results are shown in Table I.

The detection limit of galanthamine base in serum is about 5 ng/ml. The coefficient of variation at this level is 37.8% as presented in Table I.

TABLE I

PRECISION DATA

Galanthamine serum standards (ng/ml)	n	Mean galanthamine serum level assayed (ng/ml)	S.D. (ng/ml)	C.V. (%)
5	4	3.20	1.21	37.8
10	5	10.86	2.06	18.9
20	5	20.22	1.93	9.5
40	4	39.48	1.68	4.3
50	8	48.79	2.06	4.2
70	4	69.60	1.75	2.5
100	6	101.0	2.56	2.5

Recovery study

The recovery of galanthamine base was measured by assaying the galanthamine serum standard of 50 ng/ml eight times. Percentage recovery was calculated by comparing the peak height ratio galanthamine/phenacetin with the peak height ratio obtained by directly injecting solutions of the pure compounds. Galanthamine base showed an average recovery of 100.2% with a S.D. of 2.1% (n = 8).

DISCUSSION

To develop a sensitive method, the chromatographic dilution, amongst other factors, should be as small as possible. The use of 3-μm particles and keeping retention times small ($k' < 6$) are necessary to achieve this. The development of the method was started by applying reversed-phase chromatography on octadecyl phases. The influence of methanol content, pH of the eluent and ion-pairing reagents like hexylsulfonate were investigated.

In spite of the available tools of this technique we failed to develop a satisfactory procedure. We observed in these chromatographic systems a decrease in plate number for galanthamine of 75% compared to test components. This might be an indication of non-ideal behaviour of galanthamine under these conditions.

The application of normal-phase chromatography on silica using a mixture of n-hexane—dichloromethane and ethanolamine as an eluent gave much better results. In the latter case we observed a decrease of 25% in the plate number for galanthamine compared to a test mixture, indicating the better thermodynamic conditions in this system.

The influence of the dichloromethane content in the eluent and the addition of ethanolamine are shown in Figs. 2 and 3.

Other amines without a hydroxyl group failed to induce the same effect as ethanolamine. The extraction procedure of galanthamine from serum was started with Sep-Pak (sample enrichment and purification) cartridges (Waters Assoc., Milford, MA, U.S.A.). Sep-Pak cartridges are packed with liquid chromatographic separating materials which retain specific classes of compounds while allowing other materials to pass through. We have done several experi-

ments with Sep-Pak silica and C_{18} cartridges and solvents of different polarity. The extraction yield of galanthamine from Sep-Pak C_{18} after elution with dichloromethane at pH 9 was approximately 100% but HPLC chromatograms showed a high background signal due to co-extracted endogenous serum compounds. This high background of signals did not allow determination of nanogram amounts of galanthamine. From the various attempts that we have made to find an isolation method for galanthamine from serum with low background signals in HPLC chromatograms, the liquid—liquid extraction method presented was derived. The clean-up procedure in the liquid—liquid extraction method, described in this article, is laborious, but makes the assay very specific and sensitive.

The concentration of galanthamine in serum, found by us in preliminary experiments in man, varied between 500 and 60 ng/ml during 3 h, after an intravenous dose of 0.3 mg of galanthamine hydrobromide per kg body weight.

The following drugs, frequently used in patients undergoing anaesthesia, did not interfere with the HPLC determination of galanthamine: morphine, atropine, succinylcholine, thiopental, fentanyl, digoxin, gentamycin and tobramycin. The study of interferences by other components of comedication is continuing.

In our experiments we found that the assay procedure presented is also suitable for the determination of galanthamine in urine and bile.

CONCLUSION

The presented HPLC assay procedure for galanthamine in serum, urine and bile is very sensitive and therefore suitable for use in studies of pharmacokinetics and bioavailability of galanthamine in man.

ACKNOWLEDGEMENTS

We gratefully acknowledge the criticism of Prof. Dr. Ir. C.A.M.G. Cramers and Prof. Dr. H. Wesseling, the expert technical assistance of Dr. G.A. Vermeer, and the secretarial assistance of Mrs. B. Schellekens.

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