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Enantiomeric resolution of galanthamine and related drugs used in anti-Alzheimer therapy by means of capillary zone electrophoresis employing derivatized cyclodextrin selectors

Andreas Rizzi[a,*], Rudolf Schuh[a], Andrea Brückner[a], Beate Cvitkovich[a], Leopold Kremser[a], Ulrich Jordis[b], Johannes Fröhlich[b], Bernhard Küenburg[c], Laszlo Czollner[c]

[a]*Institute of Analytical Chemistry, University of Vienna, Währingerstraβe 38, A-1090 Vienna, Austria*
[b]*Institute of Organic Chemistry, Vienna University of Technology, Getreidemarkt 9, A-1060 Vienna, Austria*
[c]*Sanochemia AG, Landeggerstraβe 7, A-2491 Neufeld/Leitha, Austria*

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Abstract

An analytical assay is presented for the determination of the enantiomeric composition of galanthamine and related synthetic and natural compounds. (−)-Galanthamine is isolated from *Galanthus nivalis* and is used in this optical pure form in the therapy of Alzheimer's disease. Recent efforts for a total synthesis of unichiral (−)-galanthamine is connected with the need for a fast and reliable assay for the determination of the optical purity of the end product, as well as for optimizing and controlling the final steps in total synthesis particularly the asymmetric transformation of narwedine. In this paper the enantiomeric resolution of these compounds is reported employing a capillary electrophoretic system with β-cyclodextrin derived chiral selectors. With the proposed system a number of galanthamine and narwedine derived analogous compounds could be separated, including 1-bromo- and *N*-alkyl-substituted compounds.

Keywords: Enantiomer separation; Galanthamine

1. Introduction

(−)-[4a*S*(4aα,6β,8a*R**)]-Galanthamine (for structure see Fig. 1) is an alkaloid naturally occurring in unichiral form in the common snowdrop (*Galanthus nivalis*) and acts as a competitive inhibitor of acetylcholine esterase (AChE). Galanthamine hydrobromide, named Nivalin, has been approved as an agent for the treatment of Alzheimer's disease [1–3] in addition to preexisting approvals for the drug which include facial neuralgia and the treatment of residual paralysis in patients recovering from poliomyelitis.

Recent efforts have been directed towards the total synthesis of enantiomerically pure (−)-galanthamine [4,5] which is now performed in the range of kilograms. One of the key steps in this synthesis is a highly efficient crystallization induced chiral trans-

*Corresponding author: Tel.: +43-1-4277-52315; fax: +43-131-96312.
E-mail address: Andreas.Rizzi@univie.ac.at (A. Rizzi)

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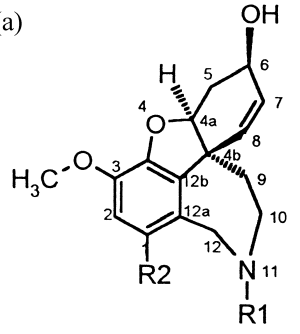
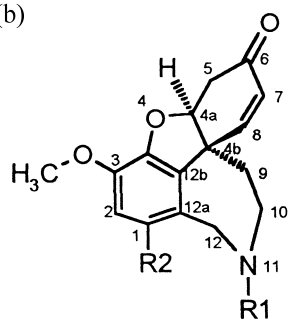
(a)



compound	R1	R2
(-) Galanthamine	CH_3	H
1-Bromo norgalanthamine	H	Br

(b)



compound	R1	R2
(-) Narwedine	CH_3	H
Ethylnarwedine	CH_2CH_3	H
Bromethylnarwedine	CH_2CH_3	Br
Bromnarwedine	CH_3	Br
Bromformylnarwedine	CHO	Br
Formylnarwedine	CHO	H
Benzylnarwedine	$CH_2C_6H_6$	H
Brombenzylnarwedine	$CH_2C_6H_6$	Br

Fig. 1. Chemical structure of (a) (−)-galanthamine, (b) (−)-narwedine and derived compounds.

formation of the final intermediate *rac*.-narwedine to homochiral narwedine crystals via a retro-Michael ring-opening and ring-closing reaction. The yield of pure enantiomers by this step was found to be highly dependent on the choice of solvent and the temperature conditions [6]. A fast and reliable assay is thus required for controlling this decisive transformation step as well as to determine the optical purity of the end product (−)-galanthamine.

Enantioseparation of galanthamine can be attained by means of high-performance liquid chromatography (HPLC) using a chiral stationary phase (CSP) based on arylcarbamate modified cellulose and organic eluents [7]. CSPs based on immobilized native β-cyclodextrin and γ-cyclodextrin were not successful in this respect. In this paper a capillary electrophoretic (CZE) method is reported for the enantiomeric resolution of galanthamine and the mentioned precursor narwedine employing heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) as chiral selector. The method is validated for the determination of enantiomeric impurities in (−)-galanthamine down to a content of 0.10% of the (+)-enantiomer. This proposed CZE method allows also the separation of various analogous compounds of potential therapeutical interest as well as their narwedine intermediates, particularly the 1-bromo-substituted and *N*-alkyl-substituted compounds. Their chemical structures are given in Fig. 1.

2. Experimental

2.1. Chemicals

Pure enantiomers of galanthamine and narwedine as well as racemic mixtures thereof were obtained by synthesis according to Ref. [5]. Pure (−)-galanthamine samples isolated from *Galanthus nivalis* were obtained from Sanochemia (Neufeld/Leitha, Austria). The chemical purity of samples was characterized by HPLC analysis using straight phase conditions. The various bromo-substituted galanthamines as well as the various *N*-substituted narwedines were prepared by the authors.

The buffers tetrabutylammoniumdihydrogen phosphate and sodiumdihydrogen phosphate were obtained from Sigma (Deisenhofen, Germany). Sodium hydroxide, hydrochloric acid and phosphoric acid were purchased from E. Merck (Darmstadt, Germany).

Native β-cyclodextrin (β-CD) was a gift from the department of chemistry of the Polish Academy of Sciences (Warsaw, Poland). Heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) containing about 14 methoxy groups per β-CD molecule), heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) containing 21 methoxy groups per β-CD molecule, (2-hydroxy)propyl-β-cyclodextrin (HP-β-CD) containing about 6.3 hydroxypropyl groups per β-CD ring, were purchased from Cyclolab R&D Laboratory (Budapest, Hungary). Native γ-cyclodextrin (γ-CD) was purchased from Fluka (Buchs, Switzerland).

2.2. Apparatus and electrophoretic conditions

All experiments were carried out using an HP-3D capillary electrophoresis instrument (Hewlett–Packard, Waldbronn, Germany), equipped with a diode array detector monitoring a wavelength of 216 nm. A non-coated fused-silica capillary (Hewlett–Packard) was used with 48.5 cm total length (40 cm effective length) and internal diameter of 50 μm which was kept at a constant temperature of 20°C±0.1°C and to which a voltage of 20 kV was applied, if not stated otherwise. For the analysis of traces of (+)-galanthamine in the range of 1% or lower, capillaries of 64.5 cm total length (56 cm effective length) were used at a voltage of 26 kV.

Injection was done by applying pressure differences of 20 to 60 mbar over a time period of 2 to 5 s. The magnitude of the electroosmotic flow (EOF) was controlled by injection of acetone.

The background electrolyte (BGE) of the validated assay consisted of an aqueous solution of 50 m*M* tetrabutylammoniumdihydrogen phosphate at pH 2.5 (adjusted with 1 *M* aqueous solution of phosphoric acid) and 30 m*M* DM-β-CD. Some of the concomitant measurements were made with a 50 m*M* sodiumdihydrogen phosphate buffer and the other cyclodextrin derived selectors mentioned above.

Washing, rinsing and reconditioning of the capillary was routinely done by flushing the capillary with 1 *M* sodium hydroxide solution for 5 min followed

by 0.05 *M* sodium hydroxide solution for 10 min each morning and with water for 5 min and BGE for 4 min previous to each run. Once a week rinsing was done with a solution of water (2 *M* in urea)–ethanol, 80:20, v/v for 3 min in order to facilitate the redissolution of cyclodextrin traces possibly adsorbed onto surfaces exposed.

The crystalline samples were dissolved in BGE (pH 2.5) to yield a concentration of about 100 μg/ml and analyzed immediately to prevent racemization.

3. Results

Electrophoretic separations of galanthamine, narwedine and of various analogues are shown in Fig. 2. With 30 m*M* DM-β-CD in the BGE and the experimental conditions given above (40 cm effective capillary length) baseline separation is achieved for most compounds within an analysis time of about 20 to 25 min.

3.1. Separation conditions

At the chosen pH of 2.5 the mentioned analytes (tertiary amines with pKa values of about 9) are fully protonated and are thus present in a cationic form. The low pH was chosen to keep the EOF low in order to avoid loss in selectivity associated with non-selective bulk flow. In the pH range between 2.3 and 3.5 the dependence of enantioselectivity on the pH was found to be insignificant. At lower pH values selectivity was diminished by too high buffer conductivity resulting in enhanced current and heat generation. At pH values higher than 4.5 the selectivity decreases and inversion of migration order is observed at pH about 6. In order to minimize electrodispersion phenomena caused by mobility mismatching between sample and BGE, tetrabutylammonium ions are used as cationic part of the

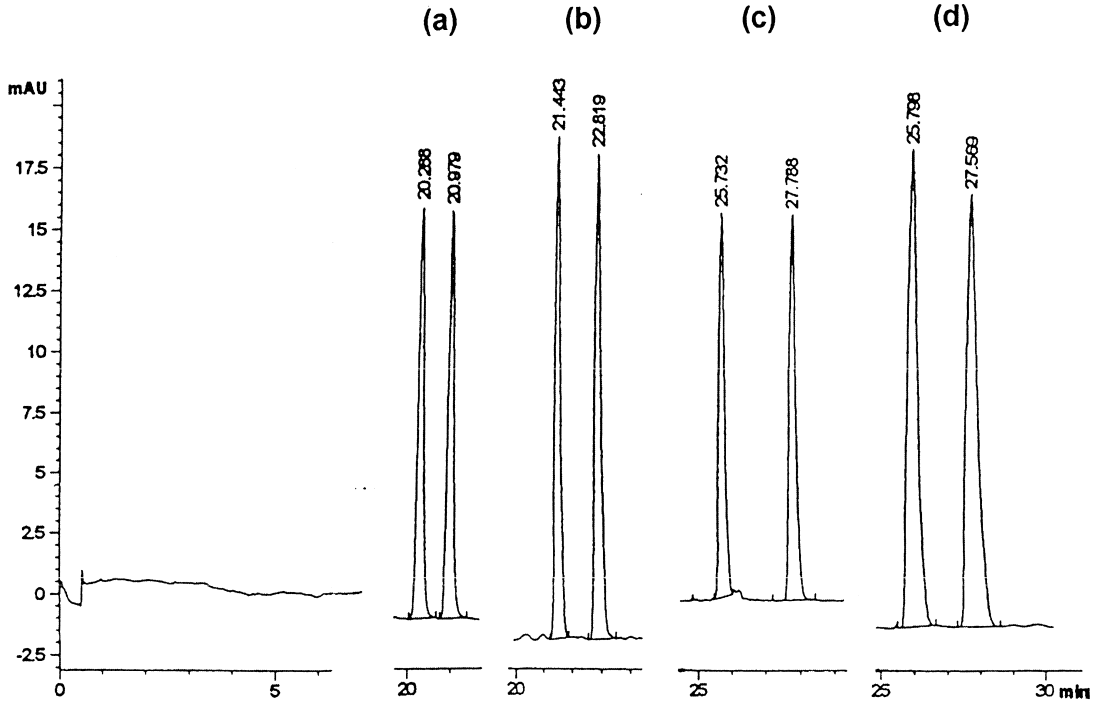


Fig. 2. Electropherograms of *rac*.-galanthamine (a), narwedine (b), *N*-ethyl-narwedine (c) and 1-bromo-*N*-ethyl-narwedine (d). Experimental conditions as described above; background electrolyte: 50 m*M* tetrabutylammoniumdihydrogen phosphate, pH 2.5, 30 m*M* DM-β-CD; effective capillary length: 40 cm,15 kV.

buffer. This buffer cation exhibits additionally a positive impact on the enantioselectivity, probably by further reducing the residual EOF or even generating a small inverted EOF.

The migration order of the enantiomers at pH 2.5 was established as (−) before (+) for galanthamine and narwedine. Although the migration order has not been determined for all analogous, the opposite order, i.e. (+) before (−), was found e.g. for 1-bromo-norgalanthamine.

Various chiral selectors were tested for the enantioseparation of galanthamine. Resolution was achieved only with DM-β-CD and to a minor extent also with HP-β-CD. These two selectors were in a few cases also successful in (partially) separating the enantiomers of some derivatized galanthamines as documented in Table 1. In no case could resolution be obtained with native-β-CD and TM-β-CD. The larger bromo-substituted compounds were in some instances resolved by γ-CD.

For some of the analyte-selector pairs the complexation constants (at pH 2.5) were determined. This was done by curve fitting the effective mobility and selectivity data as dependent on the selector concentration [8]. These mobility data (corrected for the enhanced viscosity at higher selector concentrations by using the correction factors given in [8]) are shown for DM-β-CD in Fig. 3, the complexation constants evaluated thereof are given in Table 2. The binding constants between galanthamines and cyclodextrins are low in most instances, i.e. in the order of less than 30 mol^{-1}. The knowledge of these constants enables one to approximately assess the optimum selector concentration for each pair, $[CD]^{opt}$, as the inverse (geometric) mean complexation constant [9,10]. Very high selector concentrations would be required for most selectors due to the weak interaction with the galanthamines at this pH value. On the base of these data a concentration of 30 m*M* DM-β-CD was selected for separations of galanthamine and narwedine analogous. Apparent selectivity coefficients of several narwedines obtained

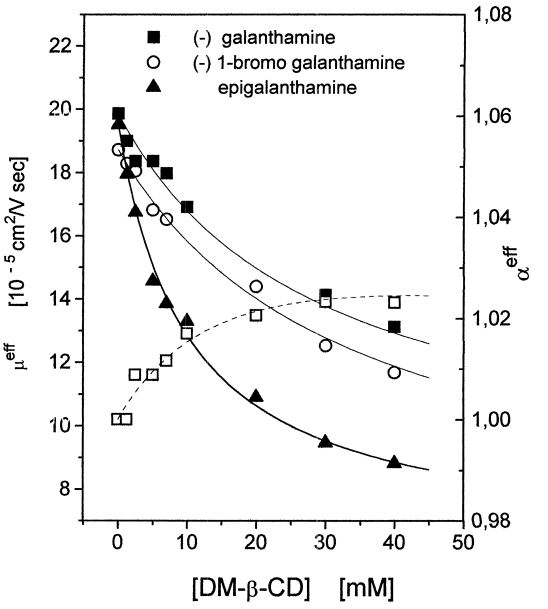


Fig. 3. Dependence of the effective mobilities of the indicated galanthamines (symbols defined in the figure corresponding to the left scale) and the effective selectivity coefficient of *rac.*-galanthamine (empty squares corresponding to the right scale) on the concentration of DM-β-CD. Experimental conditions; background electrolyte: 20 m*M* sodiumdihydrogen phosphate, pH 2.5, 20 kV.

Table 1
Effective electrophoretic mobilities of the faster migrating enantiomers, μ_1^{eff}, and effective enantioselectivity coefficients, $\alpha^{eff}=\mu_1^{eff}/\mu_2^{eff}$, for galanthamine and related compounds in the presence of the specified selector compounds. BGE: 20 m*M* sodiumdihydrogen phosphate, pH 2.5; temperature: 20°C; mobilities in 10^{-5} cm^2 V^{-1} s^{-1}

		β-CD 10 mM	DM-β-CD 30 mM	TM-β-CD 30 mM	HP-β-CD 30 mM	γ-CD 50 mM
Galanthamine	μ_1	18.6	13.2	17.9	16.0	16.6
	α^{eff}	1.0	1.023	1.0	1.012	1.0
Epigalanthamine	μ_1	15.3	8.85	16.6	11.7	15.6
	α^{eff}	1.0	1.0	1.0	1.010	1.0
1-Bromo-galanthamine	μ_1	17.8	11.7	15.7	14.7	15.4
	α^{eff}	1.0	1.0	1.0	1.009	1.0
1-Bromo-norgalanthamine	μ_1	18.1	12.3	16.7	14.7	15.7
	α^{eff}	1.0	1.022	1.0	1.011	1.023

Table 2
Approximate complexation constants of the first eluted enantiomers of galanthamine and related compounds with various cyclodextrin derived selectors as well as extrapolated optimum selector concentrations at pH 2.5 and 20°C. Complexation constants, K, in mole^{-1}, optimum selector concentrations, $[CD]^{opt}$, in mM

Analyte		Selector				
		β-CD	DM-β-CD	TM-β-CD	HP-β-CD	γ-CD
Galanthamine	K	14	35	3	8	5
	$[CD]^{opt}$	70	30	300	120	200
Epigalanthamine	K	65	100	5	40	6
	$[CD]^{opt}$	15	10	200	25	160
1-Bromo-galanthamine	K	17	30	8	14	5
	$[CD]^{opt}$	60	30	120	70	200
1-Bromo-norgalanthamine	K	10	45	4	8	5
	$[CD]^{opt}$	100	22	250	120	200

under these conditions (30 mM DM-β-CD and 50 mM tetrabutylammoniumdihydrogen phosphate) are collected in Table 3.

3.2. Inversion of elution order

As the therapeutically active enantiomer of galanthamine is the (−) enantiomer, the migration order (−) before (+) is the less desirable one when monitoring traces of the (+) enantiomer. Quantifying the trace enantiomers on a level of 0.5% and below needs strictly symmetric peaks to ensure accurate results.

The elution order is inverted when using pH values larger than 6. At pH 7, for instance, the effective selectivity is quite high, but effective mobilities are less than half the values at pH 2.5 and a considerable EOF diminishes the apparent selectivity. EOF variation upon small changes in the buffer is high as well in this pH region.

A common strategy for inverting the elution order is the inversion of the EOF by adding a cationic detergent like cetyltrimethylammonium bromide (CTAB) or a surface modifying diamine like spermidine. With both additives the EOF has been reversed. However, when applying CTAB the resolution of enantiomers disappeared. With spermidine and with permanently amine-coated capillaries (eCAP Amine; Beckman) inversion of migration order is attained at low pH values (pH 4.5) giving considerable apparent selectivities. At higher pH values (pH 7.0) the elution order was re-inverted giving again faster elution of the (−) enantiomer. The EOF inverted system at pH 4.5, however, is not as robust as the simple BGE system at pH 2.5. Particularly peak tailing was dramatically increased and reversed as well (giving fronting peaks). For the practical use in trace component quantitation no real improvement could be achieved by this inversion of elution order.

Table 3
Apparent mobilities and enantioselectivity coefficients of galanthamine, narwedine and substituted compounds; BGE: 50 mM tetrabutylammoniumdihydrogen phosphate, pH 2.5 and 30 mM DM-β-CD, 15 kV; mobilities in 10^{-5} cm^2 V^{-1} s^{-1}

Analyte	μ_1^{app}	α^{app}	Analyte	μ_1^{app}	α^{app}
Galanthamine	10.62	1.034	1-Bromo-nornarwedine	19.04	1.010
Narwedine	8.37	1.080	1-Bromo-*N*-ethyl-narwedine	8.35	1.069
N-Ethylnarwedine	10.05	1.064	1-Bromo-*N*-benzyl-narwedine	5.41	1.00
N-Benzylnarwedine	7.89	1.048	1-Bromo-*N*-formyl-narwedine	8.78	1.062
N-Formylnarwedine	10.76	1.029			

3.3. Validation of the galanthamine assay

The assay at pH 2.5 with non modified capillaries has been validated for the determination of galanthamine enantiomers. Particular emphasis is given to the quantitation of the (+) enantiomer as trace component eluted under this conditions after the therapeutic active (−) enantiomer.

3.3.1. Reproducibility of migration and separation

The reproducibility of migration times and selectivity coefficients in CZE is predominantly influenced by the stability and reproducibility of the EOF. Although the EOF is generally low at pH 2.5, a certain variation in migration times is found. It is specified by a relative standard deviation (RSD) of less than 2% in the intra-day data and about 8% in inter-day comparison. After washing with 0.05 *M* NaOH, rigorous rinsing with water and BGE solution as described in the experimental section was essential for achieving good reproducibility. Precision of enantioselectivity coefficients (as the ratio of the mobilities) is much better, characterized by a RSD of 0.1% intra-day and 0.24% inter-day.

3.3.2. Precision of quantitation

The precision of quantitation of peak area ratios is of course dependent on the signal/noise ratio. With high *S*/*N* ratios and a racemic sample the RSD lies between 0.8% (intra-day) and 1.0% (inter-day).

For traces of the (+) enantiomer (below 1.0%) the quantitation is preferably done via the peak heights. The RSD is not only dependent on the *S*/*N* ratio but also on frequently occurring peak tailing of the first eluted (−) enantiomer. The tailing of this peak becomes more significant when a high sample amount is injected. For trace amount determination the use of longer capillaries (e.g. 55–70 cm effective length) with accordingly enhanced field strengths is thus recommended in order to improve the resolution. This is, however, done on the expense of longer analysis times. Under conditions facing slight tailing of the first peak and using an effective capillary length of 56 cm and applying 26 kV the RSD (intra-day) for traces of (+)-galanthamine at the level of 0.5% (w/w) was approximately 3.3% (peak areas) and 4.7% (peak heights) and at the level of 0.1% (w/w) about 9% (peak heights and areas). Electropherograms of a sample of synthetic (−)-galanthamine spiked with different amounts of the (+) enantiomer are shown in Fig. 4.

3.3.3. Lower limit of determination, calibration function and upper limit of working range

The lower limit of determination (at a *S*/*N* ratio of 6) was approximately 2 pg galanthamine. These data are based on a baseline noise of 0.12 mAU. This limit implies that for the determination of a trace component of 0.25% at least 0.8 ng of sample has to be injected. The injected amount of 1 ng corresponded to a sample concentration of 100 μg/ml injected over a period of 5 s at 60 mbar.

Peak area based calibration lines of enantio-pure samples are linear up to an injected amount of about 5.5 ng. The linear correlation coefficient for this calibration line is 0.9958.

With injected amounts higher than 1.0 ng peak symmetry and thus the resolution of the enantiomers might be affected by beginning peak tailing. Peak height based calibration lines can be used in the range of symmetric peaks only. When facing even slight peak tailing of the major component the direct comparison of peak heights between the enantiomers will not yield accurate results. The quantitation of the trace component has to be based on calibration lines established for the trace enantiomer in the presence of the major component.

The optimum working range for the quantitation of traces smaller than 0.5% was found between 0.4 and about 1.0 ng injected sample amount.

3.3.4. Accuracy of trace component determination

With symmetric peaks there is no peak overlap even when using the shorter capillaries of 40 cm effective length thanks to the achieved selectivity. Even slight peak tailing of the main component peak, however, might influence not only the precision but also the accuracy of the trace component determination adversely. The extent of peak tailing was not only dependent on the injected amount but also on the status of the capillary wall.

Racemization of galanthamine during the electrophoretic determination is not a critical problem. No racemization has been observed when keeping the analyte dissolved in the BGE (pH 2.5) for more than eight days at room temperature. Racemization of

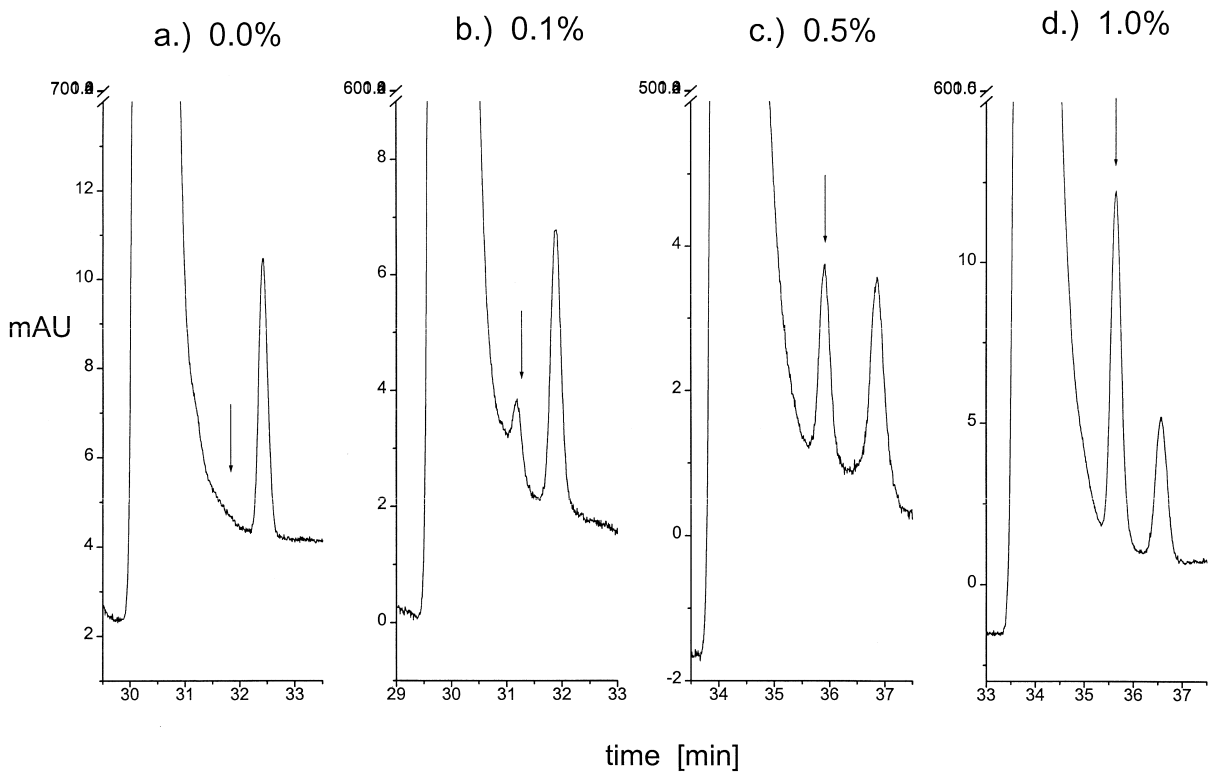


Fig. 4. Parts of the electropherograms of a sample of synthetic (−)-galanthamine spiked with different amounts of (+)-galanthamine (indicated by the arrows): (a) no addition of (+)-galanthamine; (b) addition of 0.1% (w/w); (c) 0.5% and (d) 1.0%. First peak: (−)-galanthamine; third peak: trace component present in the chosen sample. Electrophoretic conditions: total capillary length 64.5 cm, effective length 56 cm, voltage 26 kV, all other conditions as given in Section 2.2.

narwedine might be more critical, although it is very slow in acidic aqueous solution. However, it is strongly dependent on the solvent and can be significant when storing in neutral or basic solution or in acetonitrile [6]. Partial racemization of narwedine during an electrophoretic run of about 30 min at pH 2.5 and 20°C does not exceed an extent of more than 0.15%. No peak of the optical antipode is found when injecting enantio-pure narwedine even when allowing longer migration times. (One has to admit that it might be difficult to find coalescent trace peaks produced by partial racemization during the electrophoretic run.)

3.3.5. Robustness of the method

The method proposed at pH 2.5 is highly robust with respect to slight variations in pH and selector concentration. Variation of the pH within the limits 2.3 to 3.5 did not influence the separation, as discussed above, and the influence of small variations in the selector concentration was insignificant as it was chosen near its optimum value. The most decisive influence on the separation, particularly on peak shape and to a minor degree on selectivity, came from the capillary walls. Selection of the most appropriate product, careful washing and reconditioning after several runs is essential and in certain cases the replacement of the capillary after a larger number of analyses will be indicated.

4. Conclusion

The CZE assay presented allows the simple, fast and reliable determination of galanthamine enantiomers down to about 0.1%. At these trace levels the

careful control of peak tailing is decisive for the accuracy and precision of the determination. For the determination of traces below 0.5% the use of capillaries of 55 to 70 cm length applying enhanced voltages is recommended in order to achieve a precision for the trace component of better than 10% RSD.

The assay is also applicable to narwedine although in this case the residual risk of partial enantiomerization (less than 0.2%) during the electrophoretic run has to be controlled. The assay is easily applicable to a number of substituted narwedines, like 1-bromo-substituted and *N*-alkyl-substituted analogues.

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