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Chiral separation of muscarinic antagonists by capillary zone electrophoresis with cyclodextrin additives

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Abstract

The enantiomeric separation of some muscarinic antagonists bearing a quaternary ammonium group was investigated by using capillary electrophoresis. The effects of α-, β-, γ-cyclodextrin concentration were studied in an attempt to resolve all the analytes. The use of a coated capillary was found to be essential to improve resolution, efficiency and reproducibility of the separation profile. The sensitivity limit for enantiomeric concentration was as low as 1% and the response was linear after correction for time migration.

Keywords: Enantiomer separation; Buffer composition; Quaternary ammonium compounds; Muscarinic ligands; Cyclodextrins

1. Introduction

Chirality is an aspect of chemical structure which has a tremendous impact on biological response in terms of activity, organ-selectivity, toxicity and pharmacokinetic–pharmacodynamic parameters [1]. For such a reason, nowadays it has attracted the attention of both pharmaceutical companies and regulatory authorities. In the past, we used such a parameter to provide insights into the molecular requirements of the ligand–receptor interaction of compounds structurally related to muscarone (compound **1**, see Table 1) [2–4]. In the course of such an investigation we prepared and tested the racemic and the chiral forms of a series of muscarinic and antimuscarinic agents (compounds **2**–**6**) [4–6].

Interesting enough, the pharmacological data of compound **2**, designed as an antimuscarinic agent, evidenced a very low eudismic ratio (E=1.9 and 2.0) [(eudismic ratio: ratio between the potency of the most active enantiomer (eutomer) and the least active one (distomer)] in two muscarinic tests [6], in contrast to the data of the major homochiral muscarinic ligands where the eutomer is at least two order of magnitude more potent than the distomer. Such a peculiarity was further deepened through the synthesis of a series of new muscarinic antagonists i.e. **3** and **4** [De Micheli et al., unpublished results].

A common requirement for a remarkable muscarinic activity is the presence in the structure of the ligand of a quaternary ammonium head which gives rise to an ionic interaction with the aspartic acid residue of the complementary receptor subsite. Its removal or replacement with a tertiary amine moiety eliminates or sharply reduces the activity.

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The huge difference in the biological profile of the enantiomeric pairs of muscarinic ligands implies an accurate evaluation of the e.e. (enantiomeric excess) directly on the final derivatives. Despite numerous attempts, we never succeeded in the separation of the pairs of enantiomers of quaternary ammonium salts by chiral high-performance liquid chromatography (HPLC). According to the literature, the enantiomer pairs of chiral ammonium salts very seldom have been separated by chiral HPLC [7]. As a consequence, the e.e. of the chiral compounds we submitted to the biological tests was inferred on the basis of the data collected on suitable precursors by chiral HPLC or chiral gas chromatography. For example, the samples of (*R*)-(−)-**2** and (*S*)-(+)-**2** submitted to the pharmacological tests were assigned an enantiomeric excess of 98 and 91%, respectively, on the basis of the value of e.e. of the chiral precursors obtained via enzymatic resolution of a racemic substrate [6]. It has to be pointed out that chemical transformations and/or crystallizations usually modify the ratio between the enantiomers.

Since the presence of an ammonium group in the compound restricts the choice of the analytical methods suitable for an accurate evaluation of the enantiomeric excess, we investigated the possibility of applying new techniques. To the best of our knowledge, no example of analytical separations of the enantiomers of quaternary ammonium salts by capillary electrophoresis has been reported so far. Such a new methodology is now applied to the separation of a wide range of compounds besides amino acids [8].

This paper deals with the application of this new analytical method to the separation of the enantiomers of muscarinic derivatives **2–6**.

2. Experimental

2.1. Instrumentation

All experiments were performed on a Waters Quanta 4000 capillary electrophoresis system purchased from Millipore (Milford, MA, USA) equipped with Maxime 820 data evaluation software. The detection wavelength was 214 nm. For the experiments, 100 and 50 μm I.D., 370 μm O.D. capillaries, purchased from Polymicro Technologies (Phoenix, AZ, USA), coated with polyacryloylaminoethoxyethanol as described in [9] were used.

2.2. Materials

α-Cyclodextrin, β-cyclodextrin, γ-cyclodextrin and tris(hydroxymethyl)aminomethane (Tris) were bought from Sigma (St. Louis, MO, USA). The synthesis of muscarinic derivatives **1** and **2** has been previously reported [2–6,10] whereas the preparation of compounds **3–6** will be reported in due course. All solutions were prepared using highly purified water (Waters Milli-Q, Watford, UK), and filtered through 0.45-μm membranes prior to analysis.

3. Results and discussion

The chemical structure of the analytes taken into account is shown in Table 1. They contain an isoxazole, isoxazoline or isoxazolidine ring substituted at different positions by one or two aromatic rings. A common characteristic of this set of compounds is the presence of a quaternary ammonium group in position 5 of the heterocyclic ring which confers to the different molecules a positive charge in the entire pH interval. The separation of the enantiomers of muscarinic compounds by chiral HPLC is hindered by the presence in the molecule of the quaternary ammonium group which gives rise to a strong ionic interaction with the chiral matrix. Such an interaction accounts for almost the entire free energy of activation of the complex analyte–matrix rendering marginal the $\Delta\Delta G^{\neq}$ among the two enantiomers. On the other hand, the ionic character of these derivatives makes them suitable for separation by capillary zone electrophoresis.

β- and γ-Cyclodextrins are the most widely used chiral selectors in capillary electrophoresis ([8] and references therein). The inclusion of a compound, or at least part of it, into the cyclodextrin cavity is crucial for the formation of the complex and determines the choice of the proper cyclodextrin. In our study, the separation conditions were optimized by

Table 1
Chemical structure of muscarone and muscarinic antagonists

Structure	Name
1	*cis*-5-methyl-2-[(dimethylamino)methyl]-tetrahydrofuran-3-one methiodide salt
2	2-phenyl-5-[(dimethylamino)methyl]-isoxazolidin-3-one-methiodide salt
3	3-(1-hydroxybenzyl)-5-[(dimethylamino)methyl]-isoxazole methiodide salt
4	2-benzyl-5-[(dimethylamino)methyl]-isoxazolidin-3-one-methiodide salt
5	3-(1-hydroxy-1-cyclohexylbenzyl)-5-[(dimethylamino)methyl]-isoxazole methiodide salt
6	3-(1-hydroxy-1-phenylbenzyl)-5-[(dimethylamino)methyl]-Δ^2-isoxazoline methiodide salt

selecting the cyclodextrin with features appropriate to form inclusion complexes with the muscarinic antagonists under investigation. A preliminary examination of the results made it possible to correlate the size of the isoxazole, isoxazoline or isoxazolidine substituents at position 2 or 3 with the number of glucose units forming the cyclodextrin cavity. Compounds **2** and **3** (Table 1) could be separated using β-cyclodextrin in the background electrolyte, as shown in Fig. 1. The separation of compounds **5** and

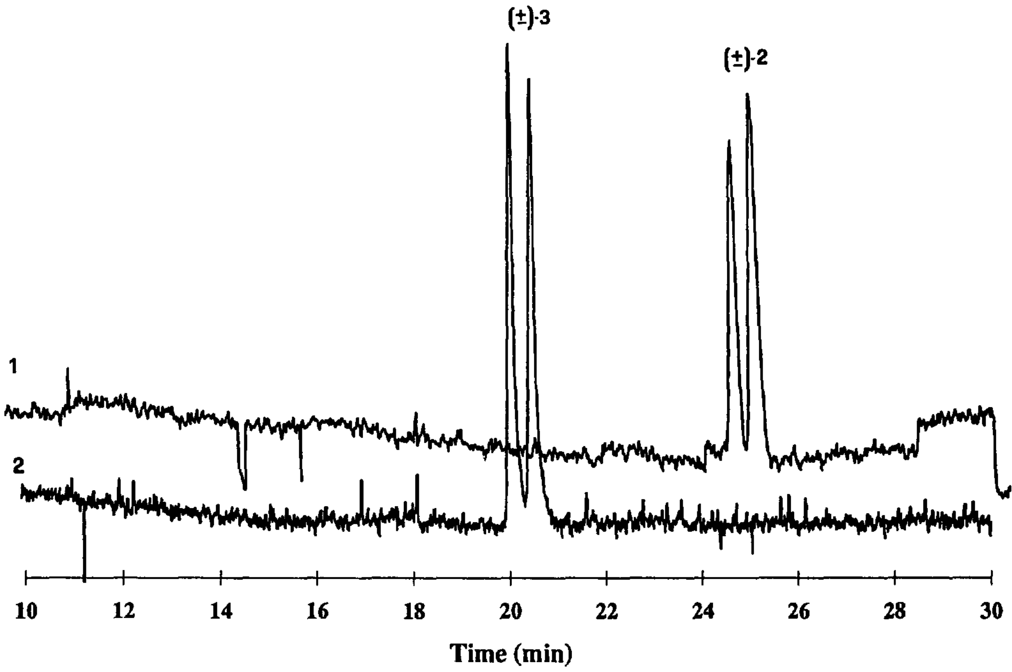

Fig. 1. CZE separation of racemates **2** and **3** in 25 m*M* Tris–acetate buffer (pH 4.2) containing β-cyclodextrin. Trace 1: compound **2**, 25 m*M* β-cyclodextrin. Trace 2: compound **3**, 10 m*M* β-cyclodextrin. Conditions: capillary, coated, 100 μm I.D., 67 cm long (59 to the detector); injection, electrokinetic mode (1 s, 20 kV); sample, 0.7 m*M* in water; applied potential, 20 kV; detector UV, 214 nm. The separation was carried out at 25°C.

6, as depicted in Fig. 2, required the use of γ-cyclodextrin which, having eight glucose units, presents a truncated cavity of increased size.

A rough guide for the choice of the appropriate cyclodextrin would suggest that β-cyclodextrin can be successfully employed in the separation of compounds with an aromatic ring, whereas γ-cyclodextrin is appropriate in the separation of enantiomers containing two aromatic rings. However, γ-cyclodextrin was the suitable selector for the separation of compound **4** (Fig. 2), which indicates that the size of the substituent is not the only parameter that must be taken into account. As an additional proof of the absence of general rules in explaining the nature of the interaction between cyclodextrin and analytes, none of the cyclodextrin was found to be able to resolve compound **1**.

3.1. Cyclodextrin concentration

The second step of the present investigation was to evaluate the resolution of each muscarine antagonist as a function of the appropriate cyclodextrin concentration. Fig. 3 and Fig. 4 show the variation of resolution at different cyclodextrin concentrations for compounds **2** and **3**. It can be seen that each compound presents a maximum in resolution at different cyclodextrin concentrations. According to theory, the difference in mobility between two enantiomers will reach a maximum at a particular selector concentration which depends on the affinity of the enantiomers for the selector [11–13].

In general, the enantiomers which are more retarded by the addition of the selector require a lower cyclodextrin concentration to be resolved as a result of their high affinity for the cyclodextrin. As can be seen from Fig. 3, the resolution of racemate **2** reaches a plateau for cyclodextrin concentrations higher than 5 m*M*, whereas compound **3** (Fig. 4) which is less retarded by the addition of cyclodextrin in the buffer, shows a gradual increase in the resolution up to a concentration of 15 m*M*. A further increase in the amount of cyclodextrin dramatically diminishes the enantiodiscrimination. The transit

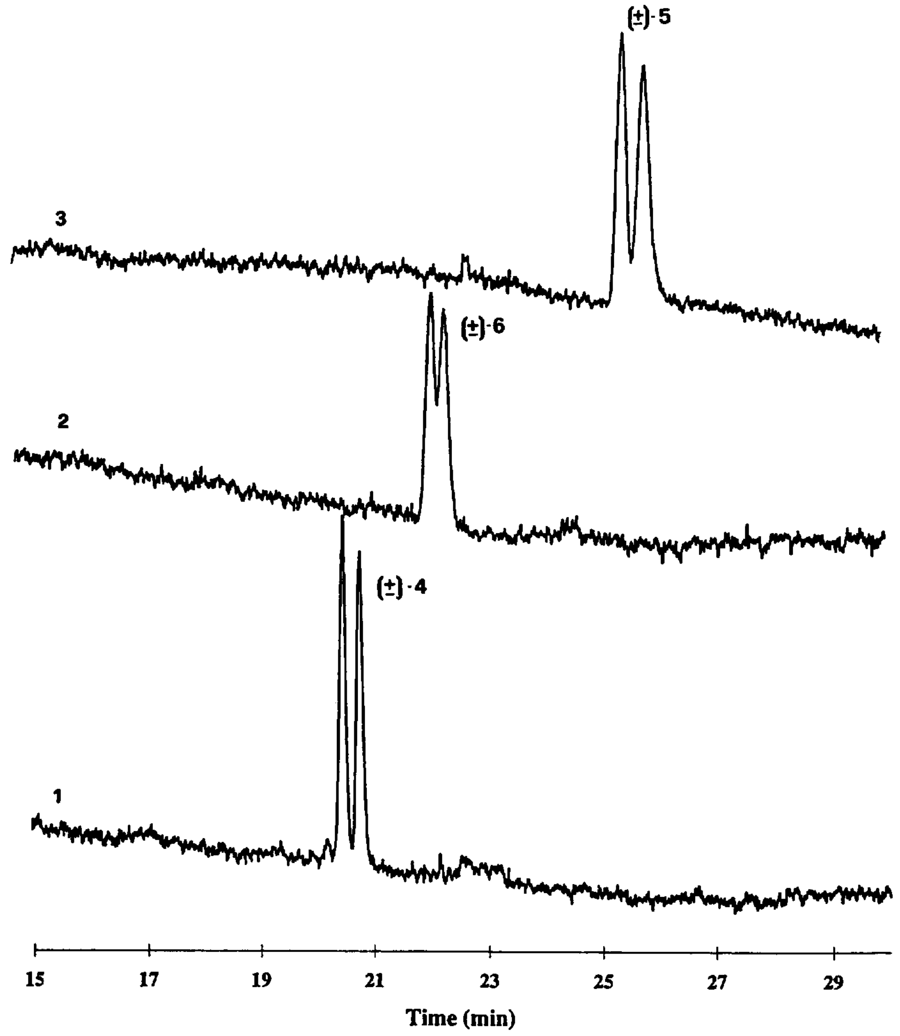

Fig. 2. CZE separation of racemates **4**, **5** and **6** in 25 m*M* Tris–acetate buffer (pH 4.2) containing γ-cyclodextrin. Trace 1: compound **4**, 30 m*M* γ-cyclodextrin. Trace 2: compound **6**, 20 m*M* cyclodextrin and 20% methanol. Trace 3: compound **5**, 15 m*M* γ-cyclodextrin and 20% methanol. Conditions: capillary, coated, 100 μm I.D., 67 cm long (59 to the detector); injection, electrokinetic mode (1 s, 20 kV); sample, 0.7 m*M* in water; applied potential, 30 kV. Other conditions as in Fig. 1.

time of both racemates, in the absence of cyclodextrin, was 12 min. The two compounds **5** and **6** containing a bulky substituent are not base-line resolved and thus, in an attempt to increase their separability we added 20% of methanol to the BGE (background electrolyte). In general, the role of organic solvents is to decrease the equilibrium constants and thus the affinity of the enantiomers for the cyclodextrin [14]. In this case, the addition of methanol only slightly influenced the resolution, possibly by modulating the hydrophobic interactions between the more apolar analytes and the cavity of the selector.

3.2. Effect of the silica wall on the separation

Due to the presence of a quaternary ammonium function, the analyte molecules are positively charged at any operative pH and therefore their complexation is insensitive to pH changes. On the other hand, the cationic group leads to electrostatic interactions with the silica wall surface. For this

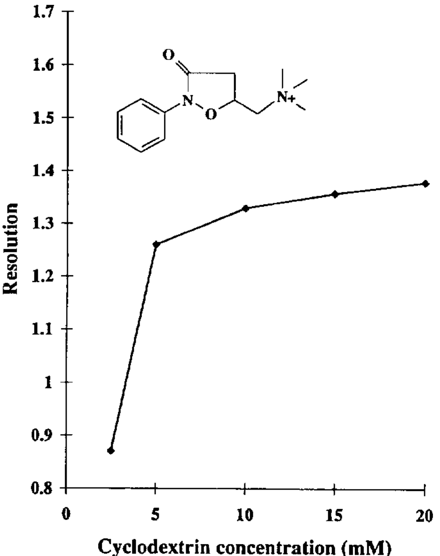

Fig. 3. Influence of β-cyclodextrin concentration on chiral resolution of compound **2**. Buffer: 2.5–20 m*M* β-cyclodextrin in 25 m*M* Tris–acetate pH 4.2. Other electrophoretic conditions as described in Fig. 1.

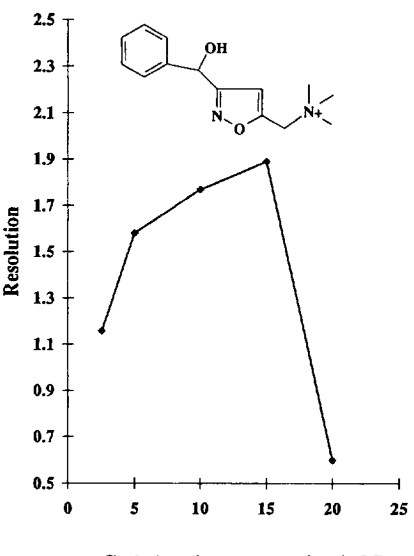

Fig. 4. Influence of β-cyclodextrin concentration on chiral resolution of compound **3**. Buffer: 2.5–20 m*M* β-cyclodextrin in 25 m*M* Tris–acetate pH 4.2. Other electrophoretic conditions as described in Fig. 1.

reason, in order to enhance the separation efficiency, we have employed capillaries coated with a linear polymer based on AAEE, a recently described highly hydrophilic monomer [9]. When the separations of the same analytes were performed in uncoated silica capillaries, using BGE of the same composition, a less efficient separation was achieved and the resolution was lower for all the samples considered. Coated capillaries of 100 and 50 μm were used in the present investigation and, generally, larger capillaries led to an increase in the sensitivity and a considerable improvement of the trace peak detectability. The separation of the examined muscarine antagonists by capillary zone electrophoresis requires an accurate optimization of the conditions since the discriminating power of cyclodextrin is not very high. The resolution, in particular, was remarkably influenced by the amount of sample injected and for this reason, the use of 100-μm capillaries was found to provide a better signal-to-noise ratio for peaks present only as a trace in the sample. A significant influence of the capillary size was found when uncoated capillaries were used in the separation. In this case, only capillaries having an internal diameter of 50 μm showed the ability to separate enantiomers, but the resolution and the efficiency of the separations were very poor while the high baseline noise rendered unreliable any quantitative evaluation of minor components. Since the analytes are driven toward the detector by the electroosmotic flow (EOF), the analysis time becomes shorter in uncoated capillaries thus eluting the enantiomers from the column before the resolution is complete. In addition, since the reproducibility of the migration time is necessary to confirm peak identity, the use of a coated capillary is of utmost importance. The EOF control is known to be critical as the influence of electroosmotic transport on analyte migration often leads to a run-to-run variation in the transit times higher than 2%, which is the highest value still suitable to confirm peak identity.

An excellent precision was demonstrated for separations occurring in the absence of EOF with a R.S.D.% lower than 0.05% for 20 consecutive injections.

3.3. Quantitative aspects

The main goal of this work was the development of a method that enables the direct determination of enantiomeric excesses of muscarine antagonists. Fig. 5 shows the chromatopherograms of the *R*- and

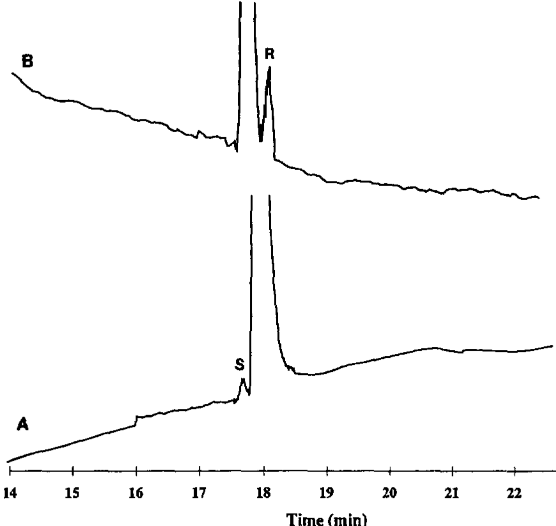

Fig. 5. CZE analysis of enantiomers *R* (A) and *S* (B) of compound **2**, contaminated with the *S* and *R* form respectively. Capillary length 58 cm, 50 to the window; other conditions as in Fig. 1.

S-forms of compound **2** contaminated by a small amount of *S*- and *R*-enantiomer respectively. The *S*-(+)-enantiomer of compound **2** was found to be contaminated by 5% (±0.5, n=6) of undesired enantiomer, whereas the *R*-(−) contained 1% (±0.3, n=6) of the *S*-(+)-form. Detection levels of <1% have been reported by several authors in capillary electrophoresis [15–18]. In capillary electrophoresis, injection precision is in general poorer that in HPLC, due to the difficulties associated with the reproducible injection of nl volumes into the capillaries. In many quantitative reports, the imprecision problem is overcome by the use of internal standards. When enantiomeric mixtures are analyzed with the aim of determining their optical purity, a good precision can be achieved as one of the two enantiomers acts as an internal standard for the other [19]. A common way to express the composition of enantiomeric mixtures in capillary electrophoresis is to give the % area/area levels for the undesired enantiomer. The area must be corrected for the time migration of the two analytes as in the absence of such a correction the analyte with low mobility would be overestimated. In order to demonstrate the accuracy of the method that allows assessment of the concentration of the undesired enantiomer, we have injected a single enantiomer spiked with known levels of its stereoisomer. The peak-area ratios obtained by capillary electrophoresis quantitatively confirmed the spiking levels which were between 1 and 20% (linearity 0.9913). These experiments also confirmed the recovery.

4. Conclusions

In summary, this paper reports a new application of the capillary electrophoresis methodology to the resolution of chiral quaternary ammonium salts provided with antimuscarinic activity. In this case capillary electrophoresis gave excellent results where other analytical methods could not be applied or gave no results. The analysis of the enantiomers of compound **2** confirmed the results previously inferred on the basis of data collected on precursors.

The possibility of evaluating the exact enantiomeric composition directly on the final derivatives is of utmost importance in medicinal chemistry due to the great difference in the pharmacological profile among the enantiomers in terms of potency, toxicity, organ-selectivity and metabolic pathways. This method will be further explored in different areas of medicinal chemistry and the results will be reported in due course.

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