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Resolution of the enantiomers of oxamniquine by capillary electrophoresis and high-performance liquid chromatography with cyclodextrins and heparin as chiral selectors

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Abstract

The methods of separation of the enantiomers of the chiral drug oxamniquine are compared, between HPLC with either cyclodextrins and their related derivatives as chiral selectors in the mobile phase or immobilised in a chiral stationary phase (as Cyclobond I and II) and between capillary zone electrophoresis (CZE) where the cyclodextrins are added to the buffer solution. The HPLC experiments, which included structured method optimisation were largely unsuccessful in resolving the enantiomers, with the exception of when a Chiral-AGP protein stationary phase was introduced into the programme. However although this chiral stationary phase provided baseline resolution of the enantiomers the stability of the method was suspect to small changes in the pH (0.2 units). In contrast the CZE method developed for both cyclodextrins and their derivatives gave good resolution of the enantiomers and method stability (R.S.D. < 1%, $n = 10$ on precision). The basis of the interaction mechanism between selector and selectand was shown as a 1:2 relationship of cyclodextrin to analyte by NMR. In addition the polysaccharide, heparin was investigated as a chiral additive and excellent resolution of the oxamniquine was achieved with 3 m*M* heparin in 50 m*M* sodium dihydrogenphosphate (pH 3.0) as buffer in CZE, which also gave a stable procedure. This method allowed the detection of each of the enantiomers in the presence of the other down to 0.23% (m/m). The overall composition of the heparin material from different sources can however be slightly variable and this can result in small differences in resolution capability.

1. Introduction

Capillary electrophoresis (CE) utilised in a free solution or micellar electrokinetic mode with a chiral selector introduced into the run buffer, has been shown to be particularly useful for the resolution of the enantiomers of chiral drugs [1]. Currently the most powerful method is through the use of cyclodextrins and their related derivatives as selectors in either CE mode, although the introduction of proteins [2] and crown ethers has been considered [3]. By this former mode, a wide range of chiral drugs have been separated for assay in pharmaceutical preparations and biological fluids often with resolution or improved resolution over the cognate procedure with mobile phase additives or the requisite chiral stationary phase in HPLC. This contrast in resolution between CE incorporating cyclic oligosaccharides and HPLC, is considered to be due to the many different contributions to the stability equilibrium and includes differences in the effects from the operational variables on the selector-selectand binding. Although the

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primary mechanisms of interaction may involve: inclusion within the cyclodextrin cavity relative to the hydrophobicity, size and shape of the analyte, multiple hydrogen bonding and other attractive and repulsive interactions which are similar in both cases. In a number of examples, however, it has been shown with these techniques and particularly in CE, that the interactions are not always concentrated around the inclusion into the cyclodextrin cavity, but it is possible that multiple complexation or the formation of diamers or triamers of either the selector or selectand in situ may complex more predominantly through interactions such as hydrogen bonding, and other attractive and repulsive effects. The accumulation of these parameters therefore combines to provide enantioselective equilibria differences in CE over those cognate separations in HPLC which are often manifest as improved resolution. In pharmaceutical analysis it is also important in method development, to consider the operational improvements of CE relative to HPLC, such as the ease of method development and optimisation through the experimental programme [4] together with generally better peak efficiency, which all go together to raise CE firmly alongside HPLC as a suitable method for examination of chiral drugs.

Both the conventional α-, β- and γ-cyclodextrins and the relatively large number of mainly alkylated derivatised cyclodextrins, which now include neutral and charged materials [5] were initially used where solubility limits in cyclodextrin concentration precluded the attainment of an optimised enantiomer resolution. However it was quickly shown that these alkylated derivatives gave the capability of achieving differences in resolution over the original cyclodextrin materials [6]. This is particularly when an extended structural addition is made to the β-cyclodextrin, such as in the formation of hydroxypropyl- and hydroxyethyl-β-cyclodextrin. In addition with the derivatives, the degree of substitution of the secondary hydroxyls (known as the RS value) on the cyclodextrin cavity can lead to marked differences in the observed enantiomeric resolutions as shown by Valko et al. [7].

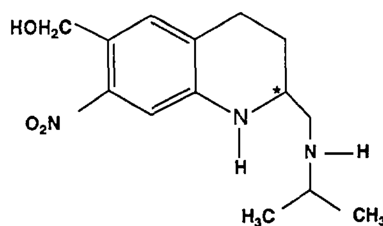

Fig. 1. Structure of oxamniquine.

In this study it was proposed to examine methods by HPLC and CE for resolution of the antischistosmiasis drug oxamniquine (Fig. 1), which is marketed in Africa and Brazil as a racemic mixture in the pharmaceutical preparation. In the case of developed HPLC methods, questions would be asked about the ruggedness of these methods for assay of the drug. Comparisons would then be made between HPLC and CE when conventional cyclodextrins and their derivatives were included within the electrolyte solution. In optimisation of the methods further, consideration would be given to the nature of the chiral selector and its ability to give a stable robust procedure for assay of the drug as a racemic mixture and as the enantiopure component. For this extension, the linear polysaccharide heparin (Fig. 2) [8] would be examined and its performance against the cyclodextrins assessed.

2. Experimental

2.1. Materials

β-Cyclodextrin was obtained from Sigma (St. Louis, MO, USA) and heparin from Sigma and Lancaster Synthesis (Morecambe, UK) and hydroxypropyl-β-cyclodextrin from Aldrich (Steinheim, Germany). Buffer materials and other general chemicals were purchased from either BDH (Poole, UK) or Hopkin and Williams (Chadwell Heath, UK).

Oxamniquine and its individual enantiomers

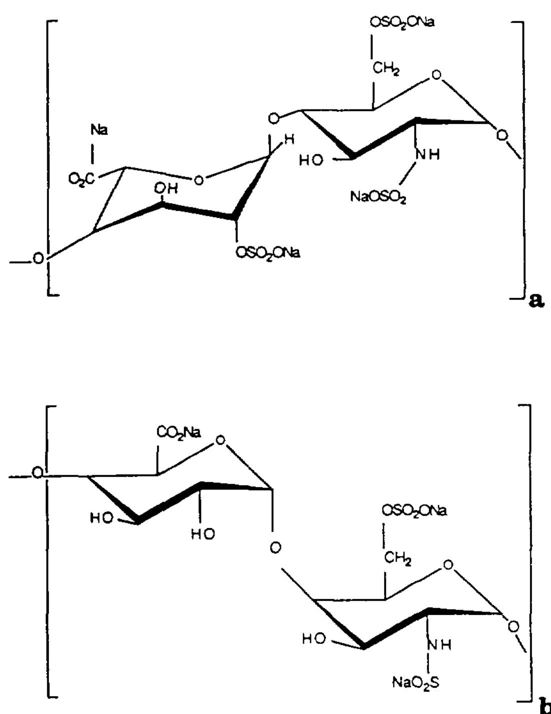

Fig. 2. Structure of heparin, showing the two forms which can vary in their proportions in heparin samples from different sources [8]. The composition of a + b is in the range 8–15 molecules in samples from different sources.

were kindly supplied by Pfizer (Pfizer Central Research, Sandwich, UK). For initial method development experiments racemic oxamniquine and the individual enantiomers were prepared at about 60 μg/ml in methanol–water (60:40, v/v) with each sample solution filtered through a 0.22-μm filter before use.

2.2. Apparatus

HPLC system

This consisted of a ConstaMetric 3000 dual reciprocating pump (LDC, Riviera Beach, FL, USA) and a Pye Unicam PU 4020 variable-wavelength UV detector (wavelength 246 nm) (ATI-Unicam, Cambridge, UK) with data manipulation through a Hewlett-Packard HP3394A computing integrator (Waldbronn, Germany). The sample was introduced through a Rheodyne 7125 injection valve, fitted with a 20-μl loop.

For mobile phase additive experiments the achiral stationary phase was either 5-μm C_{18}-Hypersil or SAS-Hypersil packed onto a 100 × 4.6 mm stainless-steel column (Shandon Scientific, Runcorn, UK).

For experiments with chiral stationary phases 100 × 4.6 mm Cyclobond I and II cyclodextrin phases (Astec, Whippany, NJ, USA) and a 100 × 4.6 mm Chiral-AGP column packing material was examined (ChromTech, Norsborg, Stockholm, Sweden). A range of mobile phases were used of variable composition, but the optimum for the assays on the Chiral-AGP column packing was: propan-2-ol–10 m*M* sodium dihydrogenphospate + 0.1 *M* sodium chloride pH 5.85 (99.5:0.5, v/v). All mobile phases were filtered through a 0.45-μm Durapore filter (Millipore, Molsheim, France) and degassed by ultrasonication under reduced pressure prior to use.

Capillary electrophoresis system

The instrument was an Applied Biosystems Model 270A (Warrington, UK) and the data was manipulated on a Model HP3396A integrator.

The uncoated fused-silica capillary was 710 × 0.05 mm I.D. (500 mm to the detector) (Chrompack, London, UK). Again a range of buffer compositions were used. But the injection time of 2 s (7 nl, hydrodynamic vacuum injection at the anode), temperature 30°C, applied voltage 15 or 20 kV and detection wavelength 246 nm were kept constant throughout the development experiments.

In order to establish reproducible, robust results, the capillary was conditioned and rinsed in a set procedure at the beginning and end of each day. For the initial capillary conditioning, a new capillary was washed for 2 h with 1 *M* sodium hydroxide, 1 h with double distilled water and then 30 min with run buffer (all solutions were initially filtered through a 0.22-μm filter). Each day the capillary was washed for 40 min with 1 *M* sodium hydroxide, water for 15 min prior to use and between runs with 0.1 *M* sodium hydroxide (2 min) and run buffer (3 min).

3. Results and discussion

The initial HPLC experiments involved β-cyclodextrin (5 mM) or hydroxypropyl-β-cyclodextrin (20 mM) introduced into the mobile phase on the C_{18} or SAS-Hypersil achiral stationary phase and the use of a Cyclobond I or II chiral stationary phase with a mobile phase of acetonitrile–water. Stepwise optimisation of the mobile phase was attempted in each case, but no appreciable resolution of the chiral oxamniquine drug was achieved with either of the chiral selector additives or the chiral stationary phases. This lack of success was slightly unexpected as the structure of oxamniquine suggests inclusion of the aromatic moiety (with the possibility of the nitro and alcohol groups passing through the hydrophobic cavity) and the likelihood of N–H hydrogen bonding. However, the retention time data for oxamniquine in the presence of the chiral selector was different from that in the same mobile phase without the cyclodextrin, indicating selector interaction but similarity in equilibrium constant binding and/or the rate of migration is faster than the achievement of differences in binding equilibrium [9].

As an extension to the HPLC work further examination of the oxamniquine structure indicated that the cyclic ring nitrogen and hydrogen bonding groups could show favourable interactions on a protein based chiral stationary phase and a number of experiments were carried out to examine the best conditions for separation. The range of recommended operating parameters for the Chiral-AGP stationary phase are slightly limited but a suitable range of mobile phase conditions was found for examination through a modified simplex, structured optimisation procedure. The starting conditions were: pH 4.0–7.0 and propan-2-ol concentration of 0.2–1.4%(v/v) with a 10 mM sodium dihydrogenphosphate buffer. After 12 experiments the global optimum was reached where the mobile phase was: propan-2-ol–10 mM sodium dihydrogenphosphate (0.5:99.5, v/v) (pH 5.85), although the chromatographic peak shape was slightly broad. But the addition of 0.1 M sodium chloride caused a sharpening of the peaks. However when this simplex was viewed as the three-dimensional response surface, it was clear that small changes in the pH would cause large differences in the enantiomeric resolution (Fig. 3). This would result in a very unstable operating basis on which to carry out regular assays on racemic oxamniquine as the bulk drug and the tablet pharmaceutical preparation [10] (differences as small as 0.2 pH units led to relatively large losses in resolution) and therefore although baseline resolution of the racemic oxamniquine was achieved the lack of ruggedness in the assay suggested further study was necessary.

The picture was completely different when CE

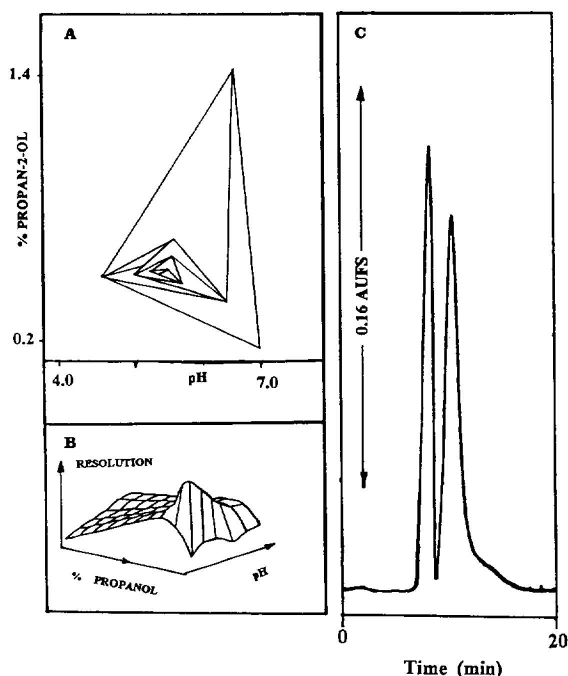

Fig. 3. Basis of the optimisation experiments for the resolution of oxamniquine (60 mg/ml) on the Chiral-AGP stationary phase. (A) Optimisation experiments based on the modified simplex procedure using a two-variable design; (B) three-dimensional response surface from the simplex procedure, indicating the steepness of the pH and propan-2-ol responses; (C) optimum chromatographic separation of the oxamniquine enantiomers from the structured optimum procedure. The conditions were: 100 × 4.6 mm stainless-steel column packed with 5-μm Chiral-AGP, the optimised mobile phase was propan-2-ol–10 mM NaH_2PO_4 + 0.1 M NaCl (0.5:99.5, v/v), pH 5.85. Flow-rate was 1.0 ml/min and the detection wavelength 246 nm.

was used in the buffer/chiral selector additive mode of capillary zone electrophoresis (CZE) with β-cyclodextrin and quite rapidly good resolution of the oxamniquine enantiomers was achieved. From this further fine tuning of the enantiomer separation was carried out along with a pH study to check method stability. These experiments gave the enantioseparation shown in Fig. 4, where the electrolyte consisted of 50 m*M* disodium hydrogenphosphate (pH 12.0) containing 25 m*M* β-cyclodextrin.

In order to describe the chiral resolution in free solution CE a number of theoretical models have been suggested. These have attempted to take into account the effects of diffusion band broadening [11], electroosmotic mobility and equilibration selector–analyte binding [12,13]. In addition the operating conditions of applied potential, electrolyte pH [14] and organic buffer additive [15] have been included in these ideal models. The importance of cyclodextrin concentration was discussed by Wren and Rowe [16] and the resolution given here followed their theoretical work with an optimum concentration reached in terms of resolution and effective reduced band broadening at 25 m*M* β-cyclodextrin (a limit of 30 m*M* β-cyclodextrin was reached before solubility was problematic). It is possible, by taking into account all these influences to suggest that the differences in observed resolution in CE over HPLC are linked directly to the many different factors influencing the electrophoretic mobility difference. As regards the sites of molecular interaction between the cyclodextrin and the oxamniquine it has been shown in early NMR studies that a 1:2 relationship of cyclodextrin to analyte is occurring and this aspect is currently being more fully studied along with the examination of the competitive binding equilibria for the HPLC and CE data. Through the 1:2 molecular interactions it is possible that the oxamniquine diamer comes closely into contact with the secondary hydroxyls on the rim of the cyclodextrin cavity and because of steric effects the higher competition factor in the selector–enantiomer binding leads to a difference in the enantiomeric mobility.

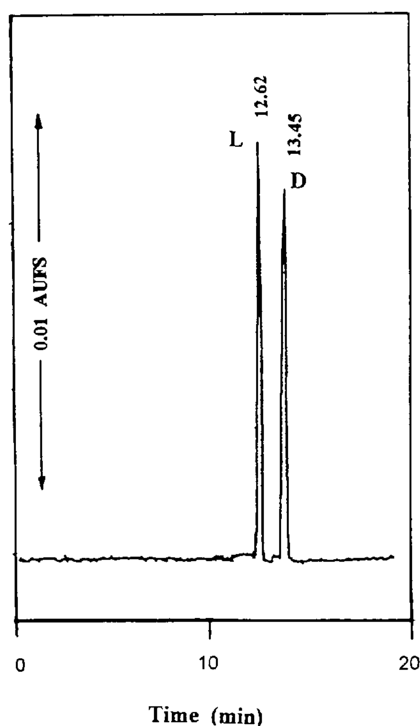

Fig. 4. Resolution of the enantiomers of oxamniquine by capillary zone electrophoresis with β-cyclodextrin in the electrolyte. The operating conditions were, applied voltage of 15 kV, temperature 30°C, wavelength 246 nm and buffer, 50 m*M* disodium hydrogenphosphate containing 25 m*M* β-cyclodextrin pH 12.0.

In examining the resolution of the oxamniquine enantiomers it is interesting to note that the migration order differs between the β-cyclodextrin and the hydroxypropyl-β-cyclodextrin (RS = 0.9), which may be a function of the differential interaction due to the alkyl spacer on the extended hydroxypropyl arm, over the conventional β-cyclodextrin.

Method stability in both these cases was very good as demonstrated in Table 1, where migration times and peak height (peak areas) gave values of R.S.D. $< 1\%$. This was also the position on examining method ruggedness on a day to day basis. In this case stability under varying buffer pH changes was much better (changes of ± 1 pH unit gave only small changes in resolution between the enantiomers) than in HPLC with the protein chiral stationary phases.

These data suggest that the method would be

Table 1
Repeatability of migration times and peak height values for oxamniquine (60 μg/ml, $n = 10$) by CZE with β-cyclodextrin (25 mM) in the buffer solution

	Migration time (min)		Peak height	
	Peak I	Peak II	Peak I (L)	Peak II (D)
Mean	12.49	13.34	28156	21505
S.D.	0.116	0.106	430.91	304.14
R.S.D. (%)	0.93	0.80	0.97 (1.53)[a]	0.73 (1.41)[a]

For experimental details: see text.
[a] Values of peak height are normalised and the untreated values are given in parentheses.

suitable for determination of the individual enantiomers in the bulk drug and the pharmaceutical tablet preparation (Vansil, Pfizer), where the drug is present in the racemic form and this is currently being completed.

In conjunction with these method development CE experiments using cyclodextrins, the chiral selector has also been considered and polysaccharides have been examined. Of these heparin, a mucopolysaccharide (extracted from bovine lung tissue and intestinal mucosa of pigs and cattle), was shown to be particularly successful in the resolution of the enantiomers of oxamniquine.

This polysaccharide (Fig. 2) is a linear sugar molecule, but with some degree of helical structure around the α-1,4 links within the molecule and as it has a relatively large number of sulphate groups present then the molecule has a strong anionic charge. Previously, the resolution of a small number of chiral chemicals has been achieved [17], but very little consideration has been given to the separation or chiral drugs [18].

In this case the oxamniquine was resolved with a very large resolution factor, in a buffer of 50 mM sodium dihydrogenphosphate (pH 3.0) containing 3 mM heparin (Fig. 5A). As with the cyclodextrin assays the method gave very stable results (method repeatability around a R.S.D. of 1%, $n = 10$) under small changes in buffer composition, and therefore it is also suggested that the procedure could be used for assay of oxamniquine in bulk drug and pharmaceutical preparation. There is a slight drawback, however, to the regular use of heparin in chiral drug assays, in that the material is heterogeneous and thus heparin from different sources can give small changes in resolution due to the variable composition of the disaccharide and trisaccharide residues. This is illustrated in the material examined in this programme from different biochemical suppliers, where the effects of variable chain length and composition have been shown to provide a difference in the degree of resolution experienced with oxamniquine (Fig. 5B).

Nevertheless it was possible to show that if oxamniquine was to be marketed as either the pure D- or L- enantiomer for related substance analysis or if a check on chiral inversion was required after pharmaceutical formulation, they could be detected in the presence of one enantiomer in the other, down to 0.23% (m/m), although for reproducible results one source of heparin is recommended for use in the electrolyte solution.

In all these experiments to obtain acceptable method validation values, it was important to condition and regularly wash the fused-silica capillary at start up, between runs and at the end of the day.

4. Conclusions

This particular example of resolution of oxamniquine with cyclodextrins demonstrates the usefulness of CE as a tool to resolve chiral drugs and illustrates its advantages over HPLC in this case. The findings in this programme are backed up generally in the literature, where it is clear

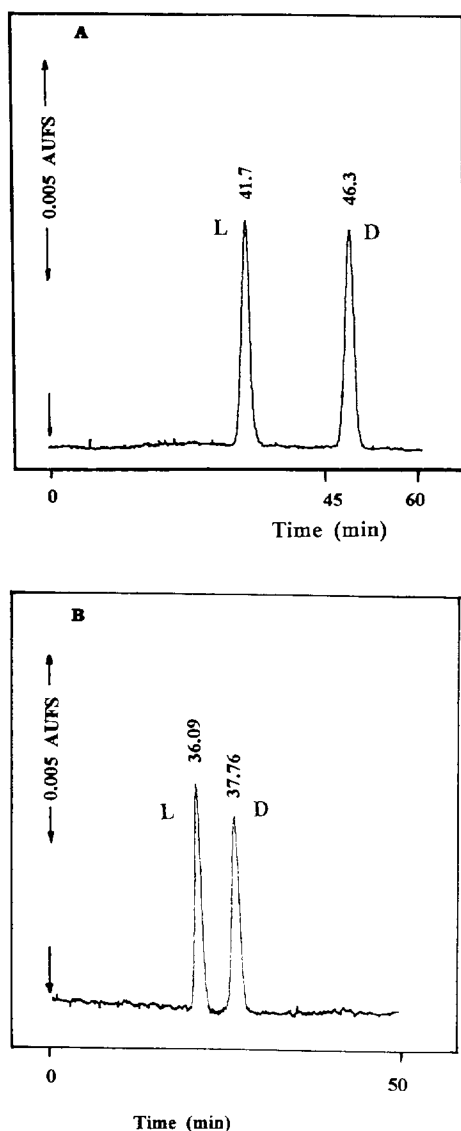

Fig. 5. Resolution of the enantiomers of oxamniquine by capillary zone electrophoresis with heparin in the electrolyte solution. (A) Resolution achieved with an electrolyte solution of 50 mM sodium dihydrogenphosphate (pH 3.0) + 3 mM heparin (from Sigma), an applied voltage of 20 kV, temperature 30°C and detection wavelength of 246 nm. (B) Resolution under the same conditions, but with 3 mM heparin from Lancaster Synthesis.

that in many instances the analyst has found that CE in capillary zone and micellar mode with chiral selectors added to the buffer solution, gives better resolution and more rapid method development over HPLC. The method was shown to be stable and robust over a period and could be applied to the assay in a pharmaceutical preparation. This was also the case with the method developed around heparin which as a newly suggested additive for chiral separation of drugs, gives very impressive resolution of oxamniquine. However the analyst would have to be careful with use of the additive on a regular basis, unless the variation in the composition of the heparin from different sources, which leads to differences in resolution of the chiral compound of interest, could be resolved.

Acknowledgement

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