Micellar Electrokinetic Capillary Chromatography of 1,4-Benzodiazepine Derivates and their Degradation Products

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The aim of this work is to elaborate a fast and reliable capillary electrophoresis method, for the separation of 1,4-benzodiazepine (BZD) derivates and their degradation products in acidic medium and in UV light. Because BZDs are neutral, hydrophobic compounds, the most efficient method proved to be micellar elektrokinetic capillary chromatography (MECC). For the separation we used a sodium tetraborate buffer with tensioactive agent (sodium dodecyl sulphate - SDS) and an organic modifier (methanol). We also optimized the analytical conditions, and studied the effects of the SDS and methanol concentrations and buffer pH on the determination efficiency.

Keywords: 1,4-benzodiazepines derivates, micellar elektrokinetic capillary chromatography, degradation products, separation

BZD have become the most commonly used psychotropic drugs and are widely prescribed throughout the world. The 1,4-benzodiazepines (BZD) derivates are known primarily for their anxiolytics effects, but are also used as sedatives, hypnotics, anticonvulsants and muscle relaxants [1].

To emphasize the importance of these compounds we can mention the fact that in the European Pharmacopoeia 5th edition (EPh 5) [2] are officinal a number of 15 BZD, while in the Romanian Pharmacopoeia (FR X) [3] are officinal 3 compounds (*chlorodiazepoxide, diazepam, nitrazepam*).

Taking in consideration the great therapeutically importance of these drugs, elaboration of new methods of analysis is a permanent necessity. A number of methods for the analysis of these drugs have been reported, including thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) [2,3]. Capillary electrophoresis (CE), an officinal method in Eph 5 [2], proved to be an alternative or complementary technique for the chromatographic separations. Due to its speed of analysis, high efficiency and low solvent and sample consumption, this technique has gained momentum in pharmaceutical research laboratories [4,5].

CE is based on the migration of charged particles dissolved or spread in an electrolyte solution and subdued to the action of an electrical field. BZDs are hydrophobic, neutral from electrophoretic point of view substances, with low pK_a values (ca. 1.3-5), so they are difficult to ionize. This means that a good separation by capillary zone electrophoresis (CZE), which relies on differences in electrophoretic mobility of analytes cannot be expected, because migration towards the detector will probably occur with the same velocity as the electroosmotic flow (EOF). Our previous studies showed that the best electrophoretic method for their separation is micellar elektrokinetic capillary chromatography (MECC), which extends the applicability of EC to neutral substances [6].

MECC, introduced by Terabe et al. is based on a micellar "pseudostationary" phase added to the buffer, which interacts with the solutes according to partitioning mechanisms, in a chromatography-like mode. In this system, EOF acts as the chromatographic "mobile phase". The MECC "pseudostationary" phase is composed of a surfactant added to the buffer above its critical micellar concentration (CMC) [7,8].

To separate neutral substances, like BZDs, it is necessary to extend the concept of using a mobile phase and a pseudostationary phase to the utilization of buffer modifiers such as organic solvents and cyclodextrins [8,9]. A few papers were published recently in leading journals, regarding the separation of 1,4-BZD derivates by means of MECC, using a tetraborate or phosphate buffer, SDS as surfactant and organic solvents as buffer additives (methanol, acetonitrile, isopropanol) [8, 10-14].

In the present work, the separation by MECC, of the 8 of the most frequently used 1,4-BZD (*alprazolam, bromazepam, chlordiazepoxide, diazepam, flunitrazepam, medazepam, nitrazepam and oxazepam*) was carried out, and the analytical conditions were optimized. An anionic surfactant (SDS) was used, with the buffer system also containing an organic modifier (methanol). Using the same method we also studied the degradation of BZD in acidic medium and UV light.

Experimental part

Materials

8 BZDs with different structures were used: *chlorodiazepoxide, diazepam, oxazepam* (Terapia, Romania), *alprazolam, bromazepam, flunitrazepam, medazepam, nitrazepam*(Labormed, Romania) (fig. 1). All the analyzed BZDs were pure.

Reagents of analytical grade were obtained from various distributors: methanol (Spektrum-3D, Hungary), sodium dodecyl sulfate (SDS) (Fluka), sodium tetraborate (Reanal, Hungary), sodium hydroxide solution 0.1 M (Fluka), concentrate hydrochloric acid (Reanal).

Instrumentation and conditions

All separations were performed on a Hewlett Packard 3D CE (Agilent, Germany) instrument. Separations were achieved using a fused-silica capillary of 64.5 cm total length 50 μm I.D. (effective length: 56 cm) (CS-Chromatographie, Germany). Each new capillary was rinsed with 0.1 M NaOH for 30 min., water for 5 min., and

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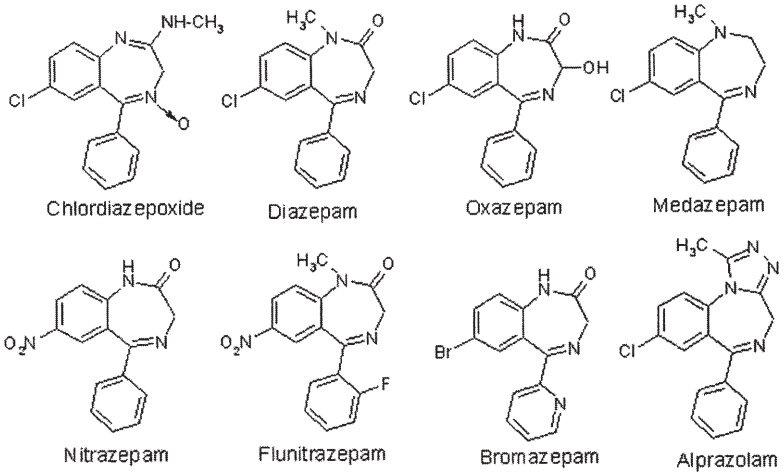

Fig. 1 The chemical structures of the eight studied BZD

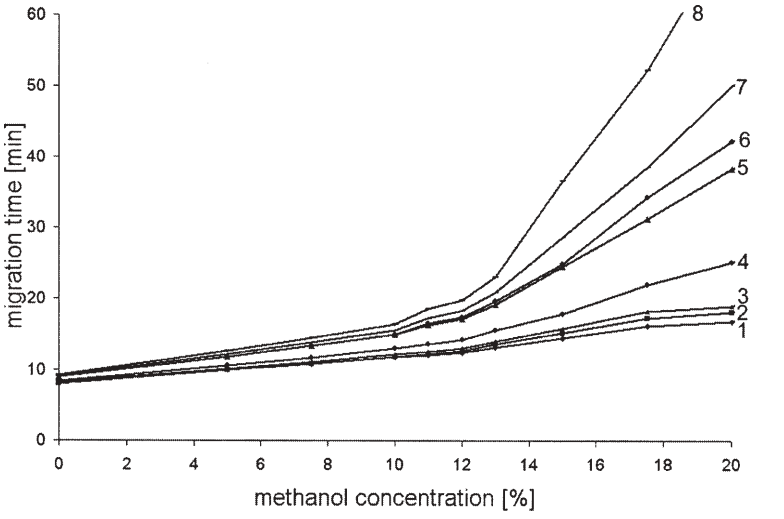

Fig. 2 Variation of migration time depending on the methanol concentration of the buffer (separation conditions: capillary 64.5 cm . 50 μm I.D., buffer: 25 mM borate, 50 mM SDS, pH: 9.3, voltage +25 kV, detection: UV absortion at 214 nm) (1: bromazepam; 2: nitrazepam; 3: flunitrazepam; 4: chlorodiazepoxide; 5: alprazolam; 6: oxazepam; 7: diazepam; 8: medazepam)

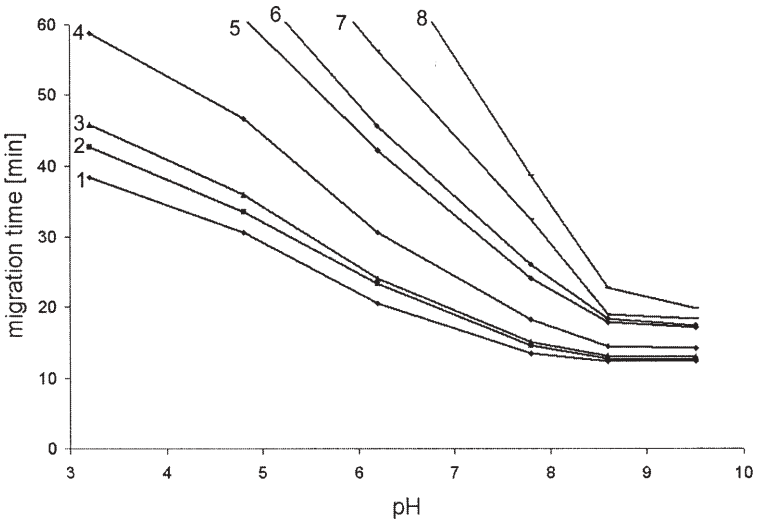

Fig. 3 Variation of the migration time depending on the *p*H of the buffer solution (The separation conditions were described in fig. 2)

buffer solution for 5 min. Between runs the capillary was flushed 5 min with buffer. In all measurements the hydrodynamic sample injections was used, by applying a precisely controlled vacuum of 50 mbar for 2 s. The cartridge temperature was kept at 20^{0}C with a run voltage of + 25 kV, the cathode being on the detector side. The UV detector was set at 214 nm. The individual compounds could be identified by taking their UV spectra. All experimental data were collected and integrated using the HP ChemStation software 7.01 version (Agilent).

Sample preparations

BZD are hydrophobic substances, therefore organic solvents such as methanol are necessary to make their sample solutions. The sample solutions (2 - 100 mg/L) were prepared by weighing the drug, then dissolving them in absolute methanol, diluting the solution with water (50:50) and storing them in the refrigerator at + 4 ˚C.

The running buffer consisted of 25 mM sodium tetraborate, 50 mM SDS (*p*H 9.5) and different concentrations of methanol (0, 5, 7.5, 10, 12, 15, 20, 30%). To prevent capillary blockage, prior to CE measurements, all the samples and buffers were filtered using a 0.45 μm syringe filter.

Results and discussion

Optimization of separation conditions

Our aim was not only to elaborate a rapid and simple procedure for simultaneous screening of eight 1,4-BZDs

using MECC, but also the optimization of the method. During the optimization the pH, the percent of organic modifier, SDS and sodium tetraborate concentration were varied.

The higher the concentrations of tetraborate ions, the later the migration times for each component, because the EOF will decrease with an increase of ionic strength [14].

The anionic SDS micelles are electrostatically attracted towards the anode, but, because of the prevalent velocity of the EOF, they slowly migrate towards the cathode, in the direction of the detector. Neutral analytes like BZD will partition between the micelle phase and buffer phase, increasing migration times [8,10,12]. The migration times increased with the increasing SDS concentration, due to the solubilization of the solutes into the micellar phase. It is usually a good practice to keep SDS concentration as low as possible to avoid problems with elevated current.

With the micelle alone, the structurally similar BZD co-eluted, because of their comparable hydrophobicity, so the eight BZD derivates have not distinguishable mobilities. This problem of the overlapping peaks is solved by the addition of an organic modifier (methanol). Methanol increases the viscosity of the buffer, reduces EOF, and also improves the solubility of drugs [11]. The migration times of the BZD increased with the increase in the methanol concentration (fig. 2). When methanol concentrations reach 30% or above, distortion of micelles and loss of current can occur.

It was established that lowering the pH increased migration time, but at a pH between 8-10, the migration times remained relatively constant (fig. 3).

Upon review of the data, the most favorable buffer was determined to be with 25mM tetraborate, 50 mM SDS at *p*H 9.5 with 12 % methanol. A baseline separation of the eight analytes was achieved in less than 20 min under the optimized conditions. The separation occurred in the following order: *bromazepam, nitrazepam, flunitrazepam, chlorodiazepoxide, alprazolam, oxazepam, diazepam and medazepam* (fig. 4).

The migration velocity of the analytes depends on the distribution coefficient between the micelle and non-micellar (aqueous) phase. The greater the percentage of analyte that is distributed into the micelle, the slower it migrates. The migration order could be explained taking into consideration the hydrophobicity, molecular mass and structure of each BZD.

Analytical performance

The proposed separation method was evaluated on the basis of precision (migration time and peak area), linearity, linear range and limit of detection (LOD) (table I, table 2). For all these measurements the optimized separation parameters were used.

BZD degradation

BZDs are relatively instable substances , because they easily hydrolyze in acidic solution and also decompose in UV light [15].

Hydrolisis of the benzodiazepine ring is one of the most frequently observed degradation routes for BZD leading generally to 2-aminibenzophenone derivates, through the split of the N_1-C_2 bond of the diazepinic ring (fig. 5).

Studying the hydrolysis concentrate hydrochloric acid was added to the stock solution of each analyte. Each sample solution was reinjected for several times over duration of 24 h. The analytical parameters were the same as for the separation.

During the stability studies an internal standard (cinnamic acid) was applied, which is stable in acidic solution, has a remarkable absorbance in UV at 214 nm, and also a shorter migration time than the BZDs. The peak areas of the components were referred to that of the inner standard to compensate the possible change in the measurements or evaporation of samples. Peak areas obtained for the first analysis were regarded as 100%.

The degradation rate for 24 hours increased in the order: *alprazolam, bromazepam, flunitrazepam, nitrazepam, chlordiazepoxide, diazepam, medazepam, oxazepam* (fig. 6). The rate of the decrease of the concentration was rather similar for BZD with related structures (*nitrazepam-flunitrazepam, diazepam-medazepam*).

BZD photostability constitutes an important subject of investigation because the photodegradation can result in a loss of potency of the drug and also in adverse effects due to formation of degradation products.

For the UV radiation exposure we used a Medium – Pressure Metal Halide Lamp HPA-400 W (Philips) as an artificial radiation system for simulating natural sunlight exposure. Distance between lamp and sample was 5 cm, likely heating of the sample was monitored, and the exposure time was 3 hour. We used 10 mg/L BZDs solutions prepared in absolute methanol. The UV spectrum of BZD generally exhibits maximas between 200 and 350 nm [2,3], therefore, the UV components of the sunlight can be

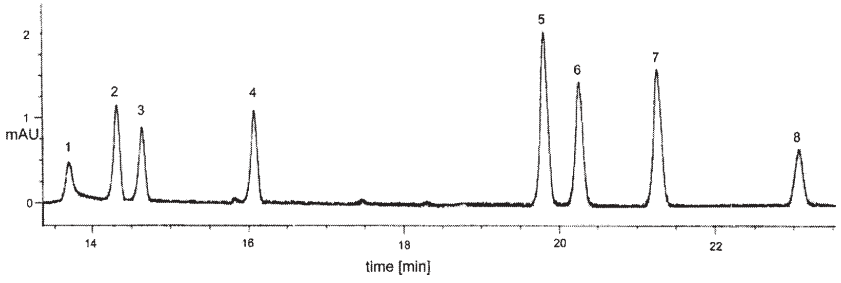

Fig. 4 Separation of the 8 BZDs with a 25 mM borate 50mM SDS 12 % methanol buffer solution (The separation conditions were described in fig. 2)

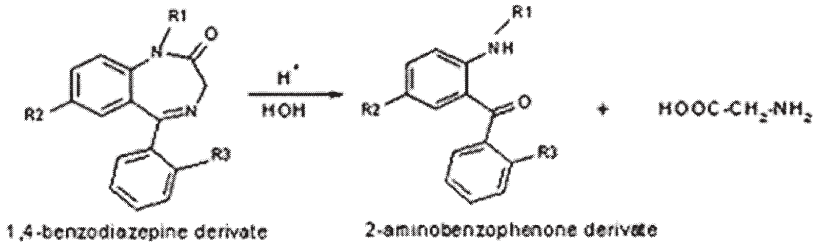

Fig. 5 Hydrolisis of BZD derivates in acidic medium

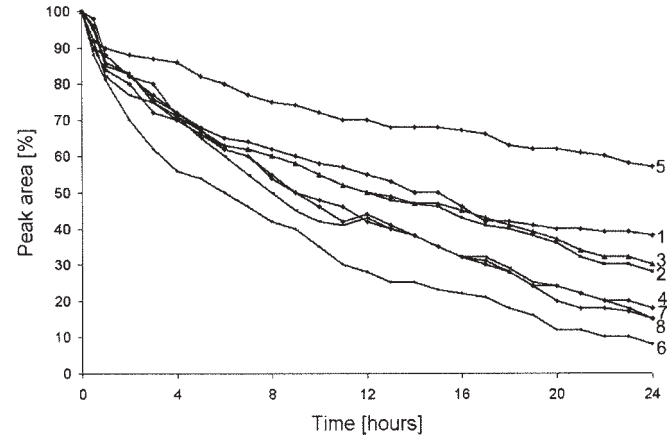

Fig. 6 Monitoring the amount of the 8 BZDs for 24 h after their hydrolysis in acidic medium ((The separation conditions were described in fig. 2.)

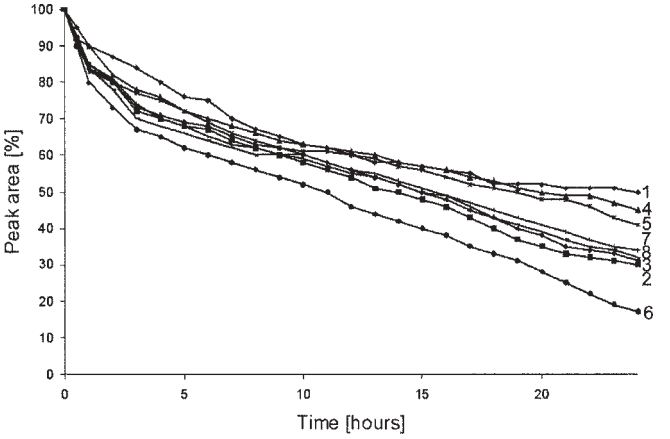

Fig. 7. Monitoring the amount of the 8 BZD for 24 h after UV exposure (The separation conditions were described in fig. 2)

considered the main responsible for the photochemical reactivity of the drug. The degradation rate was monitored over 24 h; each sample was reinjected periodically. The analytical parameters remained the same as for the separation.

The degradation rate for 24 h increased in the following order: bromazepam, chlorodiazepoxide, alprazolam, diazepam, medazepam, flunitrazepam, nitrazepam, oxazepam (fig. 7). With one exception (alprazolam) the degradation rates in UV light are smaller than in acidic medium. Also the degradation rates at least for the first 10 h are rather similar for the all-8 BZD.

Conclusions

CE is proving to be an attractive approach for the analysis of pharmaceutical substances. This approach, which is fully automated, is probably one of the most rapidly expanding analytical techniques of the last decade. The development of MECC extended the application range of CE, as it can separate both ionic and neutral substances.

In this paper we present a rapid and simple original procedure for simultaneous separation of eight of the most frequently used BZDs and their degradation products in acidic medium and UV light. Efforts were first focused on the optimization of analytical conditions, i.e. on the effects of buffer concentration, modifier concentration and buffer pH on the separation.

With a 25 mM sodium tetraborate 50 mM SDS 12 % methanol buffer solution, we managed to separate the 8 BZDs in less then 20 min. The RSD values for the migration times and peak areas were under 1, the LOD were between 0.22-0.73 mg/L and the linearity of the method proved to be good.

The method can be successfully used also for the separation of other BZDs and should find practical application in the separation of BZDs from body fluids.

Acknowledgements
This work was supported CEEPUS program (H-76 network).

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Manuscript received: 13.03.2007