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Possibilities and limitations of capillary electropherosis in pharmaceutical analysis

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Dedicated to Professor Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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Capillary electropherosis (CE) has been proved to be an important alternative to high-performance liquid chromatography (HPLC) in pharmaceutical analysis. However, when it comes to the analysis of compounds, e.g. impurities or metabolites, of very different polarity and water solubility CE and the related techniques come to its limits. This is demonstrated for the antipsychotic drug quetiapine and its impurities. A non-aqueous capillary electrophoresis (NACE) method was developed using a background electrolyte (BGE) composed of ammonium acetate dissolved in a mixture of acetonitrile and methanol including acetic acid to protonate the substances. The NACE method gave an excellent separation of all components. Since the conductivity of the BGE used in the NACE method is quite low and problems with current occurred, an additional aqueous capillary zone electrophoresis (CZE) method was developed for quetiapine and the two water soluble derivatives, using phosphate buffer as BGE. The method was validated with regard to repeatability and limit of detection.

1. Introduction

Since the introduction of capillary electrophoresis (CE) in 1967 by Hjerten and coworkers it has been largely applied e. g. for the elucidation of the human genome due to the good separation efficiency of macromolecules such as DNA and for chiral analysis (Ahuja and Jimidar 2008). However, CE techniques can also be used to separate and quantify drugs, their corresponding impurities, and metabolites (Wätzig 2002; Holzgrabe et al. 2006, 2008). Whereas in the beginning the optimism to completely replace HPLC with CE was rather high due to the tremendous selectivity, nowadays pharmaceutical industries, licensing authorities and the pharmacopoeias do not make use of CE with some very rare exceptions, e.g. levocarbastine, erythropoietin, and glutathione as well as products of recombinant DNA technology and products such as human coagulation factor VIII in the European Pharmacopoeia 7.6 (2012). Nevertheless, numerous examples of the separation and quantification of drugs and their impurities for quality assurance reasons, and of drugs and metabolites in urine, plasma and other body liquids were reported.

Especially ionic compounds or substances, which can be ionized by applying a background electrolyte (BGE) with an appropriate pH value, can be easily separated by means of capillary zone electrophoresis, if the size-to-charge ratio is different. In the case the analytes suffer from poor water solubility, the non-aqueous CE (NACE) being characterized by background electrolytes consisting of organic solvents and organic ions provides an alternatives to CZE (Ahuja and Jimidar 2008). Non-ionic compounds can often be separated by either micellar electrokinetic chromatography (MEKC) or microemulsion electrokinetic chromatography (MEEKC). Here, the separation is based either on the partition between the BGE and the micelles, formed by a surfactant, in the case of MEKC (El Deeb et al. 2011) or on the partition of the analytes between the oil droplets of the oil-in-water microemulsion and the buffer solution in the case of MEEKC. The microemulsion is formed by adding a surfactant, a co-surfactant (a short-chain alcohol) and an oil-component to the aqueous buffer (Altria 2000; Huie 2006; McEvoy et al. 2007; Ryan et al. 2011).

Even though a lot of different CE techniques are available, problems might arise when it comes to the separation of analytes of very different polarity and/or water solubility. This will be demonstrated for quetiapine (Seroquel®) (structure see Fig. 1) which is one of the most recent "atypical" antipsychotic drugs for the treatment of schizophrenia (Cheer et al. 2004), and its impurities. Quetiapine is characterized by a huge collection of impurities originated from the synthesis (Wariwari et al. 2001) and from acidic hydrolysis and oxidative degradation (Bharathi et al. 2008; Stolarczyk et al. 2009). Among them are desethanol quetiapine, *N*-formyl-quetiapine, quetiapine carboxylate, *N*-ethylpiperazinyl thiazepine, ethylquetiapine, and bis(dithiazepine) (dimer) beside the oxidation products, i.e. *N*- and *S*-oxides.

In order to show the CE separation problem of analytes of a large polarity and water solubility range, the following impurities of quetiapine were chosen: 1) quetiapine lactam, being a synthesis intermediate (Wariwari et al. 2001; Xu et al. 2007) and simultaneously a degradation product in acid media (Stolarczyk and Kutner 2010), is insoluble in aqueous systems; 2) desethanol quetiapine, being a synthesis by-product due to the use of the impure alkylation agent 2-(2-chloroethoxy)ethanol containing

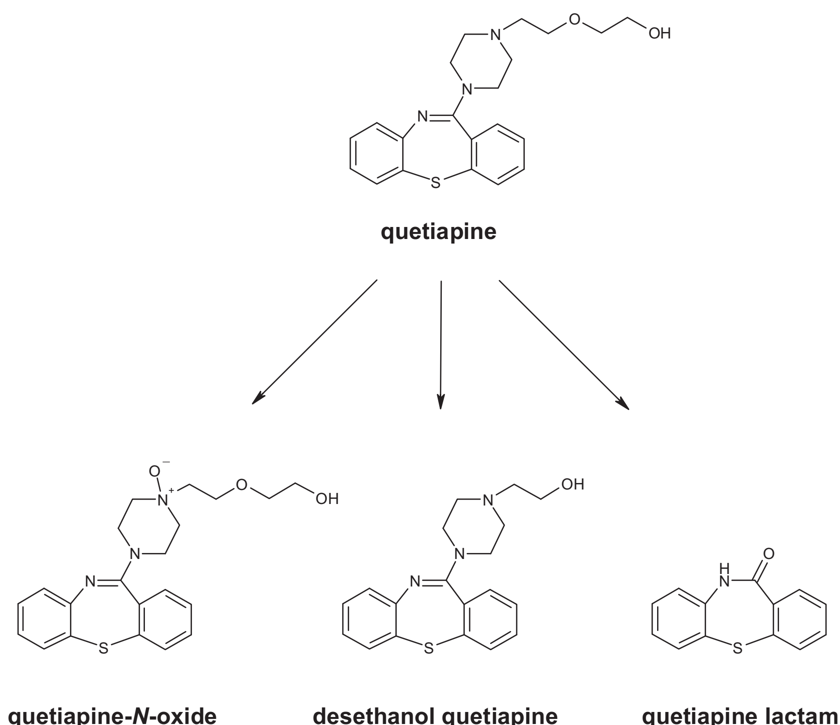

Fig. 1: Structural formulae of quetiapine and its impurities.

chloroethanol, and 3) quetiapine-*N*-oxide, a putative oxidation product (structures see Fig. 1). The latter two are water-soluble. The structures of all impurities were confirmed by mass spectrometry and the findings are in agreement with the data reported by Stolarczyk and Kutner (2010).

2. Investigations and results

Beside the content determination of quetiapine in tablets (Pucci et al. 2003) no quality-indicating CE method has been published till now.

In MEEKC, the separation of uncharged and hydrophobic substances was made available, because the oil droplets of the ME act as a pseudostationary phase and solubilize many hydrophobic analytes. Since the quetiapine lactam is not soluble in aqueous solutions, it was tried to develop a MEEKC method. However, whenever the lactam came in contact with the buffer it precipitated. Thus, aqueous solutions were completely avoided by means of a totally non-aqueous CE.

2.1. Method optimization of NACE

NACE has many advantages in comparison to aqueous CZE methods. Using organic solvents, selectivity often shows better results than using aqueous media because of their stronger autoprotolysis constants (Catai et al. 2009, Psurek et al. 2006). Furthermore, migration times of the analytes are often shorter than in aqueous solutions, because of the low viscosity of the organic buffer (Li 1998). Another important advantage of the NACE is the ability of organic solvents to solubilize lipophilic pharmaceutical substances. Quetiapine and its degradation products are readily soluble in a mixture of acetonitrile and methanol in equal parts and detectable at 254 nm.

A mixture of methanol and acetonitrile in varying proportions was chosen to study the effects of ionic strength: To the solvent mixture ammonium acetate in concentrations between 20 and 200 mM was added. Furthermore, acetic acid was added to protonate quetiapine and to some extent the impurities. Test runs revealed a non-stable current making the reproducibility of the method insufficient. Therefore, another salt, i.e. lithium chloride, was tested, but the stability of the current was not improved. The addition of water, especially with sodium chloride, had a positive effect on the current, but even small amounts of an aqueous solution resulted in precipitation of the quetiapine lactam, so this was not applicable to solve the mentioned problem. Eventually, a good baseline separation of all components was achieved within 12 min using 150 mM ammonium acetate in methanol and acetonitrile (50:50, v/v) and 1 % acetic acid as running buffer (see Fig. 2). The NACE method gave electropherograms of low baseline noise, excellent resolution values and peak shapes. Unfortunately, the reproducibility of the NACE method was not sufficient, as the current was not stable during the runs and many runs failed. This is likely caused by the low conductivity of a nonaqueous BGE. Thus, validation of the method was not possible.

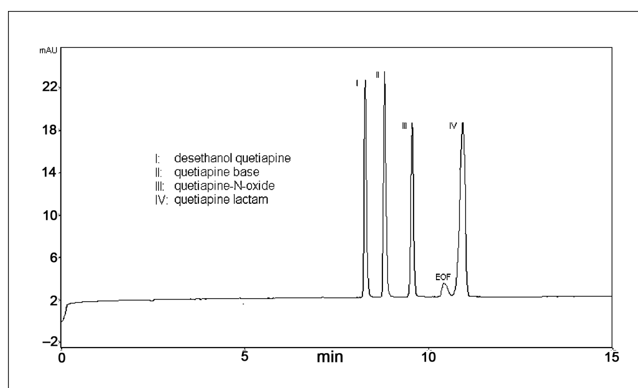

Fig. 2: NACE method: Separation of quetiapine, desethanol quetiapine, quetiapine-*N*-oxide and quetiapine lactam. Separation conditions: 150 mM NH_4Ac in MeOH/ACN/AcOH (44.5:44.5:1, v/v/v); voltage: 20 kV; temperature: 20 °C; detection wavelength 254 nm; fused-silica capillary (48.5/40 cm, 50 μm); sample conc.: ca. 0.2 mg/ml.

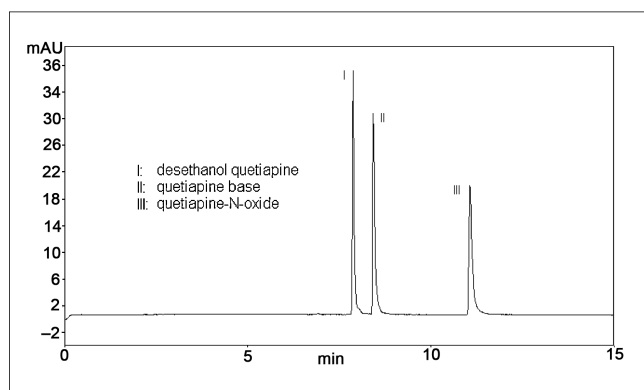

Fig. 3: Aqueous CZE method: Separation of quetiapine, desethanol quetiapine and quetiapine-*N*-oxide. Separation conditions: 80 mM phosphate buffer, pH 4.0; voltage: 20 kV; temperature: 20 °C; detection wavelength 254 nm; fused-silica capillary (50.2/40 cm, 50 μm); sample conc.: ca. 0.2 mg/ml.

2.2. Method optimization of aqueous CZE

Since the NACE method did not provide sufficient robustness, an aqueous CZE method was developed for quetiapine and the two water soluble degradation products, desethanol quetiapine and quetiapine-*N*-oxide. A phosphate buffer was initially used to determine the migration time and to optimise the resolution of the three analytes. The concentration of sodium dihydrogenphosphate was varied in the range of 20 to 100 mM and the pH values from 2.0 to 4.0. Satisfying resolution and good peak shapes within 12 min were obtained with 80 mM phosphate buffer at a pH value of 4.0, applying a capillary temperature of 20 °C and separation voltage of 20 kV (see Fig. 3).

2.3. Validation parameters of the aqueous CZE method

The aqueous CZE method was validated with respect to repeatability and limit of detection. To prove the repeatability of the developed method, the migration time and the peak areas were compared by checking the relative standard deviation (RSD) in percent. The solution of a concentration of 0.2 mg/ml was injected at least six times. The migration times of the analytes as well as the percentage of peak areas are summarized in the Table. Good results were achieved for the migration times and the peak areas, as the RSDs were lower or close to 3 %. The limit of detection (LOD) was investigated according to European Pharmacopoeia, i.e. a signal-to-noise ratio of 3:1. The LOD of quetiapine, the desethanol and *N*-oxide derivatives is 0.4 μg/ml for all components which is pretty low.

2.4. Comparison of the NACE and the aqueous CZE method

Interestingly, the order of analytes does not differ between the aqueous and the nonaqueous CE method. In fact, the resolution between quetiapine and its *N*-oxide is higher using aqueous CZE.

Table: Summary of the results of the precision for the migration time and the peak area

	t_m [min]	SD	RSD [%]	area [%]	SD	RSD [%]
Desethanol quetiapine	7.61	0.16	2.13	34.79	0.33	0.95
Quetiapine base	8.18	0.18	2.26	29.52	0.23	0.78
Quetiapine-*N*-oxide	11.42	0.32	3.23	35.69	0.53	1.49

t_m: migration time SD: standard deviation RSD: relative standard deviation

The shortest migration time has desethanol quetiapine. Because of its *O*-desalkylation, it is smaller than the base, but it is charged to the same extent. As separation occurs by size-to-charge-ratio in CZE, quetiapine base is the second analyte appearing. Using the aqueous CZE method quetiapine-*N*-oxide has the longest migration time, which is due to the zwitterionic form of the *N*-oxide in the running buffer. However, the neutral quetiapine lactam shows the longest migration time which is governed by the electroosmotic flow.
In both cases, NACE and aqueous CZE, the peaks are symmetrical, neither fronting nor tailing was observed. The greatest advantage of NACE is the ability to determine all three impurities in addition to quetiapine whereas the aqueous CZE provides a greater peak height and the method is more stable and robust.

2.5. Comparison with HPLC methods

Very few HPLC methods hyphenated with mass spectrometry are described for metabolic profiling (Pelander et al 2009; Tu et al. 2008; Li et al. 2005; Lin et al. 2004) but none is dealing with the metabolites or impurities of such different water solubility. With regard to impurity profiling, Trivedi and Patel provide an UPLC method using an RP18 column of small particle size (1.8 μm) and a mobile phase (gradient elution) consisting of aqueous triethylamine (0.1 %), methanol and acetonitrile which is able to separate quetiapine, the *N*- and *S*-oxide, piperazinyl quetiapine, desethanol quetiapine and the dimer within 4 min. However, desethanol quetiapine and quetiapine were found to be the critical pair being barely baseline separated. Similar results were reported by Bharati et al. (2008) who were able to separate 6 impurities from quetiapine using an RP18 column and 10 mM ammonium acetate buffer, acetonitrile and methanol as mobile phase. Both series of experiments did not contain the poorly water soluble lactam. Comparing the HPLCs with the aqueous CZE method presented here, CZE shows a better separation of quetiapine and the two impurities, the *N*-oxide and desethanol compound.
Belal et al. (2008) developed a stability-indicating HPLC method dealing with the *N*-oxide and the lactam. Using a phenyl-column with a mobile phase composed of acetonitrile and 20 mM phosphate buffer (pH 5.5) he was able to separate and quantify both impurities from quetiapine in bulk, tablet and human plasma samples. The method gave satisfying validation results. Interestingly, Belal did obviously not observe solubility problems with the lactam, which might be due to the fact that the molarity of the buffer was four times lower: 20 mM (HPLC) *versus* 80 mM phosphate buffer (CZE). However, the separation of the critical pair desethanol quetiapine and quetiapine itself is not reported. Taken together, in comparison to the HPLC method, the NACE method gave a nice separation of all four components but validation was not possible.

3. Discussion

Separation and quantification of compounds of different polarity and solubility by means of a CE technique is a challenge. Whereas CZE provides robust methods but suffers from the fact that all analytes have to be water soluble, the MEEKC and NACE methods, which are able to solubilise hydrophobic compounds and can provide excellent separation, are often not as robust as CZE for quality assurance in a routine environment of a production plant. This observation is rarely reported because it is a negative result. Here, HPLC methods might be superior but may suffer from less separation efficiency.

4. Experimental

4.1. Instrumentation

The development of the methods was performed on a Beckman Coulter P/ACE System MDQ (Fullerton, CA, USA), equipped with an UV-detector measuring at 254 nm. For validation parameters a HP^{3D}-CE (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) and a ChemStation 08.03 Software was used. Uncoated fused silica capillaries purchased from BGB Analytik (Schloßböckelheim, Germany) had an internal diameter of 50 μm, an effective length of 40 cm and a total length of 50.2 cm (Beckman) and 48.5 cm (Agilent). The pH value of the buffer systems was measured with a PHM 220 Lab pH meter (Radiometer Copenhagen, Lyon, France). For the preparation of homogenous sample solutions a 2510-Branson-Sonicator (Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany) was used.

4.2. Chemicals and materials

Quetiapine and its metabolites desethanol quetiapine, quetiapine lactam and quetiapine-*N*-oxide were kindly provided by Astra Zeneca (Macclesfield, UK). Acetonitrile and methanol were obtained from Fisher Scientific (Loughborough, UK), ammonium acetate from Fluka (Fluka Chemie AG, Buchs, Switzerland) and lithium chloride from VWR (VWR International GmbH, Darmstadt, Germany). Orthophosphoric acid (85%), acetic acid as well as sodium dihydrogen phosphate (NaH_2PO_4) (p.a.) were purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany). Prior to use, aqueous solutions were filtered through a 0.22 μm pore-size CME (cellulose mix ester) filter, organic solutions through a 0.22 μm pore-size PTFE (polytetrafluoroethylene) filter (Carl Roth GmbH, Karlsruhe, Germany). Aqueous buffer was prepared using ultrapure Milli-Q water (Millipore, Milford, MA, USA).

4.3. Preparation of the background electrolytes

4.3.1. NACE

For the nonaqueous capillary electrophoresis (NACE) method a mixture of the solvents acetonitrile and methanol was prepared in equal amounts. 150 mM ammonium acetate (NH_4Ac) was weighed in this solution and stirred for 5 min to aid dissolution. Finally, 1 % acetic acid (AcOH) (v/v) was added to give ACN:MeOH:AcOH = 45.5:45.5:1 (v/v/v). The background electrolyte (BGE) was stirred again for some minutes and filtered through a 0.22 μm pore-size PTFE filter.

4.3.2. Aqueous CZE

For the aqueous capillary zone electrophoresis (CZE) method the buffer solution was prepared by dissolving 80 mM of NaH_2PO_4 in Millipore water. The pH was adjusted to the desired value 4.0 with concentrated phosphoric acid (85%). This BGE was filtered through a 0.22 μm pore-size CME filter.

4.4. Sample preparation

All substances were prepared in the BGE, either in a mixture of organic solutions, ammonium acetate and acetic acid for NACE (see 2.4) or in phosphate buffer for the aqueous CZE method (see 2.4). First, 2 mg/ml stock solutions of quetiapine and its impurities desethanol quetiapine, quetiapine lactam and quetiapine-*N*-oxide were prepared in the corresponding buffer. Before measurements, the stock solutions were diluted with BGE to give 0.2 mg/ml samples or to give the required concentrations for the determination of the validation parameters, and filtered through a 0.22 μm pore-size filter and sonicated for 15 min prior to use.

4.5. Conditioning

New capillaries were conditioned at 25 °C by rinsing with 1 M NaOH for 20 min, with 0.1 M NaOH for 40 min, with water for 5 min, with 0.1 M H_3PO_4 for 10 min, and water for 5 min.

4.5.1. NACE

The capillaries for nonaqueous measurements were first flushed with 0.1 M NaOH (aqueous), then with water and acetonitrile, each for 3 min. This procedure is aimed to remove traces of water on the capillary surface (Altria 1997) and was also used to rinse the capillary after the measurements. Before a new sample was injected, the capillaries were conditioned by flushing with BGE for 3 min. While running the measurements on the Agilent CE instrument, the samples were stored at room temperature, as cooling of the sample tray is not possible. Capillary wash cycles were done at a pressure of 6 bar (Agilent).

4.5.2. Aqueous CZE

Before performing the experiments, the capillaries were conditioned at 20 °C by rinsing with 0.1 M NaOH for 3 min, with water for 3 min, and the BGE for 3 min between each run. Before a new sample was injected, the capillary was conditioned by flushing with the BGE for 3 min. At the end of each working day, the capillaries were rinsed at 30 °C with 0.1 M NaOH for 10 min, methanol for 10 min and water for 10 min. While running the measurements on the Beckman CE instrument, samples were stored at 8 °C. Capillary wash cycles were performed at a pressure of 2 bar (Beckman).

4.6. Methods

The separation methods for both CE techniques were developed individually, but based on similar conditions. For the NACE method, the BGE consists of 150 mM NH_4Ac in CAN:MeOH:AcOH, 45.5:45.5:1 (v/v/v). Using the aqueous CZE method, 80 mM phosphate buffer, pH 4.0 was applied. Separations were performed at 20 °C using a constant voltage of 20 kV in the normal polarity mode. The samples were injected electrokinetically at the anionic end of the capillary by a pressure of 50 mbar (NACE, Agilent CE) and 30 mbar (aq. CZE, Beckman CE), respectively, for 5.0 s. The detection wavelength was set at 254 nm.

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