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Short communication

Comparison of CZE, MEKC, MEEKC and non-aqueous capillary electrophoresis for the determination of impurities in bromazepam

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Abstract

The purpose of the present investigation was to develop a test for related substances in the benzodiazepine drug substance bromazepam based on capillary electrophoresis (CE). A final method for the determination of impurities in bromazepam is based on non-aqueous capillary electrophoresis (NACE).

Five modes of capillary electrophoresis were investigated and compared for the said purpose.

All the CE systems investigated make use of running buffers at low pH in order to protonate the analytes. A low pH of the running buffers was needed as the pK_a values of benzodiazepines in general are in the range from 1.3 to 4.6.

Dynamically coated capillaries were used to overcome the low electro-osmotic flow at low pH in the aqueous buffers investigated. CZE with and without dynamical coating of the internal surface of the fused capillaries was compared and also micellar electrokinetic chromatography (MEKC) as well as microemulsion electrokinetic chromatography (MEEKC) performed in dynamically coated capillaries were investigated.

The NACE was chosen as the best technique as the low solubility of the benzodiazepines in water is easily overcome. The NACE system showed good selectivity and detectability for the substances investigated and the limit of quantitation for the impurities corresponded to 0.05% of the drug substance. Linearity was good.

Keywords: Bromazepam; Impurities; Capillary electrophoresis; Dynamically coated capillaries; MEKC; MEEKC; Non-aqueous capillary electrophoresis

1. Introduction

The content of related substances (impurities) in drug substances is now-a-days most often performed using high-performance liquid chromatography. However, at present the test for related substances in the monographs for bromazepam included in the *Japanese* [1] and the *European Pharmacopoieas* [2] is performed using thin-layer chromatography. The monograph on bromazepam in the *European Pharmacopoiea* is at present under revision in order to replace TLC with an HPLC method. However, due to the high separation power of capillary electrophoresis (CE) this technique might also be applicable for this purpose.

The separation of benzodiazepines using CE has been performed at low pH [3–5] or by using micellar electrokinetic chromatography (MEKC) typically at pH values around 8–9.5 [3,6–12]. Furthermore, capillary electrochromatography has also been employed for this purpose [13,14]. Recently, the use of dynamically coated capillaries has been described for the separation of benzodiazepines at low pH [15]. None of the described methods have been

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used for the analysis of impurities at low concentrations in benzodiazepines.

The physico-chemical nature of the benzodiazepines may, however, give rise to some problems if using capillary electrophoresis as the analytical technique as they are difficult to ionise due to fairly low pK_a values. As a consequence of this lack of ionisation, and also due to the lipophilic molecular structure, many of the benzodiazepines, including bromazepam, have a very low solubility in aqueous solvents. The low solubility in water is a major drawback because a relatively high concentration of the drug substance itself in the sample solution is needed if low concentrations of impurities in the drug substance are to be detected using capillary electrophoresis.

In this paper, a number of CE modes are compared with respect to selectivity and to applicability to be used for the determination of five impurities in bromazepam (Fig. 1). For the purpose of comparison all separations were performed at low pH and as far as possible with the same electrolyte in the running buffer. The modes investigated were aqueous and non-aqueous CE in uncoated capillaries, but in order to decrease the time of analysis at low pH dynamically coated capillaries were used for micellar electrokinetic chromatography and microemulsion electrokinetic chromatography (MEEKC).

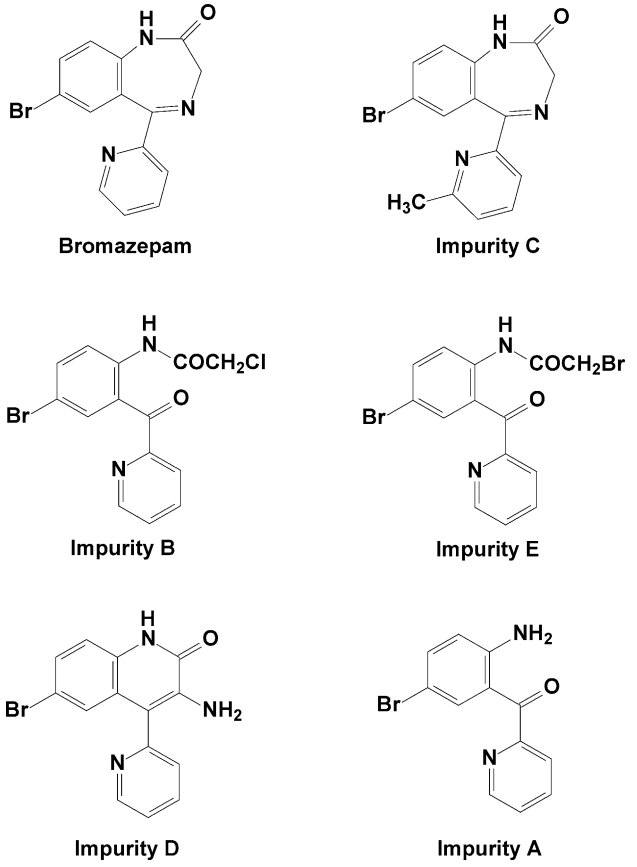

Fig. 1. Bromazepam and its five impurities.

2. Materials and methods

2.1. Instrumentation

A ^{3D}capillary electrophoresis system (Agilent Technologies, Walbronn, Germany) equipped with an on-line diode-array detector (DAD) operated at 230 nm was used.

2.2. Capillaries

The separation was performed in fused silica capillaries (48.5 cm (40 cm to detector) × 50 μm) from Polymicro Technologies (Phoenix, AZ, USA) operated at 25 °C.

Before use the capillaries were rinsed with 1 M sodium hydroxide for 20 min and distilled water for 5 min. Coated capillaries were coated using the following procedure: first by flushing with a 1% poly-(diallyldimethylammonium chloride) solution for 10 min, then distilled water for 5 min, and finally with a 1% poly-(vinylsulfonate) (PVS) solution for 10 min.

Before analysis the capillaries were flushed with the running buffer for 20 min and between each sample analysis the capillary was flushed with 0.1 M sodium hydroxide for 5 min and with the running buffer for 20 min.

A fresh running buffer was used for each analysis.

2.3. Chemicals and reagents

Poly-(diallyldimethylammonium chloride) 20% in water, poly-(vinylsulfonic acid) sodium salt 25% in water and dodecyl sulfate, lithium salt 98% were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). All other chemicals were of analytical reagent grade. Bromazepam and its impurities were obtained from the European Directorate for the Quality of Medicines (EDQM) in connection to the revision of the monograph on bromazepam for Ph.Eur.

2.4. Electrophoresis buffers

The running buffer constitutes 100 mM formic acid and 1 mM trifluoroacetic acid (TFA) for all aqueous buffers.

In MEKC, 2% of lithium dodecylsulfate and 6.6% of *n*-butanol were added and in MEEKC, 2% of lithium dodecylsulfate, 6.6% *n*-butanol and 1% *n*-octanol were added to the acidic buffer.

All aqueous running buffers were added 0.05% PVS.

In non-aqueous CE a buffer constituting methanol + acetonitrile (50:50, v/v), 25 mM of ammonium acetate and 100 mM of TFA were used.

2.5. Sample preparation

Samples for testing selectivity in the aqueous systems were dissolved in 50% methanol at a concentration of 0.1 mg/ml. For the NACE system samples were dissolved in the running buffer at 0.1 mg/ml.

Samples of bromazepam for determination of low concentration of impurities were dissolved at a concentration of 3 mg/ml with addition of the impurities at concentrations of 2, 4 or 6 μg/ml (corresponding to 0.05, 0.1 or 0.2% of the bromazepam).

Samples were injected using a pressure of 50 mbar for 2 s and a voltage of +15 kV was used for the analysis.

3. Results and discussion

3.1. Basic considerations

In order to be able to separate the benzodiazepine derivatives and impurities as cations, a fairly low pH is needed as the benzodiazepines have low pK_a values [16]. Recently, 100 mM of formic acid together with 1 mM of TFA was shown to be suitable for protonation in the LC–MS analysis of benzodiazepines [15] and this was thus chosen as the basic electrolyte in the running buffers using aqueous media. Using this system a separation of all analytes was obtained (Fig. 2a). However, the system cannot be used for the intended purpose as impurities B and E show poor efficiency resulting in high limits of detection.

3.2. Dynamically coated capillaries

It has previously been shown that dynamic coating of the capillary wall with poly-(vinylsulfonate) on top of a quaternary ammonium polymer will generate a high electroosmotic flow (EOF) at low pH [15,17]. Applying this technique resulted in major decrease in the analysis time (Fig. 2b) using the same running buffer.

Although the separation of four of the impurities and bromazepam was good the system cannot be used for the analysis of small amount of impurities in bromazepam as impurities C and D co-migrated. The migration times were too short to obtain a separation of these two impurities. Furthermore, when introducing a solution of 3 mg/ml of bromazepam made up in 50% methanol the peak for bromazepam partly covers some of the impurities (Fig. 3a).

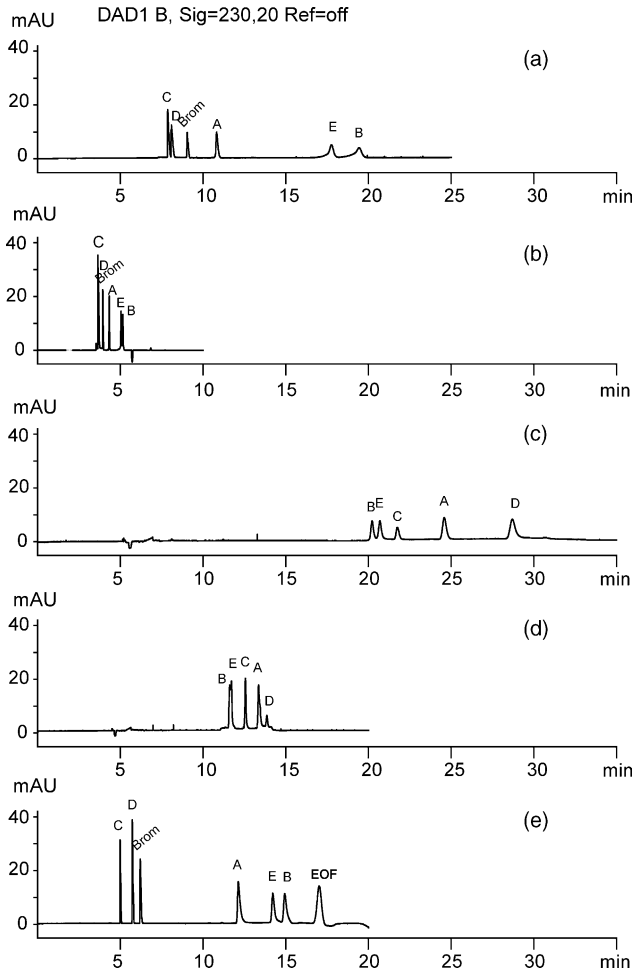

Fig. 2. Electropherograms of the separation of bromazepam and five of its impurities using fused silica capillaries (48.5 cm (40 cm to detector) × 50 μm) operated at 25 °C; voltage, +15 kV; inj., 50 mbar for 2 s; detection, 230 nm. (a) Running buffer constitutes 100 mM formic acid and 1 mM (TFA); (b) running buffer as in (a) but using a dynamically coated capillary (coating procedure, see text); (c) 2% of lithium dodecylsulfate and 6.6% of *n*-butanol in 100 mM formic acid and 1 mM (TFA); (d) 2% of lithium dodecylsulfate, 6.6% *n*-butanol and 1% *n*-octanol in 100 mM formic acid and 1 mM (TFA) and (e) methanol + acetonitrile (50:50, v/v) with 25 mM of ammonium acetate and 100 mM of TFA. In (b–d) 0.05% of PVS is added to the running buffer.

3.3. MEKC and MEEKC

Previously, MEKC has been used for the separation of benzodiazepines, and therefore this technique as well as MEEKC was investigated. In order to be able to compare the separation techniques both MECK and MEEKC were performed at low pH, and therefore capillaries dynamically coated as previously described were used. In these systems separation of the five impurities was obtained and the order of migration of the impurities in both the MEKC and the MEEKC systems was similar (Fig. 2c and d). Due to the use of the dynamically coated capillary the EOF was high and the solutes migrated after the EOF due to their relatively high lipophilicity as well as the ionic interaction with the dodecylsulfate units of the micelles or microemulsion droplets.

The selectivity observed is due to a combination of the degree of ionisation, resulting in different affinity to the surfactant molecules in the micelles and microemulsion droplets, and to the lipophilicity of the molecules themselves.

As also observed previously [18] the microemulsion system provides much faster migrations compared to the MEKC system.

However, when introducing the concentrated (3 mg/ml) solution of bromazepam into the capillary precipitation occurred and no useful results could be obtained. The precipitation may be due to very lipophilic ion-pairs between bromazepam and dodecylsulfate. Even at low concentrations

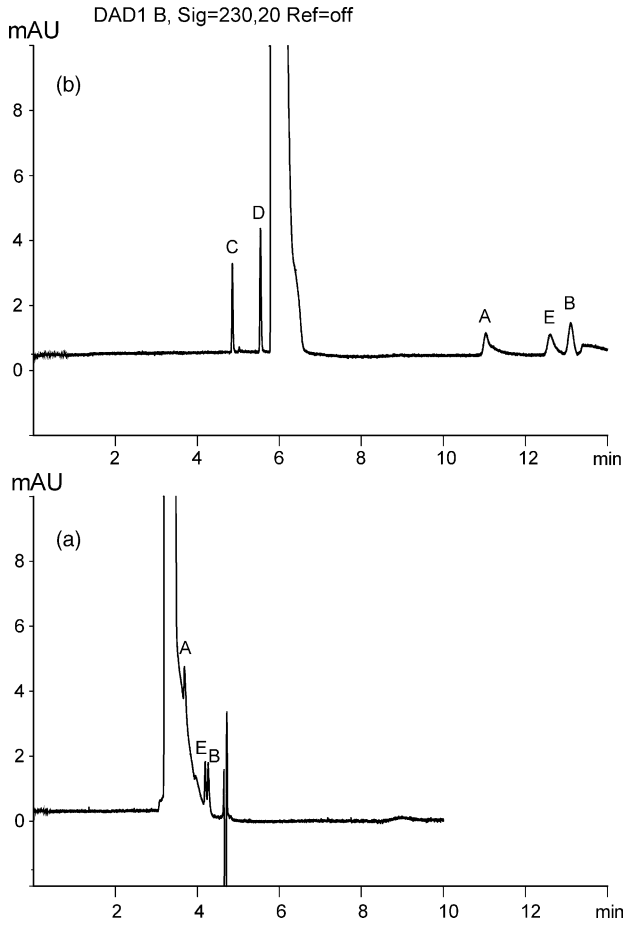

Fig. 3. Electropherograms showing the determination of impurities at the 0.2% level in bromazepam using (a) aqueous CE on dynamically coated capillary (details given in Fig. 2b) and (b) non-aqueous CE (details given in Fig. 2e).

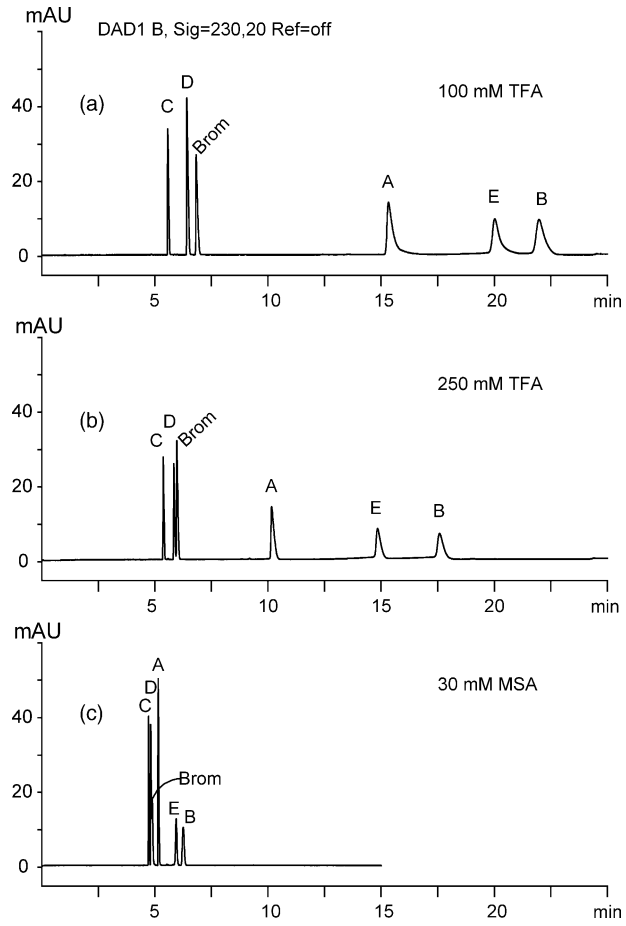

Fig. 4. Electropherograms of the separation of bromazepam and five of its impurities using NACE. Electrophoresis, fused silica capillaries (48.5 cm (40 cm to detector) × 50 μm) operated at 25 °C; voltage, +15 kV; inj., 50 mbar for 2 s; detection, 230 nm. Running buffer, methanol + acetonitrile (1:1) with 25 mM ammonium acetate and (a) with 100 mM TFA; (b) with 250 mM TFA and (c) with 30 mM MSA.

it was difficult to obtain a suitable peak corresponding to bromazepam.

3.4. Non-aqueous CE

To overcome the problem of solubility, a non-aqueous CE system was applied using a mixture of methanol and acetonitrile (1:1) for the buffer solvent. The use of 100 mM formic acid as the electrolyte did not provide a sufficiently low pH in the non-aqueous system to be able to protonate all the solutes and impurities A, E and B migrated close to the electroosmotic flow. Therefore, 100 mM of TFA was tested as the electrolyte. It is known from previous investigations [19] that TFA will lower the pH compared to formic acid. The electropherogram of the mixture of all the solutes using 100 mM TFA is seen in Fig. 2e. The order of migration of the analytes in the non-aqueous system was similar to that obtained in plain aqueous systems (Fig. 2a) but the selectivity was somewhat different. The non-aqueous system is well suited for the determination of the impurities in bromazepam (Fig. 3b) as no problems with the solubility of bromazepam are observed. As the impurities A, E and B probably are only partly ionised the pH of the running buffer was lowered further by increasing the concentration of TFA or by substituting it for 30 mM of methanesulfonic acid (Fig. 4a–c). The peak shapes were improved but now bromazepam migrated too close to some of the impurities, which made these systems less suitable for detection of small amounts of impurities in bromazepam.

As the solvent mixture used for the running buffer in NACE may have a major impact on the migration rate and selectivity [20] this was investigated using different relative amounts of methanol and acetonitrile (Fig. 5). On increasing the concentration of methanol to 75% the migration times were increased by a factor of three. With 75% acetonitrile bromazepam co-migrated with impurity D. Thus, the 1:1 mixture of the two solvents is the most appropriate for the present purpose.

In order to illustrate the applicability of NACE to the separation of benzodiazepines a number of other benzodiazepines were separated in this system (Fig. 6). Even though the benzodiazepines have fairly similar molecular weights they are nicely separated. Due to their low pK_a values (in water) they

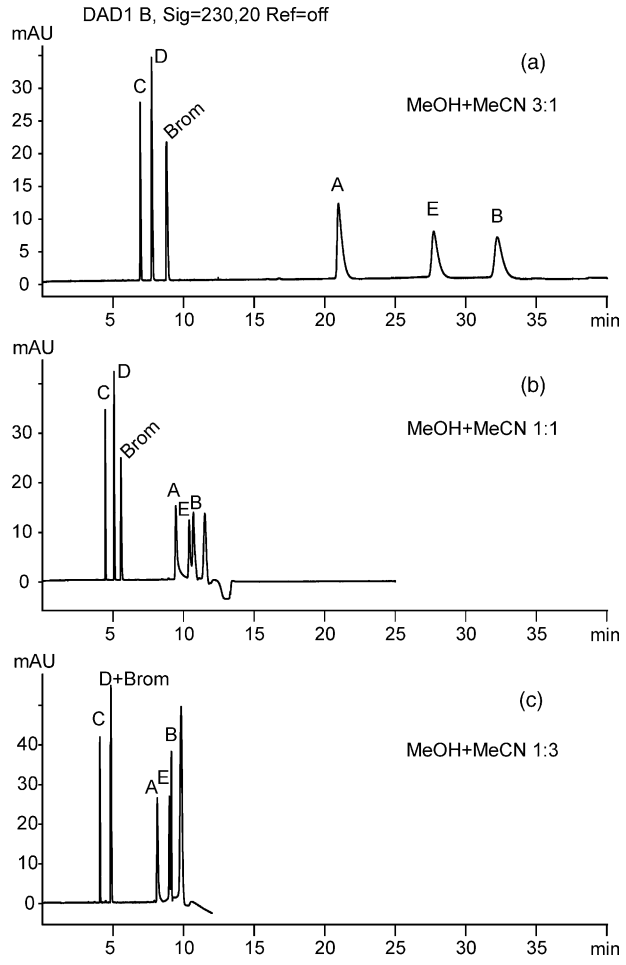

Fig. 5. Electropherograms of the separation of bromazepam and five of its impurities using NACE. Electrophoresis, fused silica capillaries (48.5 cm (40 cm to detector) × 50 μm) operated at 25 °C; voltage, +15 kV; inj., 50 mbar for 2 s; detection, 230 nm. Running buffer, methanol + acetonitrile (*x*:*y*, see text on figure) with 25 mM ammonium acetate and 100 mM TFA.

cannot be expected to be fully protonated, and furthermore they may also be solvated to a different degree. Thus, the separation is difficult to predict and it may easily be changed by changing the running buffer.

3.5. Separation selectivity

When performing the separation using a fused silica capillary and an acidic running buffer a separation of all solutes given in Fig. 1 is achieved. As shown in the electropherogram in Fig. 2a some unexpected selectivities are obtained. Impurity C having an extra methyl group compared to bromazepam has a faster electrophoretic migration rate than bromazepam and impurity B, which has a smaller mass compared to the similar impurity E, has a lower electrophoretic migration rate than impurity E. The reason for these findings is probably differences in ionisation and, possibly, also in solvation. The identity of each impurity was confirmed by electro-spray mass spectrometry in positive mode.

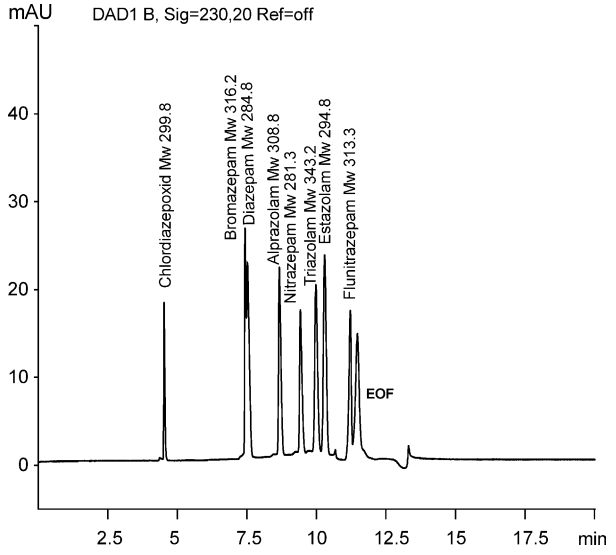

Fig. 6. Separation of a number of benzodiazepines in NACE. Separation systems as in Fig. 4a. For information the molecular weights of the benzodiazepines are given in the figure.

Applying MEKC or MEEKC all solutes migrate after the EOF. This is of course due to their affinity for the negatively charged surfaces of the micelles and microemulsion droplets, but probably also partly due the lipophilicity of the analytes, and thus their distribution into the pseudophases of these systems.

Surprisingly, bromazepam behaved very differently compared to the other solutes and it was not possible to obtain a peak corresponding to bromazepam in the MEKC system.

Using MEEKC much shorter migration times were obtained and the selectivity also changed. Impurities A and D had the highest affinities to the pseudophases. Impurity D which from Fig. 2b can be considered to be more strongly ionised also had the highest affinity, and thus the longest migration time when using running buffers with pseudophases.

In the non-aqueous system the separation selectivity was similar to the one in the aqueous system in the uncoated capillary.

3.6. Validation

Bromazepam is a very pure drug substance and the linearity of the method was tested using standard addition of the impurities to a 3 mg/ml solution of bromazepam in the running buffer. The standard addition was performed at three concentration levels 0.05, 0.1 and 0.2% of the bromazepam. Linearity, with a correlation coefficient above 0.985, was observed for all five impurities. The evaluation of the purity cannot be performed by direct comparison towards a suitable dilution of bromazepam although all six solutes have about the same UV absorption at 230 nm. This is partly due to the fact that the analytes passes the detection window at different velocities resulting in larger responses for the peaks with the largest migration times.

Thus, it is recommended for comparison to use a standard of bromazepam spiked with the relevant impurities at a level of, e.g. 0.1% is used.

4. Conclusions

The separation of the benzodiazepine bromazepam and its five impurities has been studied in five different CE systems. A major factor in controlling the selectivity of the separation of benzodiazepines is their pK_a values as they are in the range from 1.3 to 4.6. Thus, a low pH of the running buffers is needed to at least partly ionise the analytes.

When low amounts of impurities in drug substances are to be determined using CE with UV detection a relatively high concentration of the drug substance is required. Due to the low solubility of the benzodiazepines this makes aqueous running buffers unsuitable for the purpose.

Of the five tested systems, only the non-aqueous system can be used for determination of small amounts of impurities in bromazepam and the separation selectivity is very dependant on the pH and on the nature of the organic solvents used for the running buffer.

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