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Evaluation of the impurity profile of alcuronium by means of capillary electrophoresis

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Abstract

Alcuronium, a neuromuscular blocking drug, was recently introduced to the European Pharmacopoeia. A HPLC method is described to limit the impurities of alcuronium, namely the diallylcaracurine (DAC) and the allyl-Wieland-Gumlich-aldehyde (WCA), to less than 0.5%. Since alcuronium and all impurities are quaternary salts, capillary electrophoresis (CE) is highly suitable to evaluate the impurity profile. Using 12 mM *heptakis*-(2,6-di-*O*-methyl)-β-cyclodextrin in a 50 mM phosphate buffer at pH 5.5 or 50 mM diethanolamine buffer (pH 9.2)–acetonitrile 19:1 containing *heptakis*-(2,3-*O*-diacetyl-6-sulfo)-β-cyclodextrin the impurities could be baseline separated and quantified. The limit of detection for DAC and WCA was found to be in the same range as found with HPLC; thus, less than 0.1% of both DAC and WCA could be detected in the solution for injection in presence of alcuronium. In injection solutions of alcuronium which were stored at higher temperatures three additional, unidentified impurities were detected. In addition, the conversion of alcuronium to DAC, occurring under acidic condition, was monitored by means of the CE method developed.

Keywords: Impurity profile of alcuronium; Capillary electrophoresis

1. Introduction

Alcuronium, a neuromuscular blocking agent of the bisquaternary toxiferine type, was recently monographed in the European Pharmacopoeia. Two main impurities are limited in the 'related substance' section: the stability of the compound in mild acidic milieu is limited because alcuronium tends to undergo a ring-closure reaction between the allylic alcohol function and the double bond in the middle of the molecule resulting in diallylcaracurine (DAC; cf. with [1,2]). From the

Abbreviations: CD, cyclodextrin; HDAS-β-CD, *heptakis*-(2,3-*O*-diacetyl-6-sulfo)-β-CD; SBE, sulfobutylether-β-CD; sulf-β-CD, sulfated; DM-β-CD, *heptakis*-(2,3-di-*O*-methyl)-β-CD; CE, capillary electrophoresis; EOF, electroosmotic flow; DAC, diallylcaracurine; WGA, allyl-Wieland-Gumlich-aldehyde; AS, allylstrychnine; IS, internal standard.

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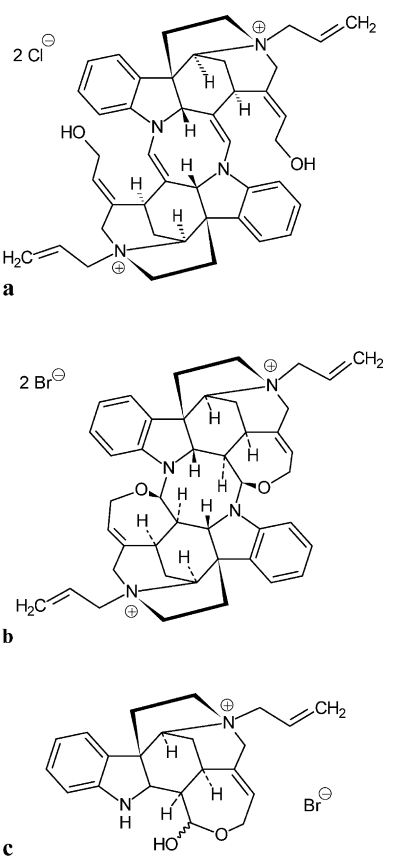
synthesis pathway, the monomeric allyl-Wieland-Gumlich-aldehyde (WGA), i.e. ((4*R*,17*R*)-4-allyl-17,18-epoxy-17-hydroxy-19,20-didehydrocuranium chloride, might appear in the product. The European Pharmacopoeia 2000 made use of a thin layer chromatography (TLC) method utilizing UV detection after spraying with ammonium cerium(IV) nitrate. This method was able to limit one impurity less than 0.5% and the second less than 0.2%, respectively. However, in the European Pharmacopoeia 2001 the TLC methods was replaced with a HPLC method utilizing the ion pair chromatography technique which produced the same limits. However, alcuronium and both impurities to be limited are permanent positively charged, and, therefore, highly appropriate to be analyzed by capillary electrophoresis (CE) which is nowadays increasingly used for impurity determination purposes of drugs [3–5] and, therefore, introduced to the European and United States Pharmacopoeia [6,7]. Thus, the purpose of the study was to check whether it is possible to separate and quantify the two impurities in both bulk material and solutions for injection, and to monitor the course of the cyclization reaction by means of CE (Scheme 1).



Scheme 1. Structural formula of alcuronium (a), DAC (b) and WGA (c).

2. Material and methods

2.1. Chemicals

Alcuronium was purchased from Promochem (Wesel, Germany) and DAC and the WGA were synthesized by D. Zlotos, Institute of Pharmacy, University of Würzburg, Germany, according to Refs. [8,9]. Alloferin® solutions for injection (Hofmann-La Roche, Basel, Switzerland and ICN Pharmaceuticals, Frankfurt, Germany), which were stored for 4 years, were provided by the German army. α-, β- and γ-CD were a gift from the Consortium für Elektrochemische Industrie (München, Germany), *heptakis*-(2,3-*O*-diacetyl-6-sulfo)-β-cyclodextrin (HDAS-β-CD) was a generous gift from Professor G. Vigh, A&M University, College Station, TX, USA. The sulfated-β-CD, having an average degree of sulfation of 9–11, was purchased from Aldrich (Steinheim), *heptakis*-(2,6-di-*O*-methyl)β-CD (DM-β-CD) from Fluka (Deisenhofen, Germany) and sulfobutylether-β-CD (SBE-β-CD) from CyDex (Overland Park KS, USA). Analytical grade KH_2PO_4, K_2HPO_4 and orthophosphoric acid purchased from Merck (Darmstadt, Germany) and diethanolamine from Fluka (Deisenhofen, Germany) were used for preparing of the buffers.

Phosphate buffer pH 3 was prepared by mixing appropriate amounts of H_3PO_4 and KH_2PO_4 solution. Phosphate buffers pH 4.5–7.4 were prepared by mixing appropriate amounts of KH_2PO_4 solution and NaOH (equivalent to PhEur). The CDs were dissolved directly into

the background electrolyte (BGE). All buffer solutions were prepared with deionized water and filtered through a 0.45 μm filter before use (Schleicher und Schüll, FRG).

2.2. CE

All experiments were performed on a Beckman P/ACE MDQ system (Beckman Instruments, Fullerton, CA, USA) using a fused-silica capillary with a total length of 60.2 cm, a detection length of 50 cm, and an internal diameter of 50 and 75 μm. Samples were loaded for 5 s of pressure injection (3448 Pa) and separated in the cationic injection mode, using a constant voltage of 20 and 30 kV.

2.2.1. Bulk material (drug substance)

The drug solution for the optimization of the separation conditions had a concentration of 51 μg/ml and was detected using a diode array detector at 254 nm. For the determination of the limit of detection (LOD) the same drug solution was used as stock solution. Detection for this purpose was carried out by an UV detector at 254 nm. The samples of the bulk material subjected to the CE were dissolved in DMSO and diluted with buffer solution of pH 7.4. The BGE used for the determination of the impurities was composed of 50 mM KH_2PO_4 buffer, pH 5.5 and 12 mM DM-β-CD. When using a new capillary, the capillary was conditioned for 20 min with 0.1 M NaOH and 10 min with water. Before each run, the capillary was washed for 2 min with 0.1 M NaOH, 1 min with water, and 2 min with the running buffer.

2.2.2. Solution for injection

Alloferin® solutions were directly loaded without any sample preparation. The diethanolamine/hydrochloride acid buffering system of the injection solution was chosen as the BGE. In order to improve the selectivity of method, acetonitrile as an organic modifier and HDAS-β-CD as selector were added. Finally, the BGE was composed of diethanolamine buffer (pH 9.2, 50 mM)–acetonitrile, 19:1, containing 30 and 46 mg/ml HDAS-β-CD, respectively.

3. Results and discussion

3.1. Optimization of the separation conditions for alcuronium, DAC and WCA in bulk material

In order to optimize the separation of alcuronium, DAC and WGA either the temperature (20 and 25 °C), the phosphate buffer concentration (50 and 100 mM) or the pH (in a range of 3.0 and 7.4) were varied whilst keeping the other parameter constant. Since the addition of cyclodextrins (CDs) to the BGE was reported to be advantageous with respect to the resolution, e.g. in the case of the separation of morphine derivatives [10], the quantification of gentamicin composition [11] and the evaluation of the stereoisomers of atracurium besylate isomers [12], a variety of neutral and negatively charged CDs was applied to enhance the resolution of the alcuronium related compounds.

The pH value has to be carefully chosen, because on the one hand the influence of the pH on the separation is very high. Especially, pH values higher than 4 create a high EOF and, thus, speed up the migration and often decrease the resolution. On the other hand, alcuronium is known to convert to DAC in acidic medium [2]. As a compromise, stock solutions of alcuronium were prepared in DMSO, the pH of 5.5 was applied as BGE. The peaks of alcuronium and DAC were baseline separated and no cyclization reaction was observed under the course of the CE measurement. The influence of the temperature on the resolution could be neglected. However, the temperature of 25 °C gave a shorter migration time and was, thus, used throughout all experiments. In addition, a buffer concentration of 50 mM was applied.

It was tried to shorten the migration time by increasing the pH (7.4; no cyclization of alcuronium!) and, along with this, the EOF. Since the separation is poor under these conditions, CD derivatives were added to the BGE in order to restore the resolution. The negatively charged CDs, HDAS-β-CD, SBE and sulf-β-CD gave longer migration times and, due to the neighborhood of the substance peaks and EOF, poor

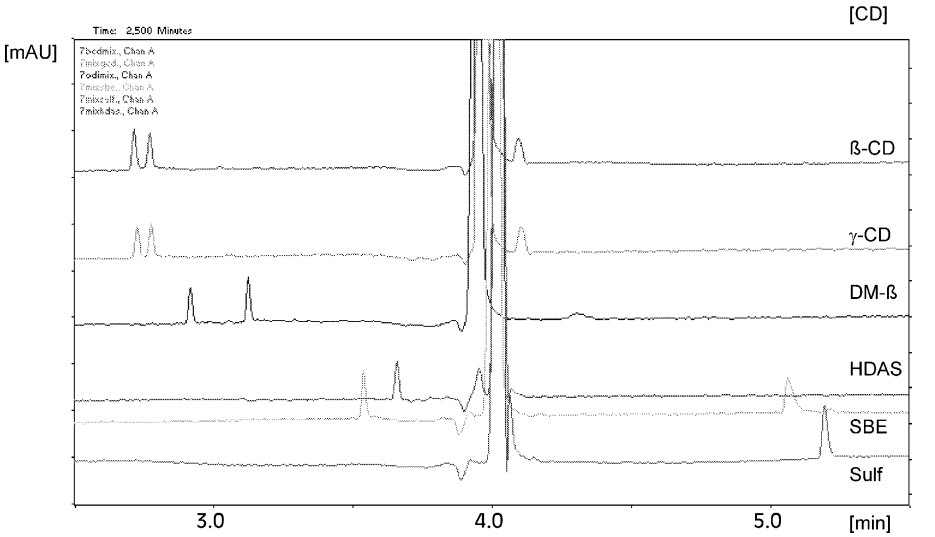


Fig. 1. Influence of various CD derivatives on the separation of alcuronium and DAC in 50 mM phosphate buffer at pH 7.4.

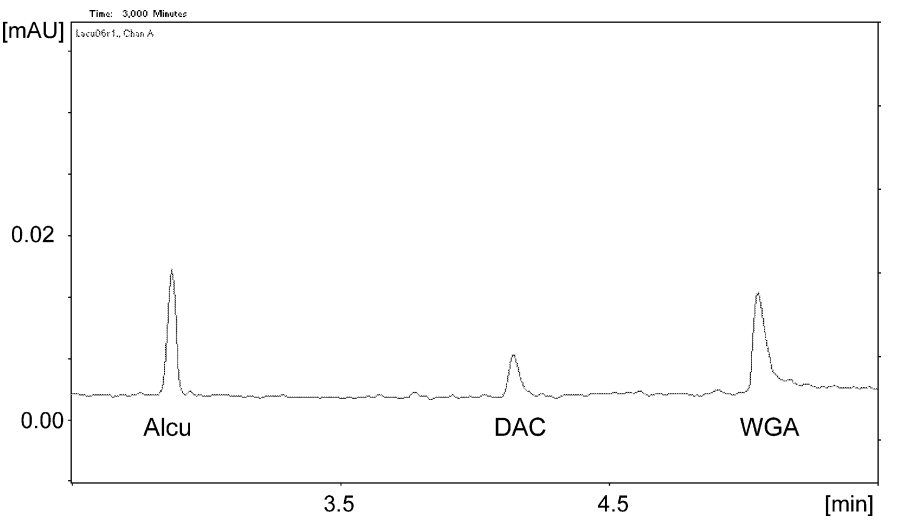


Fig. 2. Separation of alcuronium and its impurities under optimized conditions: temperature 25 °C, 50 mM phosphate buffer, pH value 5.5 and 12 mM DM-β-CD; Alc, alcuronium, DAC, diallylcaracurine and WGA, allyl-Wieland-Gumlich-aldehyde.

separations; the neutral CDs, β-CD, γ-CD and DM-β-CD, showed baseline separations in front of the EOF (see Fig. 1). Interestingly, the addition of β-CD and DM-β-CD changed the migration order (alcuronium before DAC) in comparison to experiments without or with other CDs (DAC before alcuronium). Taking the results together, the best resolution was obtained by adding 12 mM DM-β-CD.

Applying DM-β-CD, the pH value was optimized again resulting in the pH of 5.5 which was already found to be appropriate without the CD. The best separation conditions can be summarized as follows: temperature 25 °C, 50 mM phosphate buffer, pH value 5.5 and 12 mM DM-β-CD. Interestingly, the pH value, which was found to be optimal for CE experiments, is the same as described for the HPLC-method. Apply-

ing the aforementioned conditions alcuronium, the cyclization product DAC and the WGA could be separated within 5 min (Fig. 2).

In the PhEur the amount of one impurity is limited to 0.5%. The optimized separation conditions were used to determine the LOD for DAC in the presence of alcuronium. The amount of DAC was stepwise reduced while the amount of alcuronium was kept constant. The LOD was reached at 3.19 μg/ml which corresponds to 0.32%.

3.2. Stability of Alloferin® solutions under different conditions of storage

The Alloferin® ampoules (5 and 10 ml) in clinical practice consist of alcuronium 1 mg/ml, diethanolamine, sodium chloride, and hydrochloric acid in water. In order to quantify the impurities, the solutions were directly loaded in the capillary. An additional separation method was developed to study the stability Alloferin solution, because first studies revealed additional degradation products which have to be separated. In order to enhance the sensitivity of the method and to make possible a quantification below 0.5%, a capillary with internal diameter of 75 μm was applied. Using the buffer system of the injection solution, the BGE consisted of 50 mM diethanolamine (pH 9.2). The selectivity of the method was enhanced by addition of 5% v/v acetonitrile to the BGE as organic modifier and HDAS-β-CD as selector. Two concentrations of HDAS-β-CD were applied, 46 and 30 mg/ml. For the quantitative determination of the impurities an internal standard (IS) is needed. The structurally related compound allylstrychnine (AS) was chosen for this purpose. In order to estimate the content of the impurities in Alloferin® solutions, fresh solutions, solutions stored at 2–8 °C for 4 years as well as

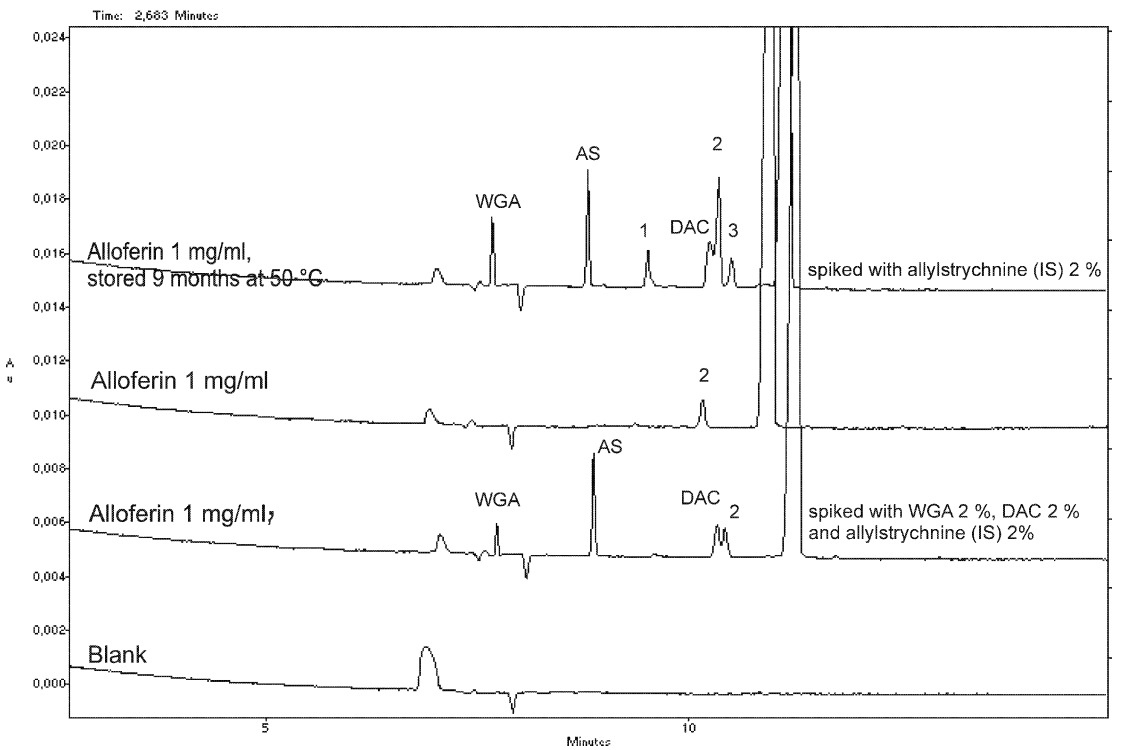


Fig. 3. Electropherograms of samples Alloferin 1 mg/ml. CE conditions: capillary: fused-silica 60.2/50 cm, 75 μm i.d.; running buffer: HDAS-β-CD 46 mg/ml in diethanolamine 50 mM, pH 9.2–acetonitrile 19:1; voltage: 20 kV; temperature: 25 °C; injection: 3448 Pa, 5 s; detection: UV, 254 nm. Peak identity: WGA, allyl-Wieland-Gumlich-aldehyde; AS, allylstrychnine; DAC, diallylcaracurine.

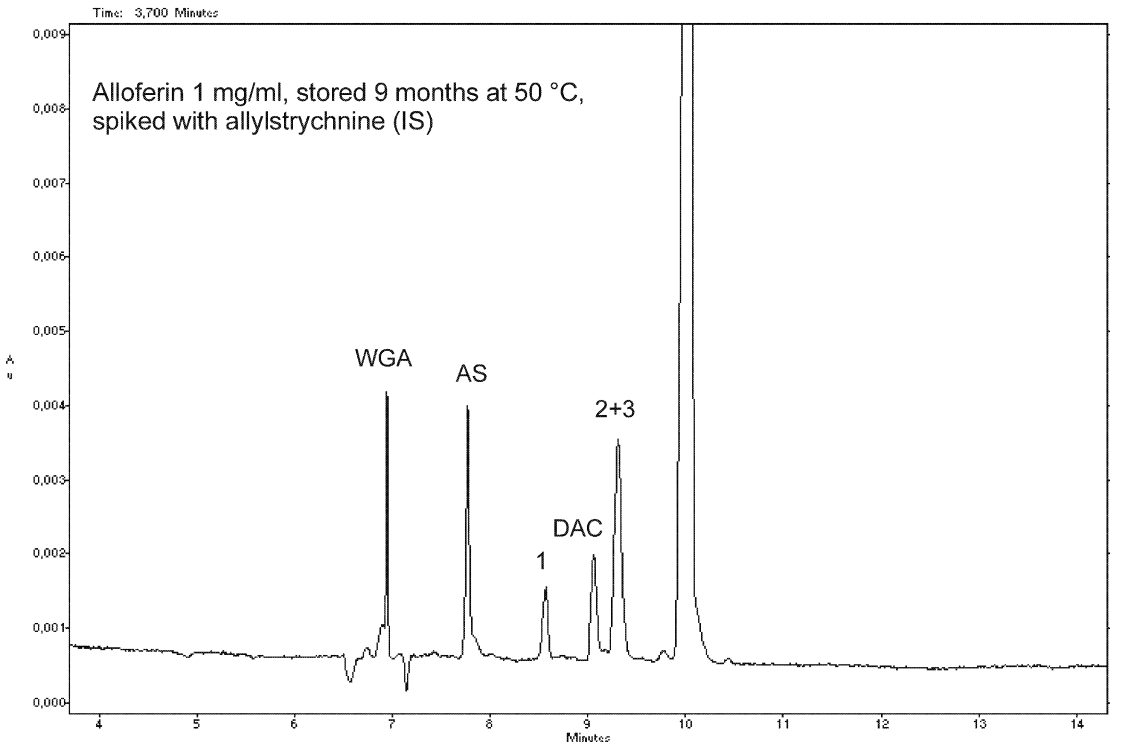


Fig. 4. Electropherograms of samples Alloferin 1 mg/ml. Running buffer: HDAS-β-CD 30 mg/ml in diethanolamine 50 mM, pH 9.2–acetonitrile 19:1. Other CE conditions and peak identity as in Fig. 3.

at room temperature and at 50 °C each for 9 months were investigated. Using 46 mg/ml of HDAS-β-CD (method 1) all impurities/degradation products and the IS were well separated from the main component alcuronium (Fig. 3). Interestingly, the Alloferin solution which was stored at 50 °C for 9 months showed three peaks in addition to DAC and WGA. Moreover, DAC and WGA were not found in the same batches of Alloferin solution which were stored at 2–8 °C or at room temperature. In order to improve the separation of DAC from peak 2, the following parameters were varied: voltage, temperature, the content of acetonitrile and the HDAS-β-CD in the running buffer. A baseline separation was achieved with 30 mg/ml HDAS-β-CD (method 2) using the same conditions as described for method 1 (see Fig. 4). Furthermore, the validation studies were performed with respect to DAC and WGA. LOD/limit of quantification, linearity, precision and the recovery were objects to study. The results, presented in Table 1, are adequate to quantification criterions of the ICH. For both impurities described in the PhEur 2001 the limit of quantification is less than 0.25%.

Both methods were applied to Alloferin solutions, stored for different periods at different temperatures. Representative results are displayed in Table 2. Only one impurity, peak 2, was found, when the samples were stored under prescribed conditions (2–8 °C). The content of this impurity remained below 1% (calculated as AS) during the shelf-life. The samples stored at higher temperatures showed two peaks corresponding to the cyclization product DAC and degradation product WGA (cf. [13]) in addition to three additional peaks (1–3, Fig. 3) whose identities are unknown. It is worth mentioning that the drug substance did not show any impurity.

Table 1
Validation data

	LOD/LOQ (% w/w)	Linearity: in the range from LOQ to 3% w/w as correlation coefficients	Precision: RSD in % from six replicate analyses at level 0.5% w/w	Recovery: confidence ($\alpha = 0.05$, $n = 9$), in % at three levels: 0.3, 1.0 and 3.0% w/w (three replicate each)
DAC (method 2)	0.05/0.17	0.996	1.83	±3.4
WGA (method 1)	0.07/0.23	0.998	2.13	±2.51

Table 2
Stability data of Alloferin 1 mg/ml solution for injection

Product	Batch/exp. date	Stored at (°C)	Impurities/degradation products (% w/w)				
			Allylated WGA	Peak 1 (calculated as AS)	DAC	Peak 2 (calculated as AS)	Peak 3 (calculated as AS)
Alloferin ICN 1 mg/ml, 5 ml	F001/07.2002 9 months before exp. date	2–8	–	–	–	0.78	–
		2–8 (20 h at 60 °C)	–	–	–	0.92	–
Alloferin ICN 1 mg/ml, 10 ml	B020/07.2000 14 months after exp. date	2–8	–	<0.1	–	1.2	–
		2–8 (9 months at 50 °C)	4.2	0.9	2.34	2.5	0.85
Alloferin Roche 1 mg/ml, 10 ml	01701/07.2000 13 months after exp. date	2–8	–	–	–	1.1	–

3.3. Interconversion of alcuronium and allylcaracurine

Amis et al. [2] already studied the ring closure and opening procedure of the alcuronium skeleton by means of the UV spectroscopy qualitatively. The aim of our CE investigations was to estimate the half lifes of alcuronium and DAC at pH 3 and 7.4, the latter is present at physiological media. It makes no difference from which compound the measurements were started. Applying acidic conditions the reaction is rather fast, the half lifes of both compounds amount to about 2 h and the equilibrium was characterized by 60% of alcuronium and 40% of DAC. In addition, after 24 h a third product of low intensity appeared which was not identified. The increase in pH slowed down the reaction. At pH 7.4 both alcuronium and DAC are stable for at least 48 h. Thus, the compounds are stable over the course of radioligand binding studies which are performed to determine the allosteric potency [9]. These half-quantitative results are in line with the findings reported by Amis. Therefore, no further investigations were performed.

4. Conclusion

Capillary electrophoretic methods were developed to quantify both impurities and degradation products in the alcuronium substance and Alloferin 1 mg/ml solution for injection. The methods can be applied to impurity tests of the drug product as 'at release' method as well as 'stability indicating' method.

Acknowledgements

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