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Determination of amlodipine enantiomers in pharmaceuticals using capillary electrophoresis separation and diode array detection

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The present work describes a capillary zone electrophoresis (CZE) separation technique coupled with on-capillary diode array detector (DAD) for highly reliable enantioselective determination of amlodipine (AML) in commercial tablets. For the separation of AML enantiomers, (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) was an appropriate chiral selector providing complete enantioresolution. Optimized separation conditions consisted of 50 mmol/l glycine-acetate buffer, pH 3.2, 50 mg/ml HP-β-CD. Hydroxyethylcellulose (HEC, 0.2% w/v) served as an electroosmotic flow (EOF) suppressor in this buffer. DAD detection was used for the characterization of the composition of separated zones according to differences in corresponding UV-VIS spectra (scanned in interval 200–800 nm). It was demonstrated, comparing reference and real spectra of the analytes, that the proposed separation method was selective enough to produce pure (non-mixed, i.e. spectrally homogeneous) analyte zones without any interfering compound. Successful validation and application of the proposed CZE-DAD method suggest its routine use in enantioselective control of AML in pharmaceuticals.

1. Introduction

In pharmacotherapy the use of single-enantiomer forms can often lead to an improvement in the efficacy of the drug or the suppression of side effects related to the other enantiomer (Daniels and Jorgensen 1982; Innes and Nickersen 1970). Amlodipine, (*RS*)-3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzenesulphonate, (AML), is a long-acting, dihydropyridine-type inhibitor of the slow calcium channel that is useful in the treatment of hypertension and coronary insufficiency. The calcium channel blocking activity resides primarily in the *S*-(−) AML enantiomer. Results of *in vitro* tests to determine the calcium antagonist activity of AML enantiomers against calcium-induced constriction of potassium-depolarized rat aorta was described by Arrowsmith et al. (1986). The authors allege that the *S*-(−) stereoisomer is twice as active as the racemic mixture in antagonizing calcium-induced constriction. Although *R*-(+)-amlodipine appears to have little activity as a calcium channel blocker, it is not pharmacologically inert, but rather it is a potent inhibitor of smooth muscle cell migration. Ideally, amlodipine should be used as *S*-(−) enantiomer being substantially free of the *R*-(+) enantiomer. It is obvious, from above mentioned facts, that the control of enantiomeric composition of pharmaceuticals containing AML is important.

Among high performance separation techniques, capillary electrophoresis (CE) is superior for the analytical separations of ionic compounds, and it can be advantageously used because of its extremely high peak efficiency, versatility, simplicity, short analysis time, good compatibility with aqueous samples and low consumption of chiral selector (low cost of enantioselective analyses). Some general concepts for CE enantioseparations of dihydropyridine

Table 1: Performance parameters of the CZE-DAD method and parameters of calibration lines of amlodipine enantiomers[a]

Parameter	AML1	AML2
t_m (min.)	8.406	8.689
s_{tm} (min.)	0.052	0.057
a (mAU)	14.67502	15.70010
s_a (mAU)	7.96881	8.52545
b (mAU · μg^{-1} · ml)	9.89880	10.59025
s_b (mAU · μg^{-1} · ml)	0.33929	0.36299
RSS	420.8	483.6
r^2	0.99708	0.99688
LOD (μg · ml^{-1})	2.41	2.53
LOQ (μg · ml^{-1})	8.05	8.42
N	20800	19000
H (μm)	7.2	7.9
Recovery (%)	97.6–99.3	97.1–98.5
Accuracy (RE) (%), mean	1.8	2.4
Robustness (ΔR) (%)	<6.7	
Enantioresolution, R	1.84	

AML1 = first migrating amlodipine enantiomer, AML2 = second migrating amlodipine enantiomer.
[a] Separating conditions as in Section 2. and other working conditions as in Experimental section. The concentration of AML in model sample was 75 μg/ml unless otherwise stated.

Table 2: Enantioselective determination of amlodipine in pharmaceuticals[a]

Parameter	Agen	Amilostad	Cardilopin	Norvasc
Determined content ± RSD (%)[b]	100.6 ± 1.2	99.4 ± 1.6	98.5 ± 1.4	99.7 ± 0.8
Enantiomeric ratio, (AML1)/(AML2)	50.5/49.5	49.2/50.8	51.1/48.9	50.7/49.3
PCC, (AML1), (AML2)[c]	0.99825, 0.99897	0.99934, 0.99872	0.99881, 0.99803	0.99789, 0.99818

AML1 = first migrating amlodipine enantiomer, AML2 = second migrating amlodipine enantiomer.
[a] Separating conditions as in Section 2., sample preparation procedures and other working conditions as in Experimental section. Concentration of AML in the injected samples was ~75 μg/ml.
[b] Average content of AML in the tested pharmaceuticals was calculated as a sum of both enantiomers (using their corrected peak areas) from six consecutive CZE runs. Declared content of AML in each pharmaceutical was 10 mg per 1 tablet.
[c] For the spectral characterization of AML peaks see Section 3.3.

derivatives by means of cyclodextrins (CDs) have been reported (Christians et al. 1999; Christians and Holzgrabe 2000). AML enantiomers have been successfully separated using CE techniques with charged and uncharged CDs as chiral selectors (Owens et al. 1998; Small et al. 1995; Wang et al. 2007; Xu and Wu 2004). Among CDs, α-CD, hydroxypropyl-β-cyclodextrin, sulphobutylether-β-CD and carboxymethyl-β-CD were effectively used (with the aid of electroosmotic transport and/or another coselector) for the enantioseparation of AML in model samples. One previous paper (Wang et al. 2007) deals with an enantioselective CE determination of AML in physiological sample (human serum). However, enantioselective determination of AML in pharmaceuticals has not been reparted so far.

The aim of the present work was to develop an enantioselective method for a highly reliable determination of AML enantiomers in pharmaceuticals (tablets), useful for routine use. To this purpose, enantioselective CZE separation was combined with diode array detection (DAD) enabling direct characterization of spectral purity (homogeneity) of separated zones and exclusion of mixed zones (spectrally inhomogeneous). Such an analytical approach (i.e. CE-DAD) is proposed as an advanced alternative for the enantioselective determination of drugs in comparison to CE combined with single wavelength UV detection.

2. Investigations, results and discussion

The principal operating parameters optimized in the present CZE separations were type and concentration of CD, carrier cation and counterion and pH of buffer. These parameters were optimized with respect to the maximal separation efficiency, ensured by the minimization of electromigration and thermal dispersion effects (the use of low-mobility/conductivity buffer constituents was favorable), and a sufficient enantioresolution of AML (neutral HP-β-CD was found to be more effective than native β-CD or ionizable CE-β-CD). Higher concentration of carrier cation helped to suppress AML adsorption onto the capillary wall. AML enantioresolution and analysis time increased with concentration of HP-β-CD. Lower pH values were favorable for increasing separation efficiency, enantioresolution and decreasing analysis time. Electroosmotic dispersion was successfully suppressed using methylhydroxyethylcellulose (m-HEC) as a capillary surface-coating additive. Optimized separating conditions, resulting in a baseline enantioseparation of AML, high separation efficiency and a short analysis time, consisted of 50 mmol/l glycine adjusted to pH 3.2 with acetic acid (168 mmol/l), 50 mg/ml HP-β-CD and 0.2% (w/v) HEC.

The optimized CZE method was validated (see Section 3.2.) and the resulting data are given in Table 1. These data clearly indicate that parameters of the proposed method, i.e. sensitivity, linearity, precision, accuracy/recovery and robustness, are favorable for its routine use in pharmaceutical analysis. This is obvious also from the good separation efficiency given by (i) the number of theoretical plates (N) as well as (ii) the height equivalent to one theoretical plate (H). The resolution of AML enantiomers (R) and overall analysis time is indicating the effective enantioseparation process.

The proposed and proven CZE-DAD method was applied in pharmaceutical analysis to determine the enantiomeric content of AML in various commercial pharmaceuticals. A representative electropherogram, shown in Fig. 1, illustrates CZE profile of sample prepared from tablets Agen® (similar results were obtained also for other pharmaceuticals tested).

The migration position of AML enantiomers in electropherograms was confirmed by spiking of real samples with the standard while the purity of zones of AML enantiomers in particular experiments (reflecting the achieved separation selectivity) was confirmed through their processed spectra (see Section 3.3.). It is shown in Fig. 2 that the spectra of AML enantiomers obtained in real pharmaceutical sample (tablets Agen) were identical to the reference spectra of AML enantiomers (similar results were obtained also for other pharmaceuticals tested). This observation was also supported mathematically via corresponding Pearson's correlation coefficients (PCCs). The PCC values higher than 0.99 (see Table 2) confirmed spectral identity of AML enantiomers present in pharmaceuti-

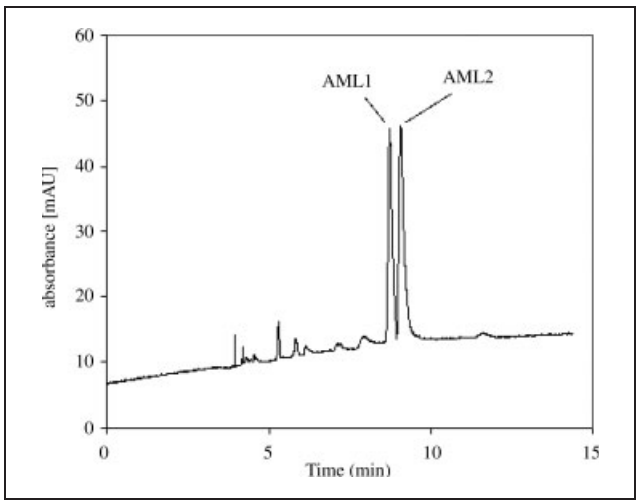

Fig. 1: Electropherogram from the enantioselective determination of AML in commercial pharmaceutical preparation (tablets Agen). The separating conditions as in Section 2. The driving current was stabilized at 150 μA and detection wavelength was set at 238 nm. The concentration of AML enantiomers in the sample was ~75 μg/ml. For the sample preparation and other working conditions see the Experimental section

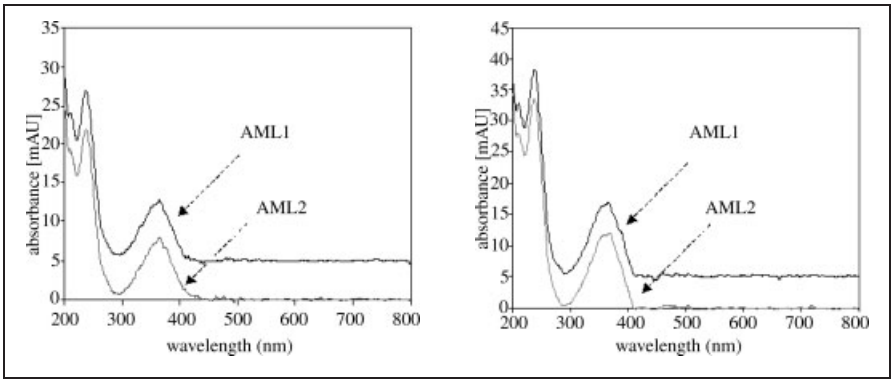

Fig. 2:
Processed UV-VIS spectra of AML enantiomers in different matrices. (a) AML present in demineralized water, serving as a reference spectrum, (b) AML present in extract of Agen tablets, at a 75 μg/ml concentration level of the drug in the samples. The upper traces belong to the first migrating AML enantiomer while the lower traces to the second migrating AML enantiomer. Separating, sample preparation and other working conditions as in Fig. 1. AML spectra were recorded in the interval of wavelengths of 200–800 nm. For the processing procedures of raw spectra see the Section 3.3

cal samples with the reference AML spectra. This reflected producing of the pure (non-mixed, i.e. spectrally homogeneous) zones of AML enantiomers in pharmaceutical matrices (i.e. no detection interferences from the sample solvent, impurities and dosage form excipients occurred in AML spectra in the interval of wavelengths of 200–800 nm) and, hence, the sufficient separation selectivity of the proposed method.

The approved method was successfully used for the determination of enantiomeric composition of AML in various commercial pharmaceuticals. The results confirmed the racemic composition of the active compound in the pharmaceuticals. The content of AML, obtained by the proposed method, was in a good agreement with that declared by the manufacturers (see Table 2).

It can be concluded that the proposed CZE-DAD method is useful for routine pharmaceutical applications with benefits of its effectivity, simplicity, cost and enhanced analytical/information value. Moreover, the method can be easily adapted to column-coupling arrangement with additional possibilities such as on-line sample purification and preconcentration and spread application range (direct ultratrace analyzes of complex samples, e.g. biological, with minimal sample preparation/handling).

3. Experimental

3.1. Instrumentation

A capillary electrophoresis analyzer EA-101 (Villa-Labeco, Spišská Nová Ves, Slovakia), assembled in the single column configuration of the separation unit, was used in this work for performing the CZE runs. The samples were injected by a 200 nl internal sample loop of the injection valve of the analyzer. The CZE column was provided with a 320 I.D. fused silica capillary tube of a 160 mm total length.

A multiwavelength photometric absorbance diode array detector Smartline PDA Detector 2800 (Knauer, Germany) was connected to an on-column photometric detection cell, mounted on the CZE column, via optical fibers. The detector operated under the following conditions: scanned wavelength range 200–800 nm; integration time 6 ms; scan interval 0.2 s; number of accumulations 1.

Prior to use, the capillary was not treated by any rinsing procedures to suppress an electroosmotic flow (EOF). A dynamic coating of the capillary wall by means of hydroxyethylcellulose (HEC 30 000; Serva, Heidelberg, Germany) in background electrolyte solution served for this purpose. The separating electrolyte in the capillary was replaced by a fresh one between each run. CZE analyses were carried out in the cationic regime of the separation (i.e. cathodic movement of the analytes) with direct injections of the samples. The experiments were performed in constant current mode at 20 °C. The driving current applied was 150 μA.

3.2. Data evaluation and performance parameters

The absorption maximum wavelength of AML (238 nm) was used for the evaluation of analytical parameters, as given in Table 1. The racemic AML standard (see Section 3.5.1.) was used for validation experiments and performance parameters were evaluated according to the ICH guideline (ICH Harmonised Tripartite Guideline). Peak areas of AML enantiomers were corrected for their migration times to compensate for their differential detector residence times (Huang et al. 1989). Parameters of calibration lines for AML enantiomers were calculated by using QCExpert ver.2.5 statistical software (Trilobyte, Prague, Czech Republic).

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as the ratio of standard deviation of y-intercept of regression line (s_a) and the slope of the regression line (b) multiplied by factor 3.3 (LOD) or 10 (LOQ).

Linearity, tested in the concentration range given in Section 3.5.1., was expressed via determination coefficient (r^2).

Precision was expressed via relative standard deviation of (i) intercept (s_a) and slope (s_b) of calibration lines, and (ii) migration times of AML enantiomers (s_{tm}) as well as via residual sum of squares (RSS). Each concentration point was measured from six consecutive CZE runs.

Recovery was evaluated by spiking of dosage form and water samples with AML at three different concentration levels, given in Section 3.5.1. (each point was measured six times), and comparing the peak areas of AML enantiomers obtained in the different matrices. Accuracy (expressed via relative error, RE) was evaluated through the recovery test.

Robustness test examined the effect that deliberate variations in operational parameters had on the analysis results, in our case, the enantioresolution (R).

3.3. Processing and comparing DAD spectra

The spectral data were acquired and processed by a EuroChrom program (version 3.05, Knauer). The raw spectra of AML enantiomers were processed in order to provide relevant spectral information (free of various noise effects).

The background correction, i.e. subtraction of background spectrum from the raw spectrum of the analyte (Strašík et al. 2003), was carried out to minimize the impact of the electrolyte system on AML spectrum. Such a corrected spectrum was further smoothed by the procedure of Savitzky-Golay (1964), implemented in EuroChrom software, with a 5-point window.

Homogeneity of spectra of AML enantiomers in real samples was expressed via PCCs (Miller and Miller 1993). The value of PCC higher than 0.99 is assumed to provide an acceptable certainty in a confirmation of the identity of the analyte (Strašík et al. 2003), i.e. a match of the tested (AML in dosage form) and reference (AML in water) spectrum.

3.4. Chemicals and samples

The electrolyte solutions were prepared from chemicals obtained from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, Arizona, U.S.A.). All chemicals used were of analytical grade or additionally purified by the usual methods (acetic acid was isothermally distilled). The solutions of electrolytes were filtered before the use through disposable membrane filters of a 0.45 μm pore size (Millipore, Molsheim, France).

Carrier electrolytes were prepared by titrating of glycine in presence of HEC by isothermally distilled acetic acid to reach a required pH. An appropriate amount of CD was then dissolved in these solutions in order to obtain the final separating buffers for the CZE stage.

Native β-cyclodextrin (β-CD) and (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), with the extent of labeling equal to 1.0 molar substitution dextrin, were obtained from Aldrich. Carboxyethyl-β-cyclodextrin (CE-β-CD), with the number of substituents on a CD ring (DS) 3 and CE purity, was obtained from Cyclolab (Budapest, Hungary).

Amlodipine mesilate monohydrate (AML) was obtained as a racemic reference substance from Zentiva (Prague, Czech Republic). Pharmaceutical tablets, Agen®, Amilostad®, Cardilopin® and Norvasc®, produced by Zentiva, STADA (Bad Vilbel, Germany), EGIS (Budapest, Hungary) and Pfizer (Kent, UK), respectively, were obtained from local drug stores. The declared content of AML in one tablet was 10 mg in every of the pharmaceuticals.

3.5. Procedures for sample and standard solution preparations

3.5.1. Standard solutions

The stock solution of AML reference substance was prepared by dissolving 10 mg of the powder in 10 ml of demineralized water and it was stored at −8 °C in the freezer. Working solutions were made by an appropriate dilution of the stock solution with demineralized water or by spiking AML from the stock solution into a dosage form.

The concentration levels of AML in the injected model calibration solutions (prepared in demineralized water) were in the range of 5–100 μg/ml (5, 25, 50, 75, 100), and each calibration point was determined six times.

For the recovery experiments, AML was spiked from its stock solution into real matrices (dosage forms). The samples with three concentration levels (10, 40 and 80 μg/ml) of AML standard were prepared.

For the robustness test, the deliberate variations in operational parameters were as follows: the concentration of complexing agent (48–52 mg/ml) and carrier cation (48–52 mmol/l), pH (3.1–3.3).

3.5.2. Sample preparation

Six pharmaceutical tablets were accurately weighed, powdered in a mortar and the amount of mass equivalent to one tablet content was dissolved in 10 ml of demineralized water. After 2 h of mechanical shaking, the mixture was ultrasonicated for 10 min and then centrifuged (8000 rpm) for 10 min. The resulting solution was appropriately diluted with demineralized water prior to the analysis and filtered through a 0.45 μm pore size (Millipore) before injection into the CE equipment.

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