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Development and validation of a capillary electrophoresis method with ultraviolet detection for the determination of the related substances in a pharmaceutical compound

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Abstract

A capillary electrophoresis (CE) method was successfully developed to quantify the impurity profile of a new substance of pharmacological interest: LAS 35917. CE method was developed in order to separate the chloromethylated, monomethylated and hydroxylated impurities (molecules with very similar chemical structures) having the three coelution in the reversed-phase LC method initially established. Taking into account the structure of the impurities of LAS 35917, separation by conventional liquid chromatography (LC) methods would be longer and tedious than separation by CE, which is an appropriate and versatile technique giving easier and quicker methods. Among the three potential impurities mentioned of LAS 35917, two are due to the synthesis route of this drug, and the third arises from degradation. These drug-related impurities were separated using a capillary of 56 cm of effective length and 50 μm I.D., a 60 mM tetraborate buffer, at pH 9.2, and a positive voltage of 20 kV. The optimised CE method was preliminary validated with regard to specificity, linearity, limits of detection and quantitation, repeatability and solution stability. The method allows the detection and quantitation of impurities above 0.04 and 0.08% level, respectively. All three related substances were separated, detected and quantified from their parent drug in the analysis of real samples of LAS 35917, stressed or not stressed, with this simple and fast CE method.
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Keywords: Pharmaceutical analysis; Validation; LAS 35917

1. Introduction

LAS 35917 (Fig. 1) is a compound belonging to the phenyltriazolopurine family, a selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5), synthesised by Almirall. This drug is intended for oral administration for the treatment of erectile dysfunction [1–3].

The development of efficient, selective and rapid separation methods requires optimisation of separation conditions especially in the preliminary steps of the development of a given drug. Likewise, fast optimisation of the separation conditions is essential in the purification and characterisation of newly synthesised products [4].

The identification and determination of impurities is an important aspect of drug analysis. An accurate analytical profile of a drug substance must fulfil the requirements of Regulatory Agencies with respect to toxicity and safety aspects from the initial stage of the development of a potential drug to the quality control of a marketed pharmaceutical product.

Liquid chromatography (LC) is an established method for the impurity profiling of drugs [5]. Chromatographic separation is based on the selective distribution of analytes between a liquid mobile phase and a stationary phase. One of the most important drawbacks of the bonded-phase materials is the limited practical pH range.

Nevertheless, the separation mechanism of capillary electrophoresis (CE) is based on differences in the charge-to-mass ratio, and is useful in a wide range of pH. Since its introduction into the analytical laboratory, CE has had to prove that it was capable of generating results

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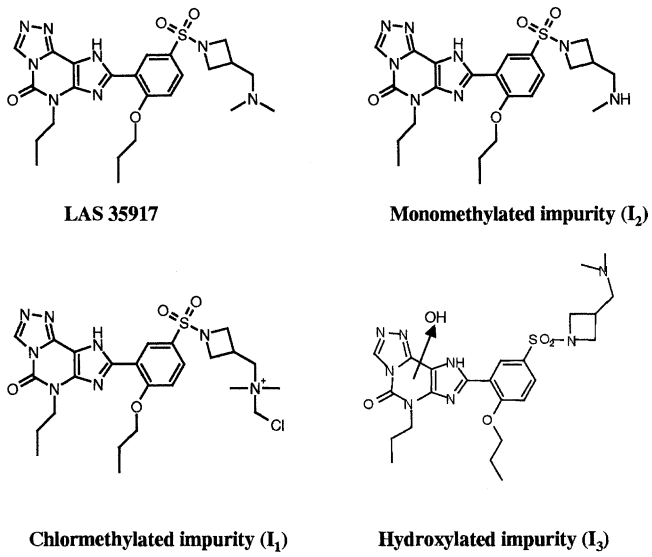

Fig. 1. Structures of LAS 35917 drug and the three impurities studied by the development of CE method.

comparable to LC technique. In some respects, this has been one of the greatest advantages of CE, and yet one of its greatest challenges. At the same time, however, LC is a well-developed tool, rugged and fully automated. Therefore, CE is presented with significant challenges relative to replacement of LC in those areas for which LC is not successful [6].

Consequently, CE is rapidly becoming a routine analytical technique for the complementary analysis of pharmaceutical drugs [7–13]. This technique is considered highly efficient, simple, selective, versatile and well-capable of simultaneously analysing both the level of the main component as well as closely related substances. Thus, nowadays CE is replacing and/or complementing conventional quality control of several products [14], and as a consequence it is now being described within the USA and European Pharmacopoeias (USP and Ph. Eur., respectively) [15,16].

The objective of this study was to develop and validate an analytical method based on CE to ensure the separation and quantitation of potential impurities arising from the parent drug synthesis (chloromethylated and monomethylated impurities, coded as I_1 and I_2, respectively) and its degradation product (hydroxylated impurity, coded as I_3) whose structure is not entirely known, having the three co-eluting in the LC method previously established. The identification of the impurities I_1, I_2 and I_3 was carried out by liquid chromatography coupled to mass spectrometry (LC–MS and LC–MS–MS). LC–MS was used in a large number of papers to identify related substances in peptides and pharmaceuticals [17–19]. Likewise, the impurity I_3 whose structure is not completely elucidated by LC–MS and LC–MS–MS is a substituted derivative with different possibilities of position in the hydroxyl group within the structure of LAS 35917.

2. Material and methods

2.1. Instrumentation

2.1.1. LC equipment

The chromatographic system used in the analysis of impurities of LAS 35917 consists of a 510 HPLC pump by Waters (MA, USA), a 486 UV detector and an WISP 917 autoinjector from the same company. Data were collected using Millennium software on a personal computer. The injection volume was 15 μl.

The chromatographic column was a 5 μm Kromasil (250 mm × 4.6 mm I.D.) (Aplicaciones Analíticas, Barcelona, Spain). The mobile phases were (A) 10 mM phosphoric acid, pH 7.0 and (B) [acetonitrile–methanol (65:35)]–water (90:10). The gradient elution is indicated in Table 2. The analyses were performed at room temperature at a flow rate of 1 ml/min. The monitoring wavelength was 220 nm.

2.1.2. LC–MS and LC–MS–MS equipment

The chromatographic system used in the identification of impurities of LAS 35917 consists of a 1100 LC/MSD Trap system by Agilent (Agilent Technologies, Waldbronn, Germany). Data were collected using LC/MSD Trap Software version 4.1 for data analysis on a personal computer. The injection volume was 15 μl. The monitoring wavelength was 220 and 334 nm.

The chromatographic column was a 5 μm Kromasil (250 mm × 4.6 mm I.D.) (Aplicaciones Analíticas). The mobile phases were (A) sodium acetate 0.2% (v/v), pH 7.0, (B) methanol and (C) acetonitrile. The gradient elution is indicated in Table 2. The analyses were performed at room temperature at a flow rate of 1 ml/min.

The mass spectrometer was equipped with an electrospray ionisation source and an ion trap as mass analyser. Optimal source working conditions for monitoring ions were as follows: capillary voltage was fixed at 4000 V, dry temperature was 350 °C. The nebuliser and drying gases were both nitrogen. The nebulising pressure and the drying gas flow rate were set at 40 p.s.i and 8.0 l/min, respectively (1 p.s.i. = 6894.76 Pa).

Full scan positive, negative and alternating ion mass spectra were acquired over the mass range *m/z* 100–1100. In all cases, the acquisition was performed in centroid mode, a target of 40.000, maximum accumulation time of 300 ms, a target mass of 400, a stability of 25 and a trap drive level of 100.

2.1.3. CE equipment

An Agilent ^{3D}CE system equipped with a diode array detector was used for the CE experiments. The detection window was positioned at 56 cm of the capillary. Separations in CE were carried out in 50 μm I.D. fused silica (FS) capillary with extended light path (Agilent Technologies), with a total length of 64.5 cm and an effective length of 56 cm.

Electrophoretic separations were performed using the Agilent ^{3D}CE (Agilent Technologies) system. It consists of a diode array detector and ChemStation Software for data analysis. The detection wavelength was set at 220 nm (bandwidth 15 nm) for data acquisition and a reference wavelength at 450 nm (bandwidth 80 nm). Samples were injected hydrodynamically at 50 mbar for 5 s. All experiments were conducted under normal polarity. The capillary was thermostated (air ventilation) at 25 °C when not indicated. The pH of the background electrolyte (BGE) was measured with a Crison pH meter, Model micropH 2002 (Crison Instruments, Barcelona, Spain). A maximum of five injections were performed from each set of electrolyte vials. The volume of electrolyte in the electrolyte vials was about 1.5 ml.

2.2. Chemicals and reagents

LAS 35917 was synthesised at Almirall, S.A. Orthophosphoric acid, triethylamine, hydrochloric acid and citric acid were supplied by Scharlau Chemie (Barcelona, Spain), triethanolamine was purchased from Aldrich Chemie (Steinheim, Germany). Sodium hydroxide and disodium tetraborate decahydrate were supplied by Merck (Darmstadt, Germany). Internal standards (IS): caffeine, 2,5-difluorophenol and 4-aminobenzoic acid were purchased from Aldrich Chemie and 2-amino-4-picoline was prepared by Almirall. α-, β- and γ-Cyclodextrins (α-, β- and γ-CDs) and derivatized CDs (β-methylated-CD, β-dimethylated-CD, β-hydroxypropylated-CD, β-carboxymethylated-CD, γ-methylated-CD, γ-hydroxypropylated-CD) were purchased from Cyclolab (Budapest, Hungary). HPLC-grade acetonitrile, methanol and isopropanol solvents were purchased from Scharlau Chemie. HPLC-grade water was provided by a Milli-Q system gradient A10 (Millipore, Bedford, MA, USA). Samples were prepared at a concentration of 2.5 mg/ml in 10 mM phosphoric acid as solvent. Related substances of LAS 35917 (intermediates of synthesis of LAS 35917 and monomethylated impurity) were supplied by Almirall, S.A.

2.3. Capillary treatment and optimal conditions

New capillaries were activated by flushing them for 20 min with aqueous 0.1 M NaOH, followed by 15 min with water and 30 min with background electrolyte. Before starting, the capillary was conditioned daily, rinsing successively for 5 min with water, 15 min with aqueous 0.1 M NaOH, 10 min with water and 20 min with BGE.

Before each run the capillary was preconditioned by flushing with water (1 min), 0.1 M sodium hydroxide (2 min), water (3 min) and BGE (3 min), in order to equilibrate it and thereby minimise hysteresis.

The optimum separation buffer was 60 mM sodium tetraborate (TB) at pH 9.2 with a positive power supply of 20 kV. Injection of the sample was achieved hydrodynamically by applying 50 mbar pressure for 5 s, followed by 1 s more of water injection (50 mbar). The analysis temperature was set at 25 °C. Direct UV detection was achieved at 220 nm (bandwidth 15 nm), with a reference at 450 nm (bandwith 80 nm). The current was monitored for the BGE evaluated, and was in the range 50–55 μA.

2.4. Preparation of buffer, sample and standard solutions

Unless otherwise noted, all analytes were dissolved in 10 mM phosphoric acid.

2.4.1. Background electrolyte

A solution of 60 mM of TB with unadjusted pH was prepared by weighing accurately 2.25 g of disodium tetraborate decahydrate. The amount indicated was transferred to a 100 ml volumetric flask and dissolved using water as solvent and the aid of an ultrasound bath (10 min).

2.4.2. Internal standard solution

Weigh accurately about 50 mg of 4-aminobenzoic acid, dissolve in and dilute to 20.0 ml with 10 mM phosphoric acid.

2.4.3. Dilute internal standard solution

Dilute 5.0 ml of internal standard solution to 50.0 with 10 mM phosphoric acid.

2.4.4. Qualitative standard

A solution of approximately 0.025 mg/ml of LAS 35917 and its monomethylated impurity, containing the internal standard at the same concentration, was prepared for qualitative purposes. This impurity was selected due to its practically identical structure with the parent drug, and its solution remains stable stored in a refrigerator for 1 month.

2.4.5. Quantitative standard

A solution of 0.025 mg/ml of LAS 35917 containing the internal standard at the same concentration was prepared.

2.4.6. Samples

For the examination of the impurity profile by CE, several batches of stressed or unstressed LAS 35917 were used. All samples were stored in the refrigerator when not in use. The samples were prepared at the concentration of 2.5 mg/ml and containing the internal standard at 0.025 mg/ml concentration. For this, approximately 50 mg of sample were weighed and transferred to a 20 ml volumetric flask. 2.0 ml of dilute internal standard solution and approximately 15 ml of 10 mM phosphoric acid were added. The sample was dissolved with the aid of ultrasound (5 min). The room temperature was allowed to be reached and was made up to volume with the same solvent. The analysis was carried out in duplicate.

Prior to use all buffer solutions and samples were filtered through a 0.45 μm poly(vinylidene difluoride) (PVDF) filter

(Millex-HV, Millipore). They were freshly prepared every day.

2.5. Calculations

Significant amounts of the impurities to make validation are not available, and in consequence the validation and the determination of the impurities is carried out with the standard sample of LAS 35917 and response factor of LAS 35917, respectively.

The response factor of LAS 35917 is calculated using the following formula:

$$F_R = \frac{WRA_{PI}t_{mp}}{D_1 A_p t_{mPI}}$$

where F_R is the response factor of LAS 35917, W the mass of the sample in the preparation of the standard solution, D_1 the volume of solvent used in the preparation of the standard in ml, R the assay relative to unity of the standard expressed as base, A_p the area of the LAS 35917 peak in the electropherogram of the standard, A_{PI} the area of the peak of the internal standard in the electropherogram of the standard, t_{mp} the migration time of LAS 35917 in the electropherogram of the standard and t_{mPI} the migration time of the internal standard in the electropherogram of the standard.

The response factor used is the mean of the initial and final standards.

The percentage (%i) of each impurity is calculated as follows:

$$\%i = \frac{F_R A_i t_{mPI} D_2}{A_{PI} t_{mi} W} \times 100$$

where %i is the percentage of impurity "i", F_R the response factor of LAS 35917, A_i the area of impurity "i" in the electropherogram of the sample, A_{PI} the area of the internal standard in the electropherogram of the sample, t_{mPI} the migration time of the internal standard in the electropherogram of the sample, t_{mi} the migration time of impurity "i" in the electropherogram of the sample, W the mass of the sample in mg expressed as base and D_2 the volume of solvent mixture used in the preparation of the sample in ml.

"Total CE" is the sum of the %i corresponding to I_1, I_2 and I_3.

3. Results and discussion

The purpose of this study was to show the method development strategies for the separation and quantitation of related substances of LAS 35917 that cannot be determined separately by conventional LC method. Taking into account the very much alike structure of LAS 35917 and its impurities together with the similarity of log D values in the overall pH scale (Fig. 2), separation by LC would be more difficult than separation by CE.

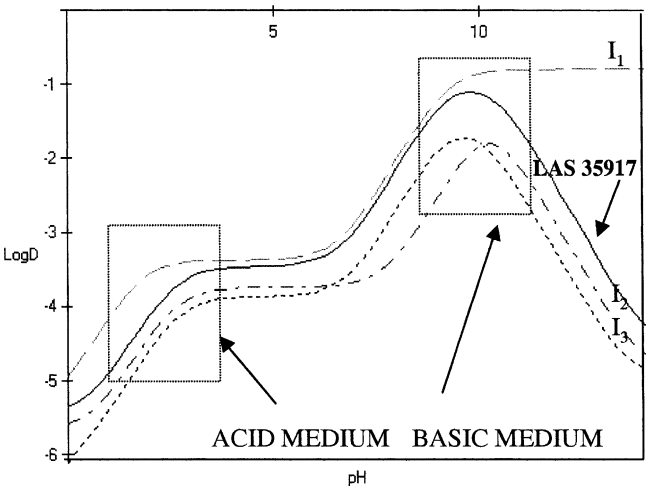

Fig. 2. log D vs. pH for LAS 35917 and its chloromethylated (I_1), monomethylated (I_2) and hydroxylated (I_3) impurities.

The structure of LAS 35917 and its impurities (pK_a and log D) renders the use of CE as an appropriate technique. The values of experimental pK_a values of LAS 35917 are shown in Table 1. The representation of theoretical log D values versus pH of the studied impurities and LAS 35917 is shown in Fig. 2. The separation of the three impurities and LAS 35917 is possible in both acid and basic media, marked in Fig. 2, because LAS 35917 and its impurities present more differences in log D. Likewise, it can be observed from Fig. 2 that the conditions of separation obtained in basic medium (pH 9.2), were slightly better than in acidic medium. CE allows work with both media, differently of the conventional HPLC columns (by degradation of the silica stationary phase at basic pH).

To check these theoretical results, the optimisation of the BGE was performed using the following acid and basic conditions:

(A) To optimise the BGE in acid conditions several parameters such as ionic strength, pH, cyclodextrin type and concentration, organic solvents and temperature were varied in order to evaluate the separation of the impurities from LAS 35917.

(B) For the optimisation of the BGE at basic conditions, parameters such as ionic strength, pH, voltage, injection time, capillary conditioning (initial and before each run) and internal standard were modified in order to optimise the separation of the impurities mentioned from LAS 35917.

Table 1
Potentiometric pK_a values of LAS 35917 and their corresponding equilibria (Sirius Analytical Instruments)

pK_a	Equilibria
2.06	$XH_3^{2+} \Leftrightarrow XH_2^{+} + H^{+}$
7.64	$XH_2^{+} \Leftrightarrow XH + H^{+}$
8.52	$XH \Leftrightarrow X^{-} + H^{+}$

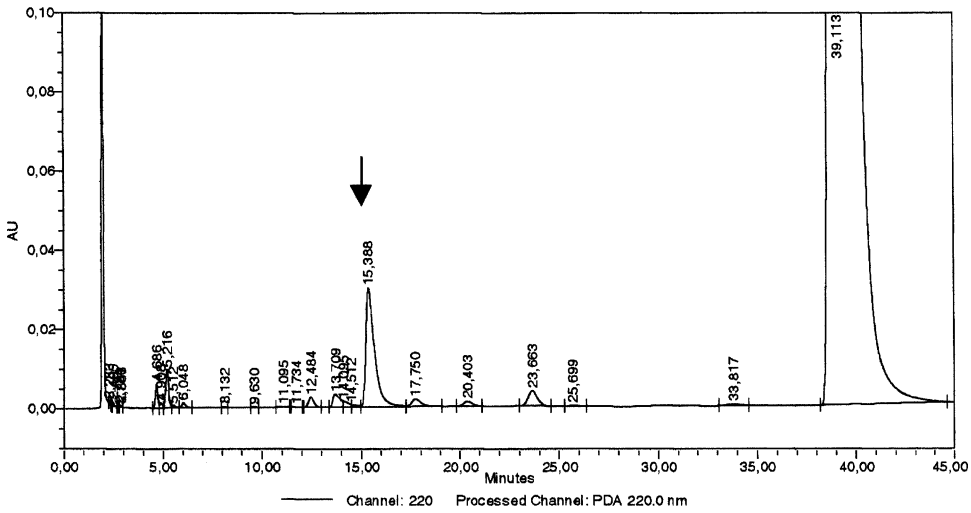

Fig. 3. Chromatogram of a LAS 35917 drug sample submitted under 80 °C of temperature for 2 months at the optimal LC conditions. Chromatographic system: column: Kromasil C_{18}, 250 mm × 4.6 mm. Mobile phase: (A) 10 mM phosphoric acid pH 7.0 and (B) [acetonitrile–methanol (65:35)]–water (90:10). Injection volume 15 μl, wavelength 220 nm. Gradient as indicated in Table 2.

3.1. Development and optimisation of the analysis method

3.1.1. LC background

The coelution of LAS 35917 impurities (monomethylated, hydroxylated and chloromethylated impurities) obtained by the LC method is shown in Fig. 3. It can be seen that all of them coelute at a relative retention time (t_{rr}) of 0.38. The chromatographic system and experimental conditions used for the analysis of LAS 35917 are indicated in Section 2.1, Table 2 and in the caption of Fig. 3. Unless otherwise noted, all samples were analysed with the same chromatographic system indicated in Fig. 3.

3.1.2. Impurities elucidation

The structure of the impurities was elucidated by LC–MS and LC–MS–MS taking into account the difference between the molecular mass of LAS 35917 and each impurity. Nevertheless, the position of the –OH group (corresponding to hydroxylated impurity) is yet not clear.

The LC–MS technique was used, on the one hand, to identify the impurities coeluting in the LC method; and on the other hand, to obtain the impurity semi-quantitation (quantitation with normalised areas of peaks). In the case of LAS 35917 it was not possible to isolate some of the impurities (such as hydroxylated and chloromethylated impurities), so making LC–MS very useful for the corroboration of the CE results.

Consequently the identification of such impurities by the CE method was performed by means of the relative migration times (t_{mr}) and with the analytical aid of semi-quantitation obtained with LC–MS. So that, samples containing only impurity I_1 or I_3 (identified by LC–MS system), respectively, were injected on CE system. In this way, the peak obtained on the LC–MS system were corresponded to the only impurity observed on CE system. Thus, the semi-quantitative analysis by LC–MS of this impurity was equivalent (in terms of normalised area of peaks) to the analysis (in terms of normalised area of peaks) obtained by CE method.

No significant amounts of I_2 were obtained as isolated form, so that, the identification of this impurity by CE was performed only by means of the relative migration time.

Table 2
Gradient A used for the LC analysis and gradient B used for the LC–MS–MS analysis of LAS 35917 drug at optimal conditions

Time (min)	Gradient A[a]		Gradient B[b]		
	Mobile phase A (%)	Mobile phase B (%)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)
0	55	45	60	14	26
40	55	45	60	14	26
45	20	80	28	25	47
55	20	80	28	25	47
57	55	45	60	14	26
70	55	45	60	14	26

[a] Chromatographic system: column: Kromasil C_{18}, 250 mm × 4.6 mm. Mobile phases: (A) 10 mM phosphoric acid, pH 7.0 and (B) [acetonitrile–methanol (65:35)]–water (90:10). Injection volume: 15 μl, wavelength 220 nm.

[b] Chromatographic system: column: Kromasil C_{18}, 250 mm × 4.6 mm. Mobile phases: (A) 10 mM sodium acetate, 0.2% (v/v), pH 7.0, (B) methanol and (C) acetonitrile. Injection volume: 15 μl, wavelength 220 and 334 nm.

Table 3
Range of optimised CE parameters in acid and basic conditions

Acid conditions, buffer: phosphate	Range	Basic conditions, buffer: borate	Range
Ionic strength	25, 50, 100 and 150 mM	Ionic strength	20, 40 and 60 mM
pH	2.5, 3.0 and 3.9	pH	8.5, 9.2 and 9.5
CD type	α-,β- and γ-CDs, β-methylated-CD, β-dimethylated-CD, β-hydroxypropylated-CD, β-carboxymethylated-CD, γ-methylated-CD, γ-hydroxypropylated-CD	Voltage	20 and 30 kV
CD concentration	10, 25 and 50 mM	Injection time	5 and 10 s
Organic solvent	5, 10 and 20%	Internal standard	Caffeine, 2,5-difluorophenol, 4-aminobenzoic acid, 2-amino-4-picoline
Temperature	15 and 25 °C		

3.1.3. Development and optimisation of the electrophoretic method

Several batches of stressed or unstressed LAS 35917 were analysed by LC–MS [20], so obtaining a semi quantitative impurity profiling for the three related substances studied [5]. The impurity profiling obtained by CE was compared with that obtained by LC–MS in order to corroborate the identification of the two impurities named as I_1 and I_3 without synthetic isolation.

Since the best signal-to-noise ratio obtained with the UV spectrum was at 220 nm, this wavelength was used throughout the study.

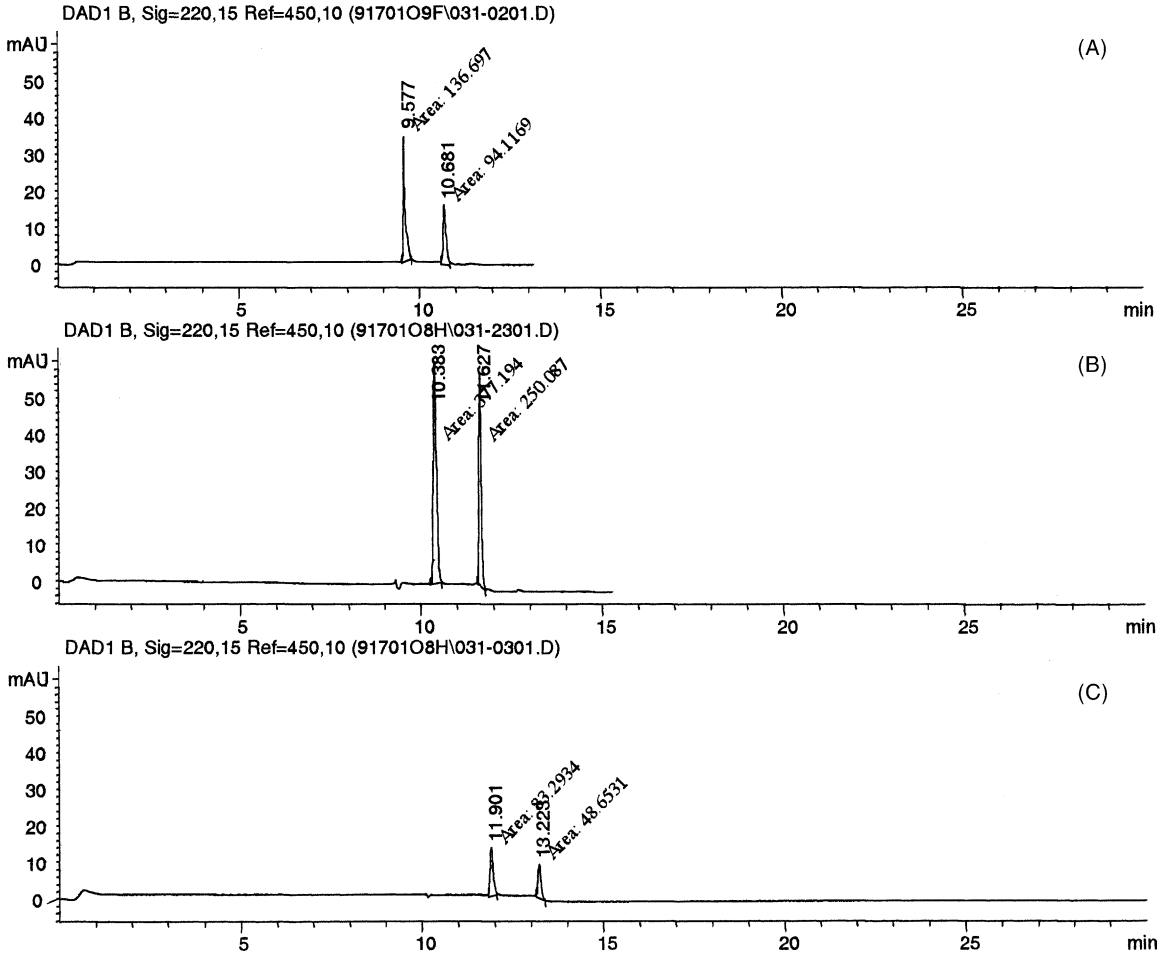

Fig. 4. Effect of the pH on the separation of a qualitative standard solution with the BGE 60 mM tetraborate at (A) pH 8.5, (B) pH 9.2 and (C) pH 9.5. Voltage 20 kV, temperature 25 °C, wavelength 220 nm, hydrodynamic injection 5 s.

The resolution between LAS 35917 and its monomethylated impurity is thoroughly discussed because this separation is probably one of the critical points in the method development. Once the separation between these two peaks was achieved, the optimised method was demonstrated to be useful for the separation of other impurities which show coelution in the common reversed phase LC method (hydroxylated and chloromethylated impurities). Consequently, for the optimisation of the CE method several variables were evaluated.

The three impurities studied were coded as I_1, I_2 and I_3 depending on the order of their migration. They correspond to chloromethylated, monomethylated and hydroxylated impurities, respectively. I_1 and I_2 are impurities formed during the synthetic process of LAS 35917. Nevertheless, I_3 appears only when the LAS 35917 is under stressed conditions of temperature (40 and 80 °C), probably due to the presence of water molecules in the hydration sphere of the LAS 35917 molecule.

Separation in acid medium was investigated by varying several parameters such as ionic strength, pH, cyclodextrin type and concentration, organic solvent type and concentration as well as temperature, Table 3. A solution consisting of LAS 35917 and monomethylated impurity was used to check the initial impurity profiling. Once the separation between these two compounds was achieved, stressed or unstressed real samples were analysed to check the separation of LAS 35917 from the other two impurities (hydroxylated and chloromethylated impurities).

A 50 mM phosphate buffer with pH 3.0 adjusted by triethanolamine was used as starting BGE. The variation in the ionic strength and pH of the buffer was unable to resolve the impurities of interest from the LAS 35917.

On the other hand, the addition of a native cyclodextrin, such as beta-cyclodextrin (β-CD) and the alkylated CD such as dimethyl-β-CD (DM-β-CD), to the BGE allowed a slight resolution by optimising the cyclodextrin concentration. These buffer additives can be employed to change the selectivity of the separation In this sense, most of the CD added to BGE only affected the migration times of LAS 35917 (increasing migration times) but did not have any effect on impurities resolution. In consequence, the interaction of the CD with the three impurities is similar and non-selective.

The addition of organic modifiers such as methanol (MeOH), acetonitrile (ACN) and isopropanol (IPA) at concentrations of up to 20% (v/v) was also tested. The addition of small amounts of acetonitrile to the background electrophoretic buffer slightly improved the separation of LAS 35917 from its methylated impurity. Similarly, the addition to the BGE of 20% (v/v) of each solvent studied resulted in resolution loss. Organic solvents interact with the capillary wall and, as a result of that slows down the electroosmotic flow (EOF). Acetonitrile is the solvent that slows down EOF less than the other alcohols, and consequently its effect on the resolution is sometimes different. This could explain

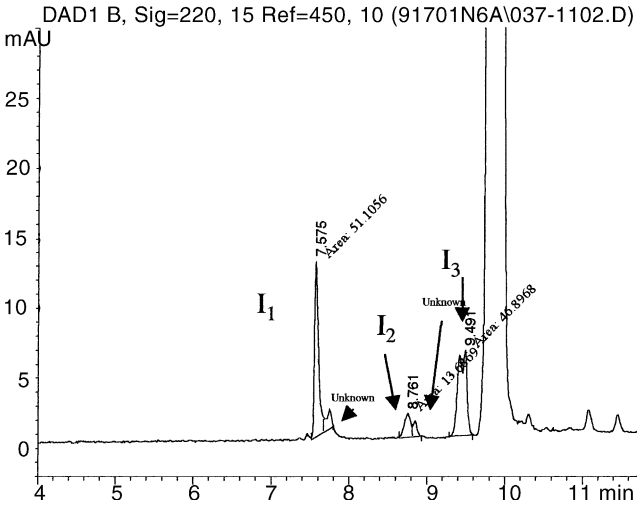

Fig. 5. Electropherogram corresponding to the stressed sample of LAS 35917 drug under 40 °C for 3 months at the optimal conditions explained in Section 2.5.

the improvement on the resolution obtained in the case of acetonitrile [6].

Furthermore, the effect of temperature on separation was studied with the BGE optimised up to date. In this way, the resolution was not improved, and consequently the initial working at 25 °C temperature was selected as the optimum temperature for sample analysis.

The resolution obtained in acid conditions of the BGE for LAS 35917 and its monomethylated impurity was only partial, and as a result of this, a BGE with basic pH was selected in order to improve separation. An excellent separation of LAS 35917 from its monomethylated impurity was obtained with a 40 mM tetraborate buffer without adjusting the pH and with a short analysis time.

In order to improve separation, the applied voltage was varied from 30 to 20 kV. The separation improved by applying the latter capillary voltage. The applied voltage was linear from 7 to 30 kV and the Joule heating was within the limits for the given type and length of capillary.

An ionic strength of 60 mM of tetraborate buffer was selected since a better symmetrical electrophoretic peak was obtained for LAS 35917 than with the other ionic strength. The effect of the ionic strength on the mobility has been reported by several authors [21,22]. But, the reason of this symmetrical peak for LAS 35917 could be related to de-

Table 4
Migration times (t_m) and relative migration times (t_{mr}) of the related substances of LAS 35917 drug

Compound	t_m (min)	t_{mr}
Chloromethylated impurity	7.7	0.35
Monomethylated impurity	9.0	0.41
Hydroxylated impurity	9.5	0.43
4-Aminobenzoic acid (IS)	22.0	1.0
LAS 35917	10.0	0.45

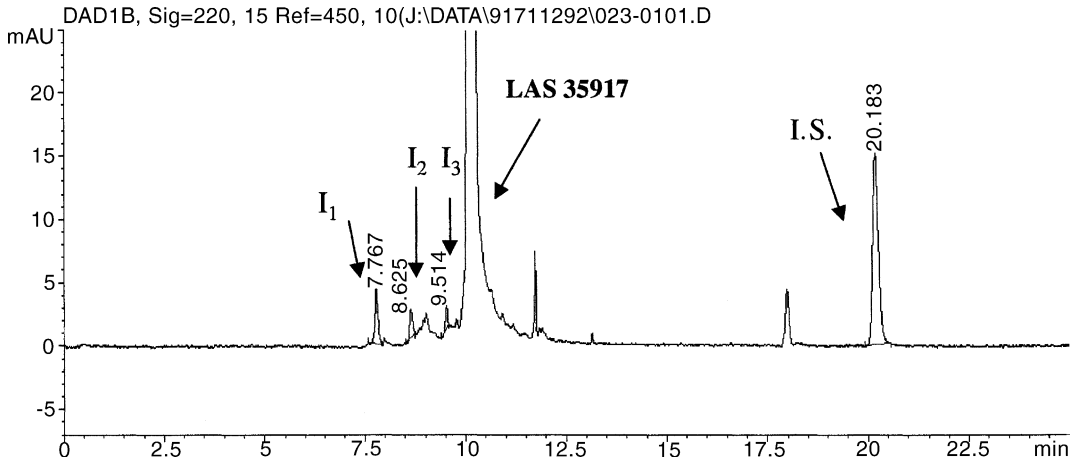

Fig. 6. Electropherogram corresponding to the unstressed batch of LAS 35917 drug at the optimal CE conditions explained in Section 2.5. I.S., internal standard.

crease of the interactions with the capillary wall when the buffer concentration increases.

Similarly, the pH of the buffer was changed from 8.5 (adjusting with HCl) to 9.5 (adjusting with NaOH). As can be seen in Fig. 4, the migration times for LAS 35917 and its monomethylated impurity decrease at a lower pH and increase at higher pH. This result could be explained taking into account the deprotonation of the cited compounds when the pH of the buffer increases.

A 60 mM sodium tetraborate buffer was selected for the separation of impurities of LAS 35917. Nevertheless, using an electrolyte with a optimum pH close of the pK_a for LAS 35917 (optimal pH 9.2 and pK_a = 8.5) could cause an unrobust method.

Therefore, a 60 mM sodium tetraborate buffer with pH unadjusted was found to provide the best option for the determination of electrophoretic profiling impurities of LAS 35917. Electropherograms of real samples of LAS 35917 where the studied impurities are separated can be observed in Fig. 5. The migration times for the identified impurities in the CE method are given in Table 4. In Fig. 5 impurity I_3 shows two overlapped peaks, corresponding to different isomers which can be explained by taking into account the different hydroxylation positions of this

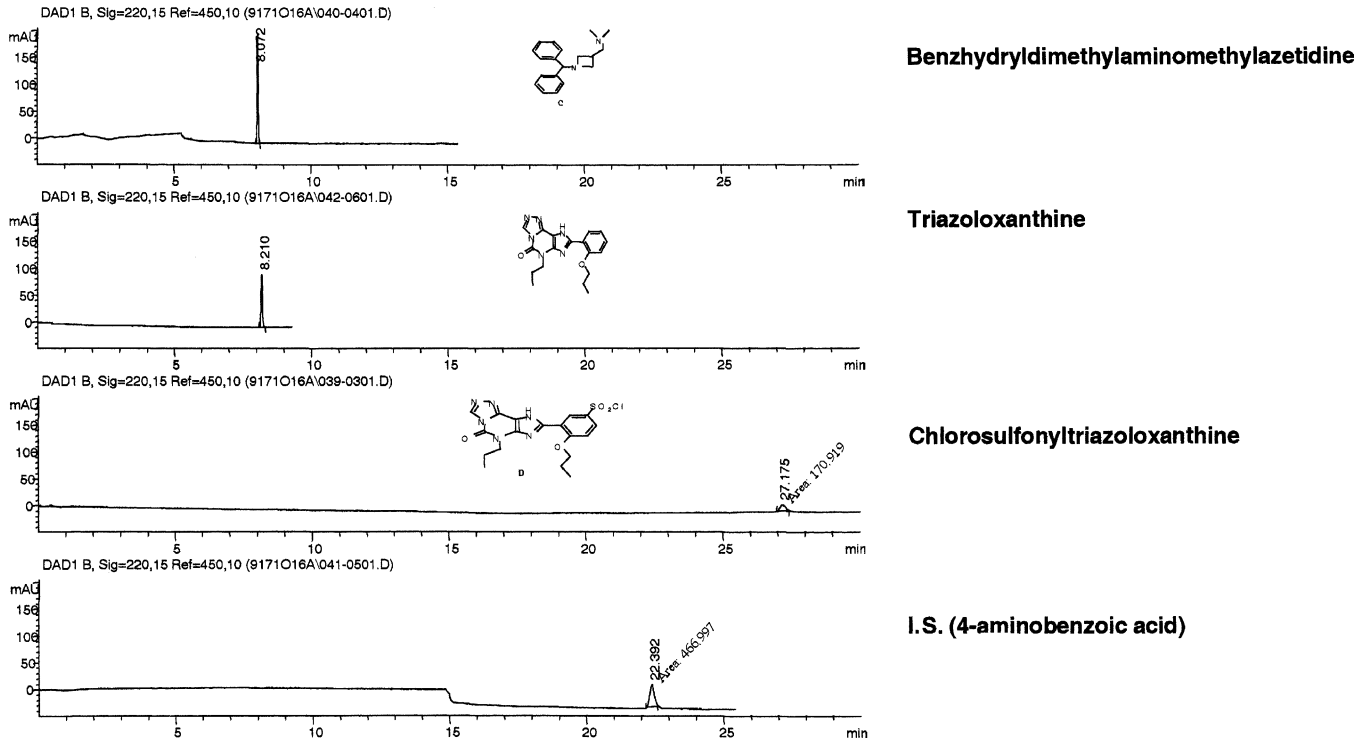

Fig. 7. Electropherograms of the synthesis intermediates of LAS 35917. I.S., internal standard.

degradation product. On the other hand, in Fig. 5 impurities I_1 and I_2 also show two overlapped peaks that would arise from another impurity present in the real samples analysed.

As it has been predicted from Fig. 2, the conditions of separation in the basic media (optimal pH) are slightly better than in acidic medium because LAS 35917 and its impurities have more differences in log *D*.

Likewise, the migration time of these three impurities could be explained by its different electrophoretic mobility. The structure of the impurities is very similar, nevertheless, the net charge differs in these impurities. In this way, the I_1 present an additional positive charge due to the position of the quaternarium amine group and this one could explain its early migration time. The impurity I_2 presents a secondary amine instead of the tertiary amine of LAS 35917, having a lower negative charge than the LAS 35917. Finally the I_3 is the impurity with the net charge more similar to LAS

Table 5
Quality parameters of CE method for the analysis of LAS 35917 drug

Linear range (μg/ml)	2–65
Correlation coefficient	0.9996
Slope	0.0271
Intercept	0.0025
LOD (S/N = 4.5) (μg/ml)	1.00
LOQ (S/N = 12.0) (μg/ml)	2.00
%R.S.D. *A* (*n* = 7)	1.51
%R.S.D. t_{mr} (*n* = 7)	1.10

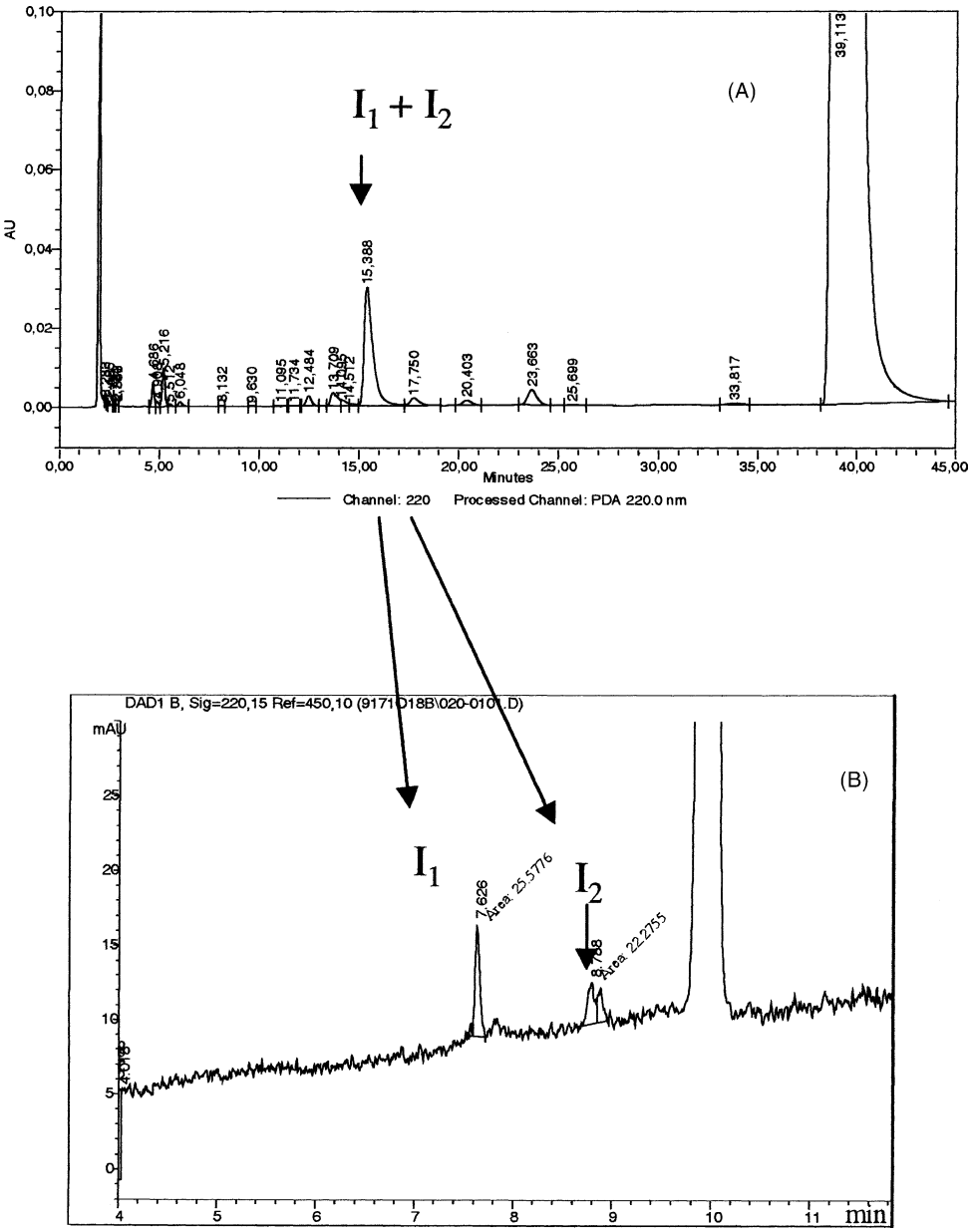

Fig. 8. Chromatogram and electropherogram of a batch of LAS 35917 drug. Optimal LC and CE conditions indicated in the caption of Fig. 3 and Section 2.5, respectively. IS, internal standard.

35917, so that would explain the migration order obtained at the optimal conditions.

Other variables such as the internal standard were also studied in order to quantify the desired impurities by means of a standard method. Several compounds were evaluated with the optimised method. For this, 2-amino-4-picoline, caffeine, 2,5-difluorophenol and 4-aminobenzoic acid were tested. 4-Aminobenzoic acid was selected as internal standard because it does not present selectivity problems with LAS 35917 or with its related substances (Fig. 6).

3.2. Method validation

The method was designed to provide analytical data on related substance determination for LAS 35917. The quality parameters considered for preliminary validation [23,24] were selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), repeatability test and stability of the samples.

Significant amounts of these impurities for validation are not available; as a consequence the validation and the determination of the impurities is carried out with the standard

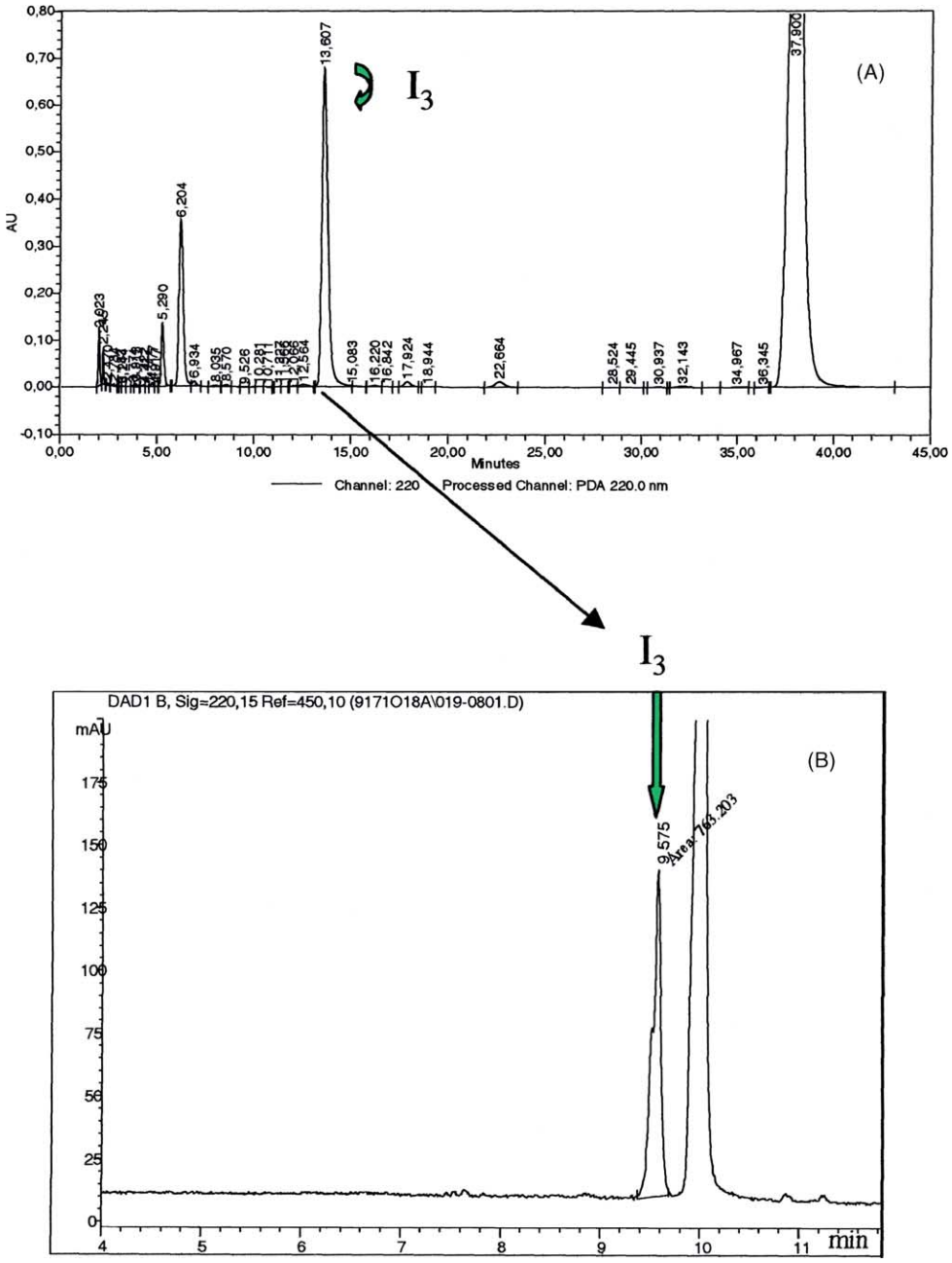

Fig. 9. Chromatogram and electropherogram of a batch of a stressed sample of LAS 35917 drug under 80 °C for 2 months. Optimal LC and CE conditions indicated in the caption of Fig. 3 and Section 2.5, respectively.

sample of LAS 35917 and response factor of LAS 35917, respectively.

3.2.1. Selectivity

Selectivity, described as the ability of a method to discriminate the analyte from all potential interfering substances, was evaluated, confirming that the signal measured is caused only by the analyte. Selectivity was demonstrated with regard to synthesis precursors, synthesis intermediates, synthesis by-products, starting materials and degradation product. As can be seen in Fig. 5, the related substances can be separated from the LAS 35917 without difficulty. The CE method is also selective with regard to the intermediate synthesis products (Fig. 7). The migration times obtained for related substances are shown in Table 4.

3.2.2. Linearity

Linearity was established by injecting solutions of LAS 35917 with concentrations ranging from the quantita-

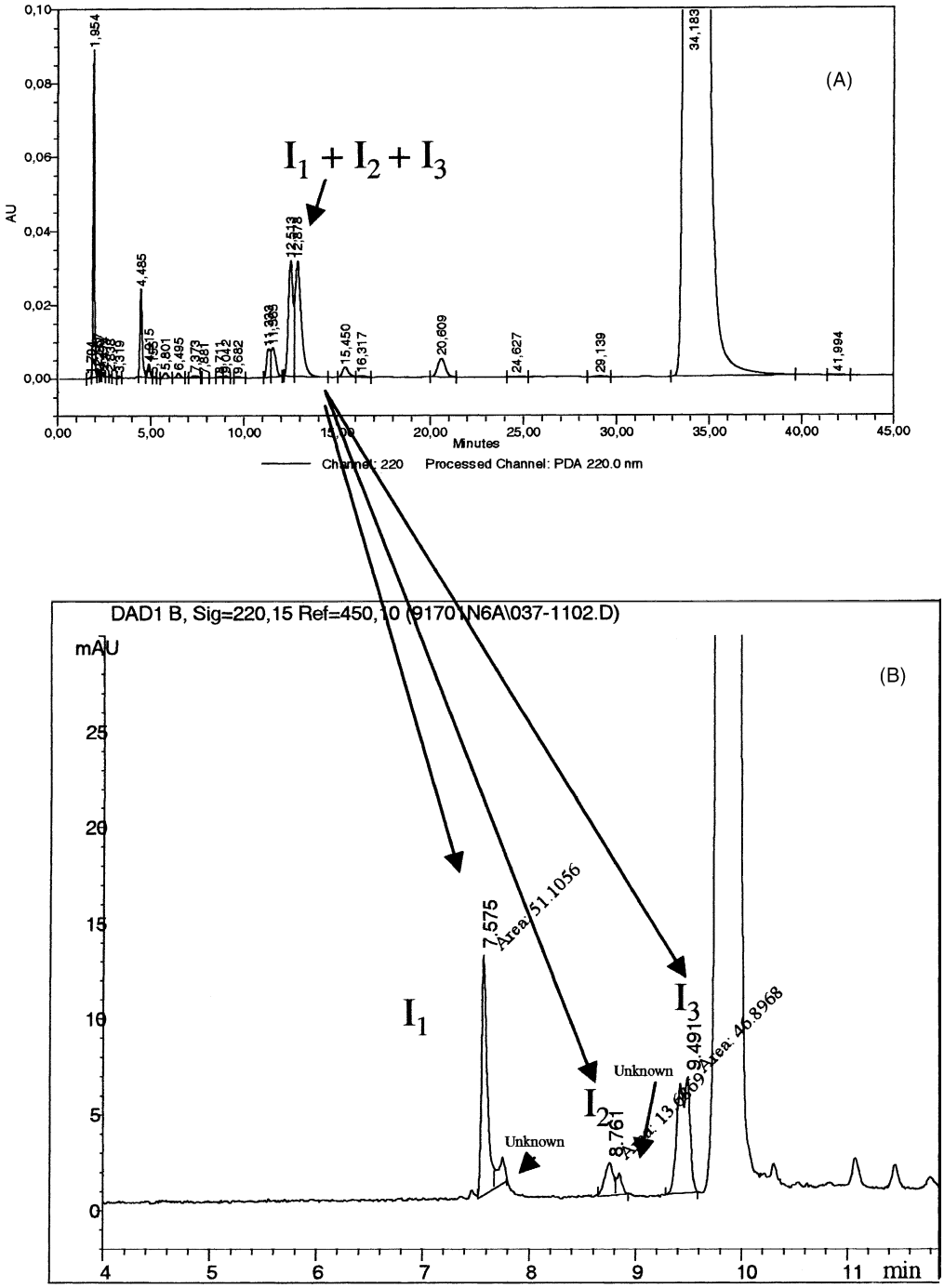

Fig. 10. Chromatogram and electropherogram of a batch of stressed sample of LAS 35917 drug under 40 °C for 3 months. Optimal LC and CE conditions indicated in the caption of Fig. 3 and Section 2.5, respectively.

tion limit up to 0.065 mg/ml (2.5% w/w of the 2.5 mg/ml target concentration). The specific concentration levels are 0.002, 0.004, 0.01, 0.015, 0.02, 0.03, 0.05 and 0.065 mg/ml and each concentration level is injected in duplicate. Resulting ratios of corrected peak areas were evaluated by linear regression analysis ($r = 0.9996$, $y = 0.0271x + 0.0025$), where y is the relative corrected area and x the LAS 35917 concentration expressed as μg/ml. For all calculations the internal standard method was applied with weighed regression of the corrected areas (Table 5).

3.2.3. Limits of detection and quantitation

The LOD calculated by using a signal-to-noise ratio of approximately 3 and the LOQ calculated by using a signal-to-noise ratio of approximately 10 are presented in Table 5. The limit of quantitation is about 2 μg/ml and the limit of detection is about 1 μg/ml.

3.2.4. Repeatability

A repeatability test was performed to determine intra-day variation in corrected areas and migration times. The statistical evaluation was carried out with the data from seven runs obtained on the same day and was determined by injecting LAS 35917 at a concentration of 0.025 mg/ml in the optimal experimental conditions. The precision was calculated as a percentage of relative standard deviation (%R.S.D.) of migration times and corrected areas obtained for LAS 35917 which were 1.51 and 1.10%, respectively (see Table 5).

3.2.5. Stability of the analytical solutions

The solution stability was established by injecting a 0.025 mg/ml standard of LAS 35917 over 24 h. The corrected areas of injected samples were between 90 and 110% (internal criterion) of the corrected area of the initial standard. No degradation of target analyte was observed.

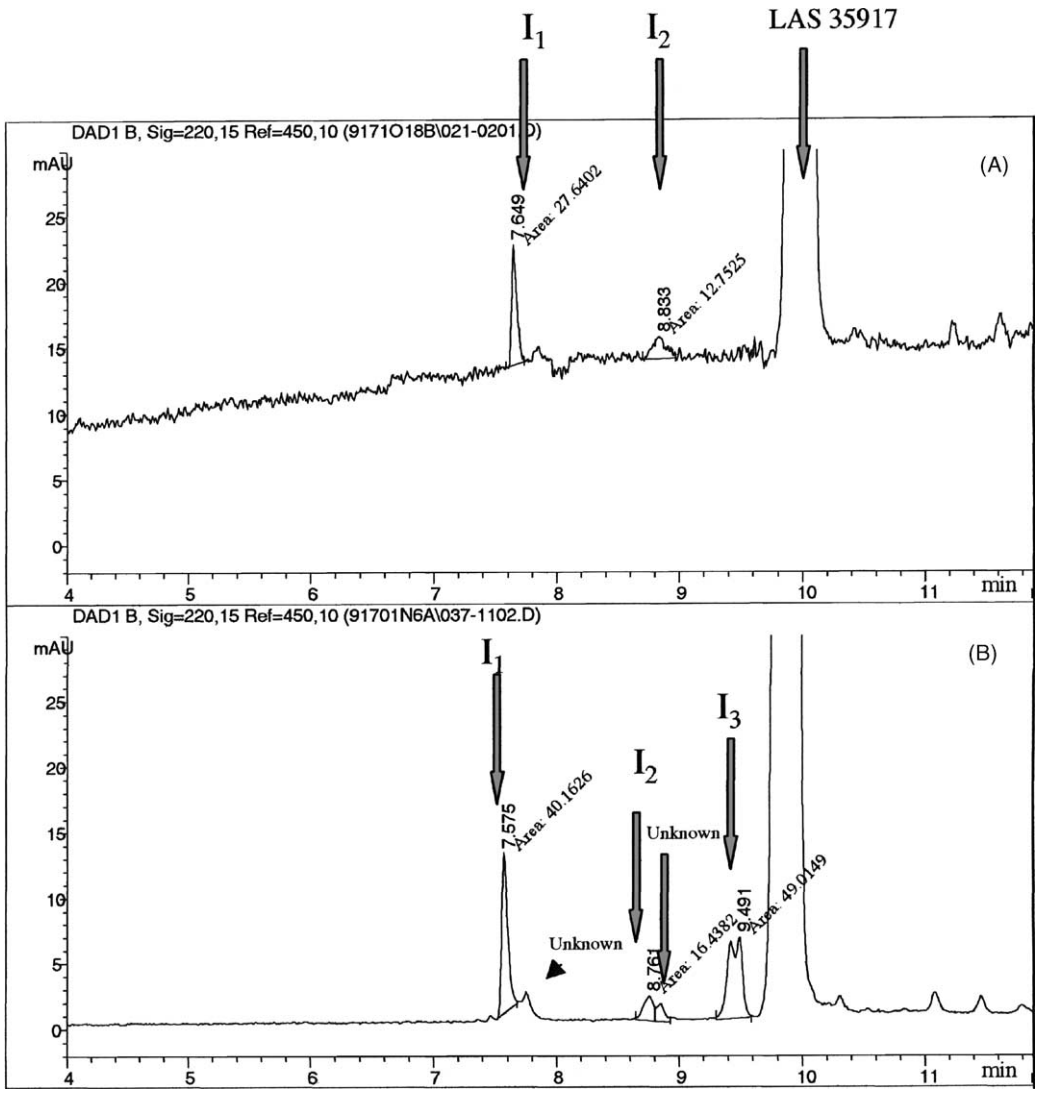

Fig. 11. Electropherograms of a LAS 35917 drug sample (A) unstressed sample and (B) stressed sample. Optimal LC and CE condition indicated in the caption of Fig. 3 and Section 2.5, respectively.

Table 6
Results of the analysis of stressed and unstressed batches of LAS 35917

Batch	I_1 (%)	I_2 (%)	I_3 (%)
Unstressed			
GR-0471-36	0.54	0.35	0.25
GR-0471-31	0.43	0.30	0.49
R002A	0.59	0.23	0.17
R002	0.44	<LOQ	<LOQ
GR-0471-40	0.40	0.36	<LOD
GR-0471-24	0.23	0.18	<LOD
GR-0471-62	0.43	0.11	<LOD
GR-0471-70	0.13	0.18	<LOD
GR-0471-67	0.16	<LOD	<LOD
GR-0471-57	0.62	0.15	0.20
R001	0.91	0.31	<LOD
Stressed			
GR-0400-19 a	<LOD	<LOD	14.21
GR-0400-19 b	0.22	<LOD	15.5
GR-0423-77	0.71	0.13	<LOD
GR-0423-53	0.61	0.23	<LOD

3.3. Application of the CE method to the analysis of several preparations of LAS 35917

The application of the CE method to the analysis of real samples of LAS 35917 was demonstrated by means of the analysis of several batches of the mentioned drug. Data about determination of impurities of these batches are shown in Table 6. Fig. 8 presents one chromatogram and one electropherogram of the same batch of LAS 35917 using optimum electrophoretic conditions. The chromatographic conditions are detailed in Section 2.1 and in the caption of Fig. 3. As can be observed, the impurity detected in the corresponding chromatogram is separated into two impurities in the electropherogram, and identified as chloromethylated and monomethylated impurity, respectively (I_1 and I_2).

Fig. 9 presents one chromatogram and one electropherogram of a stressed sample of LAS 35917. The impurity observed in the chromatogram belongs to the impurity known as I_3 (hydroxylated impurity). Fig. 10 presents one chromatogram and one electropherogram of another stressed sample of LAS 35917 where the three impurities studied are present. As can be observed from the chromatogram in Fig. 10, the coeluting impurities were slightly separated.

Finally, in Fig. 11 two electropherograms of a batch of LAS 35917 corresponding to (a) non-stressed sample and (b) stressed sample, permit corroboration of the profiling impurity. Thus, it can be concluded that impurity I_3 (hydroxylated impurity) usually appears when the sample is stressed at a higher temperature (80 °C).

4. Concluding remarks

Three impurities from LAS 35917 were separated by CE with a sodium tetraborate buffer having the three coelution in the LC method previously established. No buffer additives were needed to attain adequate resolution for the analysis performed. This is a simple, reproducible, sensitive, quick and selective method. The CE method was preliminary validated. Examples of the usefulness of the CE method have been given by means of the analysis of several stressed or unstressed samples of LAS 35917.

The identification of these impurities, coded as I_1, I_2 and I_3 was carried out by LC–MS and LC–MS–MS. Nevertheless, the structure of I_3 was not completely elucidated.

To conclude, the CE system provides high separation power and can be used in a general impurity profiling approach. Furthermore, this technique can be regarded as an excellent starting point for impurity profiling, serving as a useful complementary technique to more established techniques such as LC.

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