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# Separation and determination of nimesulide related substances for quality control purposes by micellar electrokinetic chromatography

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ABSTRACT

A micellar electrokinetic chromatography (MEKC) method has been developed and validated for the determination of nimesulide related compounds in pharmaceutical formulations. Electrophoretic separation of six European Pharmacopoeia (EP) impurities (A–F) was performed using a fused silica capillary ($L_{eff.}$ = 50 cm, $L_{tot.}$ = 57 cm, 50 μm i.d.) with a background electrolyte (BGE) containing 25 mM borate buffer (pH 9.5), 30 mM sodium dodecyl sulphate and $\varphi$ = 3% (v/v) acetonitrile. The influence of several factors (surfactant and buffer concentration, pH, organic modifier, applied voltage, capillary temperature and injection time) was studied. The method was suitably validated with respect to linearity, limit of detection and quantification, accuracy, precision and selectivity. The calibration curves obtained for the six compounds were linear over the range 5–12 μg ml$^{-1}$ (0.05–0.12%). The relative standard deviations ($s_r$) of intra- and inter-day experiments were less than 5.0%. The detection limits ranged between 0.7 and 1.6 μg ml$^{-1}$ depending on the impurity. The proposed method was applied successfully to the quantification of nimesulide impurities in its pharmaceutical formulation.



## 1. Introduction

Impurity profiling of active pharmaceutical ingredients (APIs) in both bulk material and finalized formulations is one of the most challenging tasks of pharmaceutical analytical chemists under industrial environment [1]. APIs impurities may be produced either during the synthesis process [2] or during the production and storage of the commercially available formulations [3]. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products [4]. For these reasons, all major international pharmacopoeias have established maximum allowed limits for related compounds for both bulk and formulated APIs.

From an analytical technique's point of view, impurity profiling is usually carried out by high performance liquid chromatography (HPLC) coupled to a variety of detection systems ranging from simple UV to sophisticated mass spectrometric detectors. HPLC offers readily available instrumentation in all pharmaceutical facilities and automation capabilities. However, as impurity profiling is in many cases a complicated task involving even the identification of unknown compounds, additional supportive analytical information is necessary through application of alternative separation techniques [5]. Micellar electrokinetic chromatography (MEKC) is a very interesting alternative technique that offers unique features in impurity profiling analyses, since it is capable of separating simultaneously neutral and charged compounds [6,7]. Additional advantages of MEKC include high separation efficiency, very low chemical consumption and user-friendly operation [8].

Nimesulide, a preferential COX-2 inhibitor is a non-carboxylic acid non-steroidal anti-inflammatory drug (NSAID) that has been effectively used for the treatment of a variety of inflammatory and painful conditions, including osteoarthritis in European and Asian countries for more than 15 years [9]. Although nimesulide is reported to be in the fifth place among NSAIDs in terms of market share [10], in 2007 the Irish authorities suspended its medications because of reported serious side effects mainly in the liver. However, the European Medicines Agency (EMEA) has concluded that "*the benefits of these medicines outweigh their risks, but that there is a need to limit the duration of use and to restrict their use to ensure that the risk of patients developing liver problems is kept to a minimum.*" [11].

The predominant analytical technique for the assay of nimesulide in the quality control of pharmaceutical formulations seems to be HPLC-UV coupled to either particulate-based $C_{18}$ [12–14] or monolithic columns [15]. Alternative published approaches include

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TLC [16], differential pulse [17] and adsorptive stripping voltammetry [18], capillary electrophoresis [19,20], MEKC [21] and UV–vis spectrophotometry [22,23]. All the above-mentioned methods are orientated to the determination only of the active pharmaceutical compound. On the other hand, the reports on the determination of nimesulide related substances are rather limited. The European Pharmacopoeia (EP) suggests an HPLC-UV ($\lambda_{max}$ = 230 nm) method using a reversed phase column and acetonitrile-phosphate buffer (pH 7.0) mobile phase [24]. Tubic and co-workers reported recently an HPLC-UV approach for the purity control of nimesulide also using a reversed phase column and a mobile phase consisting of acetonitrile–triethylamine–water (pH 5.2). However, only two impurities (C and D) were separated in this study [25]. Hemmateenejad et al. studied the photodegradation of nimesulide using spectrophotometry and chemometrics [26]. Although 2-phenoxy-4-nitroaniline (impurity D) was identified, it is doubtful whether this approach could perform a full screening of all potentially expected impurities. Likewise, the HPLC method proposed by Kovarikova et al. using conditions similar to that described in the EP was focused on the separation of nimesulide and impurity D [27].

The scope of the present study was to develop and validate for the first time, a micellar electrokinetic chromatographic method for the separation and quantitation of the six nimesulide related substances (A–F) that are included in the EP monograph. The proposed analytical protocol is comparable to traditional HPLC in terms of analysis time but employs 10-fold less organic solvents and produces minimum wastes. Its validated analytical performance in terms of major parameters such as selectivity, accuracy, precision and sensitivity is adequate for the routine quality control of the purity of nimesulide-containing pharmaceutical formulations.

## 2. Experimental

### 2.1. Chemicals and solutions

Nimesulide micronized reference standard (lot no. 51918, assay = 99.80%), and its impurities A–F (A, 2-phenoxy-4,6-dinitromethansulfoanilide; B, 2-phenoxymethansulfoanilide; C, 2-phenoxyaniline; D, 2-phenoxy-4-nitroaniline; E, 2-phenoxydimethansulfoanilide; F, 2-phenoxy-4-nitrodimethansulfoanilide) (Procos, Italy) were kindly donated by Cosmopharm Ltd. (Korinthos, Greece). Sodium hydroxide, sodium tetraborate decahydrate and sodium dodecyl sulphate (SDS) were of analytical grade and provided by Merck (Darmstadt, Germany). Methanol and acetonitrile (ACN) were of HPLC grade and also provided by Merck (Darmstadt, Germany). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used for preparation of all the solutions. Lizepat® tablets (lot 005) was kindly donated by Cosmopharm Ltd. (Korinthos, Greece). Pharmaceutical excipients for the preparation of the placebo mixture used in accuracy studies (sodium docusate, hydroxypropyl cellulose, lactose monohydrate, sodium starch glycolate, microcrystalline cellulose, hydrogenated vegetable oil and magnesium stearate) were obtained from domestic suppliers (Fig. 1).

Impurities and nimesulide stock solutions were prepared in ACN and kept refrigerated and protected from light. Working solutions were prepared daily by appropriate dilutions of the stock in the sample dilution medium (15 mM borate buffer pH 9.5/30 mM SDS).

A solution containing 25 mM borate buffer at pH 9.5 with 30 mM SDS and $\varphi$ = 3% (v/v) ACN was served as background electrolyte (BGE) solution in all cases. A solution of mesityl oxide $\varphi$ = 0.2% (v/v) in water was used as electroosmotic flow (EOF) marker.

All solutions were filtered through 0.45 μm membrane filters (Whatman®) and degassed by sonication for 5 min prior to use.

### 2.2. CE apparatus and analytical procedure

A Beckman P/ACE 5500 system (Fullerton, CA, USA) equipped with UV detector was used for all electrophoretic measurements. Separations were carried out in an untreated fused silica capillary (BGB Analytik) with $L_{tot.}$ = 57 cm and $L_{eff.}$ = 50 cm (i.d. 50 μm). The capillary was thermostated at 25 °C using a liquid-cooling recirculation device. Samples were hydrodynamically injected by pressure (0.5 kPa) for 5 s. Separations were carried out using normal polarity at an applied voltage of +25 kV. Detection was carried out by the on-column measurement of UV absorption at 280 nm using bandpass filter. A Beckman P/ACE control software was used for data processing.

New capillaries were conditioned by rinsing with 0.1 M NaOH for 30 min, de-ionized $H_2O$ for 10 min, and finally with the BGE solution for 10 min. At the beginning of each working day, the capillary was rinsed with 0.1 M NaOH for 10 min, water for 10 min and finally with BGE buffer for 10 min. The conditioning between runs was carried out by rinsing with BGE for 3 min, under positive pressure applied at the inlet vial. After five consequent analyses the capillary was washed with 0.1 M NaOH for 3 min, water for 5 min and finally with BGE solution with 3 min.

When analyzing real samples an extra washing step of the external surface of the capillary was adopted due to the high concentration of nimesulide (10 mg ml$^{-1}$). This washing step was carried out after sample injection and before starting the separation cycle to avoid carry-over effects. This step included immersion of the capillary inlet into water for 1 min (without applying pressure or voltage).

### 2.3. Pharmaceutical samples preparation

Ten tablets (Lizepat®, lot 005, 100 mg/tab) were powdered and an accurately weighed amount was transferred into a 50-ml volumetric flask and finally diluted to the volume with the sample dilution medium, corresponding to a 100% nimesulide concentration level of ca. 10 mg ml$^{-1}$. The sample was ultrasonicated for 30 min and then filtered through a membrane (Whatman® 0.45 μm) prior to injection into the CE system. No additional pretreatment was required prior to CE analysis.

### 2.4. System suitability

A synthetic sample was prepared containing 10 mg ml$^{-1}$ nimesulide and 10 μg ml$^{-1}$ of impurities A, D and E in the sample medium as described above. The resolution of the peaks corresponding to A–nimesulide and D–E should not be less than 1.5.

## 3. Results and discussion

### 3.1. Preliminary studies

Preliminary experiments were carried out using capillary zone electrophoresis (CZE). Separation of the six nimesulide impurities proved not feasible over a pH range of 3.0–7.0 (employing phosphate, acetate and citrate buffers to bracket effectively this range). Impurities F and E co-migrated with the EOF as expected to be neutral, while impurities C and D co-eluted before the EOF as they were positively charged. At higher pH values ranging from 7.0 to 11.5 (employing phosphate and borate buffers), sufficient separation was obtained only between impurity A and nimesulide (p$K_a$ 6.59) [28] while all other impurities were co-eluted and produced an asymmetric peak. Based on these findings, a micellar electrokinetic chromatographic system was further tested. Improved separation

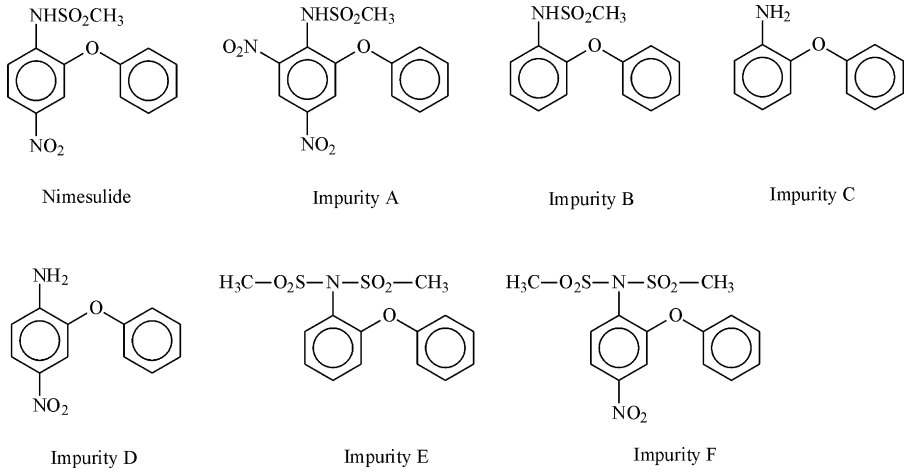


**Fig. 1.** Chemical structures of nimesulide related impurities.

and symmetric peaks were obtained when SDS was employed as anionic surfactant at basic medium.

### 3.2. Method development

The effect of several CE variables including buffer pH and ionic strength, surfactant concentration, addition of organic modifier, separation voltage, capillary temperature and sample injection time were investigated in terms of resolution, separation efficiency and analysis time.

#### 3.2.1. Effect of BGE pH

Manipulation of the pH of the BGE is a key parameter when studying the separation of ionizable species in MEKC, as it determines the degree of ionization and therefore the electrophoretic mobility of the analytes. The effect of pH was investigated in the range of 8.8–10.4 using 20 mM borate buffer and 10 mM SDS. Acidic pH values were not examined due to expected decrease of the EOF velocity [29] and therefore prolonged analysis times. Under these conditions, acceptable separation of nimesulide and impurities A–C was achieved. However, co-migration of impurities D–F was observed at the studied pH range. A pH of 9.5 was selected in subsequent experiments as it produced the best resolution between impurities A and C and higher plate numbers.

#### 3.2.2. Effect of surfactant concentration

The influence of SDS concentration on the separation was investigated in the range of 10–50 mM using 20 mM borate buffer (pH 9.5). Generally, increasing the SDS concentration, the higher amount of micelles results in a more effective separation but it also raises the current in the capillary and produces higher analysis time [29]. Impurity A and nimesulide migrated faster than the other compounds and the influence of the SDS concentration on their migration times was negligible. The highest plate numbers for impurities A–C was observed at >30 mM SDS. At these values, impurity F was also resolved from D and E. However, the resolution ($R_s$) between the impurities D and E was poor and less than 1.0 in all cases. Finally, the concentration of 30 mM was chosen for further experiments.

#### 3.2.3. Effect of buffer concentration

The effect of BGE concentration was studied by varying the borate concentration from 10 to 50 mM at 30 mM SDS and pH of 9.5. An increase in the borate concentration resulted in a slight increase in the migration time of the impurities. However, separation between impurities D and E was still not obtained. Furthermore, the plate numbers of the resolved peaks were increased at borate concentrations up to 25 mM and then decreased. Above 25 mM a higher current and also a baseline shift occurred, so the latter value was selected.

#### 3.2.4. Effect of the addition of organic modifier

In order to improve the separation between impurities D and E, the effect of organic modifiers was examined. The organic solvent influences the pH, dielectric constant and viscosity of the BGE, but also affects the zeta potential and results in decrease of the EOF [30]. When methanol was used as organic modifier in the range of $\varphi$ = 1–10%, baseline shifting and no separation between D and E was observed. On the other hand, acetonitrile gave better results, as successful separation between D and E ($R_s$ = 2) was achieved at $\varphi$ = 3% (v/v), therefore, this volume fraction was chosen for the efficient separation of nimesulide impurities.

#### 3.2.5. Effect of sample medium on the separation

Sample medium composition plays an important role in CE-based assays and particularly on the separation, current fluctuations and repeatability of migration times [31,32]. Nimesulide is freely soluble in polar organic solvents, while its solubility in aqueous solutions was reported to be in the range of 0.034 mg ml$^{-1}$ at a pH value of 7.2 and 34.6 mg ml$^{-1}$ in 0.1 M NaOH [33]. When ACN was used to dissolve the sample, the separation was poor, air bubbles were frequently observed and the R.S.D. of migration times between runs was more than 10%. For this reason, it was decided to dissolve the sample in BGE, since at the alkaline pH of 9.5 sufficient solubility of nimesulide and its impurities was achieved. It should be noted that the 100% level of nimesulide was set at 10 mg ml$^{-1}$.

The effect of borate buffer concentration was studied in the range 5–25 mM (pH 9.5/30 mM SDS). Lowering the buffer concentration to 15 mM the plate numbers were increased. Since no improvement was observed at lower buffer concentrations, thus the value of 15 mM was adopted for subsequent experiments.

#### 3.2.6. Effect of instrumental variables

The effect of critical instrumental variables such as the applied separation voltage, capillary temperature and sample volume was studied using the above-described conditions.

In order to obtain satisfactory sensitivity without peak shape deterioration, the injection time varied in the range of 3–15 s by keeping the pressure constant at 0.5 psi. When increasing the injection time, peak area also increased, but peak broadening was

**Table 1**
Linearity data, LOD, LOQ and separation parameters (resolution, peak efficiency and migration time) of the nimesulide impurities.

| Impurity | Regression equation[a] $Y=(a\pm S.D._a)x+(b\pm S.D._b)$ | $r$ | LOD[b] (μg ml$^{-1}$) | LOQ[c] (μg ml$^{-1}$) | Resolution | $N$[d] | Migration time[e] (min) |
|---|---|---|---|---|---|---|---|
| A | $Y=(436\pm8)x+(232\pm70)$ | 0.9991 | 0.9 | 3.0 | 2.11 (A–nimesulide) | 98811 | 6.95 |
| B | $Y=(1212\pm27)x+(-1350\pm237)$ | 0.9987 | 0.8 | 2.6 | 35.23 (nimesulide–B) | 115714 | 11.71 |
| C | $Y=(2062\pm42)x+(-1892\pm370)$ | 0.9989 | 0.7 | 2.3 | 12.84 (B–C) | 93898 | 12.92 |
| D | $Y=(1241\pm26)x+(-1312\pm228)$ | 0.9989 | 1.5 | 5.0 | 10.26 (C–D) | 82134 | 14.89 |
| E | $Y=(1512\pm35)x+(-2273\pm309)$ | 0.9986 | 1.4 | 4.6 | 2.29 (D–E) | 83181 | 15.24 |
| F | $Y=(903\pm22)x+(-2681\pm195)$ | 0.9984 | 1.6 | 5.2 | 4.22 (E–F) | 65808 | 16.14 |

[a] $Y$ is the peak area of each impurity, $x$ the mass concentration in μg ml$^{-1}$, $S.D._a$ and $S.D._b$ the standard deviation of slope (a) and intercept (b), respectively.
[b] Estimated limit of detection calculated as S/N = 3.
[c] Estimated limit of quantitation calculated as S/N = 10.
[d] $N$ are the number of theoretical plates calculated by the half width method.
[e] Mean of three replicates.

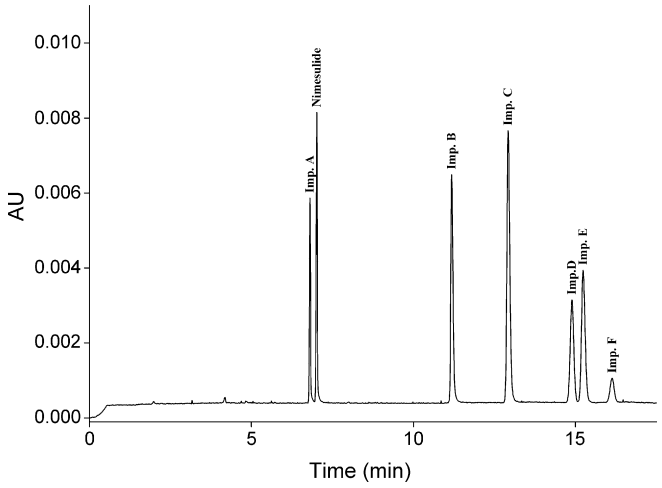


**Fig. 2.** Electropherogram of nimesulide (50 μg ml$^{-1}$) and its six impurities (A–F, 50 μg ml$^{-1}$ each). Experimental conditions—BGE: 25 mM borate pH 9.5, 30 mM SDS, $\varphi$ = 3% (v/v) ACN, $V$ = +25 kV, $\theta$ = 25 °C, hydrodynamic injection 5 s at 0.5 psi, $\lambda$ = 280 nm.

observed. Injection times longer than 5 s caused a loss of efficiency greater than 10% in terms of theoretical plate numbers, thus the latter value was adopted.

The effect of applied voltage on the separation efficiency and the analysis time was studied over the range of 20–30 kV. As expected, a voltage increase leads to shorter migration times and sharper peaks. However, higher applied voltages also result in higher current and increased joule heating phenomena. A voltage of 25 kV resulted in a current of ~51 μA and a reasonable analysis time of ca. 17 min was selected for further work.

Variation of the capillary temperature in the range of 15–35 °C had negligible effects on both the migration time and plate numbers of the peaks. The value of 25 °C was finally selected for further experiments.

#### 3.2.7. Selection of detection wavelength

The HPLC method recommended by the US Pharmacopoeia for the purity control of nimesulide formulations suggests UV detection at 230 nm [24]. Four different UV wavelengths (200, 214, 254 and 280 nm) were examined by changing the band-pass filters of the UV detector of the CE instrument. In our study the detector response and the S/N ratio for all impurities was higher at 280 nm. This wavelength was therefore selected as optimal.

### 3.3. Validation of the MEKC assay

The proposed method was validated according to parameters proposed by the ICH guidelines [34], such as linearity, limit of detection (LOD), limit of quantitation (LOQ), precision (within day and day-to-day), accuracy and selectivity.

#### 3.3.1. Linearity, limits of detection and quantitation

As mentioned above, a nimesulide concentration of 10 mg ml$^{-1}$ was set as the 100% level. The impurities' limits as defined in the United States Pharmacopoeia (USP) are not more than 0.1% (corresponding to 10 mg l$^{-1}$) for each impurity. The linearity of the proposed assay was therefore validated in the range of 0.05–0.12% (w/w) for each impurity, using seven calibration points (5, 6, 7, 8, 10, 11, 12 μg ml$^{-1}$). The peak area of each impurity was used for constructing the calibration graph. Regression equations, correlation coefficients, standard deviations of slopes and intercepts, LODs and LOQs, resolution, peak efficiencies and migration times of the

**Table 2**
Intra- and inter-day precision of the proposed MEKC method.

| Impurity | Concentration (%) | Within day ($s_r$ %, $n=6$) | Day-to-day ($s_r$ %, $n=6$) |
|---|---|---|---|
| A | 0.05 | 2.1 | 1.8 |
| | 0.1 | 2.6 | 1.6 |
| | 0.12 | 1.2 | 0.9 |
| B | 0.05 | 0.6 | 2.1 |
| | 0.1 | 1.5 | 1.0 |
| | 0.12 | 0.7 | 1.8 |
| C | 0.05 | 2.4 | 0.9 |
| | 0.1 | 3.2 | 2.3 |
| | 0.12 | 1.4 | 1.2 |
| D | 0.05 | 1.3 | 1.5 |
| | 0.1 | 1.4 | 1.1 |
| | 0.12 | 1.6 | 1.0 |
| E | 0.05 | 0.7 | 1.4 |
| | 0.1 | 0.6 | 0.8 |
| | 0.12 | 1.7 | 1.1 |
| F | 0.05 | 0.9 | 3.5 |
| | 0.1 | 3.4 | 3.7 |
| | 0.12 | 4.9 | 5.0 |

**Table 3**
Accuracy of the MEKC assay.

| Impurity | Added concentration (%) | Recovery[a] (%) |
|---|---|---|
| A | 0.05 | 98.6 |
| | 0.1 | 97.3 |
| | 0.12 | 99.4 |
| B | 0.05 | 101.5 |
| | 0.1 | 99.1 |
| | 0.12 | 102.2 |
| C | 0.05 | 98.3 |
| | 0.1 | 101.7 |
| | 0.12 | 100.4 |
| D | 0.05 | 97.8 |
| | 0.1 | 99.6 |
| | 0.12 | 100.8 |
| E | 0.05 | 102.4 |
| | 0.1 | 98.4 |
| | 0.12 | 104 |
| F | 0.05 | 98.3 |
| | 0.1 | 97.0 |
| | 0.12 | 103.6 |

[a] Three replicates per sample.

impurities are listed in Table 1. Each standard solution was injected in triplicates.

The LODs and LOQs were estimated based on the S/N approach. A typical electropherogram of a standard solution of nimesulide impurities is illustrated in Fig. 2.

### 3.3.2. Precision

The repeatability (within-day precision) of the developed assay was evaluated at three concentrations levels 5, 10 and 12 μg ml$^{-1}$ corresponding to the 0.05%, 0.1% and 0.12% levels, respectively. Six repetitive analyses were performed within a working day. The day-to-day precision was validated during a period of six consecutive days by three analyses per concentration level. The experimental results are summarized in Table 2. The precision of the assay were satisfactory in all cases, as the $s_r$ values were in the range of 0.6–5.0%.

### 3.3.3. Specificity

The specificity of the method was validated by the placebo approach. A placebo mixture (all excipients excluding the active ingredient) was therefore prepared according to the manufacturing protocol of the tablets. It consisted of *w* (%) 0.5% sodium docusate, 0.27% hydroxypropyl cellulose, 51.2% lactose monohydrate, 11.7% sodium starch glycolate, 33.3% microcrystalline cellulose, 2.7% hydrogenated vegetable oil and 0.33% magnesium stearate. Based on the fact that the 100% level of nimesulide was set at 10 mg ml$^{-1}$, the expected concentration of the excipients in the real samples is 30 mg ml$^{-1}$ (each tablet consists of 100 mg of nimesulide and 300 mg of excipients). In order to assure the selectivity of the procedure, the potential interfering effects of the excipients were tested at a concentration level of 50 mg ml$^{-1}$ dissolved in the selected sample medium. The selectivity samples were mixed ultrasonically for 15 min, filtered through 0.45 μm disposable syringe filters (Whatman®) and analyzed. As illustrated in Fig. 3, no interfering peaks were recorded.

### 3.3.4. Accuracy

The accuracy of the assay was evaluated at three concentration levels of all impurities, namely 0.05%, 0.1% and 0.12% (5, 10 and 12 μg ml$^{-1}$) by spiking placebo solutions at the desired val-

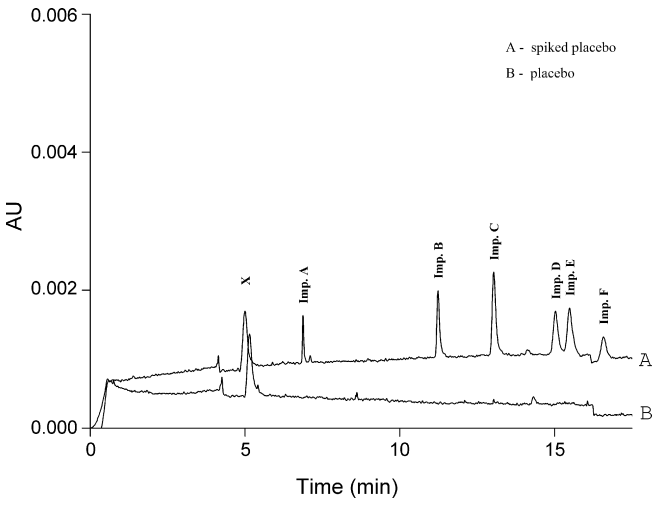


**Fig. 3.** Electropherograms of spiked placebo (A) at 0.05% level of impurities A–F and placebo (B). Experimental conditions: as described in Fig. 2.

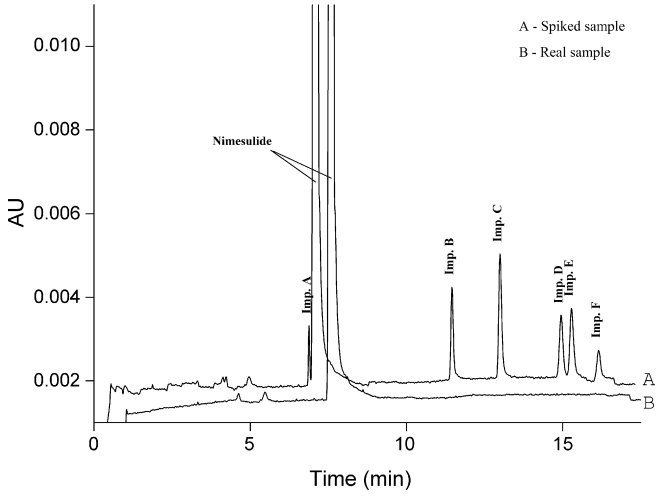


**Fig. 4.** Electropherograms of analysis of spiked Lizepat® tabs (A) at the 0.1% level of impurities A–F and Lizepat® tabs (B). Experimental conditions: as described in Fig. 2.

ues. The experimental results are presented in Table 3. The percent recoveries were satisfactory in all cases ranging between 97% and 104%.

### 3.4. Application to pharmaceutical samples

The applicability of the developed assay was evaluated by analyzing a commercially available formulation (Lizepat® tabs, 100 mg/tab, lot 005, Cosmopharm Ltd., Korinthos, Greece). Sample preparation was carried out as described in Section 2.3. Typical electropherograms of the analyzed formulation with and without spiked impurities at the 0.1% level are depicted in Fig. 4. No impurities were detected in the specific lot of the formulation, which is in accordance to in-house QC findings at Cosmopharm facilities using the EP HPLC method.

## 4. Conclusions

The first MEKC method for the separation and simultaneous quantitation of the six nimesulide impurities is reported. The proposed method was developed and adequately validated with respect to critical parameters for pharmaceutical quality control such as specificity, linearity, accuracy and precision. Compared to traditional HPLC assays it offers an environmentally friendlier alternative in terms of waste production and consumption of organic solvents without sacrificing its reliability and efficiency. The assay was applied directly to the purity control of a commercially available nimesulide-containing pharmaceutical formulation with minimal sample preparation prior to analysis.

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