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Determination of 5-aminosalicylic acid related impurities by micellar electrokinetic chromatography with an ion-pair reagent

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Abstract

A micellar electrokinetic chromatographic (MEKC) method was developed for the quantification of mesalazine or 5-aminosalicylic acid (5-ASA) and its major impurities 3-aminosalicylic acid, salicylic acid, sulfanilic acid and 4-aminophenol. The optimisation of the experimental conditions was carried out considering some important requirements: resolution, reproducibility, detection limits of 0.1% (m/m) or less, short total analysis time. Preliminary investigations employing sodium dodecyl sulfate (SDS) as surfactant did not lead to the necessary resolution of the studied compounds; the addition of tetrabutylammonium bromide (TBAB) to the SDS micellar system resulted in the complete separation of all the compounds. The effects on the separation by several parameters such as TBAB concentration, SDS concentration, background electrolyte pH and concentration, were evaluated. Using a fused-silica capillary (8.5 cm effective length) complete analysis was obtained in a very short time. Under the optimised final conditions [120 m*M* 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid buffer, pH 10.20, 65 m*M* SDS in the presence of 55 m*M* TBAB and 5% methanol] the method was validated for specificity, precision, linearity, limits of detection and quantitation, and robustness: the 5-ASA related impurities can be quantified at least at the 0.1% (m/m) level.

Keywords: Pharmaceutical analysis; Validation; Ion-pairing reagents; Aminosalicylic acid; Salicylic acid; Sulfanilic acid; Organic acids; Tetraalkylammonium salts; Aminophenols

1. Introduction

Mesalamine (5-aminosalicylic acid, 5-ASA) is a salicylate used for its local effects in the treatment of inflammatory bowel disease. The drug is poorly absorbed and is inactivated before reaching the lower intestine: it is available as a rectal suspension for treatment of mild-to-moderate proctosigmoiditis; formulations able to deliver the intact drug to the lower intestine are nowadays successfully used [1,2].

The purity evaluation of 5-ASA bulk chemical is of great importance for the pharmaceutical manufacturer; determination of active ingredient related substances would be a first step in the examination of the safety and quality of the drug. Structures of 5-ASA and its major potential impurities are shown in Fig. 1. Several high-performance liquid chromatography (HPLC) methods provide selective systems for the determination of mesalamine in biological samples [3–5]; moreover, using an ion-pairing chromatographic approach, the determination of 5-ASA related impurities was also reported [6].

Due to its high resolving power, capillary electrophoresis (CE) can offer a good alternative in drug analysis [7,8], including the determination of drug related impurities [9–12]. In this respect, the demand

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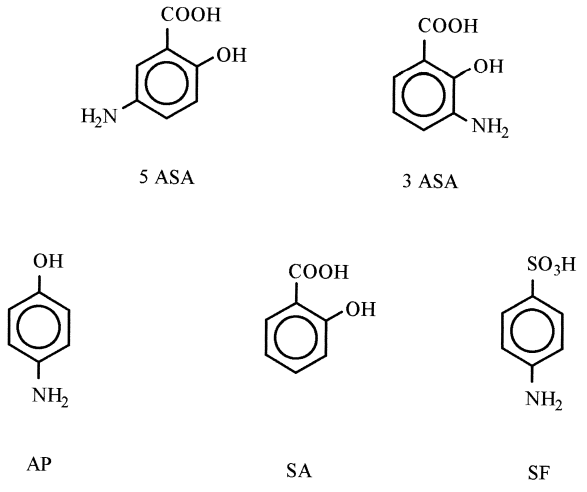

Fig. 1. Structures of 5-aminosalicylic acid (5-ASA) and related impurities: 3-aminosalicylic acid (3-ASA), 4-aminophenol (AP), sulfanilic acid (SF) and salicylic acid (SA).

for methods with high degree of selectivity can constitute a hard problem owing to the different polarities of the potentially present compounds. The use of additives in the CE running buffer can be considered a good approach to improve the selectivity: β-cyclodextrin was used to obtain the separation of zinc 5-aminosalicylate from related materials [13]. In the present paper a micellar electrokinetic chromatographic (MEKC) approach was chosen to develop a method able to determine simultaneously the drug 5-ASA and all the synthetic process impurities reported in Fig. 1. The use of sodium dodecyl sulfate (SDS) alone, as surfactant, did not provide a useful separation of the examined compounds; under alkaline electrophoretic conditions, the anionic solutes were not partitioned into the micelles even at high concentrations of SDS. Addition of tetraalkylammonium (TAA) salts, in particular tetrabutylammonium bromide (TBAB), greatly improved the separation because of the ion-pair interactions of the ammonium salts with the anionic solutes [14]. The method optimisation was carried out considering the general requirements of methods suitable for the quality control of the drug substances and related materials: resolution, reproducibility, detection limits of 0.1% (m/m) level or less, short analysis time. Under the optimised final conditions, method validation was performed leading to the opportunity of rapid applications to real samples.

2. Experimental

2.1. Materials

5-ASA (approx. 99%), 5-ASA (95–98%), sulfanilic acid (SF), 3-aminosalicylic acid (3-ASA), salicylic acid (SA), 4-aminophenol (AP) and the buffer CAPSO [3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid] were purchased from Sigma (Milan, Italy). 5-ASA (>95%), SDS, tetramethylammonium bromide (TMAB), tetraethylammonium bromide (TEAB), tetrapropylammonium bromide (TPAB), TBAB and tetrapentylammonium chloride (TPAC) were from Fluka (Buchs, Switzerland). All the other chemicals were of analytical grade and were purchased from Carlo Erba (Milan, Italy). The water used for the preparation of standard solutions and running buffers was purified by a Milli-RX apparatus (Millipore, Milford, MA, USA).

2.2. Apparatus

All separations were carried out using a ^{3D}CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector. The data were collected on a personal computer using the ^{3D}CE-ChemStation software ver. A 06. Fused-silica ''bubble'' capillaries of 48.5 cm (40 cm effective length)×50 μm I.D. (Hewlett-Packard) were used. The optimised voltage was maintained at 15 kV and the temperature was thermostatted at 30°C during the electrophoretic runs. Hydrodynamic injections were performed at 50 mbar for 5 s and the detection wavelength was 230 nm (3-ASA, SA), 254 nm (AP, SF) and 300 nm (5-ASA). When the short end of the capillary was used (8.5 cm effective length), the polarity of the system was reversed and the hydrodynamic injection was obtained by applying a vacuum (−50 mbar, 5 s) to the opposite end of the capillary.

2.3. Solutions

2.3.1. Background electrolyte (BGE) solutions

The running buffer solutions were prepared in water; for the method optimisation, the considered concentration ranges of the BGE components were: SDS (45–75 m*M*), TBAB (45–65 m*M*) and CAPSO buffer (50–120 m*M*). The effect of pH value on the

separation was studied within the range 8–11 adjusting the pH of CAPSO solutions with 0.1 *M* phosphoric acid.

2.3.2. Calibration graphs

Calibration graphs were obtained for the analysis of the impurities over the concentration range 0.75–15 μg/ml in the presence of a constant amount of 5-ASA (1500 μg/ml), corresponding to 0.05–1% (m/m). Moreover, a calibration graph was also carried out for 5-ASA in the range 450–1800 μg/ml allowing the evaluation of the response linearity for the main component assay. For each graph the corrected peak area (area/migration time) was plotted against the corresponding concentration value.

2.3.3. Standard and sample solutions

All the standard and sample solutions were prepared in sodium phosphate buffer (50 m*M*, pH 3.0). The developed method was applied to the analysis of commercially available bulk drug; sample solutions of raw materials were prepared at a concentration of 1500 μg/ml in phosphate buffer (50 m*M*, pH 3.0) and subjected to CE analysis using the described MEKC procedure. The assay of the main component (5-ASA) was carried out simultaneously to the determination of the related substances by comparison with the corrected peak area of a 5-ASA standard solution.

2.4. Analytical procedure

Prior the first use the capillary was conditioned by flushing 1 *M* NaOH at 50°C for 20 min followed by a rinse with 0.1 *M* NaOH (10 min); a final step consisted of a washing with water at 25°C during 10 min. The capillary was equilibrated (10 min) at the beginning of the day with the running buffer. The repeatability of migration times was found to be strongly dependent on the rinse procedure: the highest reproducibility of the migration times was obtained by flushing the capillary between the runs as follows: 1 min with 0.1 *M* HCl, 1 min with methanol, 1 min with 1 *M* NaOH, 1 min with water and 2 min with BGE. Vials of BGE were replaced every five injections to keep the same reservoirs level of the buffer and to avoid changes of electroosmotic flow (EOF) due to the electrolysis of the solutions.

3. Results and discussion

3.1. Method development

The structures of the studied analytes (Fig. 1) would suggest that one perform a capillary zone electrophoretic (CZE) separation under strongly alkaline conditions; accordingly, all the solutes exhibited migration toward the negative electrode due to the strong electroosmotic velocity. In an alkaline running buffer (120 m*M* CAPSO, pH 10.2), however, no successful separation of the studied compounds was obtained; particularly, the two isomers 3-ASA and 5-ASA completely overlapped one another, as well with SF. Further, under these conditions AP migrated almost simultaneously to the EOF. An MEKC approach based on the use of SDS as surfactant was also applied. Preliminary investigations using alkaline solutions containing SDS (at concentrations higher than 65 m*M*) did not lead to successful results: the distribution of the anionic solutes into the negatively charged micelles will not be possible and the obtained electropherograms were similar to that provided under CZE conditions.

The addition of TAA salts to the SDS micellar system resulted in a useful way to improve the solute–micelle interactions allowing the separation of the anionic analytes. Some TAA ions are reported to combine with the anionic SDS micelles remarkably altering their character. Also, the same TAA cations can interact with the anionic solutes forming ion pairs with a higher solubility into the micelles than the free anionic analytes [14]. Following the above described principle, several TAA salts (TMAB, TEAB, TPAB, TBAB and TPAC) were employed in combination with the SDS running buffer; the best results were obtained with TBAB which provided the complete separation of the studied compounds.

3.2. Method optimisation

The optimisation of the separation of 5-ASA related impurities was aimed to resolve the main component from four potential synthetic process

impurities in a single and short run. In order to develop a method able to meet the previous requirements, all the various parameters driving the electrophoretic process were studied in the following concentration ranges: SDS (45–75 m*M*), TBAB (45–65 m*M*) and CAPSO buffer (50–120 m*M*). The effect of pH value on the separation was studied within the range 8–11.

3.2.1. Running buffer pH

The role played by the buffer pH is very important controlling the EOF and the ionization degree of each analyte. Due to their pK_a values, 5-ASA (2.74; 5.84), 3-ASA, SA (2.97; 13.4) SF (3.23) and AP (amino group: 4.3–6.5, hydroxy group: 9.8–11.8) [15,16] were completely in the anionic form over the full studied pH range. Working at pH between 7 and 8, good solubilization of the neutral AP into the SDS micelles would be obtained allowing one to distinguish the analyte from the EOF. Under these conditions (also in the presence of TBAB) all the other analytes, bearing a carboxylate or sulfonic function, resulted in anionic form and migrated as unsymmetrical and overlapped (3-ASA/5-ASA) peaks. Using pH>8.0, likely due to the interaction anionic analyte–TBAB–SDS, better peak shapes and resolution of all the compounds were observed; furthermore the obtained high EOF allowed short analysis time. Within the studied pH range, the best results were at pH 10.20 which allowed a good compromise between the peak shapes and analysis time.

3.2.2. SDS concentration

As for each MEKC separation, SDS concentration is considered an important parameter controlling the selectivity. For concentrations lower than 60 m*M* of SDS, the separation of the studied compounds deteriorated progressively with a general loss of resolution. On the other hand the increase of SDS remarkably increased the migration times and the optimised value was chosen as 65 m*M*.

3.2.3. TBAB concentration

Similarly to the effect of SDS on the separation, high TBAB concentration led to high migration time according to the increased solubilization of the anionic solutes into the micelles via ion-pair interactions between the analytes and the TAA salt. This evidence was also supported by a constant measured EOF at the different levels of TBAB into the BGE. Therefore, the addition of TBAB can be considered of great importance to obtain conditions for a selective migration of the analytes. The separation was optimised considering the simultaneous effect on three different parameters: (a) the total analysis time (migration time of the last migrating analyte: t_m SA), (b) the resolution between the peaks of the isomers 5-ASA and 3-ASA (R_{5-3}) and (c) the tailing factor (T) of the electrophoretic peak of 3-ASA. Both resolution and tailing factor were calculated accordingly to the USP 24 [17]. Precisely, the resolution of two peaks is:

$$R = 2(t_2 - t_1)/W_1 + W_2$$

and

$$T = W_{0.05}/2f$$

where t is the migration time, W is the width of the peak measured by extrapolating the relatively straight sides to the baseline, $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

As can be seen from Fig. 2, the ratio of resolution (R_{5-3}) to the migration time of SA (t_m SA) was plotted against the TBAB concentration. The highest value of the (R_{5-3}/t_m SA) ratio was obtained at a concentration of 55 m*M* TBAB where, besides the peaks symmetry being the best; particularly, the tailing factor of 3-ASA reached unity.

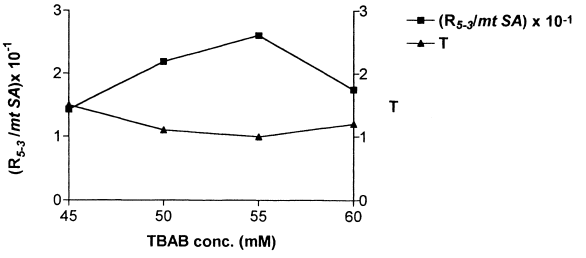

Fig. 2. Effect of TBAB concentration on the ratio R_{5-3}/t_m SA and tailing factor of 3-ASA (T).

3.2.4. CAPSO concentration

Preliminary investigations on the buffer nature effects on the examined separation, showed a better behaviour of CAPSO compared with the traditional CE buffer (borate, phosphate). The effect of the CAPSO concentration on the CE analysis showed particular interest due to the increased analyte response to the detector when relatively high levels of CAPSO were used. Precisely likely because of stacking phenomena, a significant increased detectability of SA was obtained using a CAPSO concentration of 120 m*M*. Under these conditions the recorded current was lower than 100 μA.

3.2.5. Effect of temperature, applied voltage and capillary length

The electrophoretic runs were carried out at constant temperature of 30°C; changes (±3°C) around this value did not affect the separation (peak shape, peaks resolution and analysis times). Uncoated fused-silica capillaries of 48.5 cm (40 cm length to detector window)×50 μm I.D. were used after the conditioning program described in the Experimental section. The separations were performed at a voltage of 15 kV with a current <100 μA. Under the optimised conditions (120 m*M* CAPSO buffer, pH 10.20, 65 m*M* SDS in the presence of 55 m*M* TBAB), a representative separation of the studied compounds is reported in Fig. 3. With the aim to reduce the analysis time an attempt was done shortening the capillary; precisely, it was possible to keep constant the field strength employing, for the separation, the short end of the capillary (8.5 cm effective length). A significant reduction of the analysis time was observed while the separation was maintained; however, in view of an overloading of 5-ASA necessary to allow detection limits of 0.1% or less of the related impurities higher resolution would be required. These conditions were achieved by the addition of a small percentage of methanol (5%) to the BGE, improving the resolution of the analytes with limited increase of analysis time (Fig. 4).

3.3. Method validation

The developed MEKC method was validated under the optimised experimental conditions (120 m*M*, pH 10.20 CAPSO buffer, 65 m*M* SDS in the presence of 55 m*M* TBAB and 5% methanol with an 8.5 cm length fused-silica capillary) according to the ICH guidelines [18].

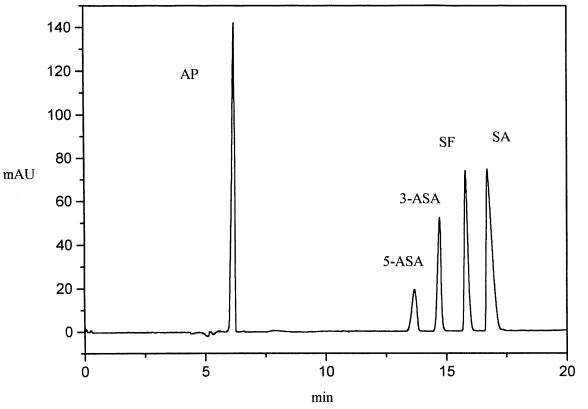

Fig. 3. Optimised MEKC separation of 5-ASA and the four related substances employing a 40 cm effective length×50 μm I.D. fused-silica capillary. Separation conditions: 120 m*M* CAPSO buffer, pH 10.20, 65 m*M* SDS, 55 m*M* TBAB. Voltage: 15 kV. Temperature: 30°C. Detection wavelength: 230 nm. Hydrodynamic injection 50 mbar, 10 s. Abbreviations as in Fig. 1.

3.3.1. Selectivity

The selectivity of the method was demonstrated by

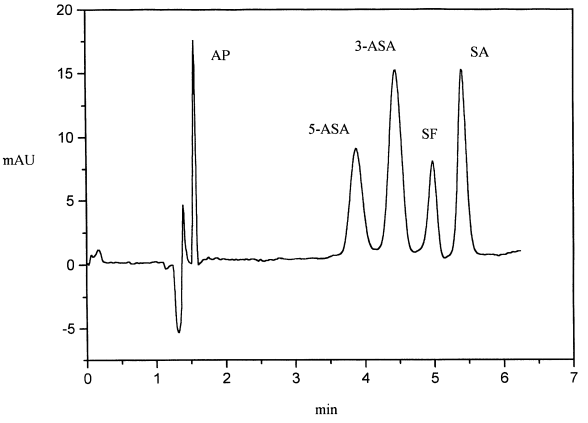

Fig. 4. Optimised MEKC separation of 5-ASA and the four related substances employing the short end capillary (8.5 cm effective length×50 μm I.D.). Separation conditions: 120 m*M* CAPSO buffer, pH 10.20, 65 m*M* SDS, 55 m*M* TBAB and 5% methanol. Voltage: 15 kV. Temperature: 30°C. Detection wavelength: 230 nm. Hydrodynamic injection 50 mbar, 10 s. Abbreviations as in Fig. 1.

the use of pure and commercially available reference compounds. The peak identity was also confirmed by the spectra recorded using the diode-array detector. Under the optimised conditions the resolution values between the adjacent peaks were: resolution 5-ASA/3-ASA, 1.85; resolution 3-ASA/SF, 1.94; resolution SF/SA, 1.90.

3.3.2. Precision

Multiple injections from a single sample solution were performed to demonstrate the repeatability of the migration times and the corrected peak area (area/migration time). Ten replicate runs of standard mixtures of 5-ASA (1500 μg/ml) and the four impurities (0.1%, m/m, of nominal 5-ASA content) were carried out; the obtained RSDs of the migration times and peak area are summarised in Table 1. It should be pointed out that adequate good precision was obtained at the trace level for the impurities.

3.3.3. Linearity

Linearity was demonstrated for 5-ASA (range 450–1800 μg/ml) measuring the absorbance at 300 nm where the detector response of the main component was low enough to avoid the possible non-linearity due to the high concentration. The linearity was also evaluated for all the related impurities, spiking a 1500 μg/ml solution of 5-ASA with 0.05, 0.1, 0.2, 0.5 and 1% (m/m) of nominal 5-ASA content for each compound. For the impurities UV detection was carried out at the respective absorption maximum wavelength. Representative electropherogram of a 5-ASA (1500 μg/ml) solution spiked with the related impurities each at 0.1% (m/m) level is shown in Fig. 5. Plots of the corrected peak area of the analytes versus the corresponding standard concentrations resulted in the equations reported in Table 2.

Table 1
Precision of the migration time and peak area (RSD, $n=10$) for the studied impurities at 0.1% (m/m) of nominal 5-ASA content[a]

Analyte	t_m (min)	RSD (%)	Corrected peak area	RSD (%)
AP	1.57	0.54	2.9	3.21
3-ASA	4.48	0.48	1.83	3.13
SF	4.97	0.98	1.63	3.44
SA	5.38	1.3	1.47	6.90

[a] Experimental conditions: 120 m*M* CAPSO running buffer, pH 10.20 with 65 m*M* SDS, 55 m*M* TBAB, 5% methanol. Fused-silica capillary (8.5 cm effective length) thermostatted at 30°C. Hydrodynamic injection (50 mbar, 5 s). UV detection at 254 nm (AP and SF), 230 nm (3-ASA and SA). Voltage: 15 kV.

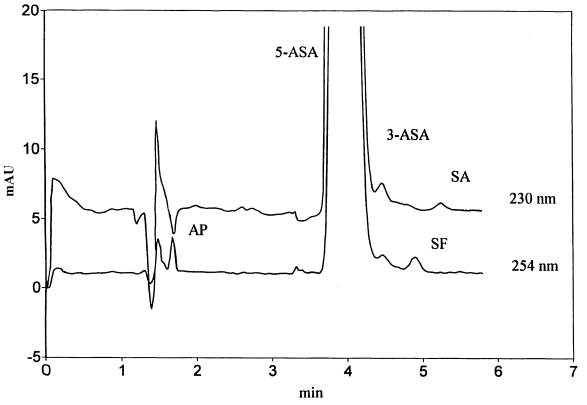

Fig. 5. Electropherogram of a 5-ASA (1500 μg/ml) solution spiked with the related impurities each at the 0.1% (m/m) level. Detection was performed at two different wavelengths. Separation conditions and abbreviations as in Fig. 4.

3.3.4. Accuracy

The accuracy of the method was evaluated for 5-ASA assuming as reference compound a highly pure standard substance (>99%) and by comparing the response of standard solution with those obtained with different bulks supplied. The mean recoveries ($n=5$) for the two tested batches (95.8% and 96.6%) were found to be in agreement with the declared content (95–98% and >95%) of 5-ASA which means that the method gives sufficient accuracy.

3.3.5. Sensitivity

The lowest detectable concentration was evaluated for the synthetic impurity 3-ASA in the presence of the main compound 5-ASA (1500 μg/ml). Solutions of 0.3 μg/ml of 3-ASA (corresponding to 0.02%, m/m) provided a signal-to-noise ratio of approximately 3, thus corresponding to the limit of detection. Ten replicate injections of the lowest level of concentration among the calibration graph solutions (0.05%, m/m, equivalent to 0.75 μg/ml) provided an RSD of the corrected peak area of 5.8%; thus 0.75 μg/ml can be considered an adequately low limit of quantitation.

Table 2
Regression curve data[a] for the studied analytes

Analyte	Concentration range (μg/ml)	*a*	*b*	r^2
AP	0.7–14	27.61 (±0.89)	0.0058 (±0.26)	0.998
5-ASA	450–1800	0.247 (±0.009)	1.52 (±11.7)	0.997
3-ASA	0.75–15	16.68 (±0.42)	−0.28 (±0.12)	0.998
SF	0.75–15	17.99 (±0.50)	−0.20 (±0.15)	0.998
SA	0.75–15	8.22 (±0.47)	−0.17 (±0.13)	0.996

[a] Regression curve data for five calibration points. $y=ax+b$, where y is the corrected peak area (area/migration time), x is the concentration (μg/ml), b is the intercept and r^2 is the correlation coefficient. Experimental conditions as in Table 1; detection of 5-ASA at 300 nm.

3.3.6. Robustness

In the present work the study of method robustness followed a traditional ''one-by-one'' approach, where individual operating parameters are separately changed over a predetermined range [9,19,20]. This approach was favoured by the obtained short analysis time. In particular, the effects on the robustness were considered for TBAB and SDS concentration, running buffer pH value and methanol percentage in the BGE. The chosen responses for the evaluation of robustness were the resolution between the peaks 5-ASA/3-ASA (R_{5-3}) and the migration time of the last migrating peak (t_m SA).

The investigation of the effects of small deliberate variations of TBAB and SDS concentrations in the BGE was carried out choosing ranges corresponding to ±1 m*M* around the optimised values. The results are summarised in Tables 3 and 4: no significant changes of R_{5-3} and t_m SA were observed.

The pH value of the BGE was varied ±0.05 units around the optimum value of 10.20 without significant change of the observed resolution and migration parameters (Table 5).

The methanol content of the BGE was varied ±0.1% around the optimum value (5%); no significant change of the studied parameters was observed (Table 6).

3.4. Applications

In order to establish the potentiality of the developed method, two batches of the bulk drug 5-

Table 3
Results of robustness experiments ($n=10$) for TBAB concentration

TBAB concentration (m*M*)	t_m[a] (min)	RSD (%)	R_{5-3}[b]	RSD (%)
54	5.41	0.88	1.86	2.10
55	5.38	0.50	1.92	2.81
56	4.98	1.65	2.12	2.58

[a] t_m: Migration time of salicylic acid (SA).
[b] R_{5-3}: Resolution between 5-ASA and 3-ASA. Other conditions as in Table 1.

Table 4
Results of robustness experiments ($n=10$) for SDS concentration

SDS concentration (m*M*)	t_m[a] (min)	RSD (%)	R_{5-3}[b]	RSD (%)
64	5.25	1.2	1.87	1.52
65	5.31	0.35	1.84	0.85
66	5.38	0.37	1.80	1.12

[a] t_m: Migration time of salicylic acid (SA).
[b] R_{5-3}: Resolution between 5-ASA and 3-ASA. Other conditions as in Table 1.

Table 5
Results of robustness experiments ($n=10$) for the running buffer pH value

pH	t_m[a] (min)	RSD (%)	R_{5-3}[b]	RSD (%)
10.25	5.58	0.44	1.92	1.58
10.20	5.39	0.30	1.90	0.85
10.15	5.38	0.24	1.97	1.82

[a] t_m: Migration time of salicylic acid (SA).
[b] R_{5-3}: Resolution between 5-ASA and 3-ASA. Other conditions as in Table 1.

Table 6
Results of robustness experiments ($n=10$) for the methanol percentage

Methanol (%)	t_m [a] (min)	RSD (%)	R_{5-3} [b]	RSD (%)
4.9	5.12	0.78	1.85	1.23
5.0	5.41	1.02	1.88	0.88
5.1	5.48	0.44	1.90	1.98

[a] t_m: Migration time of salicylic acid (SA).
[b] R_{5-3}: Resolution between 5-ASA and 3-ASA. Other conditions as in Table 1.

ASA provided by different manufactures were assayed. As described under the Section 3.3.4, the quantitation (recovery) of the main component was in excellent agreement with the declared contents for both the assayed batches.

Concerning the determination of the considered related substances, the levels found were in general very low. Precisely, in the assayed raw materials impurities of 3-ASA (<0.1%) and SA (0.26% and 0.61% for the two different batches) were found. No evidence of the presence of AP and SF was detected. A representative electropherogram of a real sample is reported in Fig. 6.

Interestingly in both batches, additional peaks with migration time shorter than 5-ASA were found. Investigations on the identity of these impurities, likely due to the photodegradative process [21], are in progress.

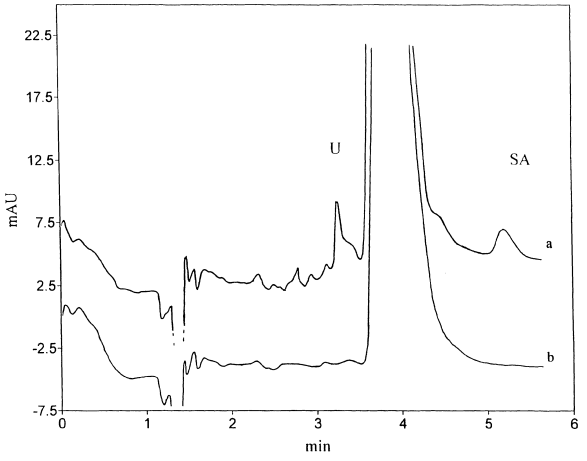

Fig. 6. Electropherogram of real sample 1500 μg/ml (5-ASA raw materials) (a), and pure standard sample (5-ASA>99%) (b). Detection at 230 nm. Conditions and abbreviations as in Fig. 4. U: Unknown compound.

4. Conclusion

A rapid CE method for the analysis of 5-ASA and related synthetic impurities was developed with a MEKC approach involving the use of an ion-pair reagent. The addition of TBAB to the BGE together with the use of short capillary (8.5 cm total length) resulted in high-speed separations with high reproducibility.

Both the optimisation and validation of the method were performed and the obtained results make the proposed MEKC method useful for applications to pharmaceutical quality control.

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