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Determination of ebrotidine and its metabolites by micellar electrokinetic capillary chromatography

S. Sentellas, L. Puignou, M.T. Galceran*

Department of Analytical Chemistry, University of Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain

Abstract

Ebrotidine and its potential metabolites were determined by micellar electrokinetic capillary chromatography (MECC) using sodium dodecylsulfate (SDS) as surfactant. The influences of buffer composition, SDS concentration and addition of a neutral surfactant such as Brij 35 were studied. A 40 m*M* phosphate buffer at pH 7.50 containing 50 m*M* of SDS was selected as carrier electrolyte, and provided the optimum separation with regard to resolution and migration time. Linear calibration curves over the range studied (5.0–50 μg ml^{-1}), limits of detection between 0.25 and 2.0 μg ml^{-1} and run-to-run precision lower than 10% were obtained. The MECC method was applied to the determination of these compounds in spiked human urine.

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Keywords: Buffer composition; H_2-Receptor antagonist; Ebrotidine

1. Introduction

Ebrotidine belongs to the H_2-receptor antagonist group of drugs. It shows an antisecretory activity comparable to other analogue drugs such as ranitidine [1,2] and, unlike other H_2-receptor antagonists, shows cytoprotective activity in animals [3,4] and humans [5,6]. Ebrotidine also inhibits the activity of *Helicobacter pylori* [7–9]. The structures of ebrotidine and its related compounds are shown in Fig. 1. Only ebrotidine, its sulfoxide and sulfone, 4-bromobenzensulfonamide, and the sulfoxide and sulfone compounds resulting from the removal of the thiazolyl ring have been identified in human urine after oral ebrotidine administration [10,11]. Other compounds were probably not found in the urine because of the degradation of these compounds to other smaller ones which are hard to detect.

Rozman et al. [12] have developed a method based on reversed-phase ion-pair high-performance liquid chromatography with UV–visible detection to determine ebrotidine and its metabolites. In order to obtain structural information, the coupling LC–MS has been optimised [11,13]. As demonstrated by the increase in the use of capillary electrophoresis, this technique is suitable for the determination of a wide range of analytes [14], including several H_2-receptor antagonists [15–20]. Capillary electrophoresis (CE) is a complementary technique for HPLC, and suitable separation methods can be developed for the analysis of closely related compounds using its high separation efficiency, short analysis time and relatively low cost. In a previous paper [21], a CE separation for ebrotidine and ionizable metabolites was described using 50 m*M* acetic acid at pH 5.7–methanol (70:30) as carrier electrolyte. Nevertheless,

*Corresponding author. Tel.: +34-93-402-1275; fax: +34-93-402-1233.

E-mail address: galceran@apolo.qui.ub.es (M.T. Galceran).

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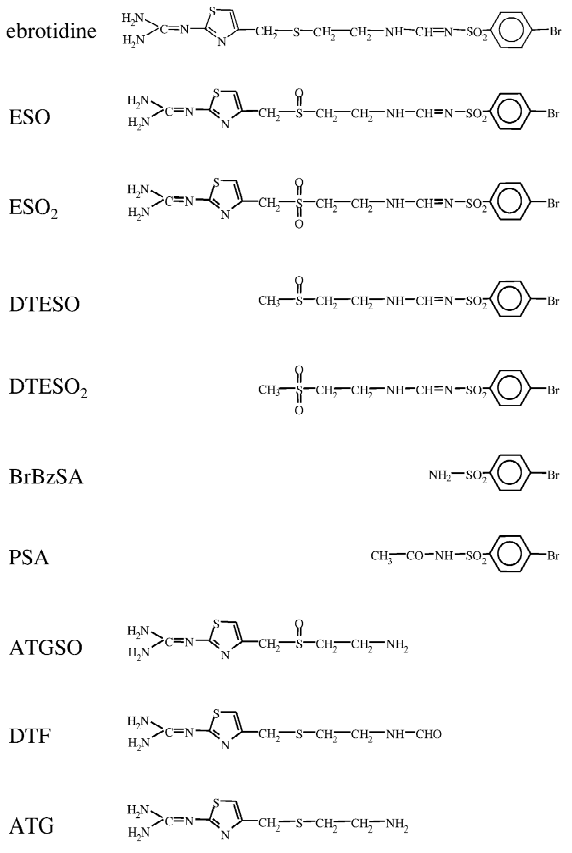

Fig. 1. Structures of ebrotidine and its metabolites.

in these electrophoretic conditions, less ionizable substances, such as the three ebrotidine-related compounds that do not contain a primary amine as functional group, could not be determined using a UV detector. Due to the neutral character of these compounds, they co-migrated by means of the electroosmotic flow and their detection was prevented by the corresponding system peak. A CE–electrospray ionization (ESI) MS method has been developed [21] allowing the detection of only two of the non-charged metabolites.

In this work, the use of micellar electrokinetic capillary chromatography (MECC) to overcome the difficult separation of non-charged ebrotidine metabolites was studied. When a micellar medium is used as carrier electrolyte in CE, the separation efficiency is increased because the apparent mobility of neutral substances can be distinguished. The mobility of neutral compounds is determined not only by the electroosmotic flow but also by the interactions with the pseudo-stationary phase constituted by the micelles. As a consequence, both charged and non-charged analytes can be separated [22,23]. The present paper reports the optimization of experimental conditions such as pH, electrolyte composition, sodium dodecylsulfate (SDS) concentration, and the addition of neutral micelles such as Brij 35 to carry out the separation of both charged and non-charged compounds related to ebrotidine. Figures of merit such as precision and limits of detection were established. Finally, a spiked human urine sample was extracted using the procedure proposed by Rozman et al. [12] and analysed by the MECC method.

2. Experimental

2.1. Chemicals

Ebrotidine (E) {*N*-[(4-bromophenyl)sulfonyl]-*N′*-[2-([{2-[(diamino-methylene) amino]-4-thiazolyl}-methyl]thio)ethyl]formamidine} and the related compounds ebrotidine sulfoxide (ESO), ebrotidine sulfone (ESO_2), 2-*N*[4-{[(2-aminoethyl)-thio]methyl}-2-thiazolyl]guanidine (ATG) and its sulfoxide (ATGSO), *N*-[2([{2-[(diamino-methylene) amino]-4-thiazolyl}methyl]thio)ethyl]-formamide (DTF), 4-bromobenzenesulfonamide (BrBzSA), *N*-[(4-bromophenyl)sulfonyl]acetamide (PSA), *N*-(2-methylsulfinylethylamino-methylene)-4-bromobenzenesulfonamide (DTESO) and *N*-(2-methylsulfonylethylamino-methylene)-4-bromobenzenesulfonamide ($DTESO_2$) were a gift from Grupo Ferrer (Barcelona, Spain) and their structures are shown in Fig. 1. Stock solutions (1000 μg ml^{-1}) of each compound were prepared in methanol (ebrotidine, ATGSO, DTF and ATG), acetonitrile (ESO_2, DTESO, $DTESO_2$, PSA and BrBzSA) and acetonitrile–dimethyl sulfoxide (90:10, v/v) (ESO). To prepare the standard solutions, methanol and acetonitrile were obtained from Merck (Darmstadt, Germany) and dimethyl sulfoxide was obtained from Panreac (Barcelona, Spain). Water purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for all solutions. Other reagents used for the preparation of carrier elec-

trolytes were analytical grade: boric acid (Panreac, Barcelona, Spain), sodium tetraborate (Carlo Erba, Milan, Italy), sodium phosphate monobasic and sodium phosphate dibasic (Probus, Barcelona, Spain), SDS (Fluka, Buchs, Switzerland) and Brij 35 (Merck, Darmstadt, Germany). Sudan III (Sigma, St Louis, MO, USA) was used as the marker for micelle migration and the sample solvent was used as the marker for the electroosmotic flow (EOF).

2.2. Electrophoretic conditions

A Beckman (Fullerton, CA, USA) P/ACE System 5500 with diode array detection capillary electrophoresis was used; electrophoretic data were processed using the P/ACE Station software. A fused-silica capillary (Supelco, Bellefonte, PA, USA), 47 cm (effective length 40 cm)×75 μm I.D. was used. The temperature was held at 25 °C. Direct detection was carried out at 235 nm. For the MECC separation, a carrier electrolyte of 40 m*M* phosphate buffer adjusted to pH 7.5 containing 50 m*M* SDS, and a voltage of 20 kV were used. New capillaries were pre-treated with 1 *M* sodium hydroxide for 30 min. Working capillaries were treated daily with 1 *M* sodium hydroxide for 5 min, with water for 15 min and with the carrier electrolyte for 30 min. For conditioning the electrophoretic system, a voltage of 20 kV and an overimposed pressure of 3.4 kPa were simultaneously applied during 30 min. Samples were loaded by pressure injection at 3.4 kPa for 4 s. Carrier electrolytes were filtered through a 0.45-μm membrane, and degassed by sonication before use.

2.3. Urine sample analysis

A liquid–liquid extraction procedure [12] was used for the analysis of ebrotidine, ESO, ESO_2, DTESO, $DTESO_2$ and BrBzSA in urine spiked at 5 μg ml^{-1} concentration level of each analyte. Briefly, after the addition of 100 μl of 1 *M* NaOH to 1 ml of urine, the sample was shaken for a few seconds. Then, 5 ml of CH_2Cl_2–2-PrOH (9:1) was added to the mixture and it was mechanically shaken for 15 min; finally, centrifugation at 4500 rpm (2000 *g*) was applied for 5 min. An aliquot of 3 ml of the organic layer was evaporated to dryness at 40 °C using nitrogen and the residue was dissolved with 150 μl of an aqueous solution of the internal standards.

3. Results and discussion

3.1. Optimization of MECC separation

To optimise the MECC method, experimental conditions such as pH, electrolyte composition, SDS concentration and the addition of a neutral micelle (Brij 35) were studied. As initial conditions, 50 m*M* SDS in 50 m*M* phosphate or borate buffer was used as carrier electrolyte.

3.1.1. Influence of pH

The effect of pH between 7.0 and 9.0 on the separation of ebrotidine and its metabolites was studied using phosphate and borate 50 m*M* buffer solutions. All the carrier electrolytes contained SDS at a concentration of 50 m*M*. When borate was used at pH 8.5 and 9.0, a short analysis time was obtained, around 7 min, and four of the compounds could not be resolved. Using phosphate as buffer solution, an important increase in migration time was observed that can be related to both the pH decrease and the change of the buffer. Moreover, the analysis time decreased from 35 to 20 min as pH increased from 7.0 to 8.0. At pH 8.0, the peaks were not totally resolved, so a phosphate solution at pH 7.5 was selected as the optimum in terms of resolution and analysis time. The electropherogram obtained in these conditions is given in Fig. 2(e). In these conditions, all the compounds were totally separated, even the less ionizable ones, i.e. BrBzSA, DTESO and $DTESO_2$, which could be totally resolved due to their selective interactions with the micelles. In a previous work [21], at pH 5.7, PSA showed contraelectroosmotic mobility due to its negative charge. Moreover, compounds BrBzSA, DTESO and $DTESO_2$ comigrated with the electroosmotic flow marker, behaving as neutral compounds, and ebrotidine and the rest of the metabolites showed cationic mobility. In the present work, working at higher pH values, ebrotidine migrated with the micelles, showing an important hydrophobic interaction. In fact, all the positively charged compounds were able to decrease their charge density and even

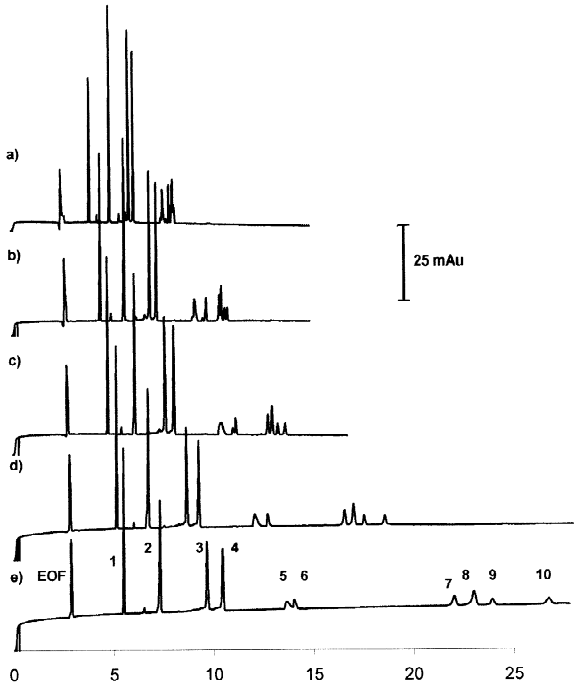

Fig. 2. Effect of the buffer concentration: (a) 10 m*M*; (b) 20 m*M*; (c) 30 m*M*; (d) 40 m*M* and (e) 50 m*M*. Other conditions: carrier electrolyte: phosphate buffer at pH 7.5, 50 m*M* SDS and applied voltage of 20 kV. Assignation: 1, PSA; 2, BrBzSA; 3, DTESO; 4, $DTESO_2$; 5, DTF; 6, ATGSO; 7, ESO_2; 8, ESO; 9, ATG and 10, E. Time scale in min.

become not-ionized, thus their mobilities could depend basically on their hydrophobic interaction with the micelles. In contrast, the negatively charged PSA migrated just after the EOF marker, as in the non-micellar conditions, and before all the other compounds, which seems to indicate non-interaction with the micelles.

3.1.2. Influence of buffer concentration

When a phosphate buffer 50 m*M* at pH 7.5, with SDS 50 m*M* was used as carrier electrolyte, all the compounds were well resolved and, moreover, the analysis time was acceptable (27 min). However, the current through the capillary was very high, around 220 μA. This is a problem because if the heat generated by the current through the capillary (Joule heat) cannot be dissipated effectively (using a liquid refrigerant in our case) peak broadening and poor reproducibility in migration times can occur [24]. In order to prevent this effect, the influence of buffer concentration between 10 and 50 m*M* on the separation was studied (Fig. 2). As expected, at lower concentrations an increase in the electroosmotic flow occurred due to an increase in the zeta potential [25], and in consequence shorter analysis times, from 26.9 to 8.2 min, were obtained. Nevertheless, this decrease in the analysis time produced a loss of resolution, which is only important for the last four peaks, those that correspond to the compounds ESO_2, ESO, ATG and E. As can be seen in Fig. 2, the peaks were totally resolved at concentrations of the buffer higher than 30 m*M*. Taking into account analysis time and resolution, a 40 m*M* phosphate buffer was chosen as the best condition (analysis time 18.7 min). Moreover, the current through the capillary at these conditions decreased to 180 μA.

3.1.3. Influence of SDS concentration

To study the influence of SDS, concentrations between 20 and 50 m*M* were tested. Lower concentrations were avoided to ensure micelle formation. As can be seen in Fig. 3, analysis time decreased from 18.7 to 15.9 min when the concentration of SDS decreased. In this case, only the resolution between BrBzSA and PSA was affected. The mobility (migrating towards the cathode) of all the compounds, except for PSA, decreased with SDS concentration. This could be due to the dependence

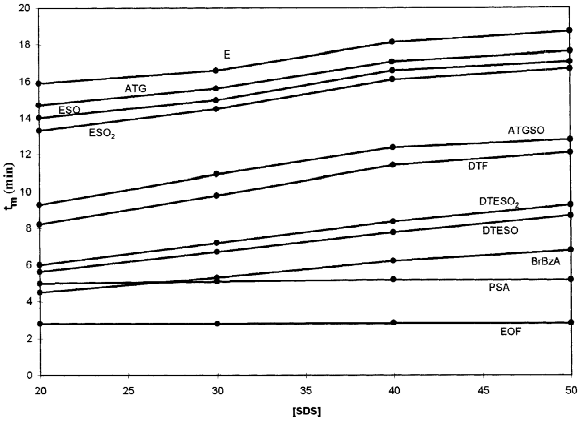

Fig. 3. Effect of the SDS concentration on the mobility of ebrotidine and related compounds. Other conditions: carrier electrolyte: 40 m*M* phosphate buffer at pH 7.5 and applied voltage of 20 kV. Concentration scale in m*M*.

of the solute's capacity factor (k') on surfactant concentration [26]:

$$k' = k_{mw} (C_{SDS} - CMC) \quad (1)$$

where k_{mw} is the solute-micelle binding constant, C_{SDS} is the surfactant concentration and CMC is the critical micellar concentration. However, the resolution was not affected because the SDS concentration has no effect on chemical selectivity. Conversely, the mobility of PSA is practically constant under all studied conditions, i.e. its migration is independent of SDS concentration. This compound has a contraelectroosmotic mobility, and taking into account its behaviour, we can suppose that its mobility only depends on its own negative charge, i.e. this compound does not interact with the micelle. The different behaviour of the compounds BrBzSA and PSA shows that the resolution between them decreased at low SDS concentration. Moreover, at 20 m*M* SDS, these two compounds changed the order of migration. As a result, SDS concentrations higher than 40 m*M* are necessary. As analysis time was not substantially affected, 50 m*M* SDS was used to obtain the maximum resolution.

3.1.4. Influence of the addition of a neutral surfactant

Finally, the addition of Brij 35 was tested. A neutral surfactant produces a decrease in analysis time because the comicelle formed is less negatively charged and its mobility toward the anode decreases. Concentrations between 0 and 6 m*M* of Brij 35 were studied and, although a reduction in analysis time to 11.1 min was obtained, a decrease in resolution was also observed. At relatively low Brij 35 concentrations, 2 m*M*, only the compounds DTF and ATGSO had poor resolution, but at higher concentrations of the neutral surfactant, losses of resolution for all the peaks were observed. Therefore, the addition of Brij 35 in the electrolyte solution was avoided.

3.2. Quality parameters

In the optimum conditions, quality parameters such as limits of detection and run-to-run precision, were determined. Calibration curves based on areas were constructed for standard solutions between 5 and 50 μg ml^{-1} and good linearity with correlation coefficients higher than 0.99 was obtained. The limits of detection for each compound, based on a signal-to-noise ratio of 3, are given in Table 1. As can be seen, these limits ranged from 0.25 to 2.0 μg ml^{-1}, which shows that limits of detection were improved from 2 to 16 times with respect to those obtained with the CZE method with UV detection [21].

To study the reproducibility of the method, a standard solution (35 μg ml^{-1} of each compound) was analysed. For this purpose, calibration curves were recorded for each compound and the standard solution was quantified in terms of concentration. The results, including precision data for migration time, are shown in Table 2. Run-to-run precision was calculated by injecting three replicates of the standard solution the same day. Results obtained for migration times and concentration were up to 1.5% and to 10%, respectively.

3.3. Application

To show the applicability of the method, a sample of human urine was spiked with compounds that had been previously identified following oral ebrotidine administration [10,11]. These compounds were ebrotidine, ESO, ESO_2, DTESO, $DTESO_2$ and BrBzSA at 5 μg ml^{-1}. Extraction and pre-concentration of the human urine sample was performed

Table 1
Limits of detection for ebrotidine and its metabolites. Comparison between the CZE and the MECC methods

	CZE (μg ml^{-1})[a]	MECC (μg ml^{-1})
Ebrotidine	4.3	0.5
ESO	3.4	0.5
ESO_2	3.5	0.5
DTESO[b]	–	0.5
$DTESO_2$[b]	–	0.5
BrBzSA[b]	–	0.5
PSA	4.0	0.25
ATGSO	3.9	2.0
DTF	3.5	2.0
ATG	3.8	2.0

[a] Data from Ref. [21].
[b] Not detectable with the CZE method.

Table 2
Run-to-run precision (RSD) for migration times and concentrations for ebrotidine and its metabolites

	RSD (%)	
	t_m	Concentration
Ebrotidine	1.39	9.91
ESO	1.37	3.63
ESO_2	1.41	4.73
DTESO	0.35	3.56
$DTESO_2$	0.43	3.60
BrBzSA	0.36	4.50
PSA	0.41	5.27
ATGSO	1.50	5.95
DTF	1.52	5.80
ATG	1.40	6.12

by the method proposed by Rozman et al. [12], as described in the Experimental section. Recoveries applied to the quantitation of the analytes were 87, 98, 97, 86, 92 and 99% for ebrotidine, ESO, ESO_2, DTESO, $DTESO_2$ and BrBzSA, respectively, as previously reported [12]. Two of the compounds not found in human urine were used as internal standards, DTF for the ebrotidine and its sulfoxide and sulfone, and PSA for compounds DTESO, $DTESO_2$ and BrBzSA. As can be seen in Fig. 4, no interferences that can prevent the analysis of a urine sample were observed. Three independent samples were processed and injected twice obtaining standard deviations between 0.5 and 3% and results in agreement with the spiked level in the original sample, with errors below 15%.

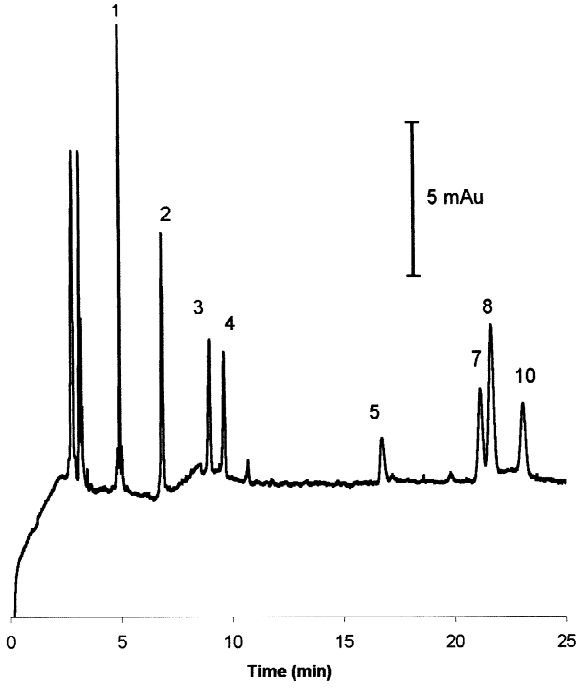

Fig. 4. Electropherogram of a sample of human spiked urine. Carrier electrolyte: 40 m*M* phosphate (pH 7.5)–50 m*M* SDS, applied voltage 20 kV. Assignation as in Fig. 2.

4. Conclusions

A MECC method has been developed for the determination of ebrotidine and its metabolites. Using a buffer of 40 m*M* phosphate with 50 m*M* of SDS as electrolyte, ebrotidine and all its related compounds can be totally resolved. Limits of detection from 0.25 to 2.0 μg ml^{-1} were obtained, which means an improvement of 2 to 16 times with respect to the previous CZE method. Good run-to-run precisions in migration time ($<1.5\%$) and in concentration ($<10\%$) were also obtained. Ebrotidine and its metabolites can be identified and quantified in human urine (errors below 15%) by the MECC method proposed.

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