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Optimization and validation of a capillary zone electrophoretic method for the simultaneous analysis of four atypical antipsychotics

S. Hillaert*, L. Snoeck, W. Van den Bossche

Laboratory of Pharmaceutical Chemistry and Drug Analysis, Department of Pharmaceutical Analysis, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

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Abstract

A capillary zone electrophoretic method has been developed and optimized for separation of four atypical antipsychotics (AAPs): clothiapine (cT), clozapine (cZ), olanzapine (O), and quetiapine (Q). A three-level full-factorial design was applied to study the effect of the pH and molarity of the running buffer on separation. Combination of the studied parameters permitted the separation of the four AAPs, which was best carried out using 80 mM sodium phosphate buffer (pH 3.5). The same system can also be applied for the quantitative determination of these compounds. The method was then validated regarding linearity, precision, and accuracy. Especially, the possibility of simultaneous quantification and identification of the active ingredient in the finished product is very attractive.

Keywords: Experimental design; Optimization; Validation; Antipsychotics; Clothiapine; Clozapine; Olanzapine; Quetiapine

1. Introduction

In the last 10 years, the treatment of schizophrenia has been improved by the introduction of a group of drugs known collectively as 'atypical antipsychotics' (AAPs). These new drugs have been proposed as alternatives to the 'classical antipsychotics' because they seem to be more effective since they can suppress positive and negative symptoms of schizophrenia and show less extrapyramidal effects [1,2]. Four AAPs are studied: clothiapine (cT), clozapine (cZ), olanzapine (O), and quetiapine (Q) (Fig. 1) [3].

Until now, high-performance liquid chromatography (HPLC) has been the major technique used for the quality control of pharmaceutical formulations containing these drugs, but these studies have usually been limited to the determination of a single component (or a few compounds) [4–8]. Capillary electrophoresis (CE) offers an alternative technique. Although analysis by means of CE has been achieved for clozapine [5,9–11] and olazapine [7,12], only two studies have reported the simultaneous determination of clozapine and olanzapine by CE [13,14]. To our best knowledge, no methods have been described so far in the literature for the simultaneous determination of the four AAPs.

The aim of the present study was therefore to develop a selective capillary zone electrophoretic method capable of separating the four above mentioned AAPs. A statistical experimental design was used for the optimization of the method [15,16]. After preliminary investigations to adjust the experimental domain under study, a three-level full-factorial design was applied to study the impact of two parameters on the retention of these compounds [17–19]. The studied parameters were the pH and the molarity of the running buffer. Afterwards, the usefulness of the system for the quantitative determination of these compounds in their pharmaceutical formulation was investigated, and the method was then validated regarding the linearity, precision (repeatability), and accuracy.

2. Experimental

2.1. Instrumentation and electrophoretic procedure

Experiments were performed on a Waters Quanta 4000 (Millipore, Milford, MA, USA). A fused-silica capillary

* Corresponding author. Tel.: +32-9-264-81-01; fax: +32-9-264-81-93.
E-mail address: sandra.hillaert@ugent.be (S. Hillaert).

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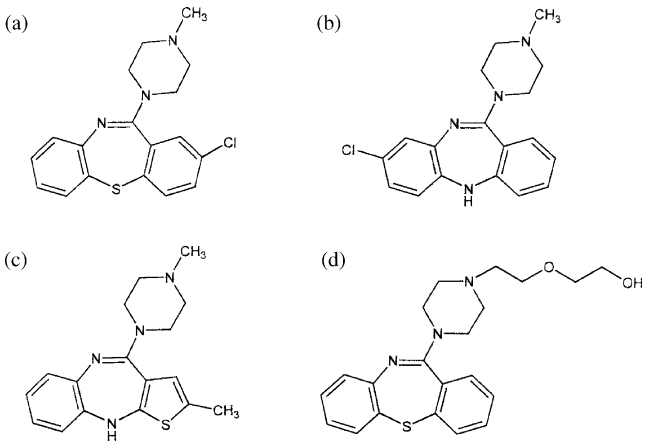

Fig. 1. Chemical structures of clothiapine (a), clozapine (b), olanzapine (c), and quetiapine (d).

was used, 37.5 cm in total length (30 cm to the detector) and 75 μm internal diameter (i.d.). Hydrostatic injections were performed by lifting the sample vial approximately 10 cm above the height of the buffer vial for 3 s. For detection, the absorbance was measured by means of an on-line fixed-wavelength UV detector with a zinc discharge lamp and a 214 nm filter. The experiments were performed at 10 kV at room temperature (20 ± 2 °C). Data were collected on a Hewlett-Packard Integrator (HP 3396 Series II, Avondale, PA, USA), which was also used for calculating the areas under the peaks. The pH measurements were performed on a calibrated Metrohm 744 pH Meter (Herisau, Switzerland).

2.2. Reagents

Sodium dihydrogenphosphate monohydrate (analytical-reagent grade) and disodium hydrogenphosphate dihydrate (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Phosphoric acid (85%, w/w) was obtained from UCB (Leuven, Belgium).

The excipients (microcrystalline cellulose, lactose, maize starch, calciumhydrogenphosphate, magnesium stearate, hydroxypropylcellulose, crospovidone, methylhydroxypropylcellulose, polyvidone, sodium starch glycolate, silicon dioxide, macrogol, polysorbate 80, titanium dioxide, gelatin, liquid paraffin, talc, and indigo carmine) are commercially available products that meet the requirements of the European Pharmacopoeia.

Clothiapine and clozapine were obtained from Novartis (Basel, Switzerland), olanzapine from Lilly (Brussels, Belgium) and quetiapine from AstraZeneca (Mölndal, Sweden). The commercially available drugs Etumine (clothiapine, Novartis), Leponex (clozapine, Novartis), Zyprexa (olanzapine, Lilly), and Seroquel (quetiapine, AstraZeneca) were used for quantitative determinations.

All solutions were prepared using distilled water obtained from deionized water.

2.3. Running buffers

During the development of the method, sodium phosphate buffers of different pH were used. In the pH range 2.0–4.5, a mixture of a phosphoric acid solution and sodium dihydrogenphosphate solution was used, while in the range 4.5–5.0, it was a mixture of a sodium dihydrogenphosphate solution and a disodium hydrogenphosphate solution. A 80 mM sodium phosphate buffer (pH 3.5) was finally chosen as the running buffer. It was prepared by adjusting the pH of a 80 mM sodium dihydrogenphosphate solution to pH 3.5 by the addition of 80 mM phosphoric acid solution.

2.4. Internal standard solutions

The use of an internal standard is only needed for quantitative determination because it compensates for differences in injection volume. Therefore, another AAP was always used as an internal standard. Selection had to be made based on the substance to be examined. Although each AAP can be combined, the nearest migrating AAP was chosen as the internal standard. An appropriate amount of the compound (Table 1) was dissolved in 20 ml 0.1 M H_3PO_4 and diluted to 100 ml with the same solvent.

2.5. Choice of solvent

The running buffer cannot be used as a solvent for the preparation of reference and sample solutions because of the poor solubility of the AAPs. Taking the acidic medium in which the experiments are performed into account, 0.1 M H_3PO_4 was added to dissolve the active substances.

2.6. Reference solutions for the experimental design

Reference solutions of clothiapine, clozapine, olanzapine, and quetiapine were prepared at 125 μg/ml in 0.1 M H_3PO_4.

2.7. Reference solutions for the quantitative determination

Reference solutions were prepared by weighing accurately an appropriate amount of the corresponding reference substance, dissolving it in 0.1 M H_3PO_4 and diluting to 50.0 ml with the same solvent. An appropriate volume of each solution was mixed with 10.0 ml of the internal standard solution and diluted to an appropriate concentration with 0.1 M H_3PO_4 (Table 1).

2.8. Sample preparations for the quantitative determination

A minimum of 20 tablets of each compound were weighed, ground, and mixed. The requisite amount of the powder was mixed with 10.0 ml of the appropriate internal standard solution and diluted to the required concentration with 0.1 M H_3PO_4 (Table 1).

Table 1
Solutions for the quantitative determination

	Diluted reference solution (mg/ml)	Internal standard	Final concentration of the internal standard (mg/ml)	Diluted sample solution (mg active substance/ml)
Clothiapine (Etumine), 40 mg tablets	0.08	Quetiapine	0.25	0.08
Clozapine (Leponex), 25 mg tablets	0.08	Olanzapine	0.65	0.08
Olanzapine (Zyprexa), 10 mg tablets	0.16	Clozapine	0.25	0.17
Quetiapine (Seroquel), 25 mg tablets	0.18	Clozapine	0.35	0.18

All samples and buffers were filtered by passing them through 0.45 μm membrane filters (Millipore, Bedford, MA, USA).

2.9. Experimental set-up and analysis of results

The set-up of the design and the statistical analysis of the response variables were supported by the statistical graphics software system STATGRAPHICS Plus Version 4.1 (STSC, Rockville, MD, USA).

3. Results and discussion

3.1. Screening phase

Several parameters were considered. From preliminary experiments, it was found that the factors most affecting the response migration time were the pH and the molarity of the running buffer. The pH of the separation buffer plays an important role, because it affects the observable migration velocity of the solutes by changing the effective electrophoretic mobility of the solutes by affecting the degree of dissociation (or protonation), and by changing the velocity of the electroosmotic flow (EOF) by affecting the zeta potential at the capillary walls. Different concentrations of the running buffer were tested to optimize the separation. Selection of the experimental domain was made from prior experience and knowledge of the separation system. The voltage initially was also considered, but it was found to have less influence on the selectivity of the separation and was kept constant at 10 kV.

3.1.1. Selection of the pH

All of the separands under investigation possess strongly basic amine groups so normal capillary zone electrophoresis (CZE) at low or moderate pH might be suitable for their determination. At these operating conditions, the analytes are mainly protonated so attention should be paid to the possible adsorption of the positively charged analytes onto the negatively charged wall of the fused-silica capillary. This interaction might cause peak broadening or even loss of resolution [20]. Therefore, a too high pH of the running buffer must be avoided. Because the best peak shapes were obtained between pH 2.0 and 5.0, the measurements were performed at three pH levels (2.0, 3.5, and 5.0).

3.1.2. Concentration of the running buffer

In earlier investigations, the molarity of the sodium phosphate buffers varied from 20 to 110 mM. When the concentration of the electrolyte increased, the selectivity of the separation improved and the migration times increased. If concentrations above 110 mM were used, high current was generated. Because of the optimum balance in ionic strength, the concentration of the running buffer was tested at three levels (50, 80, and 110 mM) for optimization purposes.

3.2. Response surface design

To establish the influence of the two parameters and their interaction on the separation, a three-level full-factorial design was applied. This design requires nine runs. The experimental matrix included two extra experiments at the central level of the design to obtain an estimate of experimental variance. Thus, the entire design required 11 runs. The individual runs of the design were carried out in a randomized sequence. Randomization offers some assurance that uncontrolled variation of factors, other than those studied, will not influence the estimations. Replicate measurements ($n = 3$) were performed to verify if migration times were stable and the capillary was well equilibrated after tuning to new electrophoretic conditions.

The measured responses were the migration times of clothiapine (t_{cT}), clozapine (t_{cZ}), olanzapine (t_O), and quetiapine (t_Q). In Table 2, the measured migration times (t) for each run of the design are compiled.

Table 2
Measured response variables

Run	pH	Molarity of the running buffer (mM)	t_{cT}	t_{cZ}	t_o	t_Q
1	2.0	50	5.21	4.86	4.82	5.39
2	2.0	80	5.32	4.97	4.92	5.48
3	2.0	110	5.32	5.07	4.96	5.50
4	3.5	50	7.18	5.75	5.06	6.74
5	3.5	80	7.38	5.96	5.35	6.91
6	3.5	80	7.38	5.96	5.35	6.91
7	3.5	80	7.37	5.95	5.34	6.90
8	3.5	110	8.80	7.03	6.25	8.22
9	5.0	50	7.89	7.56	5.73	8.38
10	5.0	80	7.29	7.00	5.50	7.67
11	5.0	110	6.86	6.67	5.50	7.22

Migration times (t) in min.

3.2.1. Regression modeling

From the 3^2 design for each response, the following model was determined:

$$y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2$$

where y is the measured response (migration time) for each compound, b_0 the intercept, b_i the regression coefficients, and X_i are the values of the independent electrophoretic variables (X_1 = pH; X_2 = molarity of the running buffer).

To obtain a good separation of compounds, an adequate difference in migration time is needed. The minimal time difference or the time difference of the two worst separated peaks (Δt_{min}) is especially important. Therefore, we were interested in the domain(s) where Δt_{min} was maximal.

First, the measured migration times for each ARA-II were modeled. Then the responses were predicted for all possible, experimentally different conditions in the studied domain. Subsequently, for each situation, the migration times of the compounds were sorted, the difference in migration time of the successive pairs of peaks (t_i) was calculated, and Δt_{min} was selected. Finally, all Δt_{min} were plotted, and the region(s) where Δt_{min} was maximal were investigated.

From preliminary results, it was found that a baseline separation of the AAPs can be expected with a predicted value of $\Delta t_{min} = 0.45$. To distinguish the regions with this value, the contour plot of Δt_{min} as a function of the pH and molarity of the running buffer was created (Fig. 2). Only one large area seemed to meet this requirement. The robustness of the selected region was also evaluated: the boundaries were not retained as optimal separation conditions, because small differences in experimental conditions can lead to inadequate separations. Therefore, the best combination seems to be pH 3.5 and 80 mM.

Not only the value of Δt_{min} is important, but the total analysis time also plays a role. The region with an optimum

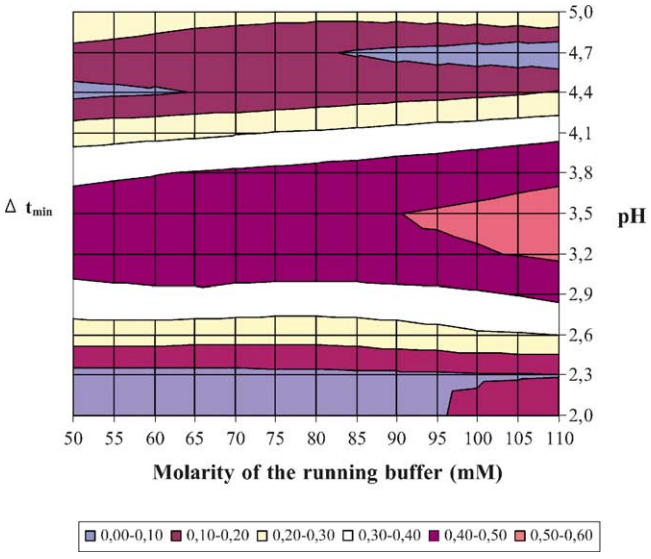

Fig. 2. Contour plot of Δt_{min} as a function of the pH and molarity of the running buffer.

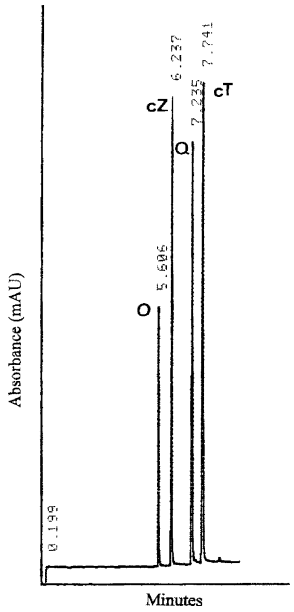

Fig. 3. Electropherogram of a mixture of several AAPs using a fused-silica capillary 37.5 cm (30 cm to the detector) × 75 μm i.d. *Conditions*: 80 mM sodium phosphate buffer (pH 3.5) as the running buffer; applied voltage, 10 kV; detection at 214 nm.

balance between Δt_{min} and the analysis time must be determined to obtain a baseline separation within an acceptable analysis time for the different AAPs. In the selected area, the longest migration time is situated between 7 and 8 min, and is thus also acceptable. Therefore, the best combination remains pH 3.5 and 80 mM. A typical electropherogram obtained applying these optimized conditions (80 mM sodium phosphate buffer, pH 3.5) is presented in Fig. 3.

3.3. Quantitative determination in pharmaceutical formulations

The same system (80 mM sodium phosphate buffer, pH 3.5) may be applied for the quantitative determination of clothiapine (Fig. 4), clozapine, olanzapine, and quetiapine in tablets. Using different placebo mixtures it was demonstrated that the following excipients do not adversely affect the results: microcrystalline cellulose, lactose, maize starch, calciumhydrogenphosphate, magnesium stearate, hydroxypropylcellulose, crospovidone, methylhydroxypropylcellulose, polyvidone, sodium starch glycolate, silicon dioxide, macrogol, polysorbate 80, titanium dioxide, gelatin, liquid paraffin, talc, and indigo carmine.

3.4. Validation of the method

3.4.1. Linearity

The detector responses were found to be linear for the different components in the concentration range, as described in Table 3. The amount of the internal standard was adjusted according to the concentration range used. Regression anal-

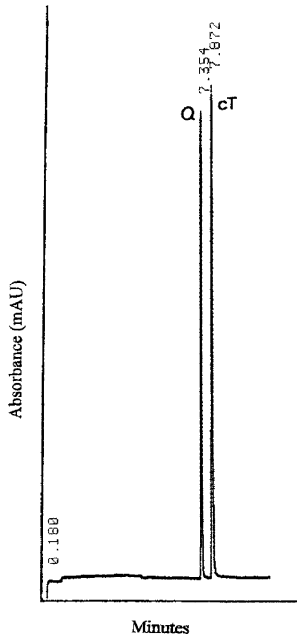

Fig. 4. Electropherogram of the quantitative determination of clothiapine (etumine) on a fused-silica capillary 37.5 cm (30 cm to the detector) × 75 μm i.d. *Conditions*: 80 mM sodium phosphate buffer (pH 3.5) as running buffer; applied voltage, 10 kV; detection at 214 nm.

Table 3
Linearity

	Concentration range (mg/ml)	Correlation coefficient (r^2)	Regression equations
Clothiapine	0.025–0.125	0.9998	$y = 0.1643x + 0.0092$
Clozapine	0.025–0.125	0.9996	$y = 0.2253x + 0.0124$
Olanzapine	0.050–0.250	0.9998	$y = 0.1412x + 0.0013$
Quetiapine	0.050–0.250	0.9994	$y = 0.1330x + 0.0142$

ysis data for the calibration curves were calculated using the peak areas.

3.4.2. Precision

The precision as repeatability of the method was determined by the total analysis of 10 replicate samples under the same operating conditions, by the same analyst, and on the same day. The mean value of the concentration and the relative standard deviation are summarized in Table 4.

Table 4
Precision (repeatability) of the total analysis of 10 replicate samples

Substance to be examined	Theoretical amount (mg/tablet)	Amount found	Relative standard deviation (%) ($n = 10$)
Clothiapine (Etumine)	40	40.11 ± 0.56 mg or 100.3%	1.39
Clozapine (Leponex)	25	25.01 ± 0.43 mg or 100.0%	1.71
Olanzapine (Zyprexa)	10	10.15 ± 0.21 mg or 101.5%	2.06
Quetiapine (Seroquel)	25	24.77 ± 0.28 mg or 99.1%	1.14

Table 5
Accuracy

	Recovery placebo + 80 (%) ($n = 3$)	Recovery placebo + 100 (%) ($n = 3$)	Recovery placebo + 120 (%) ($n = 3$)
Clothiapine	101.0 ± 0.8	101.0 ± 1.3	101.9 ± 1.5
Clozapine	99.6 ± 1.6	100.4 ± 0.7	100.7 ± 0.6
Olanzapine	99.6 ± 0.5	101.0 ± 1.0	101.8 ± 1.5
Quetiapine	97.5 ± 1.5	99.8 ± 0.8	98.9 ± 1.1

The error of the equipment, the accuracy of electrophoretic separation, and the relative standard deviations of the peak area ratios were determined by performing 10 consecutive injections of the same sample ($R.S.D._{cT} = 0.96\%$, $R.S.D._{cZ} = 0.99\%$, $R.S.D._{O} = 0.39\%$, and $R.S.D._{Q} = 1.46\%$).

3.4.3. Accuracy

The accuracy of the method was determined by investigating the recovery of each component at three levels, ranging from 80 to 120% of the theoretical concentration, from placebo mixtures spiked with the active substance (Table 5).

4. Conclusions

The above results demonstrate that a capillary zone electrophoretic separation of four atypical antipsychotics: clothiapine, clozapine, olanzapine, and quetiapine can be achieved using an 80 mM sodium phosphate buffer at pH 3.5. This method can be applied successfully to the quantitative determination of the above compounds in pharmaceutical formulations. The possibility of simultaneous identification and quantification of the active ingredients in the finished product is very attractive from the analytical viewpoint.

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