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Potentialities of ITP-CZE method with diode array detection for enantiomeric purity control of dexbrompheniramine in pharmaceuticals

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Abstract

The present work illustrates potentialities of on-line combined isotachophoresis-capillary zone electrophoresis (ITP-CZE) separation techniques coupled with on-capillary diode array detector (DAD) for enantiomeric purity testing of drugs in pharmaceuticals. The general advantages of the proposed method are its (i) high selectivity, (ii) low concentration limit of detection (LOD) obtainable, (iii) enhanced sample loadability, and (iv) enhanced reliability. For separation of brompheniramine (BP) enantiomers, serving as model analytes, carboxyethyl-β-cyclodextrin (CE-β-CD) was appropriate chiral selector providing complete enantioresolution. Given by a high sample load capacity (30 μl sample injection volume) and preconcentration of the analytes in ITP stage, concentration LOD of levobrompheniramine (LBP), serving as model impurity, was 2.5 ng/ml (8×10^{-9} mol/l). Such separation and detection conditions enabled to easily determine LBP in samples containing a 10^3 excess of dexbrompheniramine (DBP). DAD detection in comparison with single wavelength detection can enhance value of analytical information when analytes and interferents have different spectra (distinguishing impurities in analyte zone, confirmation of migration positions of migrants). In this context purity of BP zones was confirmed with higher reliability in pharmaceutical sample. Moreover, distinguishing the trace analyte signal superposed on the baseline noise was provided with sufficient reliability (for this purpose the background correction and smoothing procedure had to be applied to the raw DAD spectra). Successful validation and application of the proposed ITP-CZE-DAD method suggest its routine use for the enantiomeric purity testing of pharmaceuticals.
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Keywords: Column-coupling electrophoresis; Diode array detection; Dexbrompheniramine; Enantiomeric purity; Cyclodextrin

1. Introduction

The pharmacological activity of chiral drugs is in many cases restricted to one of the enantiomers. In pharmacotherapy the use of single-enantiomer forms can often lead to an improvement in the efficacy of the drug or the suppression of side effects related to the other enantiomer [1,2]. Production of enantiomerically pure pharmaceuticals highlights the importance of developing of new progressive analytical methods with the capability to identify and quantify enantiomers present in the sample at very high concentration ratio (active:impurity) [3,4].

Limit of detection (LOD) of 0.1% impurity is widely accepted as a minimum requirement for chiral trace impurity determinations [5]. Therefore analytical methods for the control of enantiomeric purity of the drugs are required to provide high-resolution power, high efficiencies and very low detection limits [6–8]. Among high performance separation techniques, accomplishing these criteria, capillary electrophoresis (CE) is superior for the analytical separations of ionic compounds, and it can be advantageously used because of its extremely high peak efficiency, versatility, simplicity, short analysis time, good compatibility with aqueous samples and low consumption of chiral selector (low cost of enantioselective analyzes) [9–11].

Brompheniramine (BP), *N*,*N*-dimethyl-3-(4-brom)-phenyl-3-(pyridin-2-yl)propan-1-amine, is a highly potent and widely used antihistaminic drug. Due to higher selectivity of S-

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enantiomer towards H_1-receptors, pharmaceuticals containing dexbrompheniramine (DBP) as the main active ingredient are currently used in pharmacotherapy. CE has been used for enantioseparation of BP several times, employing various chiral selectors like human serum transferrin [12], heparin [13], and native or derivatized cyclodextrins (CDs) [14–18]. These results clearly demonstrated that the CDs are more suitable for enantioseparation of BP than the biopolymers because the former selectors provided shorter analysis time and higher separation efficiency. Van Eeckhaut et al. [14] proposed CE method for enantiomeric purity testing of dexchlorpheniramine based on high enantioresolution obtained ($R > 10$), and the results suggested also potential of the method for dexbrompheniramine. However, such work has not been reported so far.

Besides enantioresolution, LOD is another factor of particular significance for enantiomeric purity testing methods [5]. Capillary zone electrophoresis (CZE) on-line coupled with capillary isotachophoresis (ITP) in the column-coupling separation system is a promising CE alternative in applications requiring low concentration LOD [19]. Fanali et al. [7,8] demonstrated successful ITP-CZE enantioseparation of trace ($\approx 10^{-7}$ to 10^{-8} mol/l) analytes (amino acids) present in model samples containing 10^2 to 10^4 excess of one of the enantiomers.

The aim of our work was to show the potentialities of ITP-CZE for enantiomeric purity testing of drugs (brompheniramine enantiomers served as an example) in pharmaceuticals (commercial tablets). The general advantages of the proposed method are expected to be its (i) high selectivity (given predominantly by appropriate chiral selector, and also by the combination of two separation mechanisms), (ii) low concentration LOD obtainable (given by high sample load capacity and preconcentration of the selected analytes in ITP stage), and (iii) possibility to remove the interfering constituents before the final CZE separation step. Combining ITP-CZE separation with diode array detection (DAD) should enhance value of analytical information when analytes and interferents have different spectra (distinguishing impurities in analyte zone, confirmation of migration positions of migrants). At a trace analyte concentration the recognition between analyte and baseline noise signals should be more reliable (due to their different spectra).

2. Experimental

2.1. Instrumentation

An EA-101capillary electrophoresis analyzer (J&M, Aalen, Germany), assembled in the column-coupling configuration of the separation unit, was used in this work for performing the ITP-CZE runs. The samples were injected by a 30 μl internal sample loop of the injection valve of the analyzer. An ITP column was provided with an 800 μm i.d. capillary tube made of FEP (fluorinated ethylene-propylene copolymer) and an on-column conductivity sensor. Its total length was 90 mm. A CZE column was provided with a 320 μm i.d. capillary tube made of fused silica (J&W, Folsom, Canada) of a 240 mm total length (180 mm to the detection cell).

The samples in single CZE runs were injected by a 200 nl internal sample loop of the CZE injection valve of the analyzer. The same CZE column as described above was used to perform the single CZE runs.

A TIDAS, multiwavelength photometric absorbance diode array detector (J&M) connected to an on-column photometric detection cell (mounted on the CZE column) via optical fibers (J&M), was used in the CZE as well as in the ITP-CZE runs. The detector operated under the following conditions: (1) scanned wavelength range −200–400 nm, (2) integration time −15 ms, (3) scan interval −0.225 s, and (4) number of accumulations −15. The spectral data were acquired and processed by a Spectralys program (version 1.82, J&M).

2.2. Chemicals and samples

The electrolyte solutions were prepared from chemicals obtained from Merck (Darmstadt, Germany), Aldrich (Steinheim, Germany), and Fluka (Buchs, Switzerland) in water demineralized by a Labconco WaterPro PS water purification system (Labconco, Kansas City, MI, USA). All chemicals used were of analytical grade or additionally purified by the usual methods. The solutions of the electrolytes were filtered before use through disposable membrane filters of a 1.2 μm pore size (Millipore, Molsheim, France).

Brompheniramine maleate (BP), *N*,*N*-dimethyl-3-(4-brom)-phenyl-3-(pyridin-2-yl)propan-1-amine maleate, was obtained from Sigma–Aldrich (Steinheim, Germany) as racemate. Dexbrompheniramine maleate (DBP), +*S*-*N*,*N*-dimethyl-3-(4-brom)-phenyl-3-(pyridin-2-yl)propan-1-amine maleate, was obtained from Sigma–Aldrich as pure enantiomer. Pseudoephedrine hydrochloride was obtained from Sigma–Aldrich. Disophrol-repetabs® produced by Schering-Plough Labo N.V. (Heist-op-den-Berg, Belgium) was obtained from the local pharmacy. The declared content of DBP in the surface layer is 3 mg and the same amount is present in the core (with prolonged release function) of one tablet. Each tablet contains also 120 mg of pseudoephedrine. Carboxyethyl-β-cyclodextrin (DS 3, CE purity), CE-β-CD, was obtained from Cyclolab (Budapest, Hungary).

2.3. Procedures for sample and standard solution preparations

2.3.1. Standard solutions

The stock solution of BP racemate was prepared by dissolving of 6.2 mg of BP standard in 20 ml of demineralized water. The stock solution of DBP was prepared by dissolving of 10.7 mg of DBP standard in 10 ml of demineralized water. The stock solution of pseudoephedrine was prepared by dissolving of 48.9 mg of its standard in 10 ml of demineralized water. Working solutions were made by appropriate dilution of the stock solutions with demineralized water.

The series of model calibration solutions for ITP-CZE runs was prepared with different concentration of BP (using racemic standard) and with constant concentration of background electrolyte (25 times diluted solution of background electrolyte).

The addition of background electrolyte to the calibration solutions eliminated the possibility to adsorb the trace BP on the labware and/or capillaries walls. The concentration levels of BP enantiomers in model calibration solutions in ITP-CZE experiments were in the range 23.16–231.6 μg l^{-1} (7.26×10^{-8} to 7.26×10^{-7} mol l^{-1}) while in CZE experiments they were in 2.5–25 mg l^{-1} (7.8×10^{-6} to 7.8×10^{-5} mol l^{-1}) range and each calibration point was measured twice.

For recovery experiments in ITP-CZE combination, standard BP solutions at three concentration levels (0.05, 0.1 and 0.2 mg/l) were prepared while for single CZE the concentration levels of BP were 5, 10 and 20 mg/l.

2.3.2. Samples preparation

Disophrol-repetabs tablets were treated in two ways to provide the samples for (i) the surface layer analysis and (ii) the whole tablet analysis.

(i) Ten tablets were put into 100 ml of distilled water and vortex for 3 min at 2000 rpm. Rest of the tablets (cores) was removed from the solution after 3 min.
(ii) Ten tablets were accurately weighed, powdered in a mortar and the amount of mass equivalent to one tablet content was dissolved in 50 ml of hydrochloric acid with the concentration 0.1 mol l^{-1}. After 2 h of mechanically shaking the solution was put into the refrigerator. After 24 h the extract was filtered through Whatman No. 42 filter paper into a 100 ml volumetric flask and filled with the distilled water.

The stock solutions of tablet extracts were kept in the refrigerator until the use and they were filtered before the use through disposable membrane filters made of Nylon of a 1.2 μm pore size (Millipore).

3. Results and discussion

3.1. Optimization of separation conditions

The buffer constituents and the driving current in the ITP stage of the ITP-CZE combination were chosen as an experimental optimum considering (i) a rapid ITP separation, (ii) sharp zone boundaries, (iii) minimization of thermal effects, and (iv) good compatibility with on-line coupled CZE separation system. Main optimizing parameters in the CZE stage were type and concentration of chiral selector and pH of the CZE buffer. Ionizable CE-β-CD was chosen as the most effective chiral selector among other CDs used (native β-CD, hydroxypropyl-β-CD, β-CD polymer). The higher was the concentration of CE-β-CD in background electrolyte, the better resolution of BP enantiomers was obtained, but also their migration times increased. Therefore, the final concentration of 5 mg/ml of CE-β-CD in the CZE step was found to provide the sufficient resolution in reasonable migration time window. Higher pH values (tested interval, pH 3.0–5.5) increased the effective charge of CE-β-CD and, by that, interactions of the selector with oppositely charged BP enantiomers (the effective charge of BP enantiomers is supposed to be slightly decreased with pH). As a result of the stronger interactions, migration times of the analytes increased, however, enantioresolution was not significantly affected. Therefore, acidic pH value (3.1) was chosen as an optimum. The final composition of electrolytes used in the ITP-CZE combination is given in Table 1 and further working conditions can be found in Experimental. The same composition of background electrolyte was also used in single column CZE arrangement.

Table 1
Electrolyte systems

Parameter	ITP	Parameter	CZE
Solvent	Water	Solvent	Water
Leading cation	Na^+	Carrier cation	Glycine
Concentration (mmol/l)	10	Concentration (mmol/l)	25
Counter ion	Acetate	Counter ion	Acetate
Concentration (mmol/l)	20	Concentration (mmol/l)	100
pH	4.75	pH	3.1
EOF suppressor	HEC	EOF suppressor	HEC
Concentration (%, w/v)	0.1	Concentration (%, w/v)	0.1
Terminating cation	Glycine	Complexing agent	CE-β-CD
Concentration (mmol/l)	5	Concentration (mg/ml)	5
Counter ion	Acetate		
Concentration (mmol/l)	10		
pH	3.5		

EOF: electroosmotic flow; HEC: hydroxyethylcellulose; CE-β-CD: carboxyethyl-β-cyclodextrin.

Number of theoretical plates (N) as well as plate height (H) given in Table 2 are indicating very good separation efficiency obtainable in single CZE as well as in ITP-CZE combination. Resolution of BP enantiomers (R) in Table 2 is also indicating the efficient enantiomeric separation when analyzed BP enantiomers at similar concentration levels.

Table 2
Validation parameters of BP enantiomers measured in CZE and in ITP-CZE combination

Parameter	CZE		ITP-CZE	
	LBP	DBP	LBP	DBP
t (min)	7.915	8.229	8.022	8.340
s_t (min)	0.0352	0.0334	0.0356	0.0359
a (mAU s min^{-1})	0.4575	0.4740	0.3166	0.3385
s_a (mAU s min^{-1})	0.0845	0.0933	0.0369	0.0643
b (mAU s min^{-1} mg^{-1} l)	0.61895	0.64270	48.8466	50.4837
s_b (mAU s min^{-1} mg^{-1} l)	0.00586	0.00646	0.26191	0.4570
RSS	0.1210	0.1474	0.0162	0.0494
r	0.99973	0.99970	0.99993	0.99980
r^2	0.99950	0.99940	0.99990	0.99960
QC	1.72038	1.82921	0.88267	1.48727
LOD (mg l^{-1})	0.450	0.479	0.0025	0.0042
LOQ (mg l^{-1})	1.365	1.451	0.0075	0.0127
N	27370	25000	33400	30450
H (μm)	6.6	7.6	5.5	6.1
R	2.79		2.94	
Repeatability (R.S.D.) (%)	2.30	2.87	0.99	1.65
Recovery (%)	97.2	96.8	98.1	97.8
Accuracy (RE) (%)	−2.8	−3.2	−1.9	−2.2
Robustness (ΔR) (%)	<3.7		<3.9	

3.2. Validation

BP is absorbing light in the range 200–300 nm wavelengths with the maximum at 261 nm wavelength. This wavelength was used for the evaluation of analytical parameters in our work. Peak areas of BP enantiomers were evaluated using Spectralys software version 1.82 (J&M) and they were corrected for the migration times [20]. Parameters of calibration line for each enantiomer were calculated by using QCExpert version 2.5 statistical software (Trilobyte, Prague, Czech Republic) and all statistical parameters are given in Table 2. For validation experiments the racemic BP standard was used.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the ICH guideline [5] as the ratio of standard deviation of *y*-intercept of regression line (s_a) and the slope of the regression line (b) multiplied by factor 3.3 (LOD) or 10 (LOQ).

Precision was evaluated according to the ICH guideline [5] as the repeatability which is expressed via relative standard deviation of peak areas measured within the concentration range of calibration line. Evaluated repeatability is acceptable and for DBP it is lower than for LBP what is clearly visible also from the standard deviations $\underline{s}_a$, s_b and also from the residual sum of squares (RSS).

Good linearity of the calibration lines is indicated by the values of correlation coefficient (r) and coefficient of determination (r^2).

Accuracy (expressed via relative error, RE) of ITP-CZE method was evaluated according to the ICH guideline [5] using the recovery of BP at three concentration levels (see Section 2). Recoveries of both enantiomers were slightly below 100% and REs calculated from these values indicated very good accuracy of the proposed CE methods.

Migration times of BP enantiomers and their standard deviations indicate high reproducibility of the separation conditions. It is supported also by data of robustness calculated for the resolution (R) of BP enantiomers. Robustness test examined the effect that deliberate variations in operational parameters (concentration of the complexing agent and carrier cation) had on the analysis results. Altering concentrations of CE-β-CD in a range of 4.9–5.1 mg/ml and glycine in a range of 24–26 mmol/l, the fluctuations of R were less than 4% of the values obtained under the standard conditions.

All performance parameters except for LOD and LOQ are similar comparing single column CZE and ITP-CZE combination. The slopes of the calibration lines in the case of CZE experiments are 76–82 times lower than the ones in the case of ITP-CZE experiments. This fact is indicating that the ITP-CZE combination provides significantly higher sensitivity in comparison to the single column CZE. The values of concentration LOD as well as LOQ obtained in ITP-CZE combination are 180 times lower (better) for the LBP and 115 times lower (better) for the DBP than are the values of concentration LOD and LOQ obtained in the single column CZE. This feature of ITP-CZE combination can be essential in the ultra trace analyzes. To see minor enantiomer in the sample the single CZE column can be overloaded by main enantiomer and the problem with the resolution of such enantiomers can arise. This situation is even more critical when the capillaries with lower i.d. are used in single CZE as their worse LODs require the use of higher sample concentrations but their load capacities are lower. From these facts it is obvious that ITP-CZE combination of electrophoretic techniques is a better choice to solve the problems connected with the testing of enantiomeric purity as its load capacity is several orders of magnitude higher than the one of single column CZE (see, e.g., literature [7] and the references given therein). Moreover, when ITP-CZE combination is performed in the column-coupling configuration of the separation unit, it provides the possibility to remove the interfering matrix constituents from the separation system so that they do not enter into the final CZE separation step [7].

3.3. Proofing the enantioseparation selectivity by DAD spectra

The signal from DAD obtained during the ITP-CZE analysis of the standard solution of DBP is shown in Fig. 1. The ratio of corrected peak areas of BP enantiomers in DBP standard was found to be 0.16: 99.84, what is indicating its good enantiomeric purity. This example showed that ITP-CZE combination was suitable technique for checking the enantiomeric purity. Migration positions of both BP enantiomers in DBP standard were confirmed by the addition of racemic BP standard, however, it need not be sufficient information (e.g., in a case of mixed zones/peaks). Analytical results concerning the identity of the analytes and/or the purity of their zones/peaks can be supported also by information from DAD spectra. In this way the reliability of the results could increase.

Raw DAD spectrum of the minor enantiomer (LBP) is shown in Fig. 1c (upper spectrum). There is practically impossible to localize the absorbance maximum of trace (5×10^{-8} mol l^{-1}) LBP because of very low signal to noise ratio. We followed the recommendation published in the literature [21] to minimize the impact of the electrolyte system on the analyte spectrum. An average spectrum calculated from the spectra acquired before the start and behind the end of the peaks of BP enantiomers in the actual ITP-CZE run was used for performing the background correction. Corrected spectrum of LBP was smoothed by the procedure of Savitzky–Golay [22] (implemented in Spectralys software) with a 21-point window and it is shown in Fig. 1c (lower spectrum). The same background correction and smoothing procedure was also applied on the raw spectrum of major enantiomer (DBP) and its both raw and corrected spectra are shown in Fig. 1d. Fig. 1c and d clearly shows the similarity in spectra of both enantiomers but this visual comparison is still not sufficient to confirm the identities of the analytes of interest. Pearson's correlation coefficient [23] served as a numerical criterion (matching factor) expressing a match of the analyte spectrum with the reference spectrum [21]. Spectra of BP enantiomers from the racemic standard acquired by the DAD in the ITP-CZE analysis served as the reference spectra in the confirmation of identity of BP enantiomers in the DBP standard sample. The reference spectra were also corrected for the background and

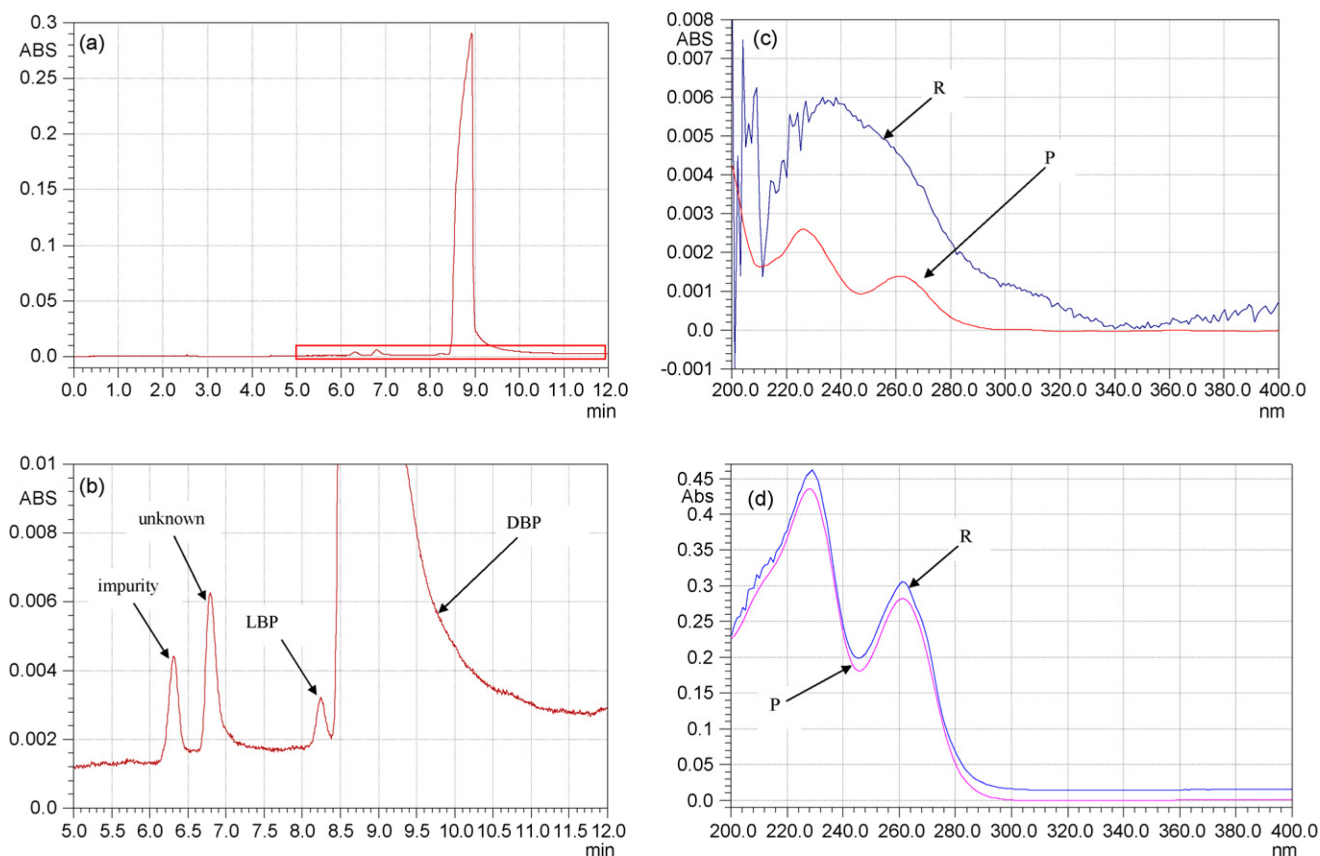

Fig. 1. ITP-CZE analysis of 30 μl dexbrompheniramine (DBP) standard. The whole electropherogram obtained in CZE step taken at 261 nm wavelength (a). Its part given in the rectangle is shown in (b). Raw (R) (unprocessed) and processed (P) spectra of levobrompheniramine (LBP) impurity (c) and DBP (d), respectively. For processing and smoothing procedure see details in text. The driving currents in the ITP and CZE stages were 200 μA and 80 μA, respectively. For the separation conditions see Table 1.

smoothed in the way described above. Matching factors of major and minor enantiomers were 0.99018 and 0.98967, respectively. It should be mentioned that the value of Pearson's correlation coefficient higher than 0.99 is assumed to provide an acceptable certainty in a confirmation of the identity of the analyte [21]. The above mentioned results clearly demonstrated the fact that DAD connected to ITP-CZE could provide valuable information about the quality of the separation even in the situation when concentration level of the analyte (enantiomeric impurity) is very low.

3.4. Enantiomeric purity testing of pharmaceutical sample

Commercial Disophrol-repetabs preparation was chosen for enantiomeric purity testing. DAD spectra and electropherogram from the analysis of the pharmaceutical sample are shown in Fig. 2. The migration positions of the BP enantiomers in the drug sample were proved by the addition of racemic BP standard. Under our separation conditions (Table 1) cationically migrating pseudoephedrine (positionally identified by spiking with its standard solution) did not interfere with the determination of BP enantiomers. These results indicated that ITP-CZE combination, providing enhanced selectivity due to CE-β-CD as ionizable chiral selector in the separation system, is suitable for the separation of BP enantiomers in the pharmaceutical sample.

DAD spectra obtained from the analysis of the pharmaceutical sample were treated in the same way as described in Section 3.3 for the standard samples (racemic BP and DBP). Pearson's correlation coefficients for the corrected spectra of BP enantiomers were 0.99825 for DBP and 0.98958 for LBP. Practically the same values of Pearson's correlation coefficients for both BP enantiomers in the sample of DBP standard (given in Section 3.3) and sample of Disophrol-repetabs indicated the identity of particular enantiomers, i.e., presence of both BP enantiomers in the pharmaceutical sample with a high probability. Enantiomeric ratios (LBP:DBP) determined by the proposed ITP-CZE method were 3.1:96.9 and 3.2:96.8 in the surface layer and whole tablet, respectively. From these results it is clear that when rapid and/or preliminary evaluation of enantiomeric purity is required, the surface layer analysis can be advantageous avoiding long-term sample preparation given by prolonged release of active compound from the core of the tablet.

The proposed ITP-CZE-DAD approach is a pragmatic solution in the field of the enantiomeric purity testing for its simplicity, cost and appropriate information value. Obviously, when the exact structure information is required the use of more selective (specific) detection, e.g., mass spectrometry (MS), nuclear magnetic resonance (NMR) has to be applied.

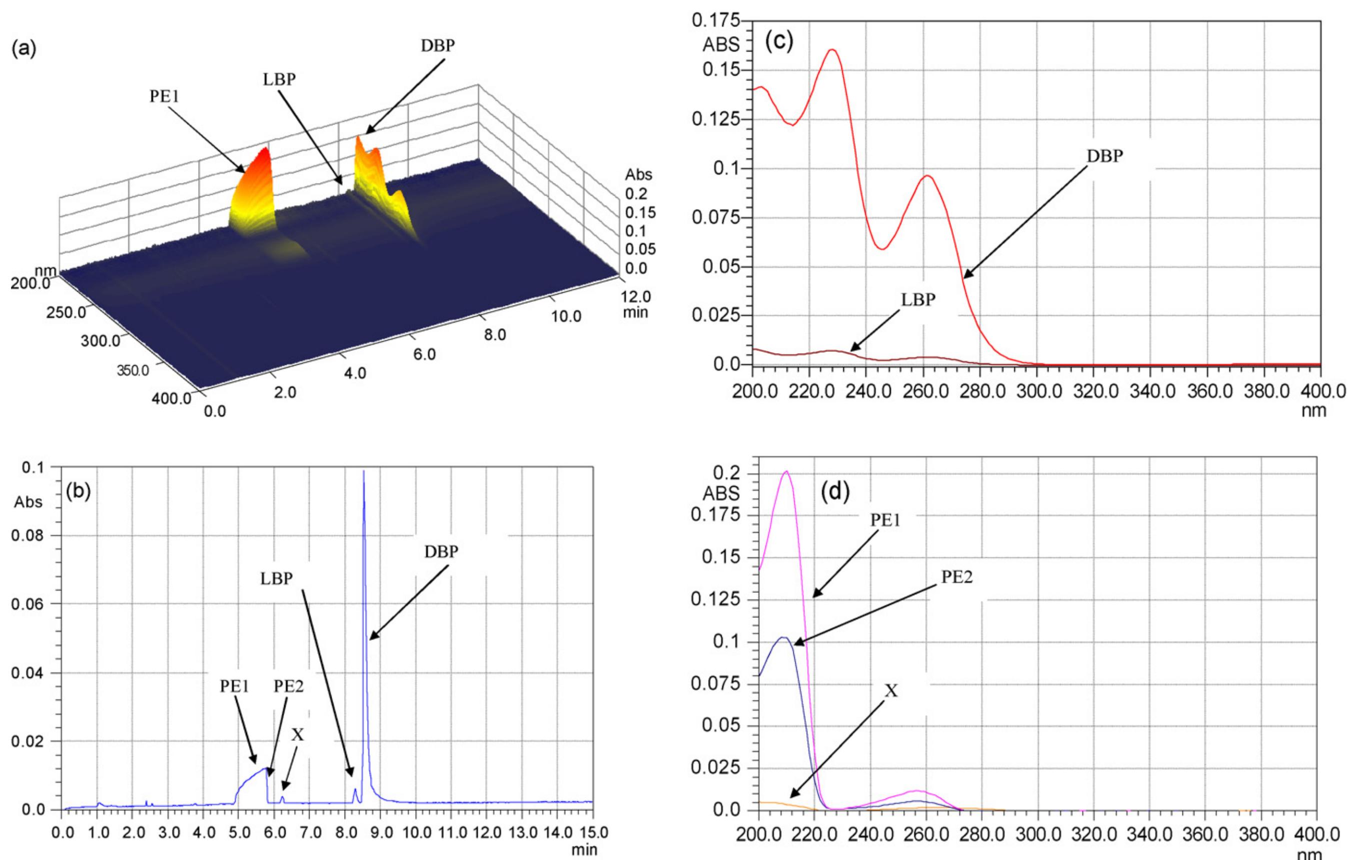

Fig. 2. Signal from DAD obtained during the CZE step of ITP-CZE analysis of Disophrol-retard extract from the surface of tablets (a) and electropherogram at 261 nm wavelength (b). Processed spectra of DBP and LBP (c), pseudoephedrine enantiomers (PE1, PE2) and unidentified compound X (d). For processing and smoothing procedure see details in text. The driving currents and separation conditions as in Fig. 1.

4. Conclusion

Experiments with model and pharmaceutical samples performed in the CE equipment with the column-coupling configuration of the separation unit and monitored by DAD showed that ITP-CZE combination provided favorable conditions for highly reliable enantiomeric purity testing of drugs.

Undoubtedly, the concentration LOD at 261 nm wavelength for LBP (enantiomeric impurity) $2.5\,\mu g\,l^{-1}$ ($8 \times 10^{-9}\,mol\,l^{-1}$) estimated from the CE runs with model samples is linked with the sample injection volume (30 μl) and preconcentration capabilities of ITP separation step. The use of CE-β-CD serving as chiral selector in the CZE carrier electrolyte was found to be advantageous in reaching the required chiral resolution of BP enantiomers and their separation from the matrix constituents. Under these working conditions trace enantiomeric impurity (LBP) present in high excess ($\sim 10^3$) of enantiomeric counterpart (DBP) could be easily quantified.

DAD spectra of BP enantiomers present in pharmaceutical samples matched their reference spectra with reasonable certainties (confirmed also by the Pearson's correlation coefficients as match factors). The background correction and smoothing procedure allowed us to obtain analytically relevant DAD spectra of LBP also when it was present in the sample at the LOD concentration level. Raw as well as smoothed DAD spectra indicated no impurity present in zones of BP enantiomers. These results demonstrated possibility of the DAD to evaluate enantioseparation selectivity/to quantify analytes more reliably in comparison with the single wavelength detection.

High analytical potential of the ITP-CZE-DAD method, given by combination of several enhanced parameters like (i) selectivity of the separation process, (ii) concentration LOD, (iii) load capacity, and (iv) reliability, does this analytical approach well suited for enantiomeric purity control of drugs in general sense. Moreover, the ITP-CZE-DAD method, capable to purify, preconcentrate and enantioselectively analyze the complex samples in one electrophoretic run, has great potential for enantiomeric purity testing of drugs when minimal sample preparation/handling is required.

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