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Application of extraction disks in dissolution tests of clenbuterol and levothyroxine tablets by capillary electrophoresis

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Abstract

Sample preparation procedures using octadecyl (C_{18}) extraction disks were developed to obtain accurate and reproducible results for determinations of clenbuterol (20 μg per dose) and levothyroxine (100 μg per dose) in dissolution media of solid oral dosage forms. Preconcentration of samples allowed final concentrations of 1.1 μg/ml of clenbuterol and 4.0 μg/ml of levothyroxine to be reached prior to CE analysis. The results obtained by CE were in good agreement with those of HPLC. The precision of the migration time, peak area, peak height and accuracy were determined in both intra-day ($n = 6$) and inter-day ($n = 18$) assays. Linearity was demonstrated over the ranges 0.5–80.0 μg/ml of clenbuterol and 1.0–30.0 μg/ml of levothyroxine. The mean recoveries were higher than 94.0%, ranging from 50 to 125% levels with respect to dose potencies. The proposed methodology may be generally applied to determine drugs at ng/ml concentrations.

Keywords: Capillary electrophoresis; Solid-phase extraction; Clenbuterol; Levothyroxine

1. Introduction

Nowadays, quality control of pharmaceutical products frequently requires that analytical methods can determine very low concentrations of active components with high accuracy and reproducibility. In vitro dissolution tests for pharmaceutical dosage forms included in many official pharmacopoeias are designed to be used during product development and quality control of finished products to assess batch to batch reproducibility or in vitro bioequivalence [1–3]. According to experimental conditions, the final concentration of the analytes in dissolution media may be at the ng/ml level. Therefore, very sensitive analytical techniques and/or an analyte preconcentration step in the sample preparation are required.

The application of solid-phase extraction disks [4–6] has increasingly been developed in environmental analysis [7–11] for enrichment and clean-up of samples, but their use is less extensive in pharmaceutical and biopharmaceutical analysis [12–14]. Even though HPLC and spectrophotometry are the most common methods of quantitation in dissolution testing, the determination of analytes at very low concentrations continues to be a difficult task to solve. In this

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paper, the use of solid-phase extraction disks is proposed as an efficient alternative for enrichment and clean-up of samples in dissolution media of oral solid dosage forms. The determination of clenbuterol (20 μg per dose) and levothyroxine (100 μg per dose) in these media is reported. The sample preparation procedure was followed by CE analysis and the results were found to be in good agreement with those obtained by HPLC.

2. Experimental

2.1. Reagents

Empore C_{18} SPE disks (Baker Bond) were supplied by Baker (Phillipsburg, NJ, USA). Clenbuterol hydrochloride, sodium levothyroxine, disodium hydrogenphosphate and 85% phosphoric acid were purchased from Sigma (St. Louis, MO, USA). Methanol, acetonitrile and potassium dihydrogenphosphate (HPLC grade) were obtained from Merck (Darmstadt, Germany). The excipients sodium citrate, gum arabic, talc, colloidal silicon dioxide, starch, polyvinylpyrrolidone and lactose were obtained from Sigma. Magnesium stearate was obtained from Aldrich (Milwaukee, WI, USA). Deionized, doubly distilled water was used. Buffer solutions and solvents were filtered through a 0.22-μm membrane (Sartorius, Göttingen, Germany) and degassed before use.

2.2. Capillary electrophoresis

A Quanta 4000 capillary electrophoresis system (Waters, Milford, MA, USA) was used. Data were collected and processed by Millenium software (Waters).

Operating conditions for clenbuterol

An uncoated fused-silica capillary [60 cm (53 cm to detector) × 75 μm I.D.] (Waters) was used. The background electrolyte was 30 m*M* phosphate buffer (pH 6.7), prepared by adding 0.26 g of potassium dihydrogenphosphate and 0.16 g of disodium hydrogenphosphate to 100 ml of water and adjusting the pH by addition of 85% phosphoric acid diluted with water (1:1). The applied voltage was +16 kV and the temperature was 25°C. Detection was effected at 214 nm using a zinc lamp and 0.002 aufs. Hydrodynamic injection (10 cm height) was performed for 15 s. At the start of the day, the capillary was rinsed with 0.2 *M* potassium hydroxide for 5 min, followed by running buffer for 15 min. Between runs, the capillary was rinsed with running buffer for 3 min. At the end of the day, the capillary was flushed with 0.2 *M* potassium hydroxide for 5 min and then with water.

Operating conditions for levothyroxine

An uncoated fused-silica capillary [35 cm (28 cm to detector) × 75 μm I.D.] (Waters) was used. The background electrolyte was 25 m*M* phosphate buffer (pH 2.5), prepared by adding 0.34 g of potassium dihydrogenphosphate to 100 ml of water and adjusting the pH by addition of 85% phosphoric acid (1:1). The applied voltage was +13 kV and the temperature was 25°C. Detection was effected at 214 nm with a zinc lamp and 0.002 aufs. Hydrodynamic injection (10 cm height) was performed for 10 s. At the beginning of each day, the capillary was rinsed with buffer running for 2 h and for 5 min between runs. At the end of the day, the capillary was flushed with 2.5% phosphoric acid for 2 min followed by water.

2.3. HPLC analysis

Chromatographic analyses were performed with a Varian Model 5020 liquid chromatograph equipped with a Varian UV-100 detector. Data were processed with a Varian Model 4270 integrator. A Rheodyne (Cotati, CA, USA) Model 7125 injector was used.

Operating conditions for clenbuterol

A LiChroCART, LiChrospher cyano column (Merck) (125 × 4 mm I.D., 5 μm) was used. The mobile phase was methanol–10 m*M* phosphate buffer [pH 6.6, adjusted by addition of phosphoric acid (1:1)] (75:25). The temperature of the column was 25°C, the flow-rate was 1.3 ml/min

and the detector was set at 214 nm and 0.03 aufs. A sample volume of 50 μl, equivalent to 55 ng of clenbuterol, was injected.

Operating conditions for levothyroxine

A LiChroCART, LiChrospher cyano column (Merck) (125 × 4 mm I.D., 5 μm) was used. The mobile phase was of acetonitrile–water–phosphoric acid (45:55:0.05). The temperature of column was 25°C, the flow-rate was 1.6 ml/min and the detector was set at 225 nm and 0.06 aufs. A sample volume of 20 μl, equivalent to 80 ng of levothyroxine, was injected.

2.4. Stock solutions

Stock solutions containing 0.2 mg/ml of clenbuterol hydrochloride and 0.2 mg/ml of sodium levothyroxine were prepared in methanol.

2.5. Standard solutions

For clenbuterol hydrochloride, a 1.0-ml aliquot of stock solution was diluted to 200.0 ml with methanol in a calibrated flask. The final concentration of the standard solution was 1.0 μg/ml of drug. For levothyroxine sodium, 1.0-ml aliquot of stock solution was diluted to 50.0 ml with methanol–50 m*M* phosphate buffer (pH 2.5). The final concentration of the standard solution was 4.0 μg/ml of drug.

2.6. Working solutions

Test dissolution samples were simulated by adding excipients to the dissolution medium, based on the composition and proportion of commercial products. The mixture was then spiked with known amounts of standard drug solutions equivalent to 50–125% of the concentration corresponding to the full dose potency [1,15].

2.7. Sample concentration and clean-up procedure

In the sample preparation procedure, Empore C_{18} extraction disks, 13 mm in diameter, were used. The extraction membranes were cut from 47 mm diameter disks with a cork borer and placed in a stainless-steel filter holder. A positive pressure technique was used during the procedure. The disks were preconditioned by washing with 2.5 ml of methanol and 2.5 ml of water at 1.5 ml/min. Sample volumes of 40 ml for levothyroxine and 50 ml for clenbuterol were passed through the disk and then the filter was washed with 2.5 ml of water and dried. Elution of the drug from the membrane was performed with 1.0 ml of methanol (0.5 ml/min). The eluting solvent was allowed to soak the disk for 3 min before pulling it through the membrane and the disk was then dried. The eluate containing clenbuterol hydrochloride was injected directly both into the CE system and into the liquid chromatograph. The eluate of levothyroxine sodium was previously diluted to 2.0 ml with 50 m*M* phosphate buffer (pH 2.5).

3. Results and discussion

The aim of this work was to develop a sample preparation procedure using extraction disks prior to the determination of clenbuterol and levothyroxine in dissolution media.

Although clenbuterol is not yet codified in International Pharmacopaeias, such as the USP XXIII and BP 1993, it is extensively used as a β_2-agonist. Taking into account that the dose in solid oral dosage forms is 20 μg, the application of the USP Convention's General Standard for Dissolution, First Case, would take clenbuterol to a final concentration range of 16–22 ng/ml. These levels are too low to be quantitated by conventional methods. On the other hand, in the USP XXIII the quantitation of levothyroxine tablets in dissolution testing is performed by injecting a sample volume of 800 μl into an HPLC system. However, it would be convenient to reduce the injection volume to optimize the chromatographic technique.

For development of the methods proposed in this work, simulated dissolution samples were prepared by addition of known amounts of drugs to obtain the working solutions (Table 1).

Table 1
Enrichment factors in simulated dissolution testing samples

Drug	Dose/comp. (μg)	Concentration (ng/ml)		Enrichment factor
		Dissolution medium	After sample preparation	
Clenbuterol hydrochloride	20	22[a]	1100	50:1
Sodium levothyroxine	100	200	4000	20:1

[a] Conditions according to General Standard, First Case, USP Policy on Dissolution Standards, USP Convention.

To optimize the sample preparation, it was necessary to evaluate the effects of pH and the nature and volume of washing and elution solvents on the recoveries. The resulting ionic strength of phosphate buffer (pH 6.7) led to optimum conditions for the analysis because a sharper peak with a short migration time was achieved.

In the CE of levothyroxine, the selected operating conditions gave symmetry of the peak and reproducibility of the peak area. The use of short capillaries and/or higher applied voltages generated deleterious heating effects.

The electropherograms of clenbuterol and levothyroxine samples are shown in Figs. 1 and 2, respectively.

The determination of levothyroxine by CE was performed by a modification of a previously reported method [16] and the chromatographic technique was based on a former report [17].

3.1. Method validation

For validation of the CE methods, sensitivity, linearity, accuracy and precision were evaluated.

Precision, expressed as the R.S.D. of the migration time, peak area and peak height of the CE systems are summarized in Tables 2 and 3. Mean values were calculated for six replicate injections of standard solutions performed each day and over three different days. The limits of detection were 0.16 μg/ml for clenbuterol and

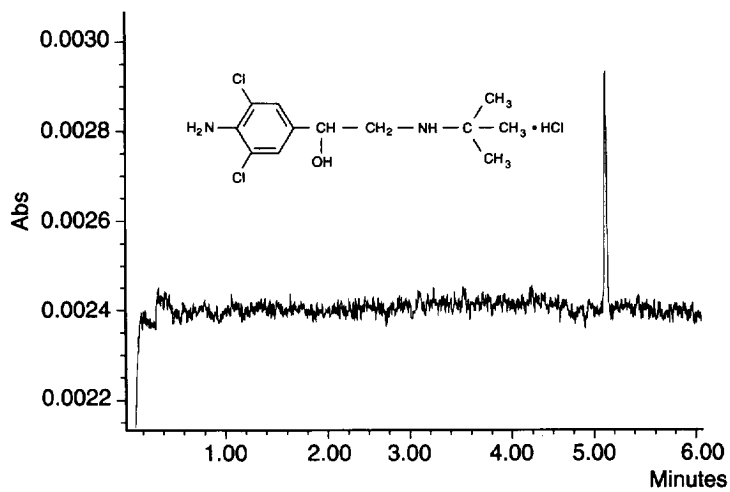

Fig. 1. Electropherogram of a sample of clenbuterol hydrochloride containing 1.1 μg/ml (29.7 pg). Experimental conditions as given in the text.

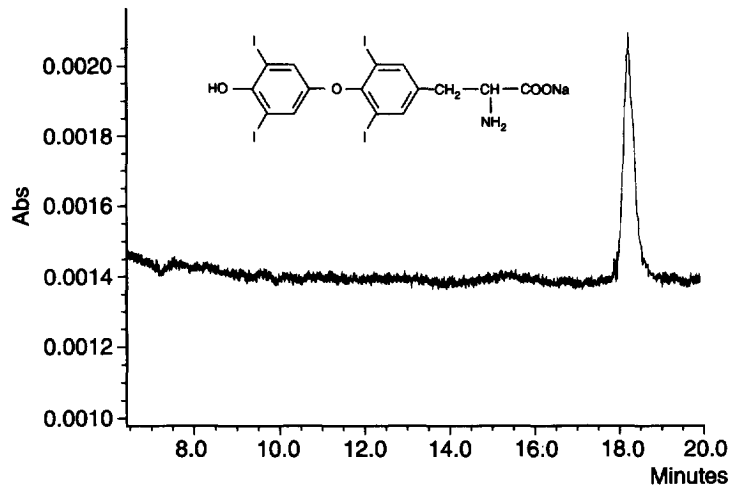

Fig. 2. Electropherogram of a sample of sodium levothyroxine containing 4.0 μg/ml (43.3 pg). Experimental conditions as given in the text.

Table 2
Precision of electrophoretic system in clenbuterol analysis

Clenbuterol hydrochloride	t_m ($\bar{x} \pm$ S.D.)	R.S.D. (%)	Area/t_m ($\bar{x} \pm$ S.D.)	R.S.D. (%)	h_p[a]/t_m ($\bar{x} \pm$ S.D.)	R.S.D. (%)
Intra-day ($n = 6$)	5.2 ± 0.4	0.7	190.3 ± 2.7	1.4	90.2 ± 0.9	1.0
Inter-day[b] ($n = 18$)	5.2 ± 0.1	1.5	193.3 ± 2.7	1.4	91.7 ± 1.5	1.6

For experimental conditions, see the text.
[a] h_p = peak height.
[b] Mean values of analyses performed on three different days.

Table 3
Precision of electrophoretic system in levothyroxine analysis

Sodium levothyroxine	t_m ($\bar{x} \pm$ S.D.)	R.S.D. (%)	Area/t_m ($\bar{x} \pm$ S.D.)	R.S.D. (%)	h_p[a]/t_m ($\bar{x} \pm$ S.D.)	R.S.D. (%)
Intra-day ($n = 6$)	18.4 ± 0.3	1.8	616.6 ± 5.2	0.8	35.7 ± 1.2	3.2
Inter-day[b] ($n = 18$)	18.7 ± 0.9	4.2	604.4 ± 23.5	3.5	36.0 ± 2.0	4.1

For experimental conditions, see the text.
[a] h_p = peak height.
[b] Mean values of analyses performed on three different days.

Table 4
Accuracy and precision of simulated dissolution samples of clenbuterol

Clenbuterol hydrochloride	Level 75%				Level 100%				Level 125%			
	CE		HPLC		CE		HPLC		CE		HPLC	
	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)
Intra-day ($n = 3$)	96.3 ± 1.9	2.0	98.3 ± 1.2	1.3	96.6 ± 1.2	1.3	95.5 ± 1.5	1.6	99.0 ± 1.6	1.6	98.3 ± 1.2	1.2
Inter-day ($n = 9$)[a]	94.8 ± 2.1	2.2	98.4 ± 1.2	1.2	96.2 ± 1.3	1.4	95.9 ± 1.5	1.5	99.2 ± 1.5	1.5	98.1 ± 1.4	1.5

[a] Mean values of recovery analyses performed on three different days.

Table 5
Accuracy and precision of simulated dissolution samples of levothyroxine

Sodium levothyroxine	Level 50%				Level 75%				Level 100%				Level 125%			
	CE		HPLC		CE		HPLC		CE		HPLC		CE		HPLC	
	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)	$\bar{x} \pm$ S.D.	R.S.D. (%)
Intra-day ($n = 3$)	94.9 ± 1.2	1.3	95.4 ± 1.3	1.4	94.4 ± 1.3	1.4	95.1 ± 1.9	2.0	95.8 ± 1.2	1.3	98.5 ± 1.3	1.3	98.5 ± 1.2	1.2	97.6 ± 0.9	0.9
Inter-day ($n = 9$)[a]	95.1 ± 1.6	1.6	95.2 ± 1.4	1.4	95.1 ± 1.4	1.5	94.0 ± 1.8	1.9	96.4 ± 1.8	1.9	97.3 ± 1.9	2.0	98.6 ± 1.5	1.5	97.6 ± 1.8	1.9

[a] Mean values of recovery analyses performed on three different days.

0.3 μg/ml for levothyroxine at a signal-to-noise ratio of 3. The limits of quantitation at a signal-to-noise ratio of 10 were 0.5 μg/ml for clenbuterol and 1.0 μg/ml for levothyroxine.

Linearity was demonstrated over a concentration range from 0.5 to 80.0 μg/ml of clenbuterol and from 1.0 to 30.0 μg/ml of levothyroxine. Calibration graphs were obtained with eight points and each concentration was measured by three replicate injections of standard solutions. The linear correlation was found to be $y = 0.04 + 0.86x$ ($r = 0.9997$, S.E. = 0.687) for clenbuterol and $y = 0.04 + 0.17x$ ($r = 0.9990$, S.E. = 0.083) for levothyroxine.

Recoveries and precision of the methods expressed as R.S.D. are given in Tables 4 and 5. Mean recoveries from spiked simulated dissolution media at 50, 75, 100 and 125% nominal concentration levels were higher than 94%. Calculations were based on peak area normalized with respect to the migration times. The data obtained confirm the validity of both the CE and HPLC methods.

4. Conclusions

The employment of solid-phase extraction disks in sample preparation allows enrichment factors of 50:1 for clenbuterol and 20:1 for levothyroxine to be obtained, making HPLC or CE analysis possible. This methodology also allows the injection volumes of sample solutions to be reduced to 20 μl in LC analyses.

The methods reported in this work may be generally applied to dissolution testing where drug concentrations are at ng/ml levels. Finally, a device for multiple sample preparation applying the procedure described could be developed.

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