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Sequential injection chromatographic determination of ambroxol hydrochloride and doxycycline in pharmaceutical preparations

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Abstract

A new separation method based on a novel reversed-phase sequential injection chromatography (SIC) technique was used for simultaneous determination of ambroxol hydrochloride and doxycycline in pharmaceutical preparations in this contribution.

The coupling of short monolith with SIA system results in an implementation of separation step to until no-separation low-pressure method.

A Chromolith® Flash RP-18e, 25–4.6 mm column (Merck, Germany) and a FIAlab® 3000 system (USA) with a six-port selection valve and 5 ml syringe were used for sequential injection chromatographic separations in our study. The mobile phase used was acetonitrile–water (20:90, v/v), pH 2.5 adjusted with 98% phosphoric acid, flow rate 0.48 ml min^{-1}, UV detection was at 213 nm.

The validation parameters have shown good results: linearity of determination for both compounds including internal standard (ethylparaben) >0.999; repeatability of determination (R.S.D.) in the range 0.5–5.4% at three different concentration levels, detection limits in the range 0.5–2.0 μg ml^{-1}, and recovery from the pharmaceutical preparation in the range 99.3–99.9%. The chromatographic resolution between peak compounds was >5.0 and analysis time was <9 min under the optimal conditions. The method was found to be applicable for routine analysis of the active compounds ambroxol hydrochloride and doxycycline in various pharmaceutical preparations.

Keywords: Sequential injection analysis (SIA); Sequential injection chromatography (SIC); Monolithic columns; Ambroxol hydrochloride; Doxycycline

1. Introduction

In the last few years, special attention was given to sequential injection analysis (SIA) as an economical and expeditious alternative for the automation of analytical procedures [1]. SIA brings many benefits for the routine analysis due to simplification of the manifold, reduction of the reagents and sample consumption, shortening analysis time, automating sampling and analytical procedures. It is a more versatile technique based on a programmable flow that allows reaction conditions to be chosen from a computer keyboard. On the other side, SIA technique has generally one important drawback—it cannot provide the separation procedure and analysis of multicomponent samples.

Over the past 5 years, the research in the field of liquid chromatography columns has tremendously grown and innumerable types of chromatography columns have been developed to solve particular problems of separation requirements. One important direction of this research area is the development of monolithic columns as new separative tools used in HPLC. In contrast to particles filled columns, monoliths can operate at high flow rates with lower back-pressure. This feature can be used for integrating these columns into a SIA manifold [2] and for extending the possibilities of SIA technique. This approach—the coupling of short monolithic column with SIA manifold is presented in this work for separation and determination of ambroxol hydrochloride and doxycycline in pharmaceutical sample.

Ambroxol (AM), *trans*-4-((2-amino-3,5-dibromobenzyl) amino)cyclohexanol hydrochloride, is an active metabolite of bromhexine, and is used as a mucolytic agent that improves

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the expectoration in the treatment of acute and chronic respiratory diseases, because it stimulates the transportation of mucus in the respiratory organs and reduces secretion stagnation [3]. Ambroxol hydrochloride can be found in pharmaceutical preparations such as drops, granules, injections, syrups and tablets.

Doxycycline (DX), 1-dimethylamino-2,4a,5,7,12-pentahydroxy-11-methyl-4,6-dioxo-1,4a,11,11a,12,12a-hexahydrotetracene-3-carboxamide, is a semi-synthetic tetracycline antibiotic derived from oxytetracycline and it can exist in two forms: doxycycline monohydrate and doxycycline hyclate. Usually this antibiotic is used in human and veterinary medicines or as feed additive, due to its activity against a wide range of Gram-positive and Gram-negative pathogens [4].

Different analytical approaches have been employed for the determination of ambroxol hydrochloride [5–9] or doxycycline [10–14] in various matrices, nevertheless there is no method describing simultaneous determination of both compounds.

This paper describes the development and validation of a new sequential injection chromatography (SIC) method for the simultaneous determination of ambroxol hydrochloride and doxycycline in pharmaceutical samples without interference of other excipients substances.

2. Experimental

2.1. Reagents

The standards of ambroxol hydrochloride (AM), doxycycline (DX) and internal standard ethylparaben (EP) were obtained from Sigma–Aldrich (Prague, Czech Republic), Stock standard solutions were prepared in methanol in concentration 1000 μg ml^{-1} and were stored at 5 °C for 1 month. The final concentrations of the sample, working standard solutions or reference standards for pharmaceutical preparations analysis were prepared by diluting the stock solution in the mobile phase. Methanol and acetonitrile (Chromasolv, for LC) were obtained from Sigma–Aldrich, phosphoric acid (98%) was from Merck (Germany). All other chemicals used were of analytical grade quality. The deionised water was purified by a Milli-Q system (Millipore Corp., Bedford, MA).

The tested pharmaceutical preparations Doxycyclin AL comp. was supplied by ALIUD® PHARMA (Germany), DOXYHEXAL® 200 TABS was supplied by HEXAL AG (Germany) and AMBROSAN® was supplied by PRO. MED. CS (Czech Republic).

2.2. Apparatus

A FIAlab® 3000 system (FIAlab® Instruments, USA), commercially produced instrument consisting of a syringe pump (syringe reservoir 5 ml) and six-port selection Chem-inert valve (Valco Instrument Co., USA) was used in our study. FIAlab® 3000 was equipped with fiber-optic UV–vis diode array detector S2000 (Ocean Optics, Inc., USA) with UV–vis tungsten lamp LS-1 (Ocean Optics, Inc.). The solarization resistant optic fibers and 10 mm Z-flow cell were from Avantes, Inc. (Colorado, USA). The whole SIA system was controlled by program FIAlab for Windows 5.0. Flow connecting lines were made of 0.75 mm i.d. PTFE tubing. Mobile phases and samples were aspirated through the selection valve and then delivered to the monolithic column and to the detector. Sample compounds separation was performed on Chromolith® Flash RP-18e, 25-4.6 mm column (Merck, Germany). The monolithic column was placed between the six-port selection valve and flow cell of the detector. The mobile phase was aspirated through the filter ending (10 μm).

2.3. Method and sample preparation

2.3.1. Mobile phase

The optimal mobile phase for separation of AM, DX and internal standard EP was acetonitrile–water (20:90, v/v), pH 2.5 adjusted with 98% phosphoric acid; flow rate 0.48 ml min^{-1} at ambient temperature. The detection wavelength was 213 nm. The sample injection volume was 25 μl for standard solutions. Mobile phase was degassed before application by means of helium.

2.3.2. Solutions and sample preparation

The pharmaceutical preparations analysed were Doxycyclin AL comp. capsules (containing 100 mg of doxycycline and 75 mg of ambroxol hydrochloride in 1 capsule), DOXYHEXAL® 200 tablets (containing 200 mg of doxycycline in 1 tablet) and AMBROSAN® tablets (containing 30 mg of ambroxol hydrochloride in one tablet).

Determination of the active substances in pharmaceuticals was done by the following procedure. One capsule of Doxycyclin AL comp. preparation was transferred to 100 ml calibrated flask; 10 ml of stock solution of internal standard ethylparaben (c = 10,000 μg ml^{-1}) in methanol, 1 ml of concentrate phosphoric acid and 50 ml of methanol were added. The sample was homogenized and dissolved by 15 min sonication; the period time 15 min was let for temperature stabilization, then the flask was filled to the mark with methanol and mixed. The volume of 1 ml of this sample was diluted 25 times to the 25 ml calibrated flask with mobile phase. The final sample solution was filtrated through the 0.45 μm filter before analysis. Sample preparation of next pharmaceuticals was carried out the same way, but different dilution ratio was used. The comparative standard solution was the same for all the analysis and it was prepared diluting stock standard solution in mobile phase. The final concentrations of the analytes in comparative standard solution were 40 μg ml^{-1} of AM, 30 μg ml^{-1} of DX and 40 μg ml^{-1} of EP.

A volume of 5 μl of sample was analysed by SIC system. Standards and samples were measured in triplicates and the mean peak height values were used for data acquisition.

3. Results and discussion

3.1. Method development and optimization

The incorporation of monolithic columns to SIA manifold has been introduced in our previous works [2,15]. The present contribution is focused on simple chromatography separation and determination of desired compounds in pharmaceutical capsules and tablets.

The selection of an appropriate internal standard was carried out from compounds of similar structure and ethylparaben was chosen as a suitable internal standard. No work dealt with simultaneous determination of AM together with DX and number of experiments concerning the type of organic phase and its proportions in relation to water was made in our study. The optimization was started with Chromolith® Flash RP-18 column 25 mm × 4.6 mm and pH of mobile phases was adjusted to 2.5 pH units with 98% phosphoric acid. The mobile phases containing acetonitrile as organic part have shown better peak symmetry than methanol containing mobile phases and the optimization was focused to make a proposal appropriate ratio of acetonitrile–water mobile phase composition.

In order to achieve a sufficient symmetry of the peaks together with a good separation of compounds and a short retention time, the optimal mobile phase for the separation of AM, DX and internal standard EP was acetonitrile/water (20:90, v/v), pH adjusted to 2.5 by means of 98% phosphoric acid, flow rate 0.48 ml min^{-1}. The proposed system enabled successful separation of target analytes in the time less than 9 min.

From the UV spectra of AM and DX in mobile phase, the optimal detection wavelength of 213 nm (the first absorption maximum of AM and the second absorption maximum of DX) was chosen. Peak height evaluation was performed using the FIAlab® software. Representative sequential injection chromatogram showing successful separation of active substances of Doxycyclin AL comp. capsules including internal standard is shown in Fig. 1.

3.2. Parameters of sequential injection chromatography process

The target compounds were successfully separated using the proposed procedure and basic chromatographic parameters were calculated from experimental data, such as peak symmetry factor, resolution factor, number of theoretical plates and retention times, and they are given in Table 1.

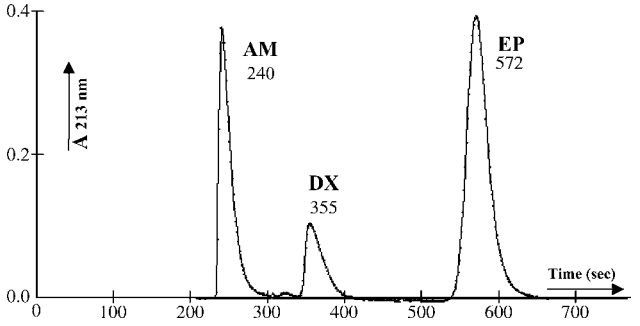

Fig. 1. SI chromatogram of the separation of active substances of Doxycyclin AL comp. capsules. Mobile phase: acetonitrile–water (20:90, v/v), pH 2.5 adjusted with 98% phosphoric acid, flow rate 0.48 ml min^{-1} at ambient temperature, UV detection at 213 nm.

3.3. Validation and analytical parameters of the method

The developed method was validated in order to evaluate if adequate linearity, sensitivity, repeatability, recovery, selectivity, precision and accuracy had been achieved.

Linearity was established with a series of working solutions prepared by diluting the stock solution with mobile phase to the final concentrations. Each concentration was injected in duplicate and the mean value of peak height was used for the calibration curve. The calibration graphs involved eight experimental points for each compound (concentration range 2, 5, 10, 20, 30, 50, 70 and 100 μg ml^{-1}) and they are described by the following equations: for AM: $A = (0.007220 \pm 0.000099)c - (0.004234 \pm 0.004818)$ (where A is the absorbance and c the analyte concentration), the correlation coefficient was 0.9994; for DX: $A = (0.0016230 \pm 0.000025)c - (0.001519 \pm 0.001211)$, the correlation coefficient was 0.9993; and for internal standard EP: $A = (0.005045 \pm 0.000081)c - (0.006316 \pm 0.003945)$, the correlation coefficient was 0.9992.

The limit of detection (LOD) was calculated by comparison of the three-fold variation of signal to noise ratio (3S/N) obtained from analysis of the standards, and the limit of quantification (LOQ) was defined as the lowest measured quantity above which the analyte can be quantified at a given statistical level of (10S/N).

The intra-day precision of the method was determined by preparing the standards of AM, DX and EP at three concentration levels and peak heights for each compound were determined after processing each standard eight times.

Table 1
Characterisation of SIC process

	AM	DX	EP
Retention time (s)	240	355	572
Peak resolution	$R_{AM,DX} = 3.38$	$R_{DX,EP} = 5.57$	
Peak symmetry	2.8	2.1	1.3
Number of theoretical plates	941	1117	1987

Table 2
Analytical parameters and method validation results

	AM	DX	EP
Calibration range ($\mu g\,ml^{-1}$)[a]	2–100	2–100	2–100
Correlation coefficient	0.9994	0.9993	0.9992
Limit of detection ($\mu g\,ml^{-1}$)	0.5	2	0.75
Limit of quantification ($\mu g\,ml^{-1}$)	1.7	6.7	2.5
System precision (%)[b]			
5 $\mu g\,ml^{-1}$	3.53	5.39	2.47
10 $\mu g\,ml^{-1}$	1.19	5.05	1.57
50 $\mu g\,ml^{-1}$	1.53	1.47	0.52
Repeatability of T_r (%)[c]	1.66	2.26	0.91

[a] Each concentration was measured in duplicate.
[b] Relative standard deviation (R.S.D.) values were calculated for intra-day repeated standard injections at three concentration levels $c = 5\,\mu g\,ml^{-1}$, $c = 10\,\mu g\,ml^{-1}$, and $c = 50\,\mu g\,ml^{-1}$.
[c] Repeatability of T_r: R.S.D. of retention times for intra-day repeated standard injections, $n = 8$.

Table 3
Determination of ambroxol hydrochloride and doxycycline in pharmaceutical capsules and tablets by SIC

Pharmaceutical preparation (capsules, tablets)	Composition	Amount of active substance in (mg)	Found amount in (%)[a] ±R.S.D.
Doxycyclin AL comp. (capsules)	AM	75	97.8 ± 3.3
	DX	100	102.8 ± 1.0
DOXYHEXAL® 200 TABS (tablets)	DX	200	99.3 ± 4.3
AMBROSAN® (tablets)	AM	30	99.9 ± 3.0

[a] Mean values of the determination of six samples for each pharmaceutical preparation.

The method validation results obtained under the final conditions are shown in Table 2. The method was found to fulfil common requirements of accuracy, precision and linearity (calibration range with correlation >0.999, R.S.D. for repeated standard injections at three concentration levels ($n = 8$) less than 5.4%).

To validate the precision of the method a number of six different pharmaceutical sample solutions were used, which were prepared from the same batch and analysed consecutively. This approach provides a means of covering the precision of the entire method, from sample preparation to data handling. The accuracy of the method was carried out measuring of the pharmaceutical samples fortified with known quantity of the analytes (addition of 100% amount of the standards to pharmaceutical preparation). Spiked sample solutions and un-spiked sample solutions were compared for recovery evaluation. The method accuracy results were tested for Doxycyclin AL comp. capsules (6 samples) and values of the recoveries were found as $99.3 \pm 0.5\%$ for AM and $99.9 \pm 1.5\%$ for DX. Assay values of recoveries show that the method allows direct determination of AM and DX in commercial dosage forms in the presence of other excipients.

3.4. Determination in pharmaceutical capsules and tablets

The method developed has been applied to the determination of AM and DX in tablets and capsules. The samples were commercially available on the Czech market. The interference effect of excipients (saccharine, gelatine, starch, shellac, TiO_2 and Fe_2O_3) was not observed. The optimal extraction medium for capsules and tablets dissolution was methanol with 1% of H_3PO_4. The procedure of sample preparation was simple, fast and achieving high precision and low sample and reagent consumption. The average amounts of AM and DX of the labelled amount in pharmaceutical preparations are given in Table 3. The results are in a good agreement with the pharmacopoeial requirements on the active compound content in tablets 95.0–105.0%.

4. Conclusion

The proposed SIC system proved to be a convenient and efficient tool for the separation and determination of ambroxol hydrochloride and doxycycline in pharmaceutical preparations. There is no research describing the usage this kind of technique in the field of analytical chemistry. SIC technique shows several advantages as short time of analysis, the production of the waste and the consumption of solvents lower than HPLC methods, and the reduced cost per analysis. The coupling of the monolithic column to SIA manifold solves the separation problems, what allows carry out simple separations without an expensive instrumentation such as the HPLC. From this point of view, the low-pressure monolithic silica columns open an area offering separation analysis in sequential injection systems.

In summary, the SIC system can be an important tool for the rapid separation and quantification of one or more compounds not only in pharmaceutical preparations, that allows the use of this technique in the routine analysis. The method described in this contribution is a good alternative to the traditional analysis with the HPLC instrumentation for pharmaceutical preparations that containing ambroxol hydrochloride and doxycycline, together or separately.

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