Simultaneous Determination of Hydrochlorothiazide and Benazepril Hydrochloride or Amiloride Hydrochloride in Presence of Hydrochlorothiazide Impurities: Chlorothiazide and Salamide by HPTLC Method

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Simple, selective and sensitive high-performance thin layer chromatographic (HPTLC) method has been developed and validated for the simultaneous determination of hydrochlorothiazide (HCZ) in the presence of its impurities (chlorothiazide (CT) and salamide (DSA)), in two quaternary mixtures with benazepril hydrochloride (BZ) or amiloride hydrochloride (AM). The separation was carried out on HPTLC silica gel 60 F254 using ethyl acetate–methanol–glacial acetic acid (85:2:0.3 v/v/v) followed by densitometric measurement of bands at 240 nm for the first mixture containing HCZ, CT, DSA, BZ and by using ethyl acetate–methanol–water–ammonia (90:10:5:3 v/v/v/v) followed by densitometric measurement at 278 nm for the second mixture containing HCZ, CT, DSA, AM. Calibration curves were constructed in the range of (0.2–1.8 μg/band) and (0.4–2.2 μg/band) with good accuracy for HCZ and BZ, respectively, for the first mixture and in the range of (0.6–1.8 μg/band) and (0.4–2.4 μg/band) with good accuracy for HCZ and AM, respectively, for the second mixture. The developed method was validated according to ICH guidelines and demonstrated good accuracy and precision. Moreover, the methods were successfully applied for the determination of HCZ and BZ and AM in pure form and pharmaceutical dosage forms. The results were statically compared with the reported methods with no significant difference, indicating the ability of the proposed method to be used for routine analysis of drug product.

Introduction

Hydrochlorothiazide (HCZ), 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide (Figure 1a) (1), is an antihypertensive diuretic agent used for management of hypertension. Benazepril hydrochloride (BZ) (Figure 1b), (3S)-1-(carboxymethyl)-[(1S)-1 -(ethoxycarbonyl)-3-phenylpropyl]-amino]-2,3,4,5-tetrahydro-1H-[1]benzazepin-2-one hydrochloride, is an angiotensin-converting enzyme (ACE) inhibitor used in the treatment of hypertension and heart failure (2). Amiloride hydrochloride (AM) (Figure 1c), 3,5-diamino-N-carbamimidoyl-6-chloropyrazine-2-carboxamide hydrochloride hydrate (3), is a potassium-conserving diuretic with antihypertensive activity. It is indicated as adjunctive treatment with thiazide diuretics or other diuretic agents in congestive heart failure or hypertension to aid in the restoration of normal serum potassium levels and to prevent the development of hypokalemia (4). Formulation of HCZ with either BZ or AM increases the antihypertensive effect. Chlorothiazide (CT) (Figure 1d), 6-chloro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide, and salamide (DSA) (Figure 1c), 4-amino-6-chlorobenzene-1,3-disulphonamide, are considered as specified impurities for HCZ which are synthetic impurity for which a maximum pharmacopeial limit is defined (5).

Literature review revealed that HCZ and BZ have been determined together by different analytical methods, such as spectrophotometric (6–8), reversed-phase high-performance liquid chromatography (RP-HPLC) (9–12), thin-layer chromatography (TLC)-densitometric (9, 10) and chemometric (13, 14) methods. While HCZ and AM have been determined by spectrophotometric (15–17), RP-HPLC (15, 18, 19) and chemometric (20, 21) methods.

Chromatographic analysis of process-related impurities and of degradation products is very important in the pharmaceutical industry. The possibility of side and toxic effects and also reduced activity of the active substances must be reduced to a minimum. For this reason, pharmacopoeias and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) have established very restrictive requirements for levels of impurities in pharmaceutical products. One of the main analytical problems is the large difference between the amounts of active substances and impurities, so a method for their simultaneous identification and quantification must be sufficiently selective. A second important problem is the structural similarity of the components analyzed, which lead to similar chromatographic behavior (22). A comprehensive literature search revealed the lack of a suitable method for determination of the studied drugs in their combined pharmaceutical formulations without interference from drug impurities. All the reported chromatographic methods failed to separate the HCZ in its combination with either BZ or AM in presence of its impurities. So, the present work deals with the determination of both active compounds and assessment of stability of the bulk drugs and of pharmaceutical dosage forms using a simple HPTLC.

Experimental

Materials and reagents

Authentic samples

Standard HCZ, BZ and AM were kindly supplied by Sigma Pharmaceuticals Industries (El Monofeya, Egypt) with certified

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purities of 99.6, 99.7 and 99.7%, respectively. Standard CT and DSA were purchased from Sigma Aldrich Chemie (Germany) with certified purities of 99.8 and 99.6%, respectively.

Pharmaceutical formulation
Cibadrex® tablets batch No. (Y0006) were manufactured by Novartis Pharma S.A.E (Cairo, Egypt). Each tablet is claimed to contain 20 mg of BZ and 25 mg of HCZ. Moduretic® tablets batch No. (110530) were manufactured by EL Kahira CO. for Pharmaceutical and Chemical Industries (Cairo, Egypt). Each tablet is claimed to contain 5 mg of AM and 50 mg of HCZ. Hydikal® tablets batch No. (210) were manufactured by Pharco Pharmaceuticals (Alexandria, Egypt). Each tablet is claimed to contain 5 mg of AM and 50 mg of HCZ.

Reagents
All reagents and chemicals used were of analytical grade and were used without further purification; they included
- (i) Methanol analar (Central Drug House Ltd., India).
- (ii) Ethyl acetate, ammonia and glacial acetic acid (El-Naser Pharmaceutical Chemical Co., Abu-Zabaal, Cairo, Egypt).
- (iii) Deionized water (Sedico Pharmaceuticals Co., Cairo, Egypt).

Instruments
- (i) CAMAG TLC scanner 3 S/N 130319 with win CATS software.
- (ii) Linomat 5 autosampler (Switzerland).
- (iii) CAMAG microsyringe (100 μL).
- (iv) HPTLC aluminum plates (20 × 20 cm) precoated with 0.25 mm silica gel 60 F254 (Merck, Germany).
- (v) Sonix TV ss-series ultrasonicator (USA).
- (v) Digital balance (Sartorius, Germany).

Standard solutions
Stock standard solutions Stock solutions of each of HCZ, BZ, AM, CT and DSA (1 mg mL⁻¹) were prepared by accurately weighing 100 mg of pure powder of each into four separate 100-mL volumetric flasks. Fifty milliliter methanol was added into each flask, and the flasks were shaken for complete mixing of contents, and then volume was made up to the mark with methanol.

Working standard solutions
Ten milliliters of stock solutions of HCZ, BZ and AM (1 mg mL⁻¹) were separately transferred into three separate 100-mL volumetric flasks and diluted to the mark with methanol to get 100 μg mL⁻¹ working solutions of each.

Procedures
Linearity and construction of calibration curves
For HCZ/BZ mixture, accurate aliquots equivalent to 0.2–2 and 0.4–2.4 mg of HCZ and BZ, respectively, were separately transferred from their respective stock solutions (1 mg mL⁻¹) into a series of 100-mL measuring flasks, then the volume of each flask was completed to volume with methanol. Ten microliters of each solution applied in triplicate on the HPTLC plates. Accurate aliquots equivalent to 0.2–2 and 0.4–2.4 mg of HCZ and AM, respectively, for HCZ/AM mixture were applied in the same way. Samples were applied in the form of bands; the band width was 4 mm and the bands were applied 20 mm apart from each other and 15 mm from the bottom edge. Linear ascending development was performed in a chromatographic tank previously saturated with ethyl acetate–methanol–glacial acetic acid (85:2:0.3 v/v/v) for the first mixture and ethyl acetate–methanol–water–ammonia (90:10:5:3 v/v/v) for the second one as a mobile phase for 30 min. The migration distance was 90 mm from the lower edge of the plate. The developed plates were air dried and scanned at 240 nm for the first mixture and 278 nm for the second one under the specified instrumental conditions. The area under the peak was recorded and calibration curves were constructed by plotting the integrated peak area/10⁴ versus the corresponding concentrations, then the linear regression equations were computed.

Application to pharmaceutical formulation
Cibadrex® tablets. Twenty tablets of Cibadrex® were powdered and mixed well. Accurately weighed amount of the powdered tablets equivalent to 100 mg of HCZ and BZ were separately transferred into 100 mL volumetric flasks. Fifty milliliters of methanol was added and ultrasonicated for 30 min, cooled and flasks were completed to volume with methanol. The solution was filtered and diluted to obtain 100 μg mL⁻¹ working solution using methanol as solvent. The procedure mentioned under Linearity and construction of calibration curves section for the first mixture was followed. By applying the corresponding regression equation, the concentrations of HCZ and BZ and their mean percent recoveries were calculated.

The same procedure was repeated by spiking tablet’s powder with known amounts of pure HCZ and BZ at different concentrations levels (standard addition technique) and the percent recoveries of the added pure drugs were calculated.
Moduretic® tablets and Hydikal® tablets. Twenty tablets of each Moduretic® or Hydikal® tablets were powdered and mixed well. Accurately weighed amount of the powdered tablets equivalent to 100 mg of HCZ and AM were separately transferred into 100 mL volumetric flask. Fifty milliliters of methanol were added and ultrasonicated for 30 min, cooled and complete to volume with methanol. The solution was filtered and diluted to obtain 100 µg mL⁻¹ working solution using methanol as solvent. The procedure mentioned under Linearity and construction of calibration curves section for the second mixture was followed. By applying the corresponding regression equation, the concentrations of HCZ and AM and then the mean percent recoveries were calculated.

The same procedure was repeated by spiking tablet’s powder with known amounts of pure HCZ and AM at different concentrations levels (standard addition technique) and the percent recoveries of the added pure drugs were calculated.

Results

The main task of this work is to apply this technique to develop a sensitive, selective and accurate analytical method for simultaneous determination of HCZ and its impurities (CT and DSA) in two quaternary mixtures with BZ and AM in bulk powder and pharmaceutical formulations with satisfactory precision for good analytical practice.

Method development and optimization

To improve separation of bands, it was necessary to investigate the effect of different variables where optimum values for maximum separation were determined. Different developing systems of different compositions and ratios were tested including the two developing system ethyl acetate–methanol–chloroform (10:3:2 v/v/v) (9) and ethyl acetate–methanol–ammonia (85:20:10 v/v/v) (10) for separation taking into consideration the similarity in physical and chemical properties of HCZ and CT which represents a challenge during the choice of a suitable developing system. Complete separation of HCZ, CT and DSA in the presence of BZ was achieved by using ethyl acetate–methanol–water–ammonia (90:10:5:3 v/v/v) as the developing system. Complete separation of HCZ, CT and DSA in the presence of AM as a mobile phase. The used mobile phases lead to good resolution, sharp and symmetrical peaks for HCZ, CT, DSA, BZ and AM (Figures 2 and 3).

Different scanning wavelengths were tried. Upon using 240 and 278 nm for the first and second mixtures, respectively, sharp er and symmetrical peaks with minimum noise were obtained. The \( R_f \) values (HCZ = 0.73, BZ = 0.36, CT = 0.56, DSA = 0.88) for the first mixture and (HCZ = 0.46, AM = 0.20, CT = 0.10, DSA = 0.62) for the second mixture. Typical densitograms are shown in Figures 2 and 3.

Method validation

Method validation was performed according to the ICH guidelines (25) for the proposed methods.

Linearity

Under optimum chromatographic conditions, linearity of the developed method was evaluated by measuring the integrated area under the peak area of different concentrations each of HCZ, BZ and AM and then plotting calibration graphs relating the peak area/10,000 against the corresponding concentration. It was evident in the range of 0.2–1.8 and 0.4–2.4 µg/band for HCZ and BZ, respectively, for the first mixture, 0.6–1.8 and 0.4–2.4 µg/band for HCZ and AM, respectively, for the second mixture. The regression equation for the proposed methods was calculated and found to be

For the first mixture

\[
Y = 0.266C + 0.059, \quad r = 0.9997 \text{ for HCZ.}
\]

\[
Y = 0.324C + 0.065, \quad r = 0.9999 \text{ for BZ.}
\]

For the second mixture

\[
Y = 0.698C + 0.467, \quad r = 0.9993 \text{ for HCZ.}
\]

\[
Y = 0.316C + 0.248, \quad r = 0.9995 \text{ for AM.}
\]

where \( Y \) is relative peak area/10,000, \( C \) is corresponding concentration in µg band⁻¹ and \( r \) is the correlation coefficient.
Good linearity is evident from the high value of the correlation coefficient and low value of intercept, Table I.

**Accuracy**

Accuracy of the proposed methods was checked by applying them for the determination of pure samples of the studied compounds. The concentrations were calculated from the corresponding regression equations and the results are shown in Table I. Accuracy was further assessed by applying the standard addition technique to Cibadrex, Moduretic and Hydikal tablets, where good recoveries were obtained revealing no interference from excipients and good accuracy of the proposed methods, Table II.

**Precision**

Precision was studied with respect to both repeatability and intermediate precision by analysis of different concentrations of pure HCZ, BZ and AM in triplicate on the same day to determine repeatability and on four consecutive days to determine intermediate precision. Good results and acceptable relative standard deviations (RSDs) were obtained (Table I).

**Selectivity**

Selectivity of the proposed methods is evident from the HPTLC densitogram in Figures 2 and 3.

**ICH and LOQ**

ICH recommendations (23) were followed using a visual noninvasive LOD and LOQ densitogram in Figures 2 and 3. Selectivity of the proposed methods is evident from the HPTLC densitogram in Figures 2 and 3. Selectivity standard deviations (RSDs) were obtained (Table I).

**Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability during normal usage. To determine the robustness of the method, the experimental conditions were deliberately changed (e.g., change in the ethyl acetate volume ± 0.5 mL and methanol ± 0.05 mL; change in the saturation time ± 5 min) and the resolutions of HCZ, BZ, AM, CT, DSA were evaluated, where there is no significant effect found on Rf values or symmetry of the peaks. However, small increase in volume of ammonia solution (0.5 mL) greatly affects separation of CT (decrease in Rf).

**System suitability**

System suitability testing for the HPTLC method is based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. The testing is used to ensure system performance before or during the analysis of the drugs. System suitability was checked by calculating the capacity factor (K') (24), selectivity factor (α), resolution (R_s) and symmetry factor (T), where the system was found to be suitable as shown in Table III.

**Statistical comparison to reference methods**

The results obtained by the developed HPTLC method were statistically compared with those obtained by the reported RP-HPLC method (9) for Cibadrex tablets and with those obtained by the reported ratio spectra derivative spectroscopic method (15) for Moduretic and Hydikal tablets using Student’s t-test and variance ratio F-test at a 95% confidence level, and no significant difference was found between the results indicating the high selectivity and accuracy of the methods (Table IV).

**Discussion**

The monitoring of in-process impurities has become a major factor in modern pharmaceutical industry. This is mainly because of the pressure for product quality, and the demand for higher standards of process reliability. Toxicological issues have also brought about a greater sensitivity to the significance of impurities at trace levels (25). As the presence of these unwanted chemicals, even in small amounts, may influence the efficacy and safety of the pharmaceutical products.

The use of chromatographic techniques for monitoring the starting materials, intermediates and the process reactions is an excellent means for controlling the purity of the final drug and thereby protecting the patient who ultimately receives it (26). TLC is a powerful tool for screening unknown materials in bulk drugs (27). It provides a relatively high degree of assurance that all possible components of the drug are separated (28). Due to structural similarity between CT and HCZ, it is difficult to be separated by spectroscopic method so it is necessary to develop a chromatographic method to separate them. The proposed method separates them with good resolution.

After optimization and validation of the developed HPTLC method, it was successfully applied for determination of HCZ and BZ in Cibadrex tablets and HCZ and AM in Moduretic and Hydikal tablets. Good results were obtained and presented in Table II.

The proposed method has advantage over reported HPLC and ratio spectra derivative spectroscopic methods in that it offer detection of both impurities of HCZ.

**Conclusion**

The proposed methods provide sensitive, accurate and reproducible means for determination of HCZ in the presence of its
impurities (CT and DSA) in two quaternary mixtures of BZ and AM using the HPTLC method.

The HPTLC method has the advantage that several samples can be run simultaneously using a small quantity of mobile phase and that it can provide high sensitivity and selectivity.

The proposed HPTLC method has the advantage over the reported methods that it offers detection of both impurities of HCZ in the presence of BZ and AM. No method was reported for the determination of HCZ and its impurities (CT and DSA) in the presence of BZ and AM.
Application of the proposed methods to the analysis of the studied drugs in laboratory-prepared mixtures and pharmaceutical formulation shows that neither the impurities (CT and DSA) nor the excipients interfere with the determination, indicating that the proposed methods could be applied for the determination of pure HCZ, BZ and AM in the presence of CT and DSA either in bulk powder or in pharmaceutical formulations.

References
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