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Simultaneous determination of dorzolomide and timolol in aqueous humor: A novel salting out liquid–liquid microextraction combined with HPLC

Abdel-Maaboud Ismail Mohamed [a], Hanaa Mohammed Abdel-Wadood [a], Heba Salah Mousa [b,*]

[a] Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Assiut University, 71526 Assiut, Egypt
[b] Drug Research Center, Assiut University, 71526 Assiut, Egypt

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ABSTRACT

A Snovel method for the simultaneous separation and determination of two antiglaucoma drugs namely, dorzolamide hydrochloride (DOR) and timolol maleate (TIM) in aqueous humor samples (AH) was developed by using salting-out assisted liquid–liquid microextraction (SALLME) combined with HPLC–UV method. Box–Behnken experimental design and response surface methodology were employed to assist the optimization of SALLME conditions, including salt concentration, the pH of sample solution and vortex time as variable factors. The optimal extraction conditions were as follows: to 50 μL of AH sample, 100 μL of phosphate buffer (100 mmol L^{-1}, pH 11.9), 90 μL of acetonitrile (ACN) and 0.11 g of $(NH_4)_2SO_4$ salt were added into an Eppendorf vial (1 mL) then vortexed for 1.1 min. As an effort to miniaturize SALLE system, a 1 mL syringe adapted with a capillary tube was employed as the phase separation device. Once the phase separation occurred, the upper layer could be narrowed into the capillary tube by pushing the plunger; thus, the collection of the upper layer solvent was simple and convenient. By miniaturization, the consumption of the organic solvent was decreased as low as possible. The chromatographic separation was achieved on Gemini C_{18} column using a mobile phase of ACN: 30 mmol L^{-1} potassium dihydrogen phosphate buffer containing 0.1% triethylamine, pH 3.5 (20:80, v/v) at a flow rate of 1 mL min^{-1} and UV detection at 254 and 295 nm for DOR and TIM, respectively. Mepivacaine hydrochloride was used as an internal standard. The described method showed better separation with enhanced sensitivities than the previously reported methods with limits of quantitation of 8.75 and 10.32 ng mL^{-1} in aqueous solution and 15.97 and 23.53 ng mL^{-1} in AH for DOR and TIM, respectively. The simple, rapid and eco-friendly SALLME–HPLC method has been successfully applied for the simultaneous pharmacokinetic studies of DOR and TIM in rabbit AH.

1. Introduction

Timolol maleate (TIM), Fig. 1, is a nonselective β-adrenergic receptor antagonist which acts by lowering the intraocular pressure (IOP) primarily by reducing the production of aqueous humor (AH) by the ciliary epithelium [1]. It is considered the first-line drug for the treatment of glaucoma [1–3].

Dorzolamide hydrochloride (DOR), Fig. 1, is the first topical carbonic anhydrase inhibitor (CAI) used for treatment of glaucoma by lowering IOP through inhibition of CA isoenzyme involved in AH production [3].

In many cases, there is a need for more than one type of medication. Hence, to improve compliance, fixed combinations of different drugs have been introduced. A combination therapy including TIM and DOR has a scientific bearing as the two drugs have complementary mechanisms of action [4]. Therefore, a fixed combination of DOR and TIM (FCDT) has been approved by the FDA and is widely used for treatment of glaucoma. Generally, it is instilled by patients themselves for months or years. Therefore, a better understanding of the pharmacokinetics of this combination in AH will help decreasing the incidence of any adverse effects.

Abbreviations: ACN, acetonitrile; AH, aqueous humor; BBD, Box–Behnken design; DOR, dorzolamide hydrochloride; CE, capillary electrophoresis; FCDT, fixed combination of dorzolamide and timolol; HPLC, high-performance liquid chromatography; IS, internal standard; LLE, liquid–liquid extraction; PPT, protein precipitation; RSM, response surface methodology; SALLME, salting-out assisted liquid–liquid microextraction; TEA, triethylamine; TIM, timolol maleate; TLC, thin layer chromatography

* Corresponding author. Tel.: +20 88 2 312821; fax: +20 88 2 332776.
E-mail address: hebasalah2001@yahoo.com (H.S. Mousa).

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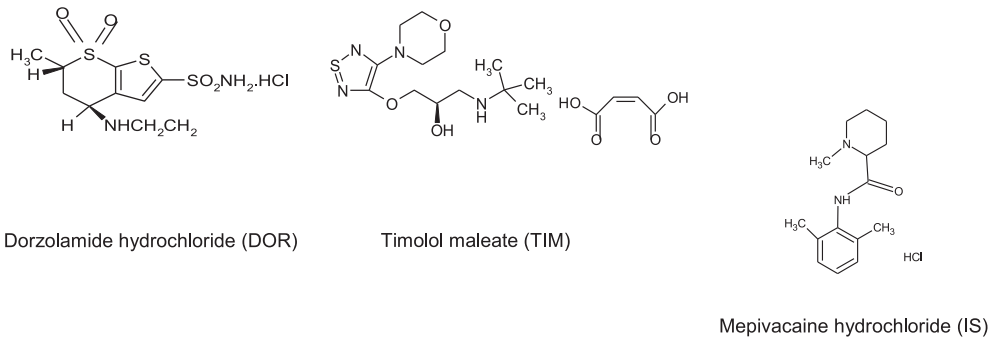

Fig. 1. Chemical structures of the studied antiglaucoma drugs and the internal standard.

In addition, recently, several preparations of this combination with novel drug delivery systems (DDS) have been developed [5,6]. Thus, analysis of their pharmacokinetics in AH becomes more important since it can help in evaluating the properties of the new DDS preparations and determining dosage schedules.

The literature survey revealed that there are few methods reported for the simultaneous analysis of DOR and TIM including spectrophotometric [7–9], thin layer chromatographic (TLC) [7], capillary electrophoretic (CE) [10] and high-performance liquid chromatographic (HPLC) [11–16] methods. All these methods lack the enough sensitivity which enables the determination of small concentrations of DOR and TIM in complex matrices such as AH in which they are typically found. Meanwhile, these methods are applied only for the analysis of the studied drugs in their pharmaceutical formulations. Moreover, although both drugs were determined in the USP 35 [17] and BP 2013 [18], there is no pharmacopeial method for their simultaneous determination so far. Therefore, the present work aimed to develop a sensitive HPLC method for their simultaneous analysis in AH samples.

Furthermore, pre-treatment and enrichment processes are crucial steps in analysis of AH samples because of the very low concentrations of the antiglaucoma drugs typically found, besides the complexity of this matrix. Methods reported for either DOR or TIM extraction from AH samples are either protein precipitation (PPT) [19–23] or liquid–liquid extraction (LLE) [24–28]. Although PPT is simple, the obtained extract still contains a significant amount of impurities which could result in relatively high background in the chromatogram and column deterioration. At the same time, although LLE provides much cleaner extracts than PPT and is the most popular choice, it is time-consuming, tedious and uses large amounts of potentially toxic organic solvents. These organic solvents pose a threat to the environment and human health and their disposal is also extremely expensive. Recently, a novel extraction technique termed as salting-out assisted liquid–liquid microextraction (SALLME) has been developed and applied for the determination of various target analytes from water, food and biological matrices [29–32]. SALLME integrates sample cleanup and preconcentation in a single step. This method is based on the extraction of analytes from the aqueous phase with water miscible organic solvent at high salt concentrations (salting-out phenomena).

The present work reports, for the first time, the development and applicability of a new vortex-assisted SALLME method for the rapid and efficient extraction of DOR and TIM from AH samples. Moreover, a 1-mL syringe adapted with a capillary tube was employed as the phase separation device to reduce the consumption of organic solvent as much as possible, while ensuring a convenient and simple operation. In addition, Box–Behnken design (BBD) approach and response surface methodology (RSM) were employed to assist finding optimal extraction conditions, quickly and reliably. To the best of our knowledge, this is the first demonstration of SALLME optimization by virtue of experimental design for antiglaucoma drug analysis. The SALLME coupled with HPLC with the aid of experimental design was developed, validated and successfully applied for the simultaneous pharmacokinetic studies of DOR and TIM in rabbit AH samples.

2. Experimental

2.1. Chemicals and reagents

DOR and TIM were obtained as a gift from Jamjoom Pharmaceuticals (Jeddah, Kingdom of Saudi Arabia). All solvents were of HPLC grade (Merck, Darmstadt, Germany) and all other materials were of analytical grade. Pharmaceutical dosage forms (Xolamol® eye drops) were kindly supplied by Jamjoom Pharmaceuticals, Egypt Scientific Office and were claimed to contain 2% and 0.5% of DOR and TIM, respectively. Double distilled water was used throughout the work.

2.2. Chromatographic system

The HPLC system consisted of a Knauer HPLC system (Knauer, Berlin, Germany), which consisted of K-500 solvent delivery pump, injector valve with a 20 μL loop and K-2600 UV detector. The HPLC system control and data processing were performed by computer integration software (EuroChrom 2000® Knauer). Digital microtransfer pipettes 5–250 μL were used (Acura, Socorex, Switzerland).

Analytes were separated using Gemini RP-C_{18} column (250 × 4.6 mm^2, 5 μm) (phenomenex, USA) protected with a precolumn (guard column with Gemini C_{18} precolumn inserts) (Phenomenex, USA). Isocratic mobile phase consisted of ACN: 30 mmol L^{-1} potassium dihydrogen phosphate buffer containing 0.1% triethylamine (TEA) at pH 3.5 (20: 80, v/v). The mobile phase was degassed in an ultrasonic cleaner (Cole-Parmer, Chicago, IL, USA) and was filtered through a 0.45 mm membrane filter (Gelman Instrument) using vacuum filtration unit (Phenomenex, USA) and delivered at a flow rate of 1 mL min^{-1}. The injection volume was 20 μL and the detector was set at 254 and 295 nm for DOR and TIM, respectively. The chromatography was performed at room temperature using mepivacaine hydrochloride as internal standard (IS).

2.3. Standard and quality control solutions

Standard stock solutions of DOR and TIM were prepared separately in water at concentrations of 50 μg mL^{-1}. Working solutions containing both drugs were prepared from the stock solutions by appropriate mixing and dilution with water. Working solution of mepivacaine HCl (IS) was prepared in ACN at a concentration of 2 μg mL^{-1}. AH standards for the calibration daily