Journal of Chromatography B, 1002 (2015) 160–168

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rat dried blood spot analysis of (*R,S*)-(−)- and (*S,R*)-(+)- enantiomers of emtricitabin on immobilized tris-(3,5-dimethylphenyl carbamate) amylose silica as a chiral stationary phase

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ARTICLE INFO

Article history:
Received 25 January 2015
Received in revised form 10 August 2015
Accepted 12 August 2015
Available online 21 August 2015

Keywords:
Emtricitabin enantiomers
Immobilized
Amylose tris-(3,5-dimethylphenyl carbamate)
Dried blood spots
Hematocrit

ABSTRACT

An enantioselective high performance liquid chromatography method has been developed and validated by evaluating the suitability of newly introduced immobilized polysaccharide chiral stationary phases, the effect of different organic modifiers and temperature including the entropy and enthalpy on resolution of the (*R,S*)-(−) & (*S,R*)-(+) emtricitabine enantiomers on rat dried blood spots. Both the enantiomers were extracted from dried blood spots using ethanol: methanol (80:20 v/v) mixture and separated on an immobilized amylose tris-(3,5-dimethyl phenyl carbamate) chiral stationary phase using *n*-hexane:ethanol (65:35 v/v) as a mobile phase at a flow rate of 0.8 mL/min. The detection was carried out at 280 nm using photo diode array detector connected to a polarimeter in series to determine their order of eluton. The method was validated with respect to limits of detection and quantification, linearity, accuracy and precision. The calibration curves were linear over the concentration range of 0.5–500 μg/mL for both enantiomers and the correlation coefficient (r^2) was >0.998. The overall recovery of (*R,S*)- & (*S,R*)-enantiomers of emtricitabin from DBS were 90.4 and 90.6%, respectively. The limits of detection and quantification of enantiomers were 0.26, 0.30 and 0.85, 0.92 μg/mL for (*R,S*)- and (*S,R*)-emtricitabin enantiomers, respectively. The assay was specific and precise (RSD <10%). The stability of emtricitabin was also performed and the results were found to be well within the limits. The effect of hematocrit on extraction of emtricitabin enantiomers from dried blood spots was evaluated and no interference from endogenous substances was observed.

1. Introduction

Emtricitabine (EMT); (−)-4-amino-5-fluoro-1-((2*R*,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl) pyrimidine-2(1H)-one), a thio analogue of cytidine (Fig. 1) is potent a dideoxycytidine nucleoside reverse transcriptase inhibitor selective against HIV types I and II and hepatitis B viruses [1–6]. EMT is phosphorylated by cellular enzymes to form emtricitabine 5′-triphosphate, which competes with deoxycytidine 5′-triphosphate and terminates the amino acid chain of newly forming viral DNA. It is approximately 20-fold more potent against HIV in peripheral blood mononuclear cells than its antipode. As *(R,S)*-(−)-EMT is exclusively used in HIV and hepatitis B therapy, it is of great importance to develop specific methods that separate and determine (*R,S*)-(−) and (*S,R*)-(+) EMT enantiomers in biological matrices.

Several methods including HPLC and LC-MS have been reported to analyse *(R,S)*-(−)-EMT in biological matrices. LC-MS/MS was used to determine both *(R,S)*-(−)-EMT and tenofovir in plasma [7] as well as dried blood spots (DBS) of human blood [8]. Quezia et al. determined the enantiomeric purity of EMT on a polysaccharide-based chiral phase using a mobile phase consisting of polar organic solvents [9]. Kromdijk et al. have reported the separation and determination of zidovudine, abacavir, EMT, lamivudine, tenofovir and ribavirin in human plasma by LC–MS [10]. A thorough literature survey revealed that determination of (*R,S*)-(−) & (*S,R*)-(+) enantiomers of EMT on DBS is not yet reported. In general, techniques such as protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE) have been used for sample preparation of biological matrices. However, these assays require a relatively large volume of blood (typically >0.5 mL) to generate sufficient plasma for analysis. DBS technology has recently drawn massive attention due to its distinctive advantages in the quantitative bioanalysis, including low cost, ease of sample collection, less invasive sampling, simpler transfer, storage and shipping [11–14].

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http://dx.doi.org/10.1016/j.jchromb.2015.08.016
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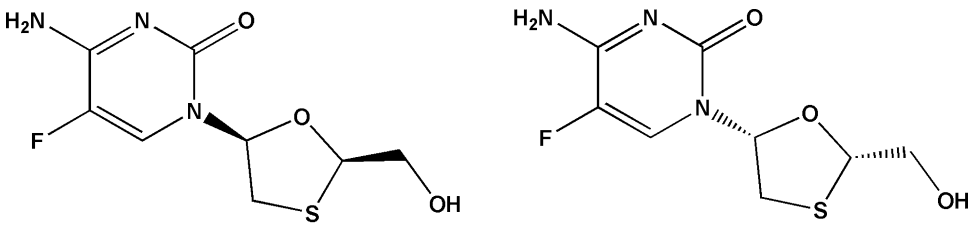

Fig. 1. Chemical structures of (A) *(R,S)*-(−) EMT and (B) *(S,R)*-(+) EMT.

There have also been a number of reports on its use in humans for therapeutic drug monitoring and pharmacokinetic (PK) studies [15–18]. In addition to the advantages associated with the clinical use of DBS, the small blood volumes used for DBS (typically ≤50 μL) facilitate increased PK data quality through the ability to take serial bleeds from the same animal particularly when coupled with sensitive and selective HPLC and LC–MS/MS techniques. Therefore it is of great importance to develop simple, rapid and selective techniques such as DBS for collection and extraction of (*R,S*)-(−)- and (*S,R*)-(+)-EMT enantiomers from biological matrices which require less amount of biological samples to find applications not only in clinical studies but also therapeutic drug monitoring in patients particularly neonates having positive signs of HIV.

The present manuscript describes the development of an economical and versatile HPLC-PDA method for evaluation of (*R,S*)-(−) and (*S,R*)-(+) EMT enantiomers on rat dried blood spots. The method was validated in accordance with USFDA guidelines [19] to assess its specificity, sensitivity, accuracy, precision, and robustness.

2. Experimental

2.1. Chemicals and reagents

Glass-distilled de-ionized water (Nanopure, Barnstead, USA), HPLC grade methanol (SD Fine Chemicals, Mumbai, India), *n*-hexane (Rankem, Mumbai, India), ethanol, 1-propanol, 2-propanol; *n*-butanol, *n*-pentanol and methyl t-butyl ether (Sisco Research Laboratories Pvt., Ltd., Mumbai, India) were used. (*R,S*)-(−) and (*S,R*)-(+) EMT enantiomers of 99.8% purity (Fig. 1) were obtained as gift samples from Neuland Laboratories Pvt., Ltd., Hyderabad, India.

2.2. Materials

Control blood used in the present study was collected from Wistar rats (Pharmacology Division of our Institute in compliance with standard animals use practices approved by Institutional Committee on animals use at Indian Institute of Chemical Technology, Hyderabad, India and stored at −20 °C just for a few minutes before spotting on the FTA cards. The multi pipette for spotting blood (Tarsons, Kolkata, India), FTA blood spot cards, the Harris punch and cutting mat (Whatmann, Sanford, ME, USA), blood collection tubes (Sarstedt Leicester, UK), heparin coated capillaries (Sangius Counting Gmbh, Nümbrecht, Germany), a centrifuge (model 2-16P, Sigma, Zurich, Switzerland) were used. Sealing plastic bags and sachets of silica gel for the storage of blood spot cards were purchased from MS Life Sciences, Hyderabad, India.

2.3. High-performance liquid chromatography

A HPLC system consisting of two LC-20AD pumps, an SPD-M20A diode array detector (PDA), an SIL-20AC auto sampler, a DGU-20A_5 degasser, a CTO-20 AC column oven and a CBM-20A communications bus module (all from Shimadzu, Kyoto, Japan) was used. The data were recorded using an HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). Chiralpak IA column (immobilized amylose tris-(3,5-dimethylphenyl carbamate) (250 mm × 4.6 mm I.D., 5 μm particle size) (Daicel, Tokyo, Japan) was used for separation.

2.4. Chromatographic conditions

Chromatographic separation was achieved on Chiralpak IA column using a mobile phase of *n*-hexane–ethanol (65:35 v/v) at a flow rate of 0.8 mL/min under isocratic mode of elution in 15 min run time. The mobile phase was freshly prepared, filtered through a Millipore filter (pore size 0.45 mm) and degassed continuously using an on-line degasser. The injection volume was 5 μL and the detection wavelength was set at 280 nm.

2.5. Stock solutions, calibration standards and quality control samples

Stock solutions of EMT enantiomers were prepared by dissolving accurately weighed quantities in methanol (20%) and then diluting using ethanol (80%) to prepare a stock of standard solution equivalent to 1.0 mg/mL. All stock standard solutions were stored in a freezer at −5 °C and analyzed periodically against fresh solutions by HPLC. Since there was no significant difference in assay (maximum <2%) observed, the stock solutions were considered to be stable and used in preparation of the next level standard solutions. The experiments conducted with QC samples containing only the *(R,S)*-(−)- and (*S,R*)-(+)-EMT enantiomers after storing for one month also did not show any significant inter conversions on rat DBS. The DBS calibration standard (CS) solutions were prepared by dilution to concentrations of 0.5, 1, 5, 10, 50, 100, 200 and 500 μg/mL of *(R,S)*-(−)- and (*S,R*)-(+)-EMT enantiomers by spiking appropriate aliquots of stock solutions to blank pooled drug-free rat blood. The DBS quality control (QC) samples (low, medium, and high concentration of 3, 200 and 500 μg/mL) were prepared following the same procedure. An external standard method was followed for quantification of *(R,S)*-(−)- and (*S,R*)-(+)-EMT enantiomers by HPLC. In this method, unlike an internal standard, a set of calibration standards containing both *(R,S)*-(−)- and (*S,R*)-(+)-EMT enantiomers at known concentrations in methanol:ethanol (20:80) were prepared, injected and the corresponding chromatograms were obtained. From the respective peak areas of the analytes, calibration curves relating peak areas and concentrations were constructed and response factors for each enantiomer were determined and used for estimation of individual enantiomers in real samples. The external standard method is not only simple and rapid but also accurate and precise when compared to an internal standard method which is generally followed in bioanalytical method development.