Journal of Chromatography B, 1008 (2016) 212–218

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Highly sensitive and selective detection of human carboxylesterase 1 activity by liquid chromatography with fluorescence detection

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ARTICLE INFO

Article history:
Received 19 August 2015
Received in revised form
20 November 2015
Accepted 25 November 2015
Available online 27 November 2015

Keywords:
Liquid chromatography-fluorescence detection (LC-FD)
Human carboxylesterase 1 (hCE1)
BMBT hydrolysis
Biological samples

ABSTRACT

Human carboxylesterases 1 (hCE1), one of the most important human drug metabolizing enzymes, catalyzes the hydrolysis of a large number of structurally diverse of endogenous and exogenous substrates. However, a practical, reliable and sensitive method for the precise measurement of hCE1 activities in complex biological samples has been rarely reported. In this study, a liquid chromatography-fluorescence detection (LC-FD) based method was developed for highly selective and sensitive measurement of hCE1 activities in human tissue and cell preparations. This method was based on the fluorimetric detection of HMBT, the hydrolyzed product of BMBT which was a newly developed specific probe substrate for hCE1. The developed LC-FD method was fully validated in terms of specificity, sensitivity, linearity, precision, recovery and stability. With the help of LC separation, most polar endogenous compounds in biological samples could be eluted in the column dead time, which is very beneficial for accurate determination of hCE1 activities in complex biological samples. The lower limit of quantification for HMBT (product of hCE1) of this LC-FD based method was as low as 20 nM, which was quite lower than other reported methods. The method also exhibited good precision, both intra- and inter- assay variances were both lower than 2.5%. Furthermore, the newly developed method was successfully applied to measure hCE1 activity in human liver preparations from individual donors ($n = 12$), as well as in homogenates from eleven different human cell lines. All these findings combined with this practical method are very helpful for the deep understanding of the expression and function of hCE1 in human biological samples.

1. Introduction

Carboxylesterases (CEs) are members of the serine esterase superfamily and are localized in multiple tissues of human and animals [1,2]. Human carboxylesterases 1 (hCE1) is one of the most abundant CEs in human, which is predominantly existed in the liver tissue [3]. In human body, hCE1 has been reported responsible for the biotransformation of a large number of structurally diverse of endogenous substrates and participated in several physiological processes, such as cholesterol hydrolysis and fatty acid metabolism [4–7]. The hydrolase activity of hCE1 may be effective in hydrolysis cholesterol ester of macrophage and relate with the formation of atherosclerosis [8,9]. It also has been reported that the expression level of hCE1 within fatty tissue is closely associated with metabolic disorders and obesity-related diseases [5,10,11]. Therefore, a highly selective and sensitive method for the measurement of hCE1 activity in tissue or cell samples will be of great importance for the investigations on its molecular functions and biological roles in cellular processes and human diseases, such as cholesterol and lipid metabolism-disease pathogenesis.

As one of the most important phase I drug metabolizing enzymes, hCE1 also plays key role in toxin detoxication and drug metabolism. The distribution and catalytic activity of hCE1 has been reported to affect the efficacy and clinical outcomes of numerous esterified drugs [12–16]. On one hand, hCE1 mediates activation of many prodrugs (such as emocapril, oseltamivil, and CPT-11 etc.), the in vivo efficacy of these prodrugs will be strongly affected by the activity of hCE1 [12,13,17,18]. On the other hand, hCE1 promotes the metabolic inactivation and clearance of

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http://dx.doi.org/10.1016/j.jchromb.2015.11.046
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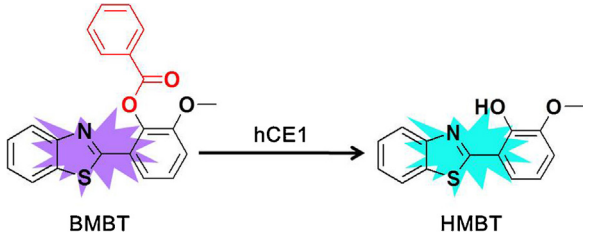

Fig. 1. BMBT hydrolysis by hCE1.
The carboxylic ester bond of BMBT was rapidly cleaved and produced HMBT upon addition of hCE1, leading to a remarkable fluorescence spectrum change.

some esterified drugs [19]. The lack of hCE1 activity may cause inadequate methylphenidate hydrolysis and lead to a significant increase of plasma concentration of methylphenidate, which easily give rise to clinical toxicity [19,20]. A new class of promising anticancer compounds, phospho-nonsteroidal anti-inflammatory drugs (phospo-NSAIDs), are inactivated by hCE1 and the inhibition of hCE1 will improve the efficacy of these phospho-NSAIDs both in vitro and in vivo [21,22]. More importantly, many factors including sex, age, drugs, and disease status, have been reported can cause individuals and tissues differences in both expression and function of hCE1 [23–25].

In the past decade, several different methods including PCR [26], western blot [27] or mass spectrometry-based proteomic techniques [28] have been developed to quantify hCE1. However, all these methods only measure mRNA or protein levels of hCE1 rather than its real activity. Thus, a practical, sensitive and selective method for the precise measurement of the real activity of hCE1 in biological samples is highly desirable. More recently, we reported a highly selective fluorescent probe (BMBT) for hCE1, which could be used to rapidly determine the activity of hCE1 by using microplate reader [29]. As shown in Fig. 1, upon addition of hCE1, BMBT could be rapidly converted to HMBT, leading to a remarkable change in fluorescence spectrum, which provides the basis for the sensitive detection of hCE1 activity. Although the selectivity of BMBT for measuring hCE1 is acceptable, the short fluorescence emission wavelength of HMBT strongly limits its widely applications on the precise measurement of hCE1 in complex biological samples directly by using microplate reader, due to the strong background fluorescence from endogenous compounds in biological matrix. Such limitation prompts us to develop a practical, highly sensitive and accurate method to monitor the real activity of hCE1 in complex biological samples, with the help of liquid chromatography.

In this study, a rapid and sensitive liquid chromatography-fluorescence detection (LC-FD) method was developed and fully validated to detect hCE1 activity in complex biological samples, by using BMBT hydrolysis as the probe reaction. With the help of LC separation, most polar endogenous compounds in biological samples could be eluted in the column dead time, which is very beneficial for accurate determination of hCE1 in complex biological samples. After method development and validation, the method was successfully applied to measure the real activities of hCE1 in complex biological samples including human cell lines and human liver microsomes.

2. Experimental

2.1. Reagents and standards

BMBT (2-(2-benzoyloxy-3-methoxyphenyl)benzothiazole) and HMBT (2-(2-hydroxy-3-methoxyphenyl)benzothiazole) were synthesized by the author (Liu et al.) as described previously [29]. The purities of both BMBT and HMBT were higher than 98%. Bis-*p*-nitrophenyl phosphate (BNPP) and loperamide (LPA) were purchased from TCI (Tokyo Japan). LC grade acetonitrile (CH_3CN), dimethylsulfoxide (DMSO), and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). HepG2, A549, THP-1, HT-29, Caco-2, K562, and MCF-7 cells were purchased from the American Type Culture Collection (Teddington, Middlesex, UK). CNE, HGC-27, HL-7702, and Tca-8113 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cell culture medium and fetal bovine serum used in this study were purchased from Hylcone (Logan, UK). Human liver microsomes (HLMs) from individual donors ($n = 12$) were purchased from the RILD Research Institute for Liver Diseases (Shanghai, China). Recombinant hCE1 was purchased from BD Biosciences (MA, USA). RNAiso Plus reagent, RNA PCR kit, and SYBR®*Premix Ex Taq*™ II Kit were purchased from Takara (Dalian, China).

2.2. Chromatographic conditions

Liquid chromatography separation was performed with a ultra-high performance liquid chromatography (UHPLC) spectrometry system equipped with a DGU-20A vacuum degasser, two LC-30AD pumps, a SIL-30AC autosampler, a RF-20A fluorescence detector, a CTO-30A column oven, and a chromatography workstation (SHIMADZU, Kyoto, Japan). The chromatographic separation was achieved using a Shim-pack XR-ODS column (75 mm × 2.0 mm, 2 μm, Shimadzu). The mobile phase consisted of CH_3CN (A) and 0.2% formic acid in water (B) with the following gradient: 0–3 min, 60–90% A; 3–4 min, 90% A; 4–5 min, 60% A. The system was operated at a flow rate of 0.4 mL/min. Column temperature was kept at 40 °C and the injection volume was 10 μL. The fluorescence detection for BMBT and HMBT was achieved with excitation wavelength at 304 nm, and the emission wavelength was set at 488 nm.

2.3. Assay validation

The method was validated according to the current recommendations for analytical method validation [30]. Specificity of the LC method was confirmed by analyzing cell homogenate and human liver microsomes and then compared with the standard samples containing HMBT and BMBT. Linearity was determined by replicate analysis of different concentrations of BMBT and HMBT. A linear regression was used to plot the fluorescence intensity (y) of analyte vs. analyte concentration (x). The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as the lowest concentrations of analyte that had a peak height ratio 3 and 10 times higher than the baseline noise, respectively. Precision was evaluated by analysis the calibration standards in 3 replicates for 1 day (intra-assay) and for 3 consecutive days (inter-assay). The overall precision of the method was expressed as % relative standard deviations (% RSD). The anti-interference ability of the probe reaction was determined by co-incubation hCE1 and probe substrate (BMBT) with each endogenous compound (such as common amino acids and metal ions), and then evaluated the change in hCE1 activity with or without endogenous compound. Sample stability was evaluated by analyzing the known amounts of HMBT in different reaction mixtures for 48 h. The concentrations of HMBT were determined using the standard calibration curve previously validated, and the results were plotted as measured concentrations versus initial concentration. Recovery was assessed by spiking known amounts of hCE1 at three different levels into the biological matrix (HLM). The results were presented as measured activities versus theoretical concentration and the recovery was calculated.

2.4. Real-time RT-PCR analysis of hCE1 expression

For real-time RT-PCR analysis, cells were cultured in 12-well plates and collected by RNAiso Plus reagent when they reached