Full-length papers

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

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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.

Keywords

Liquid chromatography-fluorescence detection (LC-FD); human carboxylesterase 1 (hCE1); BMBT hydrolysis; 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, oseltamivir, 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 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 (phospho-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 (L Feng) 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 (CH₃CN), 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 CH₃CN (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 hours. 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 90 % confluence. Then the total cellular RNA was extracted according to the following protocol. After reverse transcription synthesis of cDNA using RNA PCR kit, real-time quantitative PCR was conducted utilized SYBR[®] Premix Ex Taq[™] II Kit to detect transcript levels of hCE1 and GAPDH. The relative expression level for each target gene was normalized by the C_T value of the human housekeeping gene GAPDH ($2^{-\Delta C_T}$ formula). The data were analyzed by Applied Biosystems StepOne[™] Real-Time PCR System software version 2.0 (Applied Biosystems, Foster City, CA).

2.5. Preparation of cell homogenates

For preparation of cell homogenates, cells were cultured in 175 cm² flasks and collected when they reached 90% confluence. Then cells were harvested with trypsin, washed with ice-cold PBS, and pelleted by centrifugation. Ice-PBS was added to cell pellets and sonicated three times for 3 s (200 W) with at least 1 min on ice between two pulses. The cell lysate was centrifuged at 9000 g for 20 min and the supernatant was kept at -80°C until use.

2.6. Assay of hCE1 activity

The assay of hCE1 activity was measured by the developed LC-FD method, following the fluorimetric measurement of HMBT liberated enzymatically from the substrate BMBT. The incubation conditions and protein concentrations were used according to a previous study with slight modifications [29]. In brief, the incubation mixtures included a total volume of 200 µL PBS (100 mM, pH 7.4) with cell homogenates or human liver microsomes. After pre-incubation at 37°C for 5 min, the reaction was initiated by the addition of the stock solution of BMBT (10 µM, final concentration). After 20 min incubation, the reactions were terminated by adding 200 µL ice-cold acetonitrile (equal volume to incubation mixture) followed by centrifugation at 20,000×g for 20 min by using Allegra 64R centrifuge. The aliquots of supernatant were then taken for LC-FD analysis. To calculate the specific hCE1 activities of each sample, the concentration of HMBT (µM) was divided by the reaction time and the content of protein (mg/mL) added.

2.7. Chemical inhibition assays

In this study, chemical inhibition assays were performed by using selective chemical inhibitors as described previously [29]. The incubation mixture (200 µL total volume) consisted of PBS (100 mM, pH 7.4), enzyme sources (HLM, hCE1, or cell homogenate), inhibitor (BNPP or LPA, 100 µM) and BMBT (10 µM). Control incubations without BNPP or LPA were also performed. After incubation, the reaction was terminated by the addition of 200 µL of acetonitrile, and the resulting mixture was kept on ice. The mixture was centrifuged at 20,000 g for 20 min at 4°C. Aliquots of the supernatant were then taken for further LC-FD analyses.

The inhibition parameters were determined as our previous description [31]. Various concentrations of BNPP were used to determine the half inhibition concentration (IC_{50}). To evaluate the parameters of inhibition kinetics, various concentrations of BMBT (10 μ M, 20 μ M, 30 μ M) were added to reaction mixtures containing different concentrations of BNPP (0 μ M, 0.1 μ M, 0.2 μ M, 0.5 μ M). The second plot of slopes from Lineweaver-Burk plot vs. BNPP concentrations was utilized to calculate K_i value. All incubations throughout this study were conducted in triplicate.

3. Results and discussion

3.1. Analytical method development

To overcome the interference from biological matrix, the LC-FD based method was developed for simultaneous detection of BMBT and its corresponding fluorescent product HMBT within a very short time (5 min). With the help of LC separation on the reversed phase column, most polar endogenous compounds could be eluted in the column dead time, which strongly alleviated the interference from endogenous matrix of complex biological sample. The maximum fluorescence response for HMBT was found at 488 nm under the excitation of 304 nm, while the fluorescence detection was found much more sensitive than UV detection at the maximum absorption wavelength of HMBT. To provide the best overall separation and shorten analysis time, the LC elution method was optimized by testing different mobile phases including organic phase composition (methanol, acetonitrile) and the pH value of aqueous phase (formic acid concentration). Finally, adequate peak resolution and good peak pattern for BMBT and HMBT were observed with the following mobile phase composition, acetonitrile and 0.2% formic acid (Fig. 2, and Fig. S1). These results demonstrated that BMBT and its corresponding fluorescent product HMBT could be well separated with a short run time of 5 min.

3.2. Method validation

Specificity is the ability of assay method to determine the analyte response in the presence

of its potential impurities. The specificity of this LC-FD based method was evaluated by analyzing stock solutions of BMBT and HMBT, as well as blank PBS, blank cell homogenates and blank human liver microsomes. As shown in Fig S1, no interfering peaks from endogenous matrix were found at the retention times of both HMBT and BMBT. The LOD and LOQ for HMBT were determined as 0.005 μM and 0.02 μM , respectively (Table 1), which was quite lower than that of the microplate reader based assay for hCE1 detection. The calibration curve for HMBT demonstrated excellent linear correlation ($R^2=0.9996$) between the fluorescence intensity and concentration of HMBT in the concentration range of 0.02 – 10 μM (Fig. 3A). To evaluate the sensitivity of the mentioned method in the measurement of the real activity of hCE1, the linear fluorescence responses with enzyme concentration were investigated. With the increasing of hCE1 concentration, an excellent linear correlation between the fluorescence response of HMBT and hCE1 concentration in both PBS ($R^2=0.9993$) (Fig. 3B), HLM-containing ($R^2=0.9970$) and cell homogenate-containing incubation systems ($R^2=0.9917$) were obtained (Fig. S2). The LOQ for HMBT was 0.02 μM , which could be produced by 20 pg/L of hCE1 under optimized incubation conditions (37 °C for 20 min with 10 μM BMBT). Compared with the microplate reader based assay (BMBT as the probe substrate), the sensitivity of this newly developed LC-FD method for hCE1 detection was improved nearly 10-fold [29]. The linearity of this LC-FD based assay was also better than microplate reader based assay, which could be observed from the correlation coefficients of the corresponding calibration curves (0.9993 *VS* 0.9743, for LC-FD based assay and microplate reader based assay, respectively). Intra- and inter-assay precision for HMBT detection were summarized in Table 1. The acceptable precision results (lower than 2.5%) were obtained for HMBT over the range of concentrations tested. Notably, the newly developed LC-FD method showed good precision compared to microplate reader based assay, in which the precision expressed as RSD was about 10%. Stability of HMBT in reaction mixture was carried out by leaving samples at 4 °C for 48 h. As shown in Table 2, no apparent loss of HMBT within 48 h storage was observed. Recoveries of hCE1 in biological matrix were investigated by comparing the activities of hCE1 before and after the addition of the HLMs to the known concentrations of hCE1. To exclude the endogenous hCE1 activity already in HLMs, we tested the endogenous hCE1 activity in HLMs firstly. The enhanced

hCE1 activities were calculated by the activities of the spiked samples minus the endogenous hCE1 activity in HLMS. The recoveries results shown that the enhanced hCE1 activities were agreed well with the added concentrations of hCE1, and acceptable recoveries ($> 90\%$) were observed (Table 3). The effects of various endogenous molecules on hCE1 detection were also determined, common amino acids and metal ions showed no or slightly effects on the enzymatic activity measurement (Fig. S3). All these findings demonstrated that the newly developed LC-FD method is reliable, sensitive, and suitable for measuring the real activity of hCE1 in complex biological samples.

3.3. Measurement of hCE1 activities in human liver microsomes

The high sensitivity of the LC-FD based assay suggested that BMBT hydrolysis could be served as a marker reaction for accurate measurement of the hCE1 activities in complex tissue samples, with the help of LC separation. The proposed method was then applied to measure the hCE1 activities in human liver preparations from individual donors. To confirm the HMBT production were selectively mediated by hCE1, the inhibitory effects of 100 μM various selective esterase inhibitors BNPP (a potent inhibitor of hCEs) and LPA (a selective inhibitor of hCE2) on BMBT hydrolysis were investigated in pooled human liver microsomes (HLMS) [32]. The results showed that BNPP could strongly inhibit the formation of HMBT in HLMS while LPA displayed negligible inhibition toward the generation of HMBT (Fig. S4). After that, the catalytic activities of hCE1 towards BMBT hydrolysis in a panel of 12 human liver microsomes from different individuals were determined and depicted in Fig. 4A. These 12 individual human liver microsomes displayed varied enzymatic activities toward BMBT ranged from 227.66 to 1200.61 nmol/min/mg protein (Table S1). About 5-fold differences in hCE1 activities was observed, which agreed well with the previously reported variability in hCE1 protein levels in human liver tissues [17]. Furthermore, a correlation study was further performed to validate the specificity of this assay for measuring hCE1 activities in HLM samples. As shown in Fig. 4B, a strong correlation with a high correlation parameter ($R^2=0.9626$, $P<0.001$) was presented between BMBT hydrolysis and clopidogrel (a known specific substrate of hCE1) hydrolysis in these individual HLM samples [33]. These

findings obviously suggested that the developed method can be used for measuring the real activities of hCE1 in human tissue samples, and the quantification is highly reliable.

3.4. Measurement of hCE1 activities in human cell lines

With the purpose of exploring the applicability of the proposed method to cell samples, the LC-FD based method was further applied to measuring hCE1 activities in homogenates from kinds of human cell lines. Different cell cultures were collected and assayed for hCE1 activities and mRNA expression levels. As shown in Table 4, these cell lines displayed extremely significant differences in both mRNA expression ($2^{-\Delta C_T}$ value from 0 to 199.85×10^{-4}) and activities of hCE1 (from 0 to 352.34 nmol/min/mg protein). Among the tested cell lines, HepG-2 cells showed the highest hCE1 activity, which agreed well with the results from previous studies in which the expression of hCE1 was the highest in the liver and expressed to a lesser extent in the heart, lung, and colon [34, 35]. Specific organ-derived cell lines to a certain extent may represent the tissue expression pattern of hCE1. As shown in Fig. S5, BMBT hydrolytic activities in these cell lines positively and significantly ($R^2=0.9868$, $P < 0.001$) correlated with the relative abundance of hCE1 in gene expression detected by real-time PCR. To confirm the fluorescence response were selectively mediated by hCE1, the inhibitory effects of two selective chemical inhibitors (BNPP and LPA) on BMBT hydrolysis were also investigated in cell homogenates [32]. The results showed that BNPP could strongly inhibit the formation of HMBT in cell homogenates, while LPA have no inhibitory effect on the catalysis activity of hCE1 in the cells containing hCE1 (Fig. S6). On the basis of the results obtained above, the newly developed method could sensitively determine the real activity of hCE1 in cell preparations via measurement of the HMBT production.

3.5. Screening and characterization of hCE1 inhibitors

The LC-FD method was also provided a promising way for rapid screening and characterization of potential hCE1 inhibitors which might cause clinical DDI. To prove this potential use of the reported method, a known chemical inhibitor of hCE1 (BNPP) was used to evaluate its inhibitory effects toward hCE1 in both recombinant hCE1 and HLM. As

shown in Fig. 5A, the inhibitory tendency was similar between recombinant hCE1 and HLM and the IC₅₀ values of BNPP were closed in these two enzyme sources (32.2 nM, and 46.4 nM in hCE1 and HLM, respectively). These findings suggested that HLM could be used as the enzyme source instead of the recombinant hCE1 which is more expensive in such assays. Furthermore, inhibition kinetic analysis was also carried out for further investigation on the inhibition mechanism of BNPP against hCE1 in HLM. Both Lineweaver-Burk (Fig. 5B) and Dixon plots (Fig. S7A) demonstrated that the inhibition type of BNPP against hCE1 was best fit to a mixed model manner. The K_i value was then determined from the second plot of the slopes from Lineweaver-Burk plots vs the concentrations of BNPP. The results showed that K_i value of BNPP against hCE1 in HLM was 66 nM (Fig. S7B), which agreed well with previous reported results [36]. All findings presented here strongly confirmed the usefulness of this LC-FD method in determination and characterization of the potential inhibitory potentials of hCE1 inhibitors, implying that the newly developed method could be used for rapid screening and characterization of hCE1 inhibitors.

4. Conclusion

In summary, a LC-FD method was developed and fully validated for the precise measurement of hCE1 in complex biological samples. The LC-FD based method offers several advantages over previously reported methods, including good precision, high sensitivity, and avoiding interference by biological matrix. Furthermore, the newly developed method was successfully applied to measure the real activities of hCE1 in human liver tissue preparations and homogenates from different human cell lines. All these findings demonstrated that the LC-FD based method is practical, reliable and sensitive, **and** could serve as an efficient tool for the precise measurement of **hCE1 activities** complex biological samples. This method will be very helpful for further investigations on the molecular functions and biological roles of in hCE1 cellular processes and human diseases, for rapid screening and characterization of hCE1 modulators, as well as for the personalized medicine of esterified drugs.

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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.

Fig. 2. LC elution patterns of hCE1 activity determination in biological samples.

(A) Standard BMBT; (B) Standard incubation: hCE1 (0.1 $\mu\text{g/mL}$) was incubated with BMBT at 37°C for 20 min; (C) Standard incubation: cell homogenate was incubated with BMBT at 37°C for 20 min; (D) Standard incubation: liver microsome (0.2 $\mu\text{g/mL}$) was incubated with BMBT at 37°C for 20 min; (E) Standard HMBT.

Fig. 3. Standard curves for HMBT in PBS and acetonitrile (v/v 1:1) and plots of fluorescence intensity of BMBT response to hCE1.

(A) Standard curves for HMBT; (B) BMBT (10 μM) was incubated with different concentrations of hCE1 (0.02-6 $\mu\text{g/mL}$) in PBS or HLM at 37°C for 20 min. The supernatant of reaction mixtures were subjected to LC system to measure fluorescence intensity.

Fig. 4. Determination activities of hCE1 in human liver microsomes

(A) The catalytic activities of hCE1 in human liver microsomes determined by BMBT; (B) Correlation analysis between the hydrolytic rates of BMBT and clopidogrel in human liver microsomes (n=12).

Fig. 5. The inhibitory effects of BNPP towards hCE1

(A) Determination the inhibitory effects of BNPP towards hCE1 with HLM and recombinant expression hCE1. Various concentrations of BNPP were used to measure the half inhibition concentration (IC_{50}); (B) Lineweaver-Burk plots of the effects of BNPP on BMBT hydrolysis.

Table 1.

Intra- and inter- day precision of the method for quantitative determination of HMBT.

Theoretical concentrations (μM)	Intra-day (n=3)		Inter-day (n = 3)		Intra-day (n=3)		Intra-day (n = 3)	
	Measured concentration (μM)	% RSD	Measured concentration (μM)	% RSD	Retention time (min)	% RSD	Retention time (min)	% RSD
0.1 (Low)	0.11	0.66	0.11	0.47	2.28	1.28	2.27	1.13
1 (Middle)	1.08	0.58	1.07	0.86	2.27	0.09	2.27	0.12
2 (High)	1.85	2.31	1.83	2.34	2.27	0.15	2.27	0.24

Table 2.

Stability of HMBT in reaction mixture.

Analyte	Matrix	Conditions	Initial concentration (μM)	Measured concentration (μM)	Recovered (%)	Dev (%)
HMBT	hCE1 reaction mixture	4°C, 24 h	0.23	0.24	101.42	1.42
	hCE1 reaction mixture	4°C, 24 h	0.43	0.43	100.25	0.25
	hCE1 reaction mixture	4°C, 48 h	0.17	0.17	97.88	-2.12
	hCE1 reaction mixture	4°C, 48 h	0.50	0.50	100.77	0.77
	HLM reaction mixture	4°C, 24 h	0.23	0.24	102.49	2.49
	HLM reaction mixture	4°C, 24 h	0.43	0.43	99.83	-0.17
	HLM reaction mixture	4°C, 48 h	0.17	0.170	96.81	-3.19
	HLM reaction mixture	4°C, 48 h	0.50	0.50	100.53	0.53

Table 3.

Recoveries of the hCE1 in tissue homogenate (human liver microsome).

Added hCE1 ($\mu\text{g/ml}$)	Matrix	Theoretical activity (nM/min/mg protein)	Measured activity (nM/min/mg protein)	Recovered (%)	Dev (%)
0.1	Human liver microsome	127.22 $\pm$ 8.85	118.67 $\pm$ 0.78	93.28	-7.72
0.5	Human liver microsome	585.28 $\pm$ 10.74	530.99 $\pm$ 5.05	90.72	-9.28
1	Human liver microsome	1077.46 $\pm$ 20.70	1022.32 $\pm$ 25.74	94.88	-5.12

Table 4.

Determination of hCE1 activities and mRNA levels in human tumor cell lines

Cell lines	mRNA ($2^{-\Delta C_T} \times 10^{-4}$)	Activity (nM/min/mg protein)
HepG-2	199.85±8.34	352.34±4.96
A549	11.43±0.33	29.05±1.82
MCF-7	4.45±0.39	21.37±0.70
Caco-2	48.02±3.46	68.74±3.12
HT-29	0.29±0.07	5.17±0.20
THP-1	20.40±1.63	66.77±2.60
CNE	N. D. ^a	N. D. ^b
K562	N. D. ^a	N. D. ^b
HGC-27	N. D. ^a	N. D. ^b
Tca-8113	0.12±0.02	N. D. ^b
HL-7702	N. D. ^a	N. D. ^b

N. D.^a, C_T value ≥35 or undetermined after 40 cycles of PCR reaction.N. D.^b, The production of HMBT was lower than the LOQ.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5