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Simultaneous Absolute Protein Quantification of Carboxylesterases 1 and 2 in Human Liver Tissue Fractions using Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT:

The aims of this study were to develop a robust method for simultaneous quantification of carboxylesterases (CESs) 1 and 2 and to quantify those absolute protein levels in human liver tissue fractions. Unique peptide fragments of CES1 and CES2 in tryptically digested human liver microsomes (HLMs) and cytosol (HLC) were simultaneously quantified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using corresponding stable isotope-labeled peptides as internal standards. Bovine serum albumin was used as a blank matrix for the calibration curve samples. Our procedure showed good digestion efficiency, sensitivity, linearity of calibration curve, and reproducibility. The protein levels of CES1 and CES2 in 16 individual HLMs varied 4.7-fold (171–801 pmol/mg) and 3.5-fold (16.3–57.2 pmol/mg), respectively, that are approximately 10 times higher than the expression levels in HLC. The CES1/CES2 level ratio varied substantially from 3.0 to 25, and the correlation between the protein levels of CES1 and CES2 was negative, indicating significant interindividual variability and independence in their expression levels. CES1 levels significantly correlated with hydrolysis of the CES1 substrates, clopidogrel (5 μM) and oxybutynin (10 μM), whereas CES2 levels correlated strongly with hydrolysis of the CES2 substrate, irinotecan (1 μM), indicating that quantified protein levels are highly reliable. This is the first report to demonstrate the absolute protein levels of CESs quantified by LC-MS/MS.

Introduction

Carboxylesterases (CESs; EC 3.1.1.1) are members of the esterase superfamily that hydrolyzes various ester-bearing molecules (Imai, 2006; Hosokawa et al., 2007; Ross and Crow, 2007; Hosokawa, 2008). In humans, the majority of CESs belong to the CES1 and CES2 gene families. At present, two isoforms are available for both CES1 and CES2 in the Universal Protein Resource Knowledge Base (UniProtKB).

CESs are expressed in human liver with primary localization in the luminal side of the endoplasmic reticulum, and are also found in lower quantities in the cytosolic fraction. Quantitative immunoblot analysis of four different pooled human liver microsomes (HLMs) showed protein levels of CES1 and CES2 to be 1070 and 23.0 pmol/mg, respectively (Godin et al., 2007; Ross and Crow, 2007). However, the accuracy and precision of immunoblot analysis were not fully investigated in their reports. In addition, there seems to be a great amount of variability in the relative expression. Ross and colleagues (Ross et al., 2006; Ross and Crow, 2007) found that expression levels varied by factors of 1.3 and 2.3 for CES1 and CES2, respectively, in 11 individual HLMs, whereas Hosokawa et al. (1995) reported that the factor is actually closer to 10 for CES1 after studying 12 individual HLMs, and Xu et al. (2002) found the factor to be closer to 3 for CES2 after examining 13 individual HLMs. These differences might be due to the origin of microsome samples or the accuracy of the immunoblot-based quantification, because factors such as sample loading, blotting, and detection may affect the results.

Therefore, an alternative method that can quantify protein levels more accurately has been sought. For example, in liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS), tryptic digests of biological tissue fractions containing the desired protein are analyzed. Quantification is performed by measuring a unique tryptic peptide fragment whose sequence is specific for the protein. A peptide of the same amino acid sequence labeled with stable isotopes (^{13}C and ^{15}N) is used as an internal standard (IS). A major advantage of LC-MS/MS-based quantification is the large quantification range (>100-fold) compared with immunoblot analysis (approximately 10-fold). In addition, more samples can be measured in the same batch than with immunoblot analysis. Moreover, LC-MS/MS is capable of analyzing several proteins simultaneously.

Since 2005, MS-based protein quantification has been applied to several absorption, distribution, metabolism, and excretion-related proteins including cytochromes P450 (Alterman et al., 2005; Langenfeld et al., 2009; Seibert et al., 2009; Kawakami et al., 2011; Sakamoto et al., 2011; Williamson et al., 2011; Ohtsuki et al., 2012; Schaefer et al., 2012), transporters (Kamiie et al., 2008; Li et al., 2008,

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ABBREVIATIONS: CES, carboxylesterase; UniProtKB, Universal Protein Resource Knowledge Base; LC, liquid chromatography; MS/MS, tandem mass spectrometry; IS, internal standard; MS, mass spectrometry; BSA, bovine serum albumin; MMTS, methyl methanethiolsulfonate; TCEP, tris(2-carboxyethyl) phosphine; HLM, human liver microsomes; HLC, human liver cytosol; MRM, multiple reaction monitoring; CV, coefficient of variation; LLOQ, lower limit of quantification; PAGE, polyacrylamide gel electrophoresis.

TABLE 1

MRM transitions of CES1- and CES2-specific peptides and corresponding AQUA peptides

The precursor and product products were doubly ($[M + 2H]^{2+}$) and singly ($[M + H]^{+}$) charged ions, respectively.

	Sequence	Precursor *m/z*	Product *m/z*
CES1	GNWGHLDQVAALR	719.0	885.0
CES2	EASQAALQK	473.3	530.1
AQUA-CES1	GNWGHLDQVAALR(^{13}C ^{15}N)	724.0	895.3
AQUA-CES2	EASQAALQK(^{13}C ^{15}N)	477.2	538.1

2009, 2010; Sakamoto et al., 2011; Zhang et al., 2011), and UDP-glucuronosyltransferases (Fallon et al., 2008; Sakamoto et al., 2011; Harbourt et al., 2012; Ohtsuki et al., 2012). To our knowledge, however, similar studies have not been done for CESs.

Here, we simultaneously quantified CES1 and CES2 in human liver tissue fractions by an original, highly sensitive, accurate, and robust method using LC-MS/MS. Correlations between protein levels and hydrolysis activities for their respective substrates (clopidogrel and oxybutynin for CES1 and irinotecan for CES2) were investigated to assess the reliability of quantified protein levels.

Materials and Methods

Chemicals and Reagents. Bovine serum albumin (BSA), urea, ammonium bicarbonate, and methyl methanethiolsulfonate (MMTS) were purchased from Wako Pure Chemicals (Osaka, Japan). Tris(2-carboxyethyl) phosphine (TCEP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Sequencing grade-modified trypsin was obtained from Promega (Madison, WI). Polyclonal IgGs for human CES1 and CES2 were obtained from Proteintech (Chicago, IL). Anti-rabbit IgG (peroxidase conjugate raised by goat), nonlabeled peptides (purity of ≥95%), and AQUA peptides (labeled with stable isotopes) were purchased from Sigma-Aldrich (St. Louis, MO). Purified human CES1 and CES2 were obtained from R&D Systems (Minneapolis, MN). Pooled and individual HLMs (Reaction Phenotyping Kit version 7) and human liver cytosol (HLC) were purchased from XenoTech, LLC (Lenexa, KS). All other chemicals and reagents used were commercially available and of guaranteed purity.

Identification of Tryptic Peptides of CESs. A 2.5-μl aliquot of purified CES1 or CES2 (0.5 mg/ml) was put into a 200-μl aliquot of 50 mM ammonium bicarbonate. TCEP (20 μl, 50 mM) was added and incubated at 37°C for 1 h to perform reduction and MMTS (10 μl, 200 mM, isopropanol solution) was added to alkylate the thiol residues. A 10-μl aliquot of trypsin in 50 mM acetic acid (1 mg/ml) was added and incubated at 37°C for 16 h. A 20-μl aliquot of supernatant was injected into an LC-MS/MS system consisting of a Prominence high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) and QTRAP5500 (Applied Biosystems/MDS Sciex, Foster City, CA). LC was performed using an Inertsil ODS-3 column (150 × 2.1 mm, 3.0-μm; GL Science, Tokyo, Japan) at a flow rate of 0.2 ml/min. The HPLC mobile phases were 0.1% formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B). The gradient program was 5 to 60% B for 35 min, 60% B for 40 min, and 5% B for 40 to 50 min. MS/MS transition data obtained by the enhanced mass spectroscopy scanning were processed to identify peptide fragments based on the Paragon algorithm (Shilov et al., 2007) using ProteinPilot software (version 3.0; Applied Biosystems/MDS Sciex).

Preparation of Tryptic Digests of BSA, HLMs, and HLC. A 2-μl aliquot of BSA (20 mg/ml) or HLMs (20 mg/ml) was put into a 14-μl aliquot of 50 mM ammonium bicarbonate. A 4-μl aliquot of HLC (10 mg/ml) was put into a 12-μl aliquot of 50 mM ammonium bicarbonate. Protein concentrations of HLMs and HLC were provided by the supplier. Urea (2 μl, 10 M) and TCEP (2 μl, 50 mM) were added and incubated at 37°C for 1 h to perform reduction along with denaturation. MMTS (2 μl, 200 mM, isopropanol solution) was added to alkylate the thiol residues. A 5-μl aliquot of trypsin in 50 mM acetic acid (1 mg/ml) was added and incubated at 37°C for 16 h, as the concentration of analyte peptides reached a plateau after a 16-h incubation.

SDS-PAGE and Western Blotting. An aliquot of the digested human liver tissue fractions described above was loaded and subjected to SDS-PAGE using e-PAGEL 5 to 20% gradient gel for Coomassie Blue staining and 7% gel for Western blotting (ATTO, Tokyo, Japan). For Western blot analysis, samples without trypsin digestion were sequentially diluted (2, 5, and 10 times) and simultaneously separated by SDS-PAGE. Proteins separated by 7% gels were transferred onto polyvinylidene difluoride membranes using an iBlot Gel Transfer System (Invitrogen, Carlsbad, CA). Subsequently, membranes were incubated with polyclonal antibodies against CES1 or CES2 diluted at 1:1000. Blots were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted at 1:20,000 and 1:10,000 for CES1 and CES2, respectively. The proteins were visualized using an ECL Advance Western Blotting Detection Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Quantification of CES Proteins in Human Liver Tissue Fractions. Tryptic reactions were terminated by the addition of a 50-μl aliquot of 2% formic acid. For calibration standards, a 4-μl aliquot of reference standard solution containing CES1- and CES2-specific peptides was added to the digested BSA. To the digested HLMs and HLC, a 4-μl aliquot of 0.1% formic acid in 50% acetonitrile (vehicle of reference standard solution) was added. A 4-μl aliquot of AQUA peptides solution (IS solution) was added to all samples and mixed well. After centrifugation, a 5-μl aliquot of supernatant was injected into the LC-MS/MS system mentioned above. LC was performed using a Synergi Fusion-RP 100A column (50 × 2.00 mm, 2.5-μm; Phenomenex,

A

MWLRAFILATLSASAAWGHPSSPPVVDTVHGKVLGKFVSLEGFAQPVAIFLGIPFAKPPL
GPLRFTPPQPAEPWSFVKNATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYL
NIYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAAHENVVVVTIQYRLGIWGFF
STGDEHSR**GNWGHLDQVAALR**WVQDNIASFGGNPGSVTIFGESAGGESVSVLVLSPL
AKNLFHRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEEELL
ETTLKMKFLSLDLQGDPRESQPLLGTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQE
FGWLIPMQLMSYPLSEGQLDQKTAMSLLWKSYPLVCIAKELIPEATEKYLGGTDDTVKK
KDLFLDLIADVMFGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDEL
FSVFGAPFLKEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQI
GANTQAAQKLKDKEVAFWTNLFAKKAVEKPPQTEHIEL

B

MRLHRLRARLSAVACGLLLLLVRGQGQDSASPIRTTHTGQVLGSLVHVKGANAGVQTF
LGIPFAKPPLGPLRFAPPEPPESWSGVRDGTTHPAMCLQDLTAVESEFLSQFNMTFPS
DSMSEDCLYLSIYTPAHSHEGSNLPVMVWIHGGALVFGMASLYDGSMLAALENVVVVII
QYRLGVLGFFSTGDKHATGNWGYLDQVAALRWVQQNIAHFGGNPDRVTIFGESAGGT
SVSSLVVSPISQGLFHGAIMESGVALLPGLIASSADVISTVVANLSACDQVDSEALVGCL
RGKSKEEILAINKPFKMIPGVVDGVFLPRHPQELLASADFQPVPSIVGVNNNEFGWLIPK
VMRIYDTQKEMDR**EASQAALQK**MLTLLMLPPTFGDLLREEYIGDNGDPQTLQAQFQEM
MADSMFVIPALQVAHFQCSRAPVYFYEFQHQPSWLKNIRPPHMKADHGDELPFVFRSF
FGGNYIKFTEEEEQLSRKMMKYWANFARNGNPNGEGLPHWPLFDQEEQYLQLNLQPA
VGRALKAHRLQFWKKALPQKIQELEEPEERHTEL

FIG. 1. Complete amino acid sequence of CES1 (A) and CES2 (B). Specific peptide sequences identified and used in the present study are in bold and underlined.

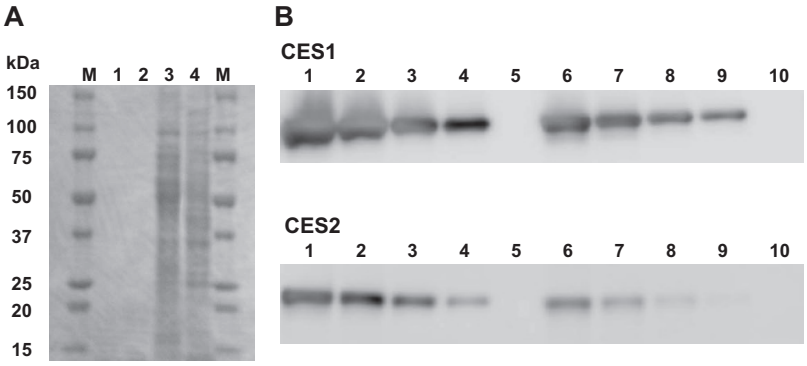

FIG. 2. Digestion efficiency of HLMs and HLC. A, Coomassie Blue staining of SDS-PAGE. Loaded samples were molecular weight markers (lane M), digested HLMs (lane 1), digested HLC (lane 2), undigested HLMs (lane 3), and undigested HLC (lane 4). B, Western blotting analysis of CES1 (top) and CES2 (bottom). Loaded samples were undigested HLMs without dilution (lane 1), undigested HLMs diluted 2 times (lane 2), 5 times (lane 3), and 10 times (lane 4), digested HLMs (lane 5), undigested HLC without dilution (lane 6), undigested HLC diluted 2 times (lane 7), 5 times (lane 8), and 10 times (lane 9), and digested HLC (lane 10).

Torrance, CA) at a flow rate of 0.4 ml/min. The HPLC mobile phases were solutions A and B (see above) with a gradient program of 5 to 30% B for 3 min, 30 to 100% B for 3.01 min, 100 to 100% B for 3.5 min, 100 to 5% B for 3.51 min, and 5% B for 6 min. The sample rack and column temperatures were maintained at 10 and 45°C, respectively. Quantification was performed in positive MRM mode (Table 1). Data were processed using the Analyst 1.5.1 software package (Applied Biosystems/MDS Sciex). Measured peptide concentrations were converted to protein levels (picomoles per milligram of protein).

Validation of Quantification. The linearity of the calibration curve was investigated in three different experiments. Relative errors (percentage) of the concentrations (back-calculated values) were obtained by applying the peak area ratio (analyte peak/IS peak) to each nominal concentration, and the correlation coefficients (r) were determined. Reproducibility of our method from the tryptic digestion step to quantification step was investigated using pooled human liver tissue fractions. Pooled HLMs and HLC were digested in six replicates and concentrations of CES1- and CES2-unique peptides were quantified by calibration curves in three different batches. Mean, S.D., and the coefficient of variation (CV) for each batch (intraday reproducibility) as well as for overall experiments (interday reproducibility) were determined.

Correlation Analysis. The protein levels of CES1 and CES2 were quantified in 16 individual HLMs. Linear regression analysis was performed using GraphPad Prism software (version 5.03; GraphPad Software Inc., San Diego, CA). Correlations between the protein levels of CES1 and CES2 and between protein levels and the respective hydrolysis activities were investigated. The

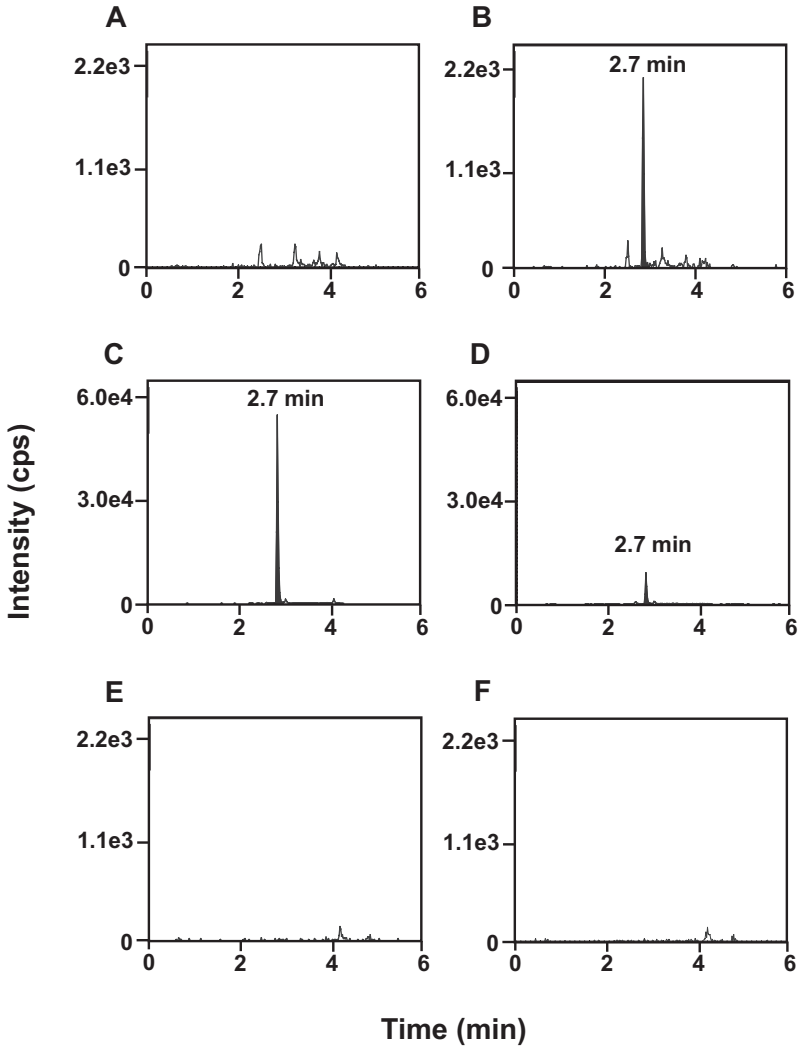

FIG. 3. Typical chromatograms of CES1-specific peptide. A, digested BSA. B, digested BSA spiked with the reference standard solution (LLOQ). C, digested HLMs. D, digested HLC. E, undigested HLMs. F, undigested HLC. Retention time of the analyte was 2.7 min.

hydrolysis rates of clopidogrel, oxybutynin, and irinotecan obtained in our previous report were used (Sato et al., 2012).

Results

Identification of Tryptic Peptides of CESs. Based on the Paragon algorithm, peptide fragments GNWGHLDQVAALR and EASQAALQK were identified for CES1 and CES2, respectively, with ≥99% confidence. These sequences are derived from Gly187 to Arg199 and Glu366 to Lys374, respectively (Fig. 1).

Digestion Efficiency in Human Liver Tissue Fractions. The results of the Coomassie Blue staining indicate that whole proteins were digested by trypsin in both HLMs and HLC (Fig. 2A). In addition, the objective bands of CES1 and CES2 in the Western blotting analysis were detectable in the lanes of undigested HLMs and HLC even after dilution by a factor of 10, whereas no bands were detected in the lanes of digested HLMs and HLC (Fig. 2B).

Development and Validation of a Simultaneous Quantification Method for CESs in Human Liver Tissue Fractions. A method to simultaneously quantify the protein levels of CES1 and CES2 in HLMs and HLC was developed using unique peptides and corresponding AQUA peptides as reference standards and IS, respectively (Table 1). A high-throughput gradient LC program with a run-time of 6 min was established by adopting a polar-embedded C18 analytical column with small particles (2.5 μm) to achieve a high signal/noise ratio and good retention of peptides. After preliminary experiments, the lower limit of quantification (LLOQ) of the calibration curves was set to 500 and 50 ng/ml for CES1- and CES2-specific peptides, respectively, when 40 μg of microsomal or cytosolic protein was used, which translates into LLOQs of 17.4 and 2.65 pmol/mg protein, respectively. Typical chromatograms of CES1 and CES2 are shown in Figs. 3 and 4, respectively. No interference peaks were observed in the chromatograms of the digested BSA samples for either analyte (Figs. 3A and 4A) on the basis of the chromatograms of the LLOQs (Figs. 3B and 4B). Both analyte peaks were confirmed in the retention times of the reference standards in the chromatograms of digested HLMs (Figs. 3C and 4C) and HLC (Figs. 3D and 4D), whereas no analyte peaks were observed in the chromatograms of undigested HLMs (Figs. 3E and 4E) or HLC (Figs. 3F and 4F). Typical calibration curves are shown in Fig. 5. The quantification range was set at 200-fold for both analytes. The relative errors (percentage) of calibration curve samples were ≤6.0% for both analytes (Table 2). Reproducibility of the process from tryptic digestion to quantification was investigated by repeating tryptic digestion of pooled HLMs and HLC in six replicates followed by LC-MS/MS analysis three times (Table 3). In HLMs, the overall averages of quantified concentrations of CES1- and CES2-specific peptides were 10,400 and 420 ng/ml (equivalent to 363 and 22.2 pmol/mg), with CV values of 4.4 and 7.8%, respectively. In HLC, these were 1570 and 52.1 ng/ml (equiv-

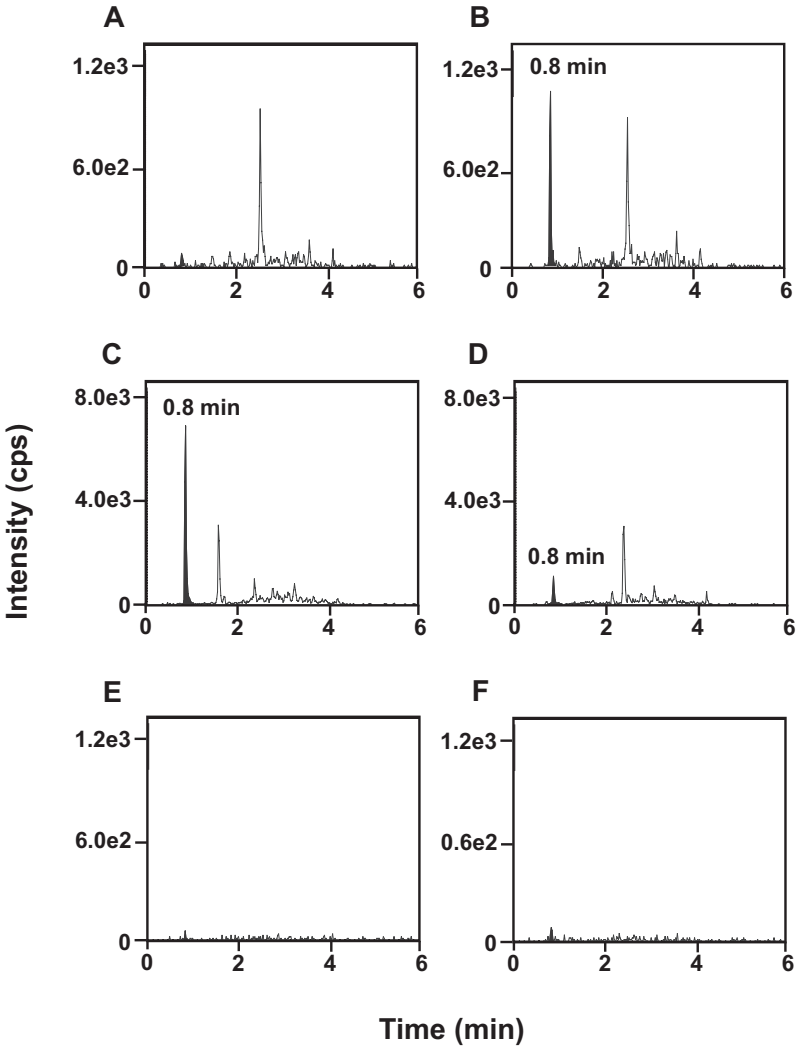

FIG. 4. Typical chromatograms of CES2-specific peptide. A, digested BSA. B, digested BSA spiked with the reference standard solution (LLOQ). C, digested HLMs. D, digested HLC. E, undigested HLMs. F, undigested HLC. Retention time of the analyte was 0.8 min.

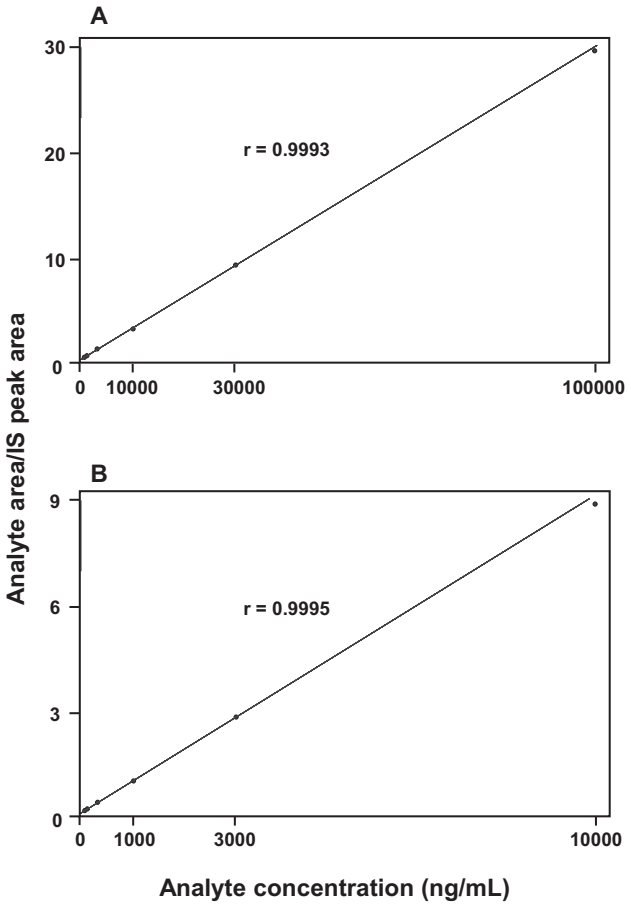

FIG. 5. Typical calibration curves of CES1-specific peptide (A) and CES2-specific peptide (B). The weighting factor was $1/x^2$ for both analytes.

alent to 54.5 and 2.76 pmol/mg), with CV values of 5.6 and 18%, respectively. The concentrations of CES2-specific peptide in 7 of 18 replicates of digested HLC were slightly below the LLOQ and calculated by extrapolation.

Quantification of CES1 and CES2 in Individual HLMs. Protein levels of CES1 and CES2 were quantified in 16 individual HLMs (Table 4). The protein levels of CES1 ranged from 171 to 801 pmol/mg with an average of 402 pmol/mg. The protein levels of CES2 ranged from 16.3 to 57.2 pmol/mg with an average of 29.8 pmol/mg. Interindividual variability in the protein levels of CES1 and CES2 was 4.7- and 3.5-fold, respectively. The CES1/CES2 level ratio substantially varied from 3.0 to 25, and the correlation between the protein levels of CES1 and CES2 was negative (Fig. 6).

Correlation between Protein Levels and Hydrolysis Activities. The protein levels of CES1 correlated well with the hydrolysis rates of clopidogrel and oxybutynin [$r^2 = 0.6810$ and $p < 0.0001$ for clopidogrel (Fig. 7A); $r^2 = 0.6285$ and $p < 0.0002$ for oxybutynin (Fig. 7B)]. The protein levels of CES2 correlated strongly with the hydrolysis rates of irinotecan (1 μM) [$r^2 = 0.8540$ and $p < 0.0001$ (Fig. 7C)]. The correlation between the protein levels of CES2 and the hydrolysis rates of irinotecan at 100 μM was found to be far less than those observed at 1 μM [$r^2 = 0.3779$ and $p = 0.0113$ (Fig. 7D)].

Discussion

To quantify protein levels in tryptic digests using LC-MS/MS, it is essential to identify a unique peptide fragment of the target protein. One approach is in silico, especially when no reference protein sample (e.g., purified protein or recombinant protein) is available. However, because purified proteins of CES1 and CES2 are commercially available, we attempted to identify tryptic peptide fragments experimentally and identified GNWGHLDQVAALR (for CES1) and EASQAALQK (for CES2) with ≥99% confidence.

Two isoforms of CES1 (isoforms 1 and 2) are available in the UniProtKB with isoform 1 being the canonical sequence. The sequences of these isoforms are almost identical, differing in only one amino acid residue (Ala18). The sequence GNWGHLDQVAALR is common to both CES1 isoforms; however, because the function of these two isoforms was expected to be identical, the above sequence was assumed to be a specific fragment representing the function of CES1. For CES2, two isoforms (isoforms 1 and 2) are also available in the UniProtKB with isoform 1 being the canonical sequence. The sequence EASQAALQK is common to both CES2 isoforms; however, because the mRNA level of isoform 2 in the total CES2 transcript was very low (Schiel et al., 2007), the above sequence was assumed to be a specific fragment representing the function of CES2. CESs are known as glycoproteins (Kroetz et al., 1993) and therefore may complicate MS-based protein quantification because of post-transcriptional modifications such as glycosylation. However, this concern does not apply to our study, because the above sequences do not possess any glycosylation sites. Moreover, there are no cysteine residues that may also compromise the MS analysis. Thus, the peptide fragments identified are considered feasible reference standards for the MS-based protein quantification of CES1 and CES2.

Reliability of quantification method was assessed by tryptic digestion efficiency, chromatograms, linearity of the calibration curve, and reproducibility. The results of Coomassie Blue staining and Western blotting analysis indicate that most CES1 and CES2 in HLMs and HLC were digested by trypsin (Fig. 2). The chromatograms of the digested BSA samples and LLOQs indicate good selectivity for MRM analysis (Figs. 3, A and B and 4, A and B). In addition, the chromatograms of the digested and undigested human liver tissue fractions indicate that the peaks observed in the digested fractions were gen-

TABLE 2

Relative errors of calibration curve samples for CES1- and CES2-specific peptides

CES1				CES2			
Nominal Peptide Concentration	Relative Error			Nominal Peptide Concentration	Relative Error		
	%			*ng/ml*	%		
	Batch 1	Batch 2	Batch 3		Batch 1	Batch 2	Batch 3
500 ng/ml	0.0	0.0	1.0	50	3.0	−0.3	−0.2
1000 ng/ml	0.0	0.0	−2.3	100	−4.1	1.0	−0.1
3000 ng/ml	−1.1	−2.8	−1.2	300	−5.9	−2.3	1.0
10,000 ng/ml	1.0	5.0	1.0	1000	−1.7	−1.8	4.0
30,000 ng/ml	1.0	−2.3	2.0	3000	3.0	0.0	−4.1
100,000 ng/ml	−0.7	−0.4	−0.1	10,000	6.0	3.0	−0.5

TABLE 3

Mean, S.D., and CV values of CES1- and CES2-specific peptide concentrations in digested pooled HLMs and HLC

Pooled HLMs and HLC were digested in six replicates, and concentrations of CES1- and CES2-specific peptides were measured. Experiments were repeated three times. Mean, S.D., and CV values were calculated from six replicates in each batch. Overall values were calculated from 18 replicates.

	CES1			CES2		
	Mean	S.D.	CV	Mean	S.D.	CV
	ng/ml		*%*	*ng/ml*		*%*
HLM						
Batch 1	10,000	311	3.1	397	10.8	2.7
Batch 2	10,700	310	2.9	446	19.9	4.5
Batch 3	10,500	503	4.8	417	40.9	9.8
Overall	10,400	461	4.4	420	32.7	7.8
HLC						
Batch 1	1510	81.2	5.4	48.7	11.1	23
Batch 2	1560	49.3	3.2	48.5	6.69	14
Batch 3	1640	79.4	4.8	59.3	6.17	10
Overall	1570	87.8	5.6	52.1	9.37	18

erated by tryptic digestion (Figs. 3, C–F, and 4, C–F). On the basis of the *r* values and relative errors of calibration curve samples, we concluded that the linearity of the curves was good for both analytes (Fig. 5; Table 2). The CV values of the quantified peptide concentrations in digested human liver tissue fractions indicate that quantification is reproducible, although the CV values for CES2 in HLC were relatively high because concentrations of CES2-specific peptide in digested HLC were as low as the LLOQ (Table 3). These results indicate that this method for the quantification of CESs in human liver tissue fractions was robust.

In pooled HLMs, the CES1 level (363 pmol/mg) was low compared with the previously reported value measured by immunoblot (1070 pmol/mg), whereas the CES2 level (22.2 pmol/mg) was comparable to the previously reported value (23.0 pmol/mg). Although the precise reason for this discrepancy remains unknown, it might be due to a difference in reference standards. In the immunoblot analysis, recombinant human CES proteins purified from baculovirus expression systems were used as reference standards for calibration curves (Godin et al., 2007). Some nonspecific bands were observed in their immunoblots of purified CES1, which might indicate impurity in the standard protein of CES1, whereas no apparent nonspecific band was shown in their immunoblots of purified CES2. In LC-MS/MS-based quantification, however, highly purified reference peptides (purity of ≥95%) were used as standards, which were able to provide highly reliable quantified values. Although the CES1 level quantified in the present study was lower than the reported value, the protein level of CES1 is considered much higher than that of CES2 in HLMs, as presented in the UniProtKB. In pooled HLC, the protein level of CES1 (54.5 pmol/mg) was markedly higher than that of CES2 (2.76 pmol/mg), and protein levels of CESs in HLCs were almost 10 times lower than those in HLMs. These results indicate that CESs are primarily expressed in the microsomal fraction, as presented in the UniProtKB.

TABLE 4

Protein levels of CES1 and CES2 in 16 individual HLMs

Sample Name	CES1 Protein Levels[a]	CES2 Protein Levels[a]	CES1/CES2 Ratio[b]
	pmol/mg protein		
1	801	31.7	25
2	467	22.5	21
3	463	29.9	15
4	435	37.4	12
5	425	18.5	23
6	418	20.9	20
7	359	30.9	12
8	294	16.3	18
9	197	32.3	6.1
10	348	30.5	11
11	407	29.4	14
12	439	33.0	13
13	171	57.2	3.0
14	352	17.6	20
15	383	22.6	17
16	467	46.0	10
Average	402	29.8	15
S.D.	139	10.8	

[a] Peptide concentrations were converted to picomoles of CES protein per milligram of microsomal protein.

[b] Calculated by dividing CES1 levels by CES2 levels.

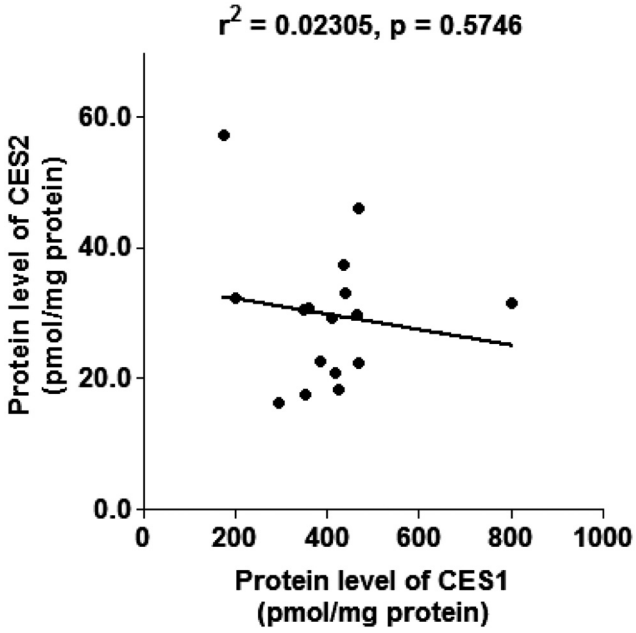

FIG. 6. Correlation between protein levels of CES1 and CES2.

Protein levels of CESs in individual samples were quantified only in HLMs, because CES levels in HLMs were confirmed to be markedly higher than those in HLC and individual HLC was not commercially available. In individual HLMs, the protein levels of CES1 and CES2 were apparently variable (4.7- and 3.5-fold, respectively), as reported in previous studies (Hosokawa et al., 1995; Xu et al., 2002). To our knowledge, however, no study has investigated the variability in relative expression levels of CESs in the same individual HLMs. In the present study, it was demonstrated that the CES1/CES2 level ratio varied substantially from 3.0 to 25 (Table 4), and the correlation between the protein levels of CES1 and CES2 was negative (Fig. 6), indicating significant interindividual variability and independence in their expression levels.

To further confirm the reliability of quantified protein levels, the correlations between the protein levels of CESs and hydrolysis activities for these substrates were investigated. Hydrolysis rates of CES1 substrates (clopidogrel and oxybutynin) and CES2 substrate (irinotecan) were determined in the same individual HLMs used in the present study (Sato et al., 2012). The protein levels of CES1 correlated well with the hydrolysis rates of clopidogrel and oxybutynin, indicating that the quantified CES1 levels are reliable. Although three samples showed relatively high deviations from the regression curve (arrows in Fig. 7, A and B), this might have been due to genetic variants of CES1, which are known to potentially alter its hydrolysis activity and could not be distinguished by our quantification method

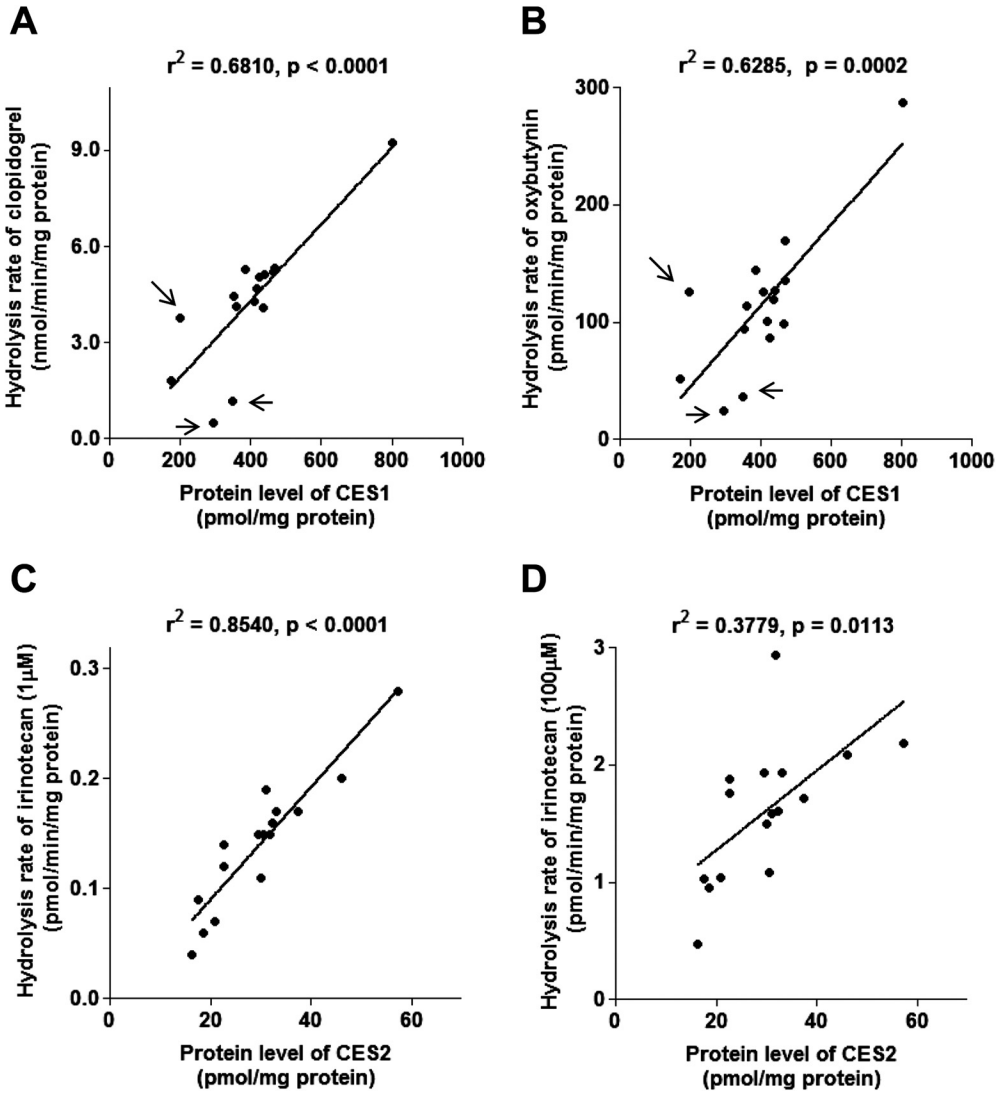

FIG. 7. Correlation between protein levels of CESs and hydrolysis activities. Linear regression analysis of protein levels of CES1 versus clopidogrel hydrolysis at 5 μM (A), protein levels of CES1 versus oxybutynin hydrolysis at 10 μM (B), protein levels of CES2 versus irinotecan hydrolysis at 1 μM (C), and protein levels of CES2 versus irinotecan hydrolysis at 100 μM (D) is shown. The hydrolysis rates used for clopidogrel (5 μM), oxybutynin (10 μM), and irinotecan (1 and 100 μM) are from our previous report. Three samples that showed relatively high deviation from the regression curve are indicated by the arrows in A and B.

(Sanghani et al., 2009). The protein levels of CES2 correlated strongly with the hydrolysis rates of irinotecan (1 μM) (Fig. 7C). It is known that both CES1 and CES2 are involved in the hydrolysis of irinotecan, but the hydrolysis at 1 μM is overwhelmingly catalyzed by CES2 in HLMs (Slatter et al., 1997). Thus, the strong correlation presented in Fig. 7C indicates the high reliability of quantified CES2 levels. At 100 μM, a concentration at which the contribution by CES1 is higher than that by CES2 (Slatter et al., 1997), the correlation between the protein levels of CES2 and the hydrolysis rates of irinotecan were far less than those observed at 1 μM (Fig. 7D).

In conclusion, we developed a highly sensitive, accurate, and robust method for the simultaneous quantification of CES1 and CES2 in human liver tissue fractions by combining tryptic digestion and LC-MS/MS analysis. The protein levels of CES1 and CES2 in HLMs were almost 10-fold higher than those in HLC, indicating that CESs are basically microsomal proteins. Furthermore, the protein levels of CES1 and CES2 in individual HLMs were found to be variable and independent of each other along with the much higher expression of CES1. Reliability of quantified protein levels was confirmed by significant correlations between the quantified protein levels and hydrolysis activities for marker substrates. This is the first report to demonstrate the absolute protein levels of CESs quantified by LC-MS/MS.

Authorship Contributions

Participated in research design: Sato.

Conducted experiments: Sato.

Contributed new reagents or analytic tools: Sato.

Performed data analysis: Sato.

Wrote or contributed to the writing of the manuscript: Sato, Miyashita, Iwatsubo, and Usui.

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