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Conventional liquid chromatography/triple quadrupole mass spectrometer-based metabolite identification and semi-quantitative estimation approach in the investigation of dabigatran etexilate *in vitro* metabolism

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Abstract

Dabigatran etexilate (DABE) is an oral prodrug that is rapidly converted by esterases to dabigatran (DAB), a direct inhibitor of thrombin. To elucidate the esterase-mediated metabolic pathway of DABE, a high-performance liquid chromatography/mass spectrometer (LC-MS/MS)-based metabolite identification and semi-quantitative estimation approach was developed. To overcome the poor full-scan sensitivity of conventional triple quadrupole mass spectrometry, precursor-product ion pairs were predicted, to search for the potential *in vitro* metabolites. The detected metabolites were confirmed by the product ion scan. A dilution method was introduced to evaluate the matrix effects of tentatively identified metabolites without chemical standards. Quantitative information on detected metabolites was obtained using ‘metabolite standards’ generated from incubation samples that contain a high concentration of metabolite in combination with a correction factor for mass spectrometry response. Two *in vitro* metabolites of DABE (M1 and M2) were identified, and quantified by the semi-quantitative estimation approach. It is noteworthy that CES1 convert DABE to M1 while CES2 mediates the conversion of DABE to M2. M1 (or M2) was further metabolized to DAB by CES2 (or CES1). The approach presented here provides a solution to a bioanalytical need for fast identification and semi-quantitative estimation of CES metabolites in preclinical samples.

Keywords

dabigatran etexilate; liquid chromatography; mass spectrometer; carboxylesterase; metabolism

Introduction

Dabigatran etexilate (DABE) is the first new oral anticoagulant to become available for the prevention of stroke and systemic embolism in patients with atrial fibrillation in over 50 years [1]. The double prodrug DABE is rapidly converted to active metabolite dabigatran (DAB) by esterase-catalyzed hydrolysis after dosing in humans, and microsomal incubation studies suggest that human carboxylesterases (CES) are likely to play an important role in the formation of the active moiety [2]. In humans, two carboxylesterases, CES1 and CES2, are important esterases in drug metabolism. The human liver predominantly contains CES1 with smaller quantities of CES2, while the small intestine contains CES2 with almost no

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CES1 [3,4]. The substrate specificity differs between the two enzymes with CES1 preferentially hydrolyzing substrates with a small alcohol group and a large acyl group, whereas, CES2 prefers substrates with a large alcohol group and small acyl group [3].

Though hepatic microsomal hydrolysis of DABE has been shown to result in formation of the active moiety (DAB), both CES1 and CES2 are present in liver microsomes so the specific role of individual carboxylesterases (CES1 and CES2) in DABE's hydrolysis could not be elucidated. Identification of the key enzymes responsible for the conversion of DABE to its active metabolite (DAB) is essential for understanding the interindividual variability, efficacy, safety, and drug interaction potential of DABE. In order to elucidate the CES-mediated portion of the metabolic pathway of DABE, a high-performance liquid chromatography/triple quadrupole mass spectrometry (LC-MS/MS)-based metabolite identification and quantitative-estimation approach was required. Recently, an LC-MS/MS-based assay for quantification of dabigatran in human plasma was reported that is simple and sensitive [5]. However, this assay is not applicable to the metabolic study of dabigatran etexilate as only DAB was quantified.

Conventional triple quadrupole mass spectrometers (MS/MS) are the most popular analytical tools due to their excellent quantitative sensitivity, stability, and low cost. However, they are not suitable for drug metabolite profiling since their full scan sensitivity is rather poor. To overcome this problem, we used a set of predicted precursor-product ion pairs instead of full-scan mode to search for the potential metabolites. In addition, we developed an approach to estimate the concentration of metabolites detected in the *in vitro* metabolism samples without the use of chemical standards. This approach is mainly based on the use of biologically generated pseudo metabolite standards from test samples. Taking advantage of this LC-MS/MS-based metabolite identification and semi-quantitative estimation approach, the contributions of CES1b, CES1c, and CES2 to the *in vitro* metabolic pathway of DABE were determined.

Materials and methods

Materials

Dabigatran etexilate was purchased from TLC PharmaChem Inc. (Mississauga, ON, Canada). Dabigatran and dabigatran-d3 were the products of Toronto Research Chemicals Inc. (North York, ON, Canada). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). LC-MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water was prepared with an in-house Milli-Q Advantage A10 Ultrapure water purification system (Bedford, MA, USA). Human carboxylesterase (CES) 1b, CES1c and CES2 SupersomesTM were obtained from BD Gentest (San Jose, CA, USA). Blank rat plasma (heparin sodium) was purchased from Innovative Research (Novi, MI, USA).

LC-MS/MS quantitative analyses for dabigatran etexilate and dabigatran

An AB SCIEX 3000 triple quadrupole mass spectrometer (Toronto, Canada) interfaced via a turbo ion spray (ESI) source with a Shimadzu HPLC separation module (including LC-10ADvp pumps and SCL-10Avp System controller) (Norcross, GA, USA) was used for metabolite profiling and quantification to support the *in vitro* metabolism study of DABE.

The LC separation for both quantification and metabolite identification was achieved on a 3.5 μ m Agilent Eclipse Plus C18 column (50 mm $\times$ 2.1 mm I.D.; Santa Clara, CA) at ambient temperature. Mobile phases were methanol/water, 1:99 (v/v), containing 2.5 mM formic acid, for A and methanol/water, 99:1 (v/v), modified with the same electrolyte, for B. A pulse gradient chromatographic method was used, which we have employed successfully

for the sensitive analysis of other drugs [6]. In brief, B 5% from 0.00 to 0.50 min; B 100% from 0.51 to 2.00 min; B 5% from 2.01 to 4.50 min.

Ionization conditions were optimized to maximize generation of the singly protonated species and to produce the characteristic product ions for the test compounds. The precursor-product ion pairs used for multiple reaction monitoring (MRM) of DABE, DAB, and DAB-d3 were m/z 628.3→289.1, 472.2→289.1, and 475.3→292.2, respectively. The parameters of the ESI source were optimized under the real chromatographic condition. The LC eluent was introduced to the ESI source at a flow rate of 0.40 mL/min over the period of 1.1–2.2 min.

One internal standard, DAB-d3, was used for quantification of both DABE and DAB. Matrix-matched (CES supersomes or rat plasma) calibration curves were constructed for DABE and DAB using weighted (1/X) linear regressions of the analyte/IS peak area ratio (Y) against the corresponding nominal concentrations of the analytes (X, nM).

Assessment of mobile phase modifier, matrix effects and assay validation

The effect of the formic acid concentration (0, 0.5, 2.5, and 25 mM) of the mobile phase on the signal intensity of the tested compounds (200 nM) in CES supersomes and rat plasma matrices was studied. Either 100 μ L CES buffer or 40 μ L rat plasma buffer was deactivated by adding 100 μ L or 120 μ L acetonitrile before spiking the DABE and DAB solution (in duplicate). The MS/MS responses of DABE and DAB under different concentrations of formic acid were compared.

In order to identify whether the CES supersomes and rat plasma components generate matrix effects on ESI-based measurement of the analytes and the internal standards, a post-extraction spike method was used [7], which assessed the sample absolute matrix effects, to validate the developed bioanalytical assay before use. Only one source of CES supersomes and rat plasma was used in the evaluation of matrix effects because the application of the current assay was limited to the same source of matrices.

Assay validation was carried out according to the Food and Drug Administration (FDA) guidelines, by assessing linearity, low limit of quantification (LLOQ), specificity, selectivity, precision (RSD), accuracy, and stability of the samples. The quality control samples were prepared from an independent weighing of the chemical standards. Calibration curves were prepared in replicates ($n = 3$). Linearity was considered acceptable when the following three criteria were met: (a) the correlation coefficient was at least 0.99, (b) calibrators had accuracies between 85% and 115%, and (c) precision was <15%. For the lowest calibration point (also the LLOQ), the criteria for accuracy were set between 80% and 120%, and for precision was <20%. For the determination of LLOQ, the peak area in each blank sample (CES supersomes or rat plasma) was compared with spiked LLOQ samples. The analytes peak areas in blank samples cannot exceed 20% of the LLOQ peak areas, with a precision of <20%. The precision and accuracy of back-calculated LLOQ replicate samples must be <20% and 80%–120%, respectively.

Application to *in vitro* metabolism of dabigatran etexilate

To demonstrate its applicability, the newly developed analytical method was applied to the *in vitro* metabolism of DABE. The metabolic stability of DABE in incubations containing human CES1b, CES1c, CES2, and their mixture was tested at 37°C. CES1b (dominant form in human liver) and CES1c are two major CES1 isoforms in humans, and their activity towards probe substrate 4-nitrophenyl acetate are similar [8]. Assays were conducted in duplicate in 96-well cluster tubes with a total assay volume of 100 μ L in each well. The assay buffer was 0.1 M potassium phosphate, pH 7.4. Incubation times were 0, 5, 15, 30,

and 60 min. Supersomal protein and substrate concentrations in the incubation were 25 $\mu\text{g}/\text{mL}$ and 200 nM, respectively. The final acetonitrile concentration was not greater than 0.2% for all assays. Assays were initiated by adding the substrate/buffer mix (50 μL) to the enzyme/buffer mix (50 μL). Both the positive and negative controls were performed. Negative control was tested to assess the chemical stability of DABE and DAB in buffer at 37°C. The reaction was terminated by the addition of an equal volume (100 μL) of ice-cold acetonitrile containing 200 nM internal standard (DAB-d3). After centrifugation at 16,000 $\times g$ for 5 min, 10 μL of supernatant (loading concentration on to the column was 100 nM DABE for the samples without incubation) were injected into the LC-MS/MS.

For the metabolism of DABE in rat plasma (containing highly active CES), the rat plasma (10 μL) was diluted by potassium phosphate buffer (29 μL), and 1 μL of DABE solution (acetonitrile) was then added to this mixture (final concentration of DABE was 2000 nM). The reaction was terminated by the addition of a 3-fold volume (120 μL) of ice-cold acetonitrile containing internal standard.

Identification of potential metabolites

A targeted approach was used in the identification of potential metabolites. In brief, a set of precursor-product ion pairs were predicted based on the probable hydrolytic pathways of DABE. Then these predicted ion pairs were used in the MRM mode to search for all of the potential metabolites. Three different values of collision energy (39, 46, and 53 V) were tested for each ion pair. These three values of collision energy were selected based on the optimized collision energy for the transition of DABE (628 $\rightarrow$ 289, 53 V) and DAB (472 $\rightarrow$ 289, 39 V). The detected metabolites were confirmed by the product ion (PI) scan using incubation samples containing a high concentration of substrate (50 μM) and CES (250 $\mu\text{g}/\text{mL}$). The collision energy that produced the highest signal intensity was used in the quantitative analysis of study samples.

Semi-quantitative estimation (SQE) of the identified metabolites

First, 200 nM ($C_{\text{DABE},0}$) of DABE was incubated with CES1b, CES1c, or CES2 individually (in triplicate). Less than 5% of parent drug was observed after incubation at 37°C for 120 min. These samples (termed ‘correction factor (CF) samples’) were analyzed by LC-MS/MS before and after the 120-min incubation. The concentration of DAB (C_{DAB}) formed was measured in three replicates. Based on the results of metabolite identification and substrate specificity of carboxylesterase isozymes, only one major hydrolyzed metabolite is produced by each CES. In addition, DABE, DAB and the tentatively identified metabolites were stable in the incubation buffer at 37°C for 2 h (data not shown). Therefore, the concentration of the formed metabolite (C_{M}) in the CF samples can be estimated using equation 1:

$$C_{\text{M}} = C_{\text{DABE},0} - C_{\text{DAB}} \quad \text{equation 1}$$

In the study samples (multiple-enzyme incubations and rat plasma), the concentrations of metabolites cannot be estimated by equation 1 because there often exist more than one metabolite in the study samples. An alternative approach is to quantify the concentrations of the metabolites (without chemical standards) in the study samples using the standard curve of the parent drug. However, the MS/MS response of an analyte obtained using an ESI source depends strongly on the chemical structure of the analyte. Accordingly, a correction factor (CF) was introduced to compensate for the differences of MS/MS response between metabolite and parent drug. CF was calculated according to equation 2:

$$CF=(C_M \times A_{DABE})/(C_{DABE} \times A_M) \quad \text{equation 2}$$

where C_{DABE} is the measured concentration of the parent drug (DABE); and A_{DABE} and A_M are the analyte/IS peak area ratio for DABE and metabolite, respectively.

The study samples (multiple-enzyme incubations and rat plasma) are then analyzed using LC-MS/MS. The metabolite in the study samples is quantified against the standard curve of the parent compound. The obtained concentration is then multiplied by the CF to yield the final estimated concentration of the metabolite in the study sample.

As the matrix effect was not considered in the CF equation, a matrix effect-free assay is the key to successful application of the SQE approach. The assessment for the matrix effects of DABE and DAB was described in the above section. However, the matrix effects on metabolites could not be directly assessed by conventional methods because we are developing a 'semi-quantitative estimation' approach. Therefore, an alternative evaluation approach (dilution method) was introduced here. Numerous studies propose that a simple dilution with matrix-free solvent can reduce or eliminate matrix effects [9–15]. The dilution method can test whether a matrix effect is present or not. In this method, a series of samples increasingly diluted with matrix-free solvent are prepared and analyzed to determine and calculate the respective 'recovery rates' of the metabolites. Step 1: Measurement of the undiluted sample (C_a); Step 2: Measurement of the diluted sample (C_b); Step 3: Calculation of the 'recovery rate' $[(C_b \times \text{dilution factor})/C_a]$. DABE (200 nM for CES, and 2000 nM for rat plasma) was incubated in CES1b, CES2, CES mixture, and rat plasma for 30 min followed by the addition of ice-cold acetonitrile. Then, the CES supersomes or plasma extracts were diluted by 2, 10, and 50-fold, respectively. The solvents used for the dilution of supersomes and plasma extracts were buffer/acetonitrile (1:1) and water/acetonitrile (1:3), respectively. Each dilution was prepared in triplicate. A 'recovery rate' of 85% to 115% was accepted as indicating no matrix effect.

The conventional method to evaluate the accuracy of the SQE approach is to compare the CF derived estimated concentration with the reference concentration obtained using a chemical standard. However, chemical standards of the metabolites were lacking in this study. Therefore, a set of 'validation samples' were used. These consisted of incubations of parent drug with single CES for different periods of time. The calculated concentrations (obtained with equation 1) of the metabolites in the validation samples were used as the 'reference concentration'. 'Reference concentration' is close to the actual concentration because only one metabolite was formed in a single CES incubation, and both the parent drug and the metabolite are stable in the incubation buffer at 37°C. Then, the estimated concentration in the same sample (obtained with the SQE approach) was compared with the 'reference concentration'. A summary of the SQE scheme is shown in Fig. 1.

Results and discussion

LC-MS/MS quantitative analyses for dabigatran etexilate and dabigatran

The effect of the formic acid concentration of the mobile phase on the signal intensity of the tested compounds is shown in Fig. 2A and 2B. The signal intensities for DABE and DAB were satisfactory when the mobile phase was fortified with 2.5 mM formic acid. Therefore, 2.5 mM formic acid was used in the subsequent validation and application study. It is noteworthy that the optimal concentration of formic acid was 10 times lower than that of the reported assay for DAB in human plasma [5].

The post-extraction spike-based assessment indicated that the absolute matrix effects of CES supersomes and rat plasma on ESI-based measurement of DABE, DAB, and DAB-d3 were between 87% and 112% (Table 2).

The calibration curves for quantification of DABE and DAB in CES and rat plasma incubation samples showed a good linear relationship, ranging from 1.37 to 1000 (CES) or 1.37 to 3000 nM (rat plasma) with correlation coefficients >0.99 , respectively. The within-run ($n = 5$) and between-run ($n = 3$) precision of the assay were 1.3–18.7% and 1.5–14.4%, respectively, while the assay accuracy was also satisfactory, i.e., 93–115% and 99–108%, respectively (Table 1). The LLOQ was 1.37 nM for both analytes (Fig. 2C). The on column sensitivity was 6.9 fmol for CES incubations and 3.4 fmol for rat plasma incubations.

Identification of potential metabolites

There are four potential metabolic sites in the chemical structure of DABE (red and blue bonds, Fig. 3) and they may lead to the formation of seven metabolites. According to the product ions of DABE and DAB (Table 3), all of the potential metabolites were predicted to have the product ion of m/z 289 with high relative abundance. Therefore, the calculated precursor ions of the potential metabolites and the product ion m/z 289 were used as the precursor-product ion pairs in the MRM detection mode. The precursor-product ion pairs included 600.3 $\rightarrow$ 289.1 (M1), 544.3 $\rightarrow$ 289.1 (M3), 516.3 $\rightarrow$ 289.1 (M4), 500.3 $\rightarrow$ 289.1 (M2), 452.2 $\rightarrow$ 289.1 (M5), 368.1 $\rightarrow$ 289.1 (M6), and 324.1 $\rightarrow$ 289.1 (M7). Three values of collision energy (40, 47, and 55 V) were tested for each ion pair. Accordingly, 21 MS/MS channels were monitored in a single analysis. The scan time for each ion pair was 25 ms, which was calculated based on the peak width and the number of ion pairs. Upon analysis of the CES incubation samples, two potential metabolites (M1, 600.3 $\rightarrow$ 289.1 and M2, 500.3 $\rightarrow$ 289.1) were found while the remaining theoretical metabolites were not observed. The two potential metabolites were characterized by the product ion scan of incubation samples which contain a high concentration of metabolites. A summary of the product ions and chromatographic behaviors of the metabolites M1 and M2 is shown in Table 2. Based on the detection of diagnostic product ions m/z 365 and m/z 434, the chemical structures of M1 and M2 were proposed (Fig. 3). The targeted metabolite identification approach presented here is sensitive and efficient with only one visit to the laboratory. The sensitivity was as low as several fmol on column, and the total LC-MS/MS running time was about 10 min, comprising only two injections.

Semi-quantitative estimation (SQE) of the identified metabolites

The results of dilution method showed no sign of matrix effects for M1 and M2 in CES supersomes or rat plasma matrices (Table 4). The derived values of CF for the metabolite M1 and M2 was 1.07 and 0.57, respectively. The results for the validation of the SQE approach are shown in Fig. 4A. In general, the concentrations of the metabolites obtained by the SQE approach were very similar to the 'reference concentrations'. For all 24 validation samples (eight samples each for CES1b, CES1c or CES2), a good correlation was observed between the 'reference concentrations' and concentrations estimated by SQE (Fig. 4A4). The accuracy of the SQE approach for the validation samples was $105 \pm 26\%$ (mean $\pm$ SD). After excluding one sample with low reference concentrations (14 nM, the reference concentration of M2 in CES2 after incubation for 5 min), the accuracy was improved to $101 \pm 15\%$. According to the metabolic profiles of DABE in single CES1b, CES1c or CES2 incubations (Fig. 4A1, 4A2, and 4A3), the formation profiles for the metabolite M1 or M2 estimated by SQE correlate well with the profiles produced using the 'reference concentrations'.

It is noteworthy that CES1b and CES1c convert DABE to M1 while CES2 mediates the formation of M2 (Fig. 4A1, 4A2, and 4A3). Furthermore, the hydrolysis rate must be very slow for the metabolic conversion of M1 to DAB by CES1 and M2 to DAB by CES2 because only a small amount of DAB is formed in single CES1 or CES2 incubations.

Next, the validated SQE approach was applied to the metabolism of DABE in a mixture of CES (containing CES1b, CES1c, and CES2) and in rat plasma. With respect to the metabolism of DABE in the CES mixture, the formation of M1 was 17-fold higher than that of M2, suggesting that CES1 is the major CES enzyme responsible for the hydrolysis of DABE. In addition, the amount of DAB produced in the CES mixture was at least 5 times higher than that in single CES incubations. This means that CES2 is possibly the major enzyme responsible for the hydrolytic pathway of M1 to DAB. For the metabolism of DABE in rat plasma, DABE was completely and rapidly hydrolyzed to M1. Then, a small portion of M1 was metabolized to DAB.

In our SQE approach, the concentrations of metabolites in study samples cannot be correctly estimated if the matrix effects (caused by CES supersomes or rat plasma) on metabolites vary between the CF samples (used to generate the CF value) and study samples. A matrix effect-free assay is the key to successful application of the SQE approach. In our study, the matrix effects on metabolites could not be directly assessed by conventional methods. However, there were two findings demonstrating that the metabolites were free of matrix effect. First, there was no sign of matrix effect using an indirect dilution method (Table 4). Secondly, the results of study samples (Figure 4B1 and 4B2) showed the concentration of initially added DABE (200 or 2000 nM) was close to the summed concentrations of all the compounds after incubation (DABE, DAB, M1 and M2).

Although a same product ion (m/z 289) was monitored, there was no cross-talk between the analytes. The mass spectrometric effect of cross-talk may occur if several mass transitions with identical product ions are acquired. If the collision cell is not emptied completely within the very short time between different transition settings (the interscan delay), spurious signals are recorded that will appear in the subsequently acquired mass transition trace [16]. Based on this theory, it was found that changing the order of the SRM experiments successfully prevents crosstalk effects from occurring [16]. In our experiment, the order of the SRM experiments is as follows: DABE (628→289), DAB (472→289), DAB-d3 (475→292), M1 (600→289), M2 (500→289). According to the retention times in Table 3, DABE and DAB is chromatographically separated, as well as M1 and M2. Therefore, there was no possibility of cross-talk between all the analytes. Actually, we found 1000 nM of DABE and DAB showed no interference on the detection of M1 and M2, respectively (see Electronic Supplementary Material Fig. S1).

Due to its sensitivity, selectivity and high throughput, LC-MS/MS using an ESI source has become the method of choice for qualitative and quantitative analysis of drugs and their metabolites in biological matrices. However, the MS response of an analyte obtained using an ESI source depends strongly on the chemical structure of the analyte and the matrix, which necessitates the use of a calibration curve prepared from an authentic standard for quantitation. Unfortunately, synthetic metabolite standards for quantitative analysis are usually not available in the early stages of drug development.

In order to obtain quantitative information on metabolites when chemical standards are not available, two MS-based SQE approaches (1 and 2) have been reported. Approach 1 often incorporates the use of a universal detector to correct for the non-universal MS response between parent compounds and metabolites. This approach mainly includes the radiometric calibration technique [17,18], the ultraviolet (UV) [19] or nuclear magnetic resonance

(NMR) [20–22] correction method, and the ultra-low flow nanospray technique [23]. The major limitation of the radiometric approach is that it relies on the availability of radiolabeled parent compounds. The UV correction method is not accurate when the UV properties of metabolites are significantly different from their parent compounds. NMR has been found to be more accurate than UV, but it requires the isolation of a few micrograms of metabolites and the availability of an NMR instrument. The nanospray method also requires the availability of a nanospray source. Approach 2 is based on the prediction of ionization efficiency *in silico* using capillary electrophoresis-ESI-MS [24]. Although this work demonstrates for the first time the feasibility of virtual quantification of polar endogenous metabolites in biological samples, the applicability of this approach in drug metabolism studies using LC-MS/MS is still unknown. The SQE approach presented here is simple, accurate, and no sophisticated instrumentation is required.

Our approach provided a new option for metabolite quantification when other reported approaches are not applicable or practical for the specific study. Although our application of the SQE approach was limited to *in vitro* metabolism samples, this approach may also be used in the *in vivo* samples from pharmacokinetic studies. The limitations of our SQE approach need to be considered, however. Our approach is limited to drugs that have only one major metabolite without a chemical standard, for each recombinant drug-metabolizing enzyme, microsomal or other system. Generation of a CF value is not feasible when this requirement is not met for the studied drugs (Fig. 1, CF samples).

Conclusions

A targeted metabolite identification and SQE approach was presented here. The targeted metabolite identification approach was sensitive and efficient with only one visit to the laboratory. Our SQE approach provided a new option for metabolite quantification when metabolite standards are not available. Quantitative information on detected metabolites was obtained using ‘metabolite standards’ generated from incubation samples that contain a high concentration of metabolite in combination with a correction factor for mass spectrometry response. Taking advantage of the developed approach, the role of CES in the *in vitro* metabolic pathway of DABE was elucidated. The approach presented here provides a solution to a practical bioanalytical need for identification and semi-quantitative estimation of drug metabolites in drug metabolism samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

The scheme of the semi-quantitative estimation (SQE) approach. DABE, dabigatran etexilate; DAB, dabigatran; M, metabolites; A, analyte/IS peak area ratio; C, concentration (nM); CF, correction factor; CES, carboxylesterase; Correction factor (CF) samples, the samples used to generate the correction factor; Validation samples, the samples used to evaluate the accuracy of the proposed approach.

Fig. 2.

The effect of the formic acid concentration of the mobile phase on the signal intensity of the tested compounds (A, analytes in CES supersomes; B, analytes in rat plasma), example chromatograms of dabigatran etexilate (DABE) and dabigatran (DAB) at the LLOQ in a matrix-matched sample (C), and example chromatograms of M1 and M2 in CES1 and CES2 incubations, respectively (D).

Fig. 3.
Proposed structures of diagnostic product ions for dabigatran etexilate and its metabolites.
CE, collision energy in the collision induced dissociation.

Fig. 4. Validation and application of the semi-quantitative estimation (SQE) approach. Figure A1, A2, and A3 illustrate the metabolism of dabigatran etexilate (DABE) in single carboxylesterase (CES) 1b, CES1c, and CES2 incubations, respectively ($n = 2$). Figure A4 is the correlation between reference concentrations of metabolites M1/M2 and the concentrations obtained by SQE. Figure B1 and B2 show the metabolism of DABE in a CES mixture and rat plasma ($n = 2$), respectively. Closed blue is DABE; closed red is DAB; closed green and orange are the reference concentrations of M1 and M2, respectively; open green and orange are the concentrations of M1 and M2 obtained by SQE, respectively.

Table 1

Precision (RSD) and accuracy for assay of dabigatran etexilate (DABE) and dabigatran (DAB) in carboxylesterase (CES) and rat plasma incubations

Nominal concentration (nM)	Within-run (n = 5)	Between-run (n = 3)		
	Measured concentration (nM) mean $\pm$ SD (RSD)	Accuracy	Measured concentration (nM) mean $\pm$ SD (RSD)	Accuracy
DABE in CES incubations				
1.37 (LLOQ)	1.28 $\pm$ 0.06 (5.1%)	93%	1.48 $\pm$ 0.18 (12.0%)	108%
12.3	12.1 $\pm$ 0.7 (5.9%)	98%	12.4 $\pm$ 1.2 (9.3%)	101%
111	119 $\pm$ 12 (10.1%)	107%	111 $\pm$ 9 (8.5%)	100%
1000	973 $\pm$ 20 (2.1%)	97%	1053 $\pm$ 111 (10.6%)	105%
DAB in CES incubations				
1.37	1.47 $\pm$ 0.18 (12.0%)	107%	1.43 $\pm$ 0.21 (14.4%)	104%
12.3	11.9 $\pm$ 0.8 (6.8%)	97%	12.3 $\pm$ 0.5 (4.1%)	100%
111	112 $\pm$ 2 (1.8%)	101%	110 $\pm$ 3 (2.4%)	99%
1000	999 $\pm$ 18 (1.8%)	100%	1033 $\pm$ 15 (1.5%)	103%
DABE in rat plasma incubations				
1.37	1.57 $\pm$ 0.05 (3.2%)	115%	— ^a	—
12.3	11.7 $\pm$ 0.3 (2.5%)	95%	—	—
111	110 $\pm$ 3 (3.1%)	99%	—	—
1000	1012 $\pm$ 36 (3.5%)	101%		
DAB in rat plasma incubations				
1.37	1.32 $\pm$ 0.25 (18.7%)	96%	—	—
12.3	12.3 $\pm$ 0.5 (3.9%)	100%	—	—
111	114 $\pm$ 3 (2.7%)	103%	—	—
1000	997 $\pm$ 13 (1.3%)	100%		

^a Because the number of rat plasma incubation samples was limited, between-run accuracy and precision data were not available for rat plasma incubations.

Table 2

Absolute matrix effects (ME) of dabigatran etexilate (DABE) and dabigatran (DAB) in CES supersomes and rat plasma matrices (n = 3)

Compounds	Matrices	Concentration (nM)	Absolute ME (%) ^a	SD (%)
DABE	CES	4.1	106	6.3
		333	97	3.5
DAB	CES	4.1	112	5.8
		333	100	8.1
DABE	rat plasma	4.1	87	5.5
		333	103	11.8
DAB	rat plasma	4.1	95	9.7
		333	103	5.4

^aDeviation of the mean peak areas of set 2 versus those of set 1 was used to indicate the possibility of ionization suppression or enhancement for the analytes; this is called an absolute matrix effect. In set 1, the tested analytes (DABE and DAB) were added to a matrix component-free solvent (buffer:acetonitrile = 1:1 for CES; buffer:acetonitrile = 1:3 for rat plasma). In set 2, the tested analytes were added to a matrix solvent, which was prepared using the protein precipitation method (CES supersomes in buffer:acetonitrile = 1:1; rat plasma in buffer:acetonitrile = 1:3). The details of the protein precipitation method are shown in the method section.

Table 3

Summary of the product ions and chromatographic behaviors of dabigatran etexilate (DABE), dabigatran (DAB), dabigatran-d3 (DAB-d3), and the identified metabolites M1 and M2

Compound	t_R , range (min)	Peak width (s)	Precursor ion ^a (m/z)	Product ion ^b (m/z)
DABE	1.59, 1.57–1.61	11	628.3	131, 144, 159, 172, 189, 261, 273, <u>289</u> , 306, 332, 365 , 434 , 526
DAB	1.42, 1.41–1.44	9	472.2	131, 144, 159, 172, 189, <u>289</u> , 306, 324, 337 , 400
DAB-d3	1.42, 1.41–1.44	9	475.3	134, 147, 162, 175, 192, 292, 309, 327, 340, 403
M1	1.56, 1.54–1.58	10	600.3	145, 149, 172, 189, 273, <u>289</u> , 306, 324, 332, 337 , 434
M2	1.45, 1.44–1.47	10	500.3	145, 159, 172, <u>289</u> , 306, 365

^a [M+H]⁺ generated in the positive ion ESI mode.

^b Produced from the [M+H]⁺ by collision induced dissociation (CID). The numbers in bold are the diagnostic product ions, and the product ions of relative abundance <5% are not shown. Product ion 289 is the base peak. The collision energy was set from 30 to 60 V.

Table 4

Matrix effects (ME) of metabolites M1 and M2 in CES supersomes and rat plasma matrices assessed by the dilution method (n = 3)

Metabolites	Matrices	Dilution factor	'Recovery rate' (%)	SD (%)	Summary ^b
M1	CES1b	2	95	5.5	No
		10	105	7.2	
		50	103	12.3	
M2	CES2	2	94	2.4	No
		10	98	6.6	
		50	107	8.4	
M1	CES mixture	2	96	8.1	No
		10	97	4.3	
		50	104	9.4	
M2	CES mixture	2	102	8.5	No
		10	— ^a	—	
		50	—	—	
M1	rat plasma	2	101	1.9	No
		10	95	5.5	
		50	110	6.1	

^aM2 cannot be detected in these dilution samples.

^bSummary for the assessment of matrix effects. "No" indicates no sign of matrix effects was found.