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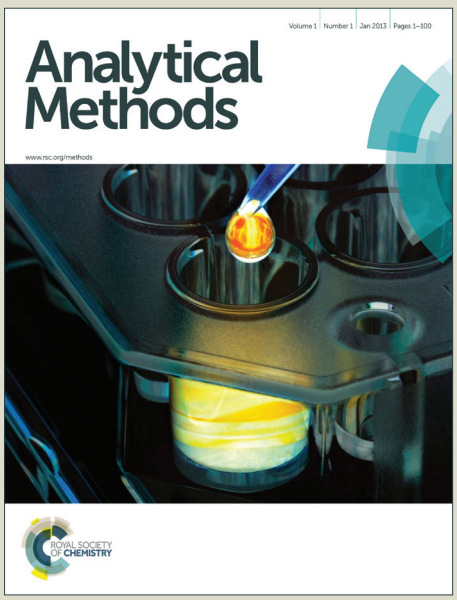

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A new calibration curve calculation method for absolute quantification of drug metabolizing enzymes in human liver microsomes by stable isotope dilution mass spectrometry

Huanhuan Wang[a,b], Haifeng Zhang[c]， Jiabin Li[b], Junying Wei[c,d], Rui Zhai[b], Bo Peng[b], Hailing Qiao[c], Yangjun Zhang[a, b*], Xiaohong Qian[b*]

Accurate quantification of Cytochrome P450 (CYP) enzymes and uridine 5-diphospho- glucuronosyltransferase (UGT) enzymes is essential for the reliable assessment of new drug safety and individual medicine. Stable isotope dilution-multiple reaction monitoring-mass spectrometry (SID-MRM MS) has been used for the determination of drug metabolizing enzymes in complex biological samples, in which a working curve is often established by adding a series of light peptide into aliquots of a blank sample matrix and stable isotope labeled (SIS) peptide. But when multiple proteins are simultaneously quantified, a blank sample matrix devoid of targeted proteins is difficult to prepare. To solve the problem, a linear curve was established by adding a series of SIS peptides to an actual sample instead of a heterologous or artificial sample as a matrix, and a new calibration curve calculation method was proposed to calculate the concentrations of endogenous peptides or proteins in a biological sample as follows: the linear curve was first plotted with peak area ratios (SIS peptides /endogenous peptides) on the y-axis and the corresponding concentrations on the x-axis, and then the concentrations of endogenous peptides in a biological sample could be accurately obtained according to our mathematic formula. Finally, a working curve was built with peak area ratios on the y-axis and the corresponding concentration ratios on the x-axis, and when the peak area ratio of a transition of a peptide in a biological sample was measured and substituted into the working curve, the corresponding concentration ratio could be obtained to calculate the peptide's concentration. Experimental results demonstrated that the established method was reliable and sensitive with recovery of 97.0% and limit of quantification (LOQ) lower than 20 fmol, a linear range from 5 fmol, 10 fmol or 20 fmol～1000

fmol for different peptides and coefficient of variation lower than 10%. The established method was applied to the determination of 21 drug metabolizing enzymes in five human liver microsomal samples, and the results are in agreement with the reported data, which proves that this method can be applied to the determination of targeted proteins in biological samples.

Key Words: Cytochrome P450 (CYP) enzymes, Uridine 5-diphospho-glucuronosyltransferase (UGT) enzymes, Matrix interference, MRM

1. Introduction

Cytochrome P450 (CYP) enzymes and uridine 5-diphospho-glucuronosyltransferase (UGT) enzymes play an important role in Phase I and Phase II metabolism of endogenous and exogenous compounds.[1, 2] The disparate expression of CYP and UGT enzymes among different individuals is due to geneticpolymorphisms.[3-5] Inter-individual differences in the expression levels of CYP and UGT enzymes strongly influence the bioavailability of drugs, leading to differences in their pharmacokinetic and toxicological actions.[6] For example, clopidogrel hydrogen sulfate tablets can be used to treat coronary thrombosis, which is effective due to metabolism by CYP2C19 enzymes in human liver microsomes. However, because the expression level of CYP2C19 varies among different individuals, this drug has a good therapeutic effect for some patients but no effect for other patients. This fact shows that the expression level of drug metabolizing enzymes is a key to new drug development, including evaluations before clinical trials and during clinical application. Researchers have developed several methods for determining the expression levels of drug metabolizing enzymes. Western blot methodologies have been applied for the quantification of drug metabolizing enzymes, and they are characterized by high sensibility but suffer from the availability of specific antibodies and a narrow linear dynamic range.[7, 8] For example, when Shimada used a western blot method to determine the content of CYPs in 60 liver microsomal samples in 1970, for the lack of specific antibodies, the total amount of CYP3A and CYP2C family enzymes were separately determined, but the amounts of CYP3A4 versus CYP3A5 and CYP2C9 versus CYP2C19 were not distinguished.[9] Recently stable isotope dilution-multiple reaction monitoring-mass spectrometry (SID-MRM MS) has been applied in the determination of drug metabolizing enzymes with high sensibility, wide dynamic range, and do not require specific antibodies.[10-14] For example, Kawakami et al. used SID-MRM MS to quantify 11 CYPs in 10 human liver microsomal (HLM) samples.[12] In the method, stable-isotope labeled internal standard peptides were prepared by chemical synthesis. The drawbacks of the method are that

the reaction and separation processes are complex and the cost is high; moreover, every peptide should be individually quantified. Achlour et al.[4] applied the QconCAT technique proposed by Pratt JM et al.[15] to prepare stable isotope-labeled internal standard peptides, combining SID-MRM MS to quantify 13 CYP and 8 UGT enzymes in 24 human liver microsomal samples, which is a high-through technique for preparing internal standard peptides, and the contents of all other peptides can be known only if one or two peptides are accurately quantified. In the method for protein quantification based on signature peptides in tryptic digests of targeted proteins in biological samples mentioned above, its limit of quantification (LOQ), linear dynamic range and accuracy may be affected by complex biological matrices.[16] Considering matrix interference in the establishment of a LC-MRM MS method, a calibration curve can be built on a blank sample matrix devoid of all target proteins, but preparation of a blank sample matrix is difficult due to the complexity of biological samples containing hundreds of thousands of proteins and other biological molecules when a number of target proteins are quantified.[17,18] Some researchers have reported preparation of calibration curves based on a solution without a blank sample matrix or heterologous matrices such as BSA or human serum albumin.[12, 19-21] These matrices were too simple to simulate complex biological sample environment well. For example, microsomal fractions of the HepG2 cell was also used as matrices similar to HLMs to quantify CYP450, [22] and Zhao et al. developed an alternative method to build working curve in a matrix prepared by mixing a high concentration of proteins except target proteins when determining the amount of vitronect in and cluster in human serum samples.[23] Although these matrices just mentioned above were similar to a blank matrix, it is not a real blank sample matrix. In another methods, Wang Xueying et al. used a metal–tag multiple labeling method to prepare a calibration curve in a sample matrix for the quantification of a protein, but the labeling process of this method is complex for actual applications.[24] Michael et al. [17] used an alternative method to plot a calibration curve by preparing serial sample dilutions and subsequently adding a defined amount of stable-isotope labeled (SIS) peptides with known concentrations, in which matrix effects were considered, but the

concentrations of endogenous peptides in the samples were calculated at a single concentration point, and this will cause deviation. Gradually diluted matrices are varied at different concentration points, which would affect the linearity and accuracy of the established methods.

To address the problems of blank sample matrices in plotting a working curve during establishing SID-MRM MS method, a reverse linear curve was plotted by preparing a series of dilutions of isotope labeled peptides added each with aliquots of human liver microsomal sample digest, and a new calibration curve calculation method that can accurately calculate the concentrations of endogenous peptides digested from the proteins in the sample was proposed for the first time. Quantitative results by our method compared to "a single concentration point" calculation method showed the advantage over that of the classical stable isotope dilution method. This calibration curve calculation method based on QconCAT technique combine with SID-MRM MS was applied to the determination of 21 drug metabolizing enzymes in 5 human liver microsomes, and the quantitative results are in agreement with those compared with the literatures.[4, 25]

2. Experimental

2.1 Chemicals and reagents

Sequencing grade modified trypsin, dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Promega Corporation (Madison, WI). Acetonitrile (ACN), formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLM) were obtained from Zhengzhou University (China). The peptides ASGNLIPQEK and TILDELVQR were synthesized by GL Biochem Ltd (Shanghai, China).Glutathione Sepharose™ 4B was purchased from GE Healthcare Bio-Sciences AB. A Pierce SILAC protein Quantitation kit-DMEM was purchased from Thermo Fisher Scientific Inc. (Waltham, MA USA). Ziptip C18 pipette tips for sample preparation were purchased from Millipore corporation (Billerica, MA).

2.2 Purity assessment of synthetic peptides ASGNLIPQEK and TILDELVQR

Two peptides ASGNLIPQEK and TILDELVQR composing a QconCAT protein were selected to quantify QconCAT protein. The purity of these two synthetic peptides were determined to be 89.03% for ASGNLIPQEK, and 37.98% for TILDELVQR by SID-MRM MS after each peptide was hydrolyzed.[26]

2.3 Preparation of a QconCAT protein

To quantify 21 drug metabolizing enzymes in human liver microsomes, QconCAT proteins were designed as a concatemer of 57 stable isotope-labeled peptides, and two or three peptides were selected for each targeted protein.[27] The genes encoding the QconCAT protein were first inserted into prokaryotic expression plasmids by Sangon Biotech Ltd. (Shanghai China), and then the plasmids were transformed and expressed in *E.coli* in DMEM medium containing $^{13}C_6$ L-Lysine and$^{13}C_6$ L-Arginine. The QconCAT protein was purified using affinity chromatography and confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) similarly to methods described in the literature. [4, 28]

2.4 In-solution digestion

Human liver microsome proteins were denatured with 8 M urea, reduced with dithiothreitol at the final concentration of 10 mM at 37°C for 4 h, then alkylated with 40 mM iodoacetamide away from light for 30 min, diluted with 7 volumes of 50 mM NH_4HCO_3 solution, and finally digested with trypsin at a trypsin to substrate ratio of 1:50 at 37°C for 26 h.

The recombinant QconCAT protein was digested the same as above and its digestion completeness was examined by FT-LTQ-ICR MS (Thermo Fisher Scientific Inc. Waltham, MA USA).

2.5 Quantification of the QconCAT protein

The peptides ASGNLIPQEK and TILDELVQR were chosen to determine the concentration of the QconCAT protein. Standard peptide series dilutions (10 fmol, 20 fmol, 50 fmol, 100 fmol, 200 fmol, 500 fmol) were prepared, and subsequently the same amount of the QconCAT digest was separately added and diluted to the same volume. After nano-high performance liquid chromatography coupled to multiple reaction monitoring mass spectrometry (nanoHPLC-MRM MS) analysis, linear

regression was performed to test the linearity between the peak area ratios of the stable-isolated labeled peptides to natural peptides and the concentrations of the stable-isolated labeled peptides. LOQ and linear range were evaluated, and the concentration of the QconCAT protein was calculated according to the linear curve equation.

2.6 Nano LC-MS/MS methodology

Biological samples were analyzed by nano LC-MRM MS using an easy nano-LC (Thermo Fisher Scientific Inc. Waltham, MA USA) coupled to a TSQ vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc. Waltham, MA USA). The samples were first separately loaded on a trap column (100 μm×20 mm, packed with SP-300-ODS-AP, 5 μm diameter particles, 100 nm pore size in house)at a flow rate of approximately 3~4 μm/min under the pressure of 200 bar, then each sample was brought into an analytical column (75 μm×11 mm, the same as the trap column just mentioned above), where peptides were eluted at the flow rate of 300 nl/min with an elution gradient of mobile phase B (100% ACN, 0.1% formic acid) and mobile phase A (100% H_2O, 0.1% formic acid): 2% to 10% Bin 5 min, 10% to 40% B in 60 min, 40% to 95% in 5 min, afterwards automatically equilibrating the LC system with mobile phase A for approximately 10 min for the next analysis. The effluent from the analytical column was continuously directed into a TSQ vantage triple quadrupole mass spectrometer by a nano-electrospray ionization (nano-ESI) source at capillary temperature: 240 ℃ and spray voltage: 1900 V. Three transitions were selected per peptide. MS conditions were as follows: the resolution for both Q1 and Q3: 0.7 Da FWHM; the pressure in Q2: 1.5 mTorr (Ar), cycle time: 1.5 s; collision energy: 0.034 x precursor ion m/z + 3.314.

3. Results and Discussion

3.1 A new calibration curve calculation method

When biological samples are analyzed to determine the absolute contents of proteins by SID-MRM MS, stable isotope dilution calibration curves need to be plotted with peak area ratios (SIS peptides /light peptides) on the y-axis and the corresponding concentration ratios on the x-axis. In SID-MRM MS analysis, there are

two types of calibration curves as follows: one is forward linear curve in which SIS peptides are varied and the light peptides are constant; the other is a reverse linear curve in which the light peptides are varied and SIS peptides are constant.[29] The reverse linear curve is better than a forward linear curve considering endogenous interference for target peptides.[29] To establish a reverse linear curve, ideally, SIS peptides serial dilutions are added into aliquots of blank sample matrix and the light peptides, but a blank sample matrix devoid of targeted proteins is difficult to obtain. In our method, as showed in Figure 1, to establish a reverse linear curve, a series of dilutions of SIS peptides were prepared, and separately added into aliquots of human liver microsomal sample digests and finally diluted to the same volume. When the mixtures were analyzed by MRM MS, a linear curve was firstly plotted with peak area ratios ($\frac{Y_H}{Y_L}$, SIS peptides/endogenous peptides) on the y-axis and the corresponding concentration of SIS peptides (X_H) on the x-axis: $\frac{Y_H}{Y_L} = C_1X_H + C_2$. In this equation, C_1 and C_2 are constant. To calculate concentration ratios, the concentrations of endogenous peptides in sample matrix need to be estimated, as their actual concentrations are unknown. To accurately calculate the concentration of each endogenous peptide (X_L), a new calculation method is proposed in our method: $X_L = \frac{1-C2}{C1}$, and this equation is derived as follows:

After one peptide is analyzed by MRM MS, the relationship of mass spectrometric signal intensity (Y) and concentration (X) of the peptide can be generally expressed as the equation:

$$Y = aX + b \quad (1)$$

Where a is the slope, the response factor of the analyte； b is the intercept, which is associated with instrument noise and matrix interference.

According to equation (1), the mass spectrometric signal intensity (Y) and the concentration (X) of an SIS peptide can be expressed as equation (2):

$$Y_H = aX_H + b \quad (2)$$

Whereas the mass spectrometric signal intensity (Y) and the concentration (X) of its

corresponding endogenous peptide can be expressed as equation (3):

$$Y_L = aX_L + b \quad (3)$$

In equations (2) and (3), Y_H is the peak area of an SIS peptide, X_H is the concentration of SIS peptide; Y_L is the peak area of its corresponding endogenous peptide, and X_L is the concentration of its corresponding endogenous peptide.

Because both the SIS peptide and its corresponding endogenous peptide have the same chemical properties, their chromatographic retention behavior on a cHPLC system is nearly identical in the same chromatographic conditions. The same situation occurs except for a mass shift when these two types of peptides are analyzed by MS, therefore, a and b are identical.[30]

When equation (2)/(3), equation (4) is obtained as follows:

$$\frac{Y_H}{Y_L} = \frac{a}{aX_L+b}X_H + \frac{b}{aX_L+b} \quad (4)$$

As aliquots of sample matrix were added with a series of SIS peptides, this means that X_L is constant, therefore equations (5) and (6) can be obtained:

$$C_1 = \frac{a}{aX_L+b} \quad (5)$$

$$C_2 = \frac{b}{aX_L+b} \quad (6)$$

Equations (5) and (6) can be substituted into (4) to generate equation (7):

$$\frac{Y_H}{Y_L} = C_1X_H + C_2 \quad (7)$$

Equation (7) is a linear regression equation in which the constants C_1 and C_2 can be obtained through the linear regression equations of peak area ratios of an SIS peptide to its corresponding endogenous peptide on the y-axis and the concentrations of an SIS peptide on the x-axis, as shown in Figure S1 for three transitions from the peptide ASGNLIPQEK of CYP1A2.

Dividing equation (5) by (6), we get equation (8):

$$\frac{C_1}{C_2} = \frac{a}{b}$$

$$\mathrm{b} = \frac{C_2}{C_1}a \quad (8)$$

Substituting equation (8) into (6)，we get equation (9):

$$C_2 = \frac{\frac{C2}{C1}a}{aX_L + \frac{C2}{C1}a}$$

$$X_L = \frac{1-C2}{C1} \quad (9)$$

The concentration of an endogenous peptide (X_L) can be accurately calculated by equation (9) when the values of C_1 and C_2 are known, and the concentration ratios can be calculated, finally, a linear curve can be established with observed peak area ratios (SIS peptides/light peptides) on the y-axis and the corresponding concentration ratios on the x-axis.

To compare this new calibration curve calculation method with the general stable-isotope dilution calibration-MS method in accuracy, for example, our calculation method and the single concentration point calculation method, 136.4 fmol of the synthetic peptide ASGNLIPQEK was first added into 2 μg of a human liver microsomal digest, then the prepared sample and the original digest of the human liver microsomal sample were separately supplemented with a series of stable-isotope labeled peptides of the QconCAT protein with known concentrations (25.4 fmol, 63.6 fmol, 127.2 fmol, 254.4 fmol, 636.0 fmol), and finally they were analyzed separately two times and the data were processed using two calculation methods. The amount of the ASGNLIPQEK peptide added was calculated by subtracting the amount in the original digest of the human liver microsomal sample from the total amount in the prepared sample, as listed in Table 1. The average recovery of the ASGNLIPQEK peptide calculated using our method was 97.0%.

When the single concentration point calculation method was used to calculate the amount of the added ASGNLIPQEK peptide at five concentration points just mentioned above within the linear range, the calculated amounts obtained had large errors, and the recoveries are listed in Table 2.

Table1 shows that the recovery of the ASGNLIPQEK peptide using our calculation method was 97.0%, and Table 2 shows that the recovery of the same peptide was between 61.0%-140.0% when using the single concentration point calculation method.

These results show that if the concentrations of endogenous peptides are calculated by the single concentration point calculation method when establishing a linear curve, large deviation would be produced, which would exert a detrimental influence on the accuracy of analytical results.

3.2 Linear response of MRM quantification

To evaluate the performance of the established method, LOQ and the dynamic concentration range for each transition were determined. LOQ can be generally defined as the lowest concentration of an analyte calculated from a linear curve when the relative standard deviation is less than 10%.[31] The dynamic concentration range is determined from LOQ as the highest concentration point on the linear curves. Based on these definitions, LOQ and the dynamic concentration range for each transition was calculated and shown in Table S1.

For the quantification of drug metabolizing enzymes, two or three signature peptides were selected to quantify a protein, and three transitions were selected to quantify each signature peptide. Ideally, for each transition from all these signature peptides, a linear working curve should be established. However, MS response signals for some peptides or transitions was poor, CYP3A4, CYP3A5, CYP2C19, UGT1A1, UGT1A3, UGT1A9, UGT2B10 and UGT2B11 were quantified using only single peptides.

3.3 Reproducibility of the established method

To evaluate the reproducibility of the established method, each sample was prepared and repeatedly analyzed five times. As shown in Table 3, the coefficients of variation (CV) of the five measurements for each peptide of 10 cytochrome P450 enzymes in human liver microsomes are lower than 10%, and the fold difference across minimal and maximal values of the five replicate measurements for a drug metabolizing enzyme is within the range of 1.08-1.21. The good reproducibility suggests that the established method is reliable for application in protein quantitation in complex biological samples.

3.4 Absolute quantification of 21 CYP and UGT enzymes in 5 human liver microsomal samples

The established method was applied to determine absolute quantification of 21 drug metabolizing enzymes in 5 human liver microsomal samples, each sample was analyzed for two times. Firstly, defined amounts of SIS peptides were added into human liver microsomal samples, then the mixtures were analyzed by nano LC-MRM MS, the peak area ratios were obtained. The peak area ratios and the amounts of SIS peptides were substituted into the linear equation to obtain the amount of endogenous peptides in the samples, then the concentrations of the corresponding proteins can be calculated from the chemical stoichiometric relation between the signature peptides and the corresponding proteins. In data processing, most quantification results of two or three signature peptides from one protein were equivalent, but for CYP2D6 and UGT2B7, their two peptides didn't give the same results, more accurate quantification results were given by peptides with high MS response signals.

The absolute amount ranges for 21 drug metabolizing enzymes in five human liver microsomal samples were listed in Table 4. The quantification results of 18 drug metabolizing enzymes were in agreement with the reported data in the literatures[4, 25] except UGT 2B10, UGT 2B11and POR which weren't studied in these two literatures.

4. Conclusions

In the determination of drug metabolizing enzymes by SID-MRM MS methods, to address the problems related to establishing a linear curve, a new calculation method was proposed. The linear curve was first plotted with peak area ratios (SIS peptides /endogenous peptides) on the y-axis and the corresponding concentrations on the x-axis, and then the concentrations of endogenous peptides in standard samples could be accurately obtained. Finally, a working curve was built with peak area ratios on the y-axis and the corresponding concentration ratios on the x-axis, and the peak area ratio of a transition of a peptide in a sample can be substituted into the working curve, and the corresponding concentration ratio can be obtained to calculate the peptide's concentration. Experimental results demonstrated that the established method is reliable and sensitive with recovery of 97.0% and LOQ was lower than 20 fmol, the linear range was from 5 fmol, 10 fmol or 20 fmol~1000fmol for different peptides and

the coefficient of variation lower than 10%. The established method was applied to the determination of 21drug metabolizing enzymes in five human liver microsomal samples, and the results are in agreement with the reported data, proving that this method can be applied to the determination of targeted proteins in biological samples.

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Notes and references

[a]*Guangxi Medical University, Nanning Guangxi, 530021, China E-mail:*
[b]*State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, No. 33 Life Science Park Road, Changping District, Beijing, 102206, China*
[c]*Basic Medical College of Zhengzhou University, Zhengzhou, 450052, China*
[d]*Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing 100700, China*
[*]*Corresponding authors: qianxh1@163.com (Xiaohong Qian); 13683167093@163.com (Yangjun Zhang)*

1. W. E. Evans and M.V. Relling, 1999, *Science*, **286**, 487-91.
2. D. F. Lewis, Pharmacogenomics, 2004, **5**, 305–318.
3. R. K. Tukey and C. P. Strassburg, *Annu Rev Pharmacol Toxicol*, 2000, **40**, 581-616.
4. B. Achour, M. R. Russell, J. Barber and A. Rostami-Hodjegan, *Drug Metab Dispos*, 2014, 42, 500-510.
5. S. Ohtsuki, O. Schaefer and H. Kawakami, *Drug Metab Dispos*, 2012, **40**, 83-92.
6. M. Ingelman-Sundber, *Naunyn Schmiedebergs Arch Pharmacol*, 2004, **369**, 89–104.
7. U. K. Laemmli, *Nature*, 1970, **227**, 680-685.
8. H. Towbin, T. Staehelin and J. Gordon, *Proc Natl Acad Sci USA*, 1979, **76**, 4350-4354.
9. T. Shimada, H. Yamazaki, M. Mimura, Y. Inui and F. P. Guengerich, *J Pharmacol Exp Ther*, 1994, **270**, 414-423.
10. A. M. Yu, J. Qu, M. A. Felmlee, J. Cao and X. L. Jiang, 2009, **37**, 170–177.
11. C. Seibert, B. R. Davidson, B. J. Fuller, L. H. Patterson, W. J. Griffiths and Y. Wang, *J Proteome Res,* 2009, **8**, 1672–1681.
12. H. Kawakami, S. Ohtsuki, J. Kamiie, T. Suzuki, T. Abe and T. Terasaki, *J Pharm Sci*, 2011, **100**, 341-352.
13. J. K. Fallon, D. E. Harbourt, S. H. Maleki, F. K. Kessler, J. K. Ritter and P. C. Smith, *Drug*

Metabolism Letters, 2008, **2**, 210-222.

14. X. Liu, L. Hu, G. Ge, B. Yang, J. Ning, S. Sun, L. Yang, K. Pors and J. Gu, *Proteomics*, 2014, **14**, 1943-1951.
15. J. M. Pratt, D. M. Simpson, M. K. Doherty, J. Rivers, S. J. Gaskell and R. J. Beynon, *Nat Protoc*, 2006, **1**, 1029-1043.
16. F. Zhou, Y. Lu, S. B. Ficarro, G. Adelmant, W. Jiang, C. J. Luckey and J. A. Marto, *Nat Commun*, 2013, **4**, 2171.
17. M. A. Kuzyk, D. Smith, J. Yang, T. J. Cross, A. M. Jackson, D. B. Hardie, N. L. Anderson and C. H. Borchers, *Mol Cell Proteomics*, 2009, **8**, 1860-1877.
18. D. J. Janecki, K. G. Bemis, T. J. Tegeler, P. C. Sanghani, L. Zhai, T. D. Hurley, W. F. Bosron and M. Wang, *.Anal Biochem*, 2007, **369**, 18-26.
19. Y. Sato, A. Miyashita, T. Iwatsubo and T. Iwatsubo, *Drug Metab Dispos*, 2012, **40**, 1389-1396.
20. Y. Sato, M. Nagata, A. Kawamura, A. Miyashita and T. Miyashita, *Xenobiotica*, 2012, **42**, 823–829.
21. M. A. Miyashita , B. Kornilayev, T. Duzhak and D. Yakovlev, *Drug Metab Dispos Biol Fate Chem*, 2005, **33**, 1399–1407.
22. A. Al, D. Touboul, J. P. Le Caër , I. Schmitz-Afonso, J. P. Flinois, C, Marchetti, I. De Waziers , A. Brunelle, O. Laprévote, P. Beaune., *Anal Bioanal Chem*, 2014, **406**, 4861–4874.
23. Y. Zhao, W. Jia, W. Sun, W. Jin, L. Guo, J. Wei, Y. Zhang, Y. Xie, Y. Jiang, F. He and X. Qian, *Journal of Proteome Research*, 2010, **9**, 3319–3327.
24. X. Wang, W. Qin, H. Lin, J. Wang, J. Wei, Y. Zhang and X. Qian, *Analyst*, 2013, **138**, 5309–5317.
25. S. Ohtsuki, O. Schaefer, H. Kawakami, T. Inoue, S. Liehner, A. Saito, N. Ishiguro, W. Kishimoto, E. Ludwig-Schwellinger, T. Ebner and T. Terasaki, *Drug Metab Dispos*, 2012, **40**, 83–92.
26. X. Wang, W. Qin and Y. Zhang, Chinese Chromatography 2012, **30**, 239-244.
27. J. Li, L. Zhou, H. Wang, H. Yan, N. Li, R. Zhai, F. Jiao, F. Hao, Z. Jin, F. Tian, B. Peng, Y. Zhang and X. Qian, *Analyst*, 2015, **140**, 1281-1290.
28. R. J. Beynon, M. K. Doherty, J. M. Pratt and S. J. Gaskell, *Nature methods*, 2015, **2**, 587-589.

29. J. R. Whiteaker, C. Lin, J. Kennedy, L. Hou, M. Trute, I. Sokal1, P.Yan, R. M. Schoenherr, L. Zhao, U. J. Voytovich, K. S. Kelly-Spratt, A. Krasnoselsky, P. R. Gafken, J. M Hogan, L. A Jones, P.Wang, L. Amon, L.A. Chodosh, P. S. Nelson, M. W. McIntosh, C. J. Kemp and A. G. Paulovich, *nature biotechnology*, 2011, **29**, 625-634.

30. S. Pan, R. Aebersold, R. Chen, J. Rush, D. R. Goodlett, M. W. McIntosh, J. Zhang and T. A. Brentnall, *Journal of Proteome Research*, 2009, 8, 787–797.

31. A. Gustavo and M. Angeles, *Trends in Analytical Chemistry*, 2007, 26, No. 3.

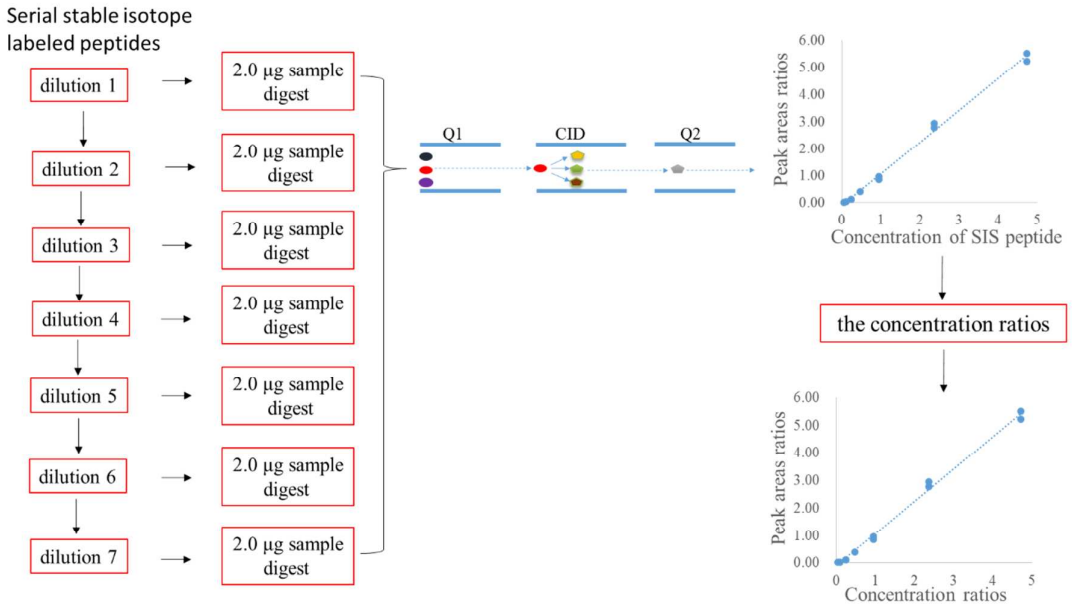

Fig. 1 A scheme of plotting a linear curve

Table 1 The recovery of the peptide ASGNLIPQEK using our method

transitions	measured amount (fmol)		difference	the average of measured amount (fmol)	the theoretical amount (fmol)	recovery
	original sample digest	prepared sample digest				
528.8/507.3	90.1	222.5	132.4			
528.8/620.4	112.0	248.0	136.0	132.6	136.4	97.0%
528.8/904.5	99.7	229.2	129.5			

Three transitions of the peptide ASGNLIPQEK: 528.8/507.3, 528.8/620.4 and 528.8/904.5

Table 2 The recovery of the peptide ASGNLIPQEK calculated by the single concentration point calculation method for five concentration points

measured average amounts(fmol)	added amount (fmol)	recoveries
111.2		82.0%
173.6		127.0%
86.2	136.4	63.0%
82.9		61.0%
191.0		140.0%

Table 3 Precision of measured concentrations of 10 cytochrome P450 enzymes in human liver microsomes

drug metabolizing enzymes	peptides	CVs(%)[a]	fold differences[b]
CYP 1A2	IGSTPVLVLSR	4%	1.08
	ASGNLIPQEK		
CYP 2A6	GYGVVFSNGER	5%	1.14
	GTGGANIDPTFFLSR		
	GTEVYPMLGSVLR		
CYP 2B6	IAMVDPFFR	10%	1.11
	DLIDTYLLHMEK		
CYP 2C8	EALIDNGEEFSGR	6%	1.16
	DQNFLTLMK		
	VQEEIDHVIGR		
CYP 2C9	GIFPLAER	7%	1.20
	SHMPYTDAVVHEVQR		
CYP 2C19	NLAFMESDILEK	3%	1.05
CYP 2E1	GDLPAFHAHR	9%	1.20
	EAHFLLEALR		
	FITLVPSNLPHEATR		
CYP 2D6	AFLTQLDELLTEHR	4%	1.09
	DLTEAFLAEMEK		
	DIEVQGFR		
CYP 3A4	GVVVMIPSYALHR	9%	1.21
CYP 3A5	DTINFLSK	5%	1.10

[a] Coefficient of variation(CV) of five measurements.

[b]Fold differences between largest and lowest value of five replicate measurements.

Table 4 Measured concentrations of 21 drug metabolizing enzymes and comparison with reported data

Drug metabolizing enzymes	This paper mean±SD (fmol/ μ g)	This paper range (fmol/ μ g)	Reference [4] range (fmol/μg)	Reference [25] range (fmol/ μ g)
CYP450 1A2	46.67±27.35	23.21-88.91	2.9 – 103.9	3.26-65.5
CYP450 2A6	48.82±9.37	42.6-64.91	13.6 – 190.8	5.45-168
CYP450 2B6	15.34±7.00	10.22-27.59	1.1 – 173.7	4.05-14.9
CYP450 2C8	35.33±4.11	31.52-41.66		5.66-83.5
CYP450 2C9	103.17±23.94	71.69-137.87	4.4 – 79.4	40.2-115
CYP450 2C19	14.27±9.41	7.05-18.00		2.02-22.2
CYP450 2E1	155.94±31.79	121.94-190.84		36.3-147
CY P450 2D6	12.03±2.49	7.89-13.90	0.1 − 62.4	6.16-36.4
CYP450 3A4	86.78±9.00	74.87-96.62	10.4 − 262.1	6.22-270
CYP450 3A5	29.50±198.27	8.04-458.82	0.6 − 57.2	2.48-17.1
UGT 1A1	153.37±42.21	103.58-202.80	8.9 − 137.9	20.8-59.7
UGT 1A3	112.06±58.87	43.33-182.78	27.0 − 487.7	8.16-37.1
UGT 1A4	68.70±16.54	48.83-90.54	14.4 − 105.6	
UGT 1A6	27.23±5.74	20.98-36.68	31.5 − 285.4	45.0-277
UGT 1A9	49.01±23.91	31.81-84.39	13.4 − 122.6	15.5-38.0
UGT 2B4	73.52±14.82	47.93-83.74	22.8 − 135.8	
UGT 2B7	124.60±11.08	109.28-135.55	33.0− 162.9	53.1-146
UGT 2B10	211.92±118.87	63.22-395.72		
UGT 2B11	<LOQ	<LOQ		
UGT 2B15	204.40±57.88	111.11-260.14	18.4 − 130.2	24.2-103
POR[a]	75.41±12.47	59.27-90.73		

[a] cytochrome P450 oxidoreductase