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**ARTICLE** *in* ANALYTICAL CHEMISTRY · MARCH 2014

Impact Factor: 5.64 · DOI: 10.1021/ac500123z · Source: PubMed



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# Click Chemistry Mediated Eu-Tagging: Activity-Based Specific Quantification and Simultaneous Activity Evaluation of CYP3A4 Using $^{153}$Eu Species-Unspecific Isotope Dilution Inductively Coupled Plasma Mass Spectrometry

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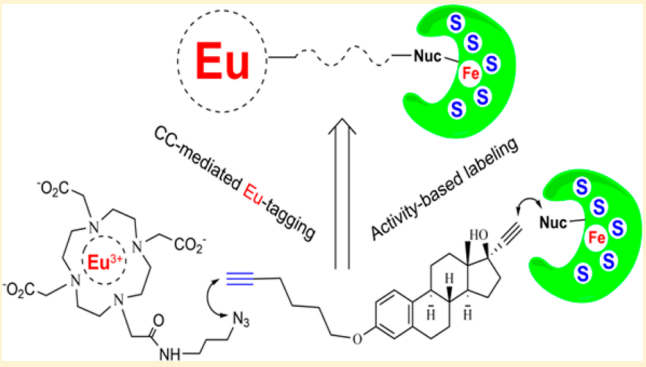
Supporting Information

**ABSTRACT:** P450 3A4 (CYP3A4) is one of the most important isoforms in the human cytochrome P450 superfamily. It was used as an example in this proof-of-concept study in order to demonstrate an activity-based labeling and then click chemistry (CC) mediated element-tagging strategy for simultaneously specific quantification and activity measurement of an enzyme using species-unspecific isotope dilution inductively coupled plasma mass spectrometry (SUID ICPMS). A dual functional hexynylated 17$\alpha$-ethynylestradiol activity-based probe was synthesized for specifically labeling CYP3A4 and then CC-mediated Eu-tagging with an azido-DOTA-Eu complex for CYP3A4 quantification and activity measurement in human liver microsome and serum samples using $^{153}$Eu SUID ICPMS. The LOD (3$\sigma$) of CYP3A4 reached 20.3 fmol when monitoring $^{151/153}$Eu ICPMS signals, in addition to the merits of specificity and simultaneous activity measurement achieved. We believe that this activity-based CC-mediated element-tagging strategy will liberate more potential advantages of ICPMS in bioanalysis.

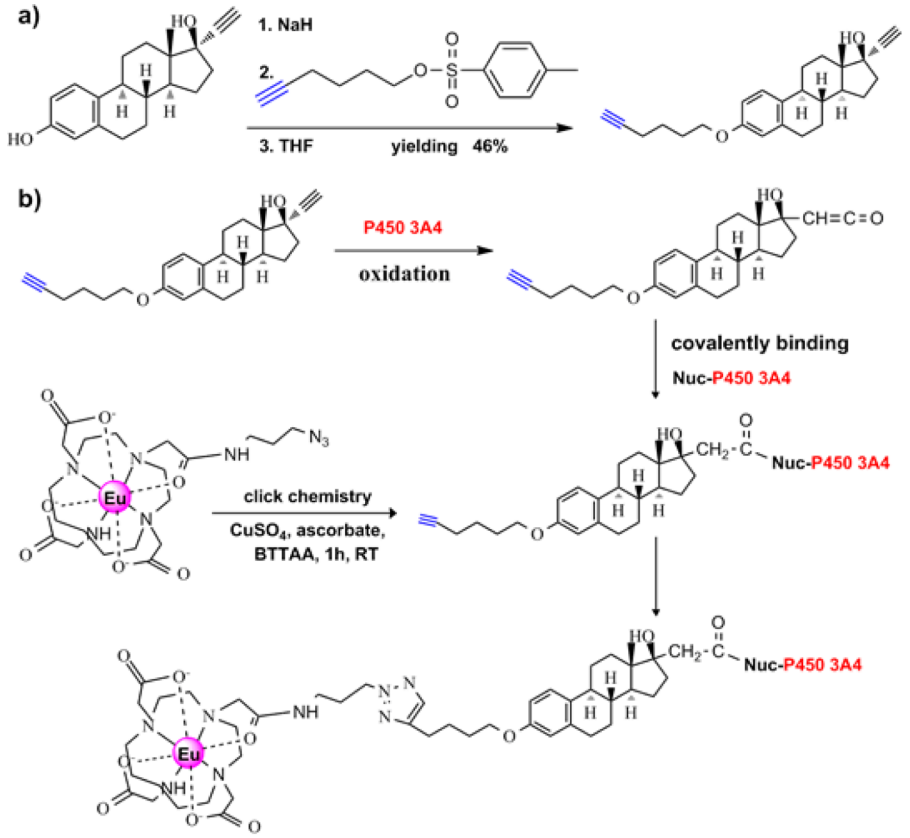



Selectivity of an analytical method is always the objective that analysts try to achieve. After obtaining the general information on a group (or a family) of biomolecules that are similar in both physicochemical and biological properties, the individual information (e.g., quantity and bioactivity) of a targeted member is often desired in understanding its unique role during a certain life process and the diagnosis of a disease and subsequent drug development.[1,2] As a typical example of such a family of biomolecules, cytochrome P450 (a superfamily of heme-thiolated low-expressed monooxygenase) contains more than 50 homologous enzymes of functional significance, which are responsible for the metabolism of a variety of xenobiotics[3] and play key roles in their activation and then oxidation of the inactive C−H bonds in drugs and exogenous compounds.[4] The regulation of their quantities and activities are closely connected to a well-balanced homeostasis and/or an abnormal physiological process of metabolic disturbance, diseases, and even carcinoma.[5−7] As one of the most important members among this family, cytochrome P450 3A4 (CYP3A4) occupies the central position during drugs and xenobiotics oxidative metabolism, accounting for approximately 50% of the total expressed P450 content in the human liver and participating in greater than half the hepatic toxicological processes.[8,9] Therefore, its quantification and activity measurement are important for the diagnosis of liver abnormality and have a great significance for metabolomic study and target-directed drug design.[10−12] Its assay can be performed using traditional protocols such as the Bradford method[13] and enzyme-linked immunosorbent assay (ELISA)[14] through isotope-coded affinity tag (ICAT)[15] and isobaric tags for relative and absolute quantitation (iTRAQ)[16] based electrospray ionization/matrix-assisted laser desorption ionization-mass spectrometry (ESI/MALDI-MS). ELISA using monoantibodies is usually used in P450 detection dependent mainly on the certain structural interactions between an antigen and its antibody while cross-reactivities of antibodies recognization occur frequently;[14,17] the ESI/MALDI-MS techniques and corresponding methods greatly depend on precise protein digestion and the synthesis of isotopic signature peptide standards.[18] In combination with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation and then electroblotting onto membranes, laser-ablation inductively coupled plasma mass spectrometry (ICPMS) was recently used for a simultaneous detection of CYP2B1, CYP2C6, CYP2E1, CYP3A1, and CYP1A1 in rat liver microsomes via the determination of lanthanides (Ln)

**Received:** January 11, 2014
**Accepted:** March 21, 2014
**Published:** March 21, 2014

   dx.doi.org/10.1021/ac500123z | *Anal. Chem.* 2014, 86, 3688−3692

**Scheme 1. Design and Synthesis of the Dual Functional Hexynylated 17α-Ethynylestradiol Activity-Based Probe (a) and Activity-Based Labeling of CYP3A4 then CC-Mediated Eu-Tagging (b)**




immunolabeled on these CYP isoforms,[17,19] and most recently, a multiparametric microarray combining with element-tagged antibodies to simultaneously detect eight CYP isoforms using LA-ICPMS.[20] Along with the development of chemically selective and biospecific element/isotope-tagging strategies, ICPMS is playing an ever more important role from the absolute quantification of proteins/enzymes to tumor cells, DNA, and viruses.[21−32] Considering the fact that 17α-ethynylestradiol (a derivative of 17α-estradiol) can irreversibly and specifically inhibit CYP3A4 through the formation of a ketene intermediate by alkynyl oxidation,[33,34] we introduced a hexyne into 17α-ethynylestradiol at its phenol hydroxyl end via nuclear substitution using hex-5-yn-5-yl-4-methylbenzenesulfonate (Scheme 1a) in this study. After the dual functional hexynylated 17α-ethynylestradiol activity-based probe specifically labeled the CYP3A4, an azido-DOTA-Ln complex was conjugated to the alkyne in the hexynylated 17α-ethynylestradiol molecule to achieve Ln-tagging (Scheme 1b) through click chemistry (CC) under the catalysis of Cu(I). In this way, we could specifically quantify CYP3A4 and evaluate its activity via the signals from the tagged Eu instead of the naturally occurring sulfur and iron in CYP3A4 using $^{153}$Eu species-unspecific isotope dilution (SUID) ICPMS. Sulfur and iron can be theoretically determined but not adept elements on a conventional quadrupole ICPMS because they have relatively low ionization efficiency and suffer from serious spectral interferences from the polyatomic ions formed by oxygen and argon in the ICP. Besides this, we are unable to discriminate which ICPMS signals are coming from which of the CYP isoforms detected.

We first synthesized the dual functional hexynylated 17α-ethynylestradiol activity-based probe toward CYP3A4 (Scheme 1a), and the product was purified using high-pressure liquid chromatography (HPLC) to give a white solid with a yield of 46% (purity >99.5%). It was characterized using ESI-ion trap (IT)-MS and NMR (see Figures S1 and S2 in the Supporting Information). CC of (3 + 2) cyclization between the alkyne from the hexynylated 17α-ethynylestradiol and the azido from azido-DOTA-Eu (Scheme 1b) was confirmed using ESI-IT-MS (Figure S6 in the Supporting Information), in which the more water-soluble ligand BTTAA (Figures S3−S5 in the Supporting Information) was used as a Cu(I) stabilizer in order to avoid the use of TBTA which requires organic solvent (e.g., *tert*-butanol) to dissolve in Tris-HCl buffer and also to increase the efficiency of the cyclization reaction (Figure S6 in the Supporting Information).[35] Moreover, the activity-based hexynylated 17α-ethynylestradiol probe exhibited a pseudo first order kinetics in inactivation of the testosterone (a typical CYP3A4 substrate) hydroxylation under a NADPH-dependent mechanism-based manner (Figure 1). The apparent inactivation constant of the hexynylated 17α-ethynylestradiol $K_I$ = 17.2 ± 1.4 μM, with a maximum inactivation rate constant of $k_{inact}$ = 0.039 ± 0.005 min$^{-1}$ and an inactivation half-life $t_{1/2}$ = 18 ± 2.3 min. Compared with 17α-ethynylestradiol ($K_I$ = 18 μM, $k_{inact}$ = 0.04 min$^{-1}$, $t_{1/2}$ = 16 min),[33] modification of a hexyne at its hydroxybenzene end did not affect the inhibitory ability of the activity-based hexynylated 17α-ethynylestradiol probe toward CYP3A4.

We next tested the specificity of the activity-based hexynylated 17α-ethynylestradiol probe toward CYP3A4. Seven CYP isoforms (CYP3A1, 1A1, 2C6, 2E1, 2D6, 2A2,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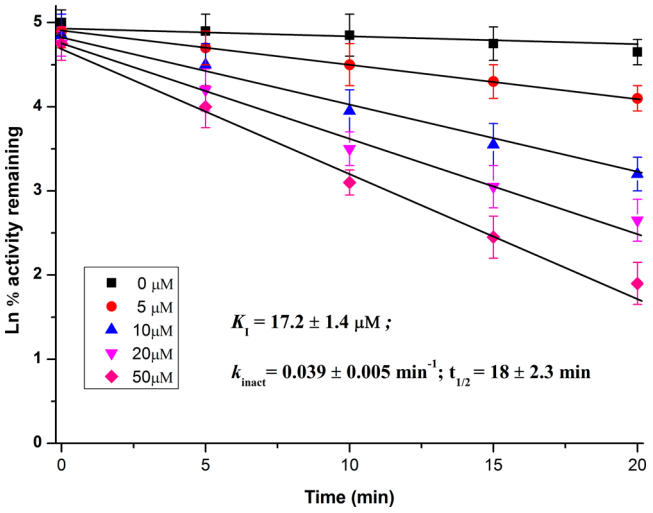


**Figure 1.** Time and concentration dependent inactivation of testosterone 6$\beta$-hydroxylation activity of CYP3A4 by the hexynylated 17$\alpha$-ethynylestradiol probe at concentrations of 0, 5, 10, 20, and 50 $\mu$M under 0, 5, 10, 15, and 20 min inhibition. Each point shown represents the mean from three separate experiments. From the figure, $k_{obs} = [I]k_{inact}/(K_I + [I])$ and $t_{1/2} = 0.693/k_{inact}$,[36] where $k_{obs}$ indicates the apparent inactivation rate constant of CYP3A4 at the initial concentration of the hexynylated 17$\alpha$-ethynylestradiol inhibitor ([I]), which was the slope of the plot between the logarithm of remaining enzymatic activity of CYP3A4 and precubation time $t$; $K_I$, the apparent inactivation constant; and $k_{inact}$, the maximum inactivation rate constant.

and 2B1) from BD Supersomes were selected in addition to CYP3A4, while azido-DOTA-Ln complexes (Ln = Y, La, Pr, Gd, Ho, Er, Yb, and Eu) were prepared in a Tris-HCl buffer (pH = 7.0) for Ln-tagging.[37] After labeling the CYP isoforms in an NADPH-dependent mechanism-based manner with the hexynylated 17$\alpha$-ethynylestradiol at 37 °C for 1 h, Ln-tagging was performed via the click reaction. After the extra free azido-DOTA-Ln complex and hexynylated 17$\alpha$-ethynylestradiol probe were separated from the CYP isoforms using a size-exclusion column, the CYP isoforms tagged with and without azido-DOTA-Ln were subjected to UV and ICPMS determination sequentially (Figure S7 in the Supporting Information). The results obtained are shown in Figure 2. Although all eight CYP isoforms could be detected using a UV detector ($\lambda$ = 214 nm) (Figure 2a) and ICPMS when monitoring $^{57}Fe^+$ and $^{32}S^{16}O^+$ (Figure 2b), only CYP3A4 could be determined based on $^{151/153}Eu$ ICPMS signals together with those of $^{57}Fe^+$ and $^{32}S^{16}O^+$ (Figure 2c,b), and the other seven CYP isoforms that were intended to be tagged using the hexynylated 17$\alpha$-ethynylestradiol and azido-DOTA-Ln loaded with other lanthanides could not be detected. These results indicated that the synthesized hexynylated 17$\alpha$-ethynylestradiol activity-based probe interacted specifically with CYP3A4 and the azido-DOTA-Eu was successfully tagged via the CC.

Clearly, the LOD (3$\sigma$) for CYP3A4 was remarkably improved down to 20.3 fmol when considering the labeling efficiency (74%) (see the Supporting Information) of the activity-based hexynylated 17$\alpha$-ethynylestradiol probe and quantitative click cyclization Eu-tagging, more than 1 and 3 orders of magnitude lower than those obtained when monitoring $^{32}S^{16}O^+$ (0.4 pmol) and $^{57}Fe^+$ (11 pmol), with an RSD of 0.12% at a concentration of 0.1 $\mu$M ($n$ = 6). Linear calibration range between the concentrations of CYP3A4 and

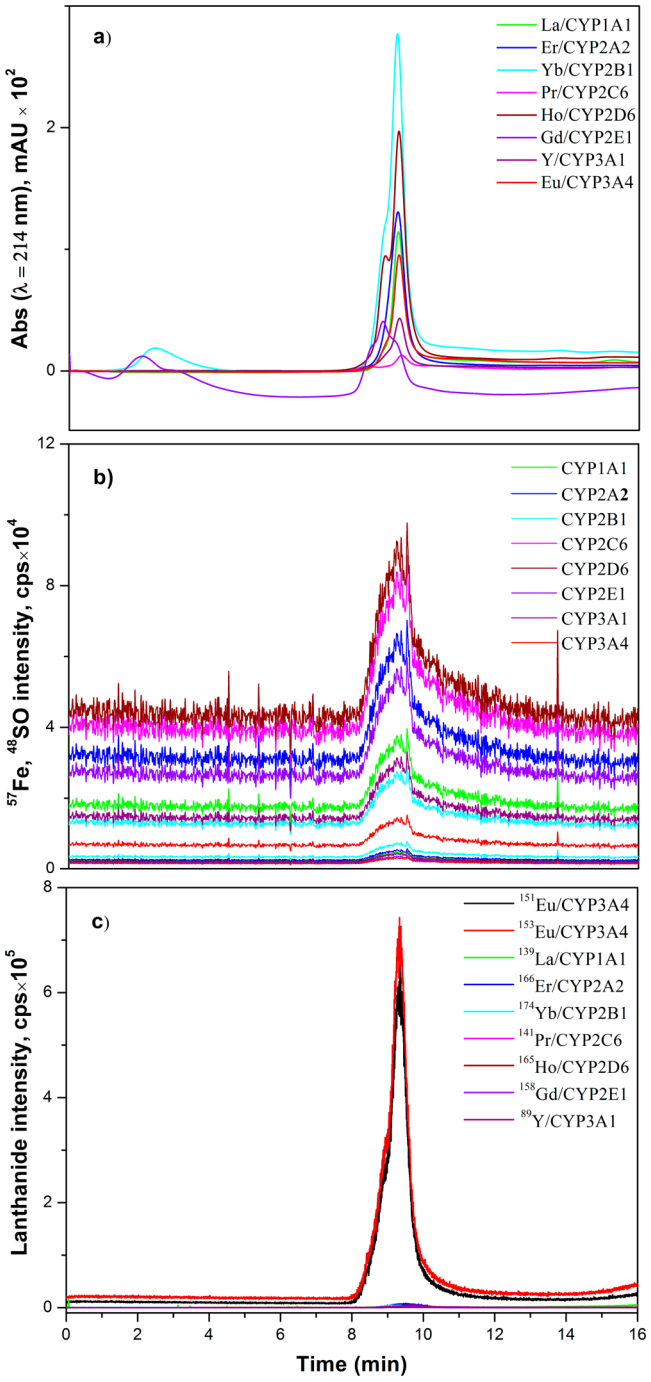


**Figure 2.** UV detection ($\lambda$ = 214 nm) of CYP2D6, 1A1, 2A2, 2B1, 2C6, 2E1, 2A1, and 3A4 (a) and ICPMS when monitoring $^{48}SO^+$ and $^{57}Fe^+$ (b), as well as monitoring $^{89}Y^+$, $^{139}La^+$, $^{141}Pr^+$, $^{158}Gd^+$, $^{165}Ho^+$, $^{166}Er^+$, $^{174}Yb^+$, and $^{151/153}Eu^+$ (c).

the tagged Eu were obtained from 0 to 0.5 $\mu$M (higher concentrations were not tested) and 0 to 0.3 $\mu$M with the correlation coefficient $R^2$ = 0.9894 in Tris-buffer and 0.9375 in phosphate buffer after the Eu mass flow conversion (Figure S8 in the Supporting Information).[37,38] Regardless of these merits, a much lower background of Eu in the biological system and $m/z$-based resolution for Eu isotopes ($^{151/153}Eu$) on ICPMS together with the specificity of the activity-based hexynylated 17$\alpha$-ethynylestradiol probe and high CC-based azido-DOTA-Eu tagging efficiency should facilitate the specific quantification


dx.doi.org/10.1021/ac500123z | Anal. Chem. 2014, 86, 3688−3692

and activity evaluation of CYP3A4 among the P450 superfamily. We then applied the developed activity-based specific labeling and Eu-tagging strategy to absolute quantification of CYP3A4 in human liver microsome samples from BD Supersomes and its activity measurement using $^{153}$Eu SUID ICPMS. The concentration of CYP3A4 in the human liver microsome samples was determined to be (1.04 ± 0.05) $\mu$M ($n$ = 6) (Figure S9 in the Supporting Information), which corresponded well with the value of 1 $\mu$M CYP3A4 that the producer provided. For the evaluation of CYP3A4 activity, the traditional testosterone 6$\beta$-hydroxylase activity assay was conducted using HPLC/ESI-IT-MS (Figure S10 and Table S1 in the Supporting Information). In parallel, instead of testosterone, the hexynylated 17$\alpha$-ethynylestradiol probe was used to inhibit CYP3A4 and then Eu-tagging for activity evaluation of CYP3A4 using $^{153}$Eu-SUID ICPMS. The enzymatic activity of CYP3A4 toward testosterone determined using HPLC/ESI-IT-MS was plotted against the Eu concentration determined using $^{153}$Eu-SUID ICPMS. They were fitted to a linear curve ($R^2$ = 0.9887) (Figure 3), suggesting that we

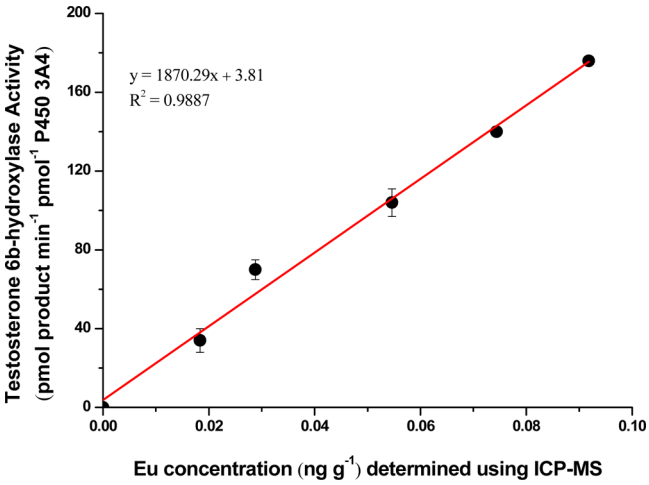


**Figure 3.** Plot of 6$\beta$-testosterone hydroxylase activity of CYP3A4 vs the tagged Eu concentration determined.

are able to directly readout the CYP3A4 activity. We further validated the specificity and overall recovery of this method developed by adding a known amount of CYP3A4 (50 pmol) together with Cytochrome $b_5$ and reductase into the serum samples (Supporting Information). The results obtained indicated that the recovery of CYP3A4 was (96.2 ± 3.8)% while those of other CYP isoforms detected were below 1% (Figures S9 and S10 in the Supporting Information), and the activity of 50 pmol CYP3A4 was measured to be (36.2 ± 2.6) pmol 6$\beta$-testosterone min$^{-1}$ pmol$^{-1}$ CYP3A4 (Figure S13 in the Supporting Information).

In conclusion, we developed a novel dual functional activity-based probe followed by CC-mediated element-tagging strategy. It allows ICPMS to be capable of quantifying a targeted enzyme and simultaneously measuring the enzyme activity. We believe that this activity-based labeling and CC-mediated element-tagging strategy will pave the way forward for the specific quantification and simultaneous activity measurement of biologically significant enzymes using ICPMS, not limited only to CYP3A4 as demonstrated here, but others when more suitable activity-based probes are designed and synthesized. Such research is ongoing on in our laboratory.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This study was financially supported by the National Basic Research 973 Program (Grant 2014CB932004) and the National Natural Science Foundation of China (Grants 21035006 and 21275120). We thank Professor John Hodgkiss for his help with the English.

## ■ REFERENCES

(1) Bunnage, M. E.; Chekler, E. L.; Jones, L. H. *Nat. Chem. Biol.* **2013**, *9*, 195−199.
(2) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Bachovchin, D. A.; Mowen, K.; Baker, D.; Cravatt, B. F. *Nature* **2010**, *468*, 790−795.
(3) Lewis, D. F. *Pharmacogeoemics* **2004**, *5*, 305−318.
(4) Ortiz de Montellano, P. R. *Chem. Rev.* **2009**, *110*, 932−948.
(5) McFadyen, M. C. E.; Melvin, W. T.; Murray, G. I. *Mol. Cancer Ther.* **2004**, *3*, 363−371.
(6) Guengerich, F. P. *Mol. Interventions* **2003**, *3*, 194−204.
(7) Pikuleva, I. A.; Waterman, M. R. *J. Biol. Chem.* **2013**, *288*, 17091−17098.
(8) Rostami-Hodjegan, A.; Tucker, G. T. *Nat. Rev. Drug Discovery* **2007**, *6*, 140−148.
(9) Isin, E. M.; Guengerich, F. P. *J. Biol. Chem.* **2007**, *282*, 6863−6874.
(10) Zanger, U. M.; Turpeinen, M.; Klein, K.; Schwab, M. *Anal. Bioanal. Chem.* **2008**, *392*, 1093−1108.
(11) Neve, E. P.; Ingelman-Sundberg, M. *Anal. Bioanal. Chem.* **2008**, *392*, 1075−1084.
(12) Oesch-Bartlomowicz, B.; Oesch, F. *Anal. Bioanal. Chem.* **2008**, *392*, 1085−1092.
(13) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248−254.
(14) Gelboin, H. V.; Krausz, K. *J. Clin. Pharmacol.* **2006**, *46*, 353−372.
(15) Jenkins, R. E.; Kitteringham, N. R.; Hunter, C. L.; Webb, S.; Hunt, T. J.; Elsby, R.; Watson, R. B.; Williams, D.; Penningtonand, S. R.; Park, B. K. *Proteomics* **2006**, *6*, 1934−1947.
(16) Wang, Y.; Al-Gazzar, A.; Seibert, C.; Sharif, A.; Lane, C.; Griffiths, W. J. *Biochem. Soc. Trans.* **2006**, *34*, 1246−1251.
(17) Waentig, L.; Jakubowski, N.; Roos, P. H. *J. Anal. At. Spectrom.* **2011**, *26*, 310−319.
(18) Langenfeld, E.; Meyer, H. E.; Marcus, K. *Anal. Bioanal. Chem.* **2008**, *392*, 1123−34.
(19) Roos, P. H.; Venkatachalam, A.; Manz, A.; Waentig, L.; Koehler, C. U.; Jakubowski, N. *Anal. Bioanal. Chem.* **2008**, *392*, 1135−1147.
(20) Waentig, L.; Techritz, S.; Jakubowski, N.; Roos, P. H. *Analyst* **2013**, *138*, 6309−6315.
(21) Nuevo Ordoñez, Y.; Montes-Bayón, M.; Blanco-González, E.; Sanz-Medel, A. *Anal. Chem.* **2010**, *82*, 2387−2394.
(22) Mounicou, S.; Szpunar, J.; Lobinski, R. *Chem. Soc. Rev.* **2009**, *38*, 1119−1138.
(23) Wang, M.; Feng, W. Y.; Zhao, Y. L.; Chai, Z. F. *Mass Spectrom. Rev.* **2010**, *29*, 326−348.
(24) Yan, X. W.; Yang, L. M.; Wang, Q. Q. *Angew. Chem., Int. Ed.* **2011**, *50*, 5130−5133.

(25) Sanz-Medel, A.; Montes-Bayón, M.; Bettmer, J.; Ferández Sánchez, M. L.; Ruiz Encinar, J. *Trends Anal. Chem.* **2012**, *40*, 52−63.
(26) Yan, X. W.; Yang, L. M.; Wang, Q. Q. *Anal. Bioanal. Chem.* **2013**, *405*, 5663−5670.
(27) Liu, R.; Wu, P.; Yang, L.; Hou, X. D.; Lv, Y. *Mass Spectrom. Rev.* **2013**, *9999*, 1−21.
(28) Zhang, Z. B.; Luo, Q.; Yan, X. W.; Li, Z. X.; Luo, Y. C.; Yang, L. M.; Zhang, Z.; Haifeng Chen, H. F.; Wang, Q. Q. *Anal. Chem.* **2012**, *84*, 8946−8951.
(29) Han, G. J.; Zhang, S. C.; Xing, Z.; Zhang, X. R. *Angew. Chem., Int. Ed.* **2013**, *52*, 1466−1471.
(30) Li, F.; Zhao, Q.; Wang, C.; Lu, X. F.; Li, X. F.; Le, X. C. *Anal. Chem.* **2010**, *82*, 3399−3403.
(31) Luo, Y. C.; Yan, X. W.; Huang, Y. S.; Wen, R. B.; Li, Z. X.; Yang, L. M; Yang, C. Y. J.; Wang, Q. Q. *Anal. Chem.* **2013**, *85*, 9428−9432.
(32) Brückner, K.; Schwarz, K.; Beck, S.; Linscheid, M. W. *Anal. Chem.* **2014**, *86*, 585−591.
(33) Lin, H. L.; Kent, U. M.; Hollenberg, P. F. *J. Pharmacol. Exp. Ther.* **2002**, *301*, 160−167.
(34) Wright, A. T.; Song, J. D.; Cravatt, B. F. *J. Am. Chem. Soc.* **2009**, *131*, 10692−10700.
(35) Besanceney-Webler, C.; Jiang, H.; Zheng, T.; Feng, L.; Soriano del Amo, D.; Wang, W.; Klivansky, L. M.; Marlow, F. L.; Liu, Y.; Wu, P. *Angew. Chem., Int. Ed.* **2011**, *50*, 8051−8056.
(36) Zhou, S. F.; Chan, S. Y.; Goh, B. C.; Chan, E.; Duan, W.; Huang, M.; Mcleod, H. L. *Clin. Pharmacokinet.* **2005**, *44*, 279−304.
(37) Yan, X. W.; Xu, M.; Yang, L. M.; Wang, Q. Q. *Anal. Chem.* **2010**, *82*, 1261−1269.
(38) Yan, X. W.; Luo, Y. C.; Zhang, Z. B.; Li, Z. X.; Luo, Q.; Yang, L. M.; Zhang, B.; Chen, H. F.; Bai, P. M.; Wang, Q. Q. *Angew. Chem., Int. Ed.* **2012**, *51*, 3358−3363.