Rapid Communications in Mass Spectrometry

Received: 30 September 2015 Revised: 15 November 2015 Accepted: 17 November 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2016**, *30*, 491–496
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7465

Elucidation of a CGP7930 *in vitro* metabolite by liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry

Haidong Wang, Wenxiang Zhang and Xian Wang*

Key Laboratory of Catalysis and Materials Science of the State Ethnic Affairs Commission and Ministry of Education, College of Chemistry and Materials Science, South-Central University for Nationalities, Wuhan, Hubei 430074, P.R. China

RATIONALE: γ-Aminobutyric acid-B ($GABA_B$) receptors are widely expressed in the nervous system and have been implicated as targets for various neurological and psychiatric disorders. CGP7930 is a positive allosteric modulator of $GABA_B$ receptors. It has been demonstrated to reduce drug self-administration and has gained increased research as a potential psychotropic treatment.
METHODS: An *in vitro* metabolic system with liver microsomes of SD rats has been conducted and evaluated by probe drugs. The predominant *in vitro* metabolite of CGP7930 was identified and elucidated using liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry (LC/ESI-QTOF-MS/MS). Its structure was determined by comparing the characteristic ions of CGP7930 and those of the metabolite, based on the accurate mass measurement by MS and the fragmentation pattern obtained by MS/MS.
RESULTS: We found that the main metabolic pathway of CGP7930 was via a monohydroxylation reaction and the hydroxylation site located at the terminal butyl-carbon. The collision-induced dissociation (CID) fragmentation of the hydroxylated metabolite underwent McLafferty rearrangement and α-cleavage.
CONCLUSIONS: This work provides an understanding of the *in vitro* metabolism of CGP7930, which is helpful for the further study of the development of potential drug candidates targeting $GABA_B$ receptors, for the treatment of depression. The work also demonstrates that the LC/ESI-QTOF-MS/MS method has the advantage of possibly determining the structures of drug metabolites without the use of standards. Copyright © 2016 John Wiley & Sons, Ltd.

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the mammalian central nervous system. Because GABA regulates neuronal excitability and GABA receptors are widely distributed throughout the brain and the nervous system, drugs targeting GABA receptors can profoundly regulate behaviour.[1] $GABA_B$, one of the major GABA receptors, belong to the G protein-coupled receptor family and are generally considered to be excellent drug targets.[2] The $GABA_B$ receptors play an important role in the treatment of depression and anxiety.[3] However, conventional drug development targeting $GABA_B$ receptors mainly focuses on agonists and antagonists which have the limitations of side effects, poor pharmacokinetics, and the development of tolerance.[4] For example, baclofen, a $GABA_B$ agonist, is currently a commercial drug for treating depression and other various mental disorders, but it can result in adverse reactions and drug fastness.[5,6] Therefore, recent studies have tended to explore allosteric modulators as potential drug candidates, instead of agonists and antagonists for drug development.

* *Correspondence to:* X. Wang, College of Chemistry and Materials Science, South-Central University for Nationalities, Wuhan, Hubei 430074, P.R. China.
E-mail: xwang27@hotmail.com

3,5-Bis(1,1-dimethylethyl)-4-hydroxy-β,β-dimethylbenzene-propanol (CGP7930) is a positive allosteric modulator of $GABA_B$ receptors.[7,8] Compared to baclofen, CGP7930 has the advantage of treating drug abuse and positive psychotropic effects,[9] which may be very useful in the treatment of depression and anxiety. The literature contains reports of research on the pharmacological profile[10] and effects of CGP7930 on food intake,[8] anxiolytic[11] and depression,[12] but the *in vitro* metabolism of CGP7930 has rarely been studied.

The investigations of *in vivo* and *in vitro* metabolites are very critical in the development of drug candidates.[13,14] Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has nowadays become a very important technique to probe and characterize metabolites for drug study.[15,16] Tandem mass spectrometry has been successfully applied to the structural elucidation of metabolites by coupling a HPLC (high-performance liquid chromatography) column with a triple-quadrupole (QQQ),[17,18] a quadrupole ion trap (QIT),[19,20] or a quadrupole time-of-flight (QTOF)[21,22] mass spectrometer. In LC/MS/MS analysis, the chromatograms of complex metabolite profiles are resolved by HPLC separation, and full scan MS and product ion scan MS/MS spectra are obtained online for structural characterization.[23,24] Therefore, LC/MS/MS has shown the advantage of making it possible to speculate the structure of drug metabolites rapidly and systematically without the use of standards.

 Copyright © 2016 John Wiley & Sons, Ltd.

In order to predict the *in vivo* drug metabolism, the *in vitro* drug metabolism by using human liver microsomes or hepatocytes is often studied prior to animal experiments.[25,26] Such *in vitro* studies are significantly useful for the explanation of metabolite generation and the development of toxicology studies. Furthermore, it also provides data for predicting pharmacokinetic variability and potential drug-drug interactions before clinical trials in humans.[14,27,28]

In this work, we have developed a LC/ESI-QTOF-MS/MS (liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry) method to investigate the major CGP7930 *in vitro* metabolite by a liver microsomal incubation system. The accurate molecular weight of CGP7930 metabolite was measured by a TOFMS full scan with high resolution. The fragmentation pattern of the target metabolite ion was obtained from collision-induced dissociation (CID) MS/MS analysis for structural elucidation. We have determined the structure of the monohydroxylation metabolite of CGP7930 and proposed the fragmentation mechanism including a McLafferty rearrangement. The present study helps us understand the *in vitro* metabolism of CGP7930, and also assists the further *in vivo* metabolism study of CGP7930 and safety evaluation of anti-depressant drugs.

EXPERIMENTAL

Chemicals and materials

CGP7930 (purity ≥98%) was purchased from Tocris Bioscience Co. Ltd. (Bristol, UK). Midazolam (purity >97%) and testosterone (purity >98%) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Liver microsomes of SD female rats (20 mg/mL) were purchased from Nuojia Biological Technology Co., Ltd. (Guangzhou, China). D-Glucose 6-phosphate (purity ≥98%), NADP (purity ≥98%), glucose-6-phosphate dehydrogenase (200–400 units/mg) and phosphate-buffered saline tablets were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA). Analytical reagent (AR) grade $MgCl_2$ and HPLC grade ethyl acetate were obtained from Kermel Chemical Reagent Co. (Tianjin, China). Ultra-purified water was obtained from a water purification system (Mole Scientific Instrument Co., Ltd., Shanghai. China).

In vitro incubation with liver microsomes and sample preparation

The *in vitro* incubations were carried out in 1.5 mL polystyrene vials with 360 μL of a mixture which contained 200 μL potassium phosphate buffer (pH 7.4, 0.1 M), 50 μL liver microsomes (20 mg/mL), 10 μL substrate (i.e. 0.2 mM midazolam, testosterone or CGP7930), 40 μL $MgCl_2$ (5 mM), 20 μL glucose-6-phosphate dehydrogenase (200–400 units/mg), and 40 μL D-glucose 6-phosphate (10 mM). The mixture was shaken for 5 min for equilibration in a water bath at 37 °C before adding NADP to initiate the reactions. Anaerobic incubation was performed as described in the literature.[29] Briefly, the incubation was carried out at 37 °C in the dark for 30 min and the mixture was extracted using 500 μL ice-cooled ethyl acetate. The sample was vortexed and centrifuged for 10 min. The organic layer was collected and evaporated by a stream of nitrogen gas at 37 °C. The dried residue was constituted with 100 μL of acetonitrile and water (v/v, 1:1) prior to injection onto the HPLC column for LC/MS and LC/MS/MS analysis. Sample preparations were conducted in four replicates for testing the reproducibility.

LC/ESI-MS and LC/ESI-MS/MS analysis

HPLC experiments were conducted on a model 1200 LC system (Agilent, Santa Clara, CA, USA). A 2.1 × 150 mm, ZORBAX Eclipse XDB-C18 reversed-phase column was used for separation and the injection volume was 5 μL. The mobile phases were water (A) and acetonitrile (B). The solvent gradient was started from 25%B, programmed to 50%B in another 5 min, and raised to 65%B in another 5 min, then increased to 98%B at 20 min, and finally returned to 25%B in 10 min. All mass spectra and collision-induced dissociation (CID) MS/MS spectra were acquired from a model 6520 quadrupole time-of-flight mass spectrometer (Agilent, Santa Clara, CA, USA) with a standard ESI source. The samples were injected by an autosampler and introduced into the ESI source by a quaternary pump at a flow rate of 0.3 mL/min. The source parameters were optimized as follows: gas temperature, 300 °C; flow rate of the drying gas, 12 L/min; nebulizer pressure, 40 psi; capillary voltage, 3500 V; fragmentor voltage, 125 V. The collision energy used for the MS/MS scan was 40 eV.

RESULTS AND DISCUSSION

Confirmation of *in vitro* microsomal incubation system using probe drugs

Because CYP3A4 was the most important isoform in the P450 enzyme system, its specific probe drugs, namely testosterone and midazolam, were chosen in this work to evalutate the *in vitro* microsomal incubation system.[30] The metabolites of testosterone and midazolam are known to be 6β-OH-testosterone[31,32] and 1-OH-midazolam,[33–35] respectively. Hence, the signal responses for these probe drugs and their metabolites were measured by LC/ESI-QTOFMS to assess the activity of CYP450 enzymes in the used liver microsomes. In addition, the accurate masses of probe drugs and their metabolites were measured and compared with theoretical values to assess the mass accuracy of the QTOFMS analysis of CGP7930 and its metabolites for quality assurance (QA).

In vitro incubation of these probe drugs followed the same protocol as that for CGP7930 described above, and the ESI-MS analysis was performed in positive ion mode. The extracted ion chromatogram (EIC) and ESI-MS spectra of testosterone and the metabolite species are given in Supplementary Fig. S1 (see Supporting Information). The protonated ion of testosterone was detected at *m/z* 289.2186 and its metabolite ion was seen 16 m/z higher than that, which was the monohydroxylated testosterone. Testosterone and its metabolite (i.e. 6β-OH-testosterone) were assumed to have the same ESI-MS responses and so the metabolic rate of testosterone was calculated to be 65.1% according to the ratio of their peak areas.

Similar to the testosterone system, midazolam and its main metabolite, 1-OH-midazolam, were analyzed by LC/ESI-QTOFMS (the spectra are given in Supplementary Fig. S2,

 Copyright © 2016 John Wiley & Sons, Ltd.

see Supporting Information). The metabolic rate of midazolam was calculated as 40.3%. These experiments demonstrated that the incubation system using such liver microsomes was valid to metabolize the probe drugs and thus could be used for the further study of CGP7930 metabolism *in vitro*.

Characterization of CGP7930 and its metabolite by LC/ESI-MS

The CGP7930 *in vitro* metabolism was carried out by using the same incubation system as the probe drugs. LC/ESI-QTOFMS analysis was performed to separate and identify CGP7930 and its metabolite, for which their EIC and negative ion ESI-MS spectra are given in Fig. 1. The deprotonated CGP7930 at *m/z* 291 (the exact mass *m/z* 291.2393 ± 5 ppm) was eluted at the retention time of 17.5 min and the deprotonated metabolite at *m/z* 307 (the exact mass *m/z* 307.2390 ± 5 ppm) was eluted at 14.2 min. Comparing the MS data of both ions, we found that the mass-to-charge ratio of the metabolite was 16 Da higher than that of CGP7930 (Fig. 2). Because cytochromes P450 (P450s) in liver microsomes function as terminal oxidases in multi-component mono-oxygenase systems,[36,37] CGP7930 can be oxygenated by P450s to produce hydroxylation or epoxidation metabolites. Hence, the metabolite at *m/z* 307 could be generated from the mono-oxidation of CGP7930 catalyzed by P450s, resulting in the addition of one oxygen atom with a *m/z* shift of 16 Da. This metabolism could occur either via an epoxidation or a monohydroxylation. However, all the possible epoxidized sites on the phenyl ring of CGP7930 were occupied by alkyl or phenol groups and so CGP7930 was unlikely to be epoxidized. Therefore, we presumed that *m/z* 307 was the deprotonated ion of the monohydroxylated metabolite that was formed by the *in vitro* incubation of CGP7930 with liver microsomes. This metabolite detected by ESI-MS was also subjected to subsequent ESI-MS/MS analysis for structural confirmation.

Identification of metabolite structure and fragmentation profile by ESI-MS/MS

In order to determine the molecular structure of the metabolite, MS/MS spectra of the deprotonated CGP7930 and the metabolite ions were examined. Due to the simple

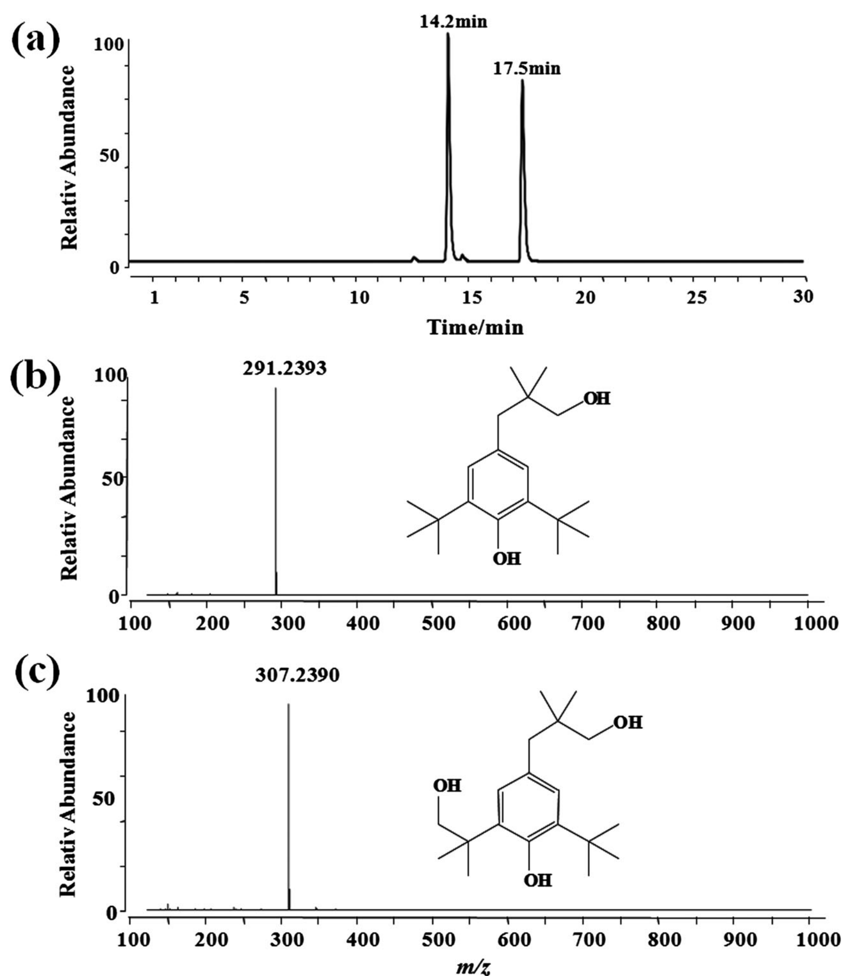

Figure 1. (a) The extracted ion chromatogram (EIC) of *in vitro* CGP7930 metabolism. CGP7930 eluted at 17.5 min and the metabolite eluted at 14.2 min. (b) The ESI-MS spectrum of CGP7930. (c) The ESI-MS spectrum of the monohydroxylation metabolite of CGP7930.

 Copyright © 2016 John Wiley & Sons, Ltd.

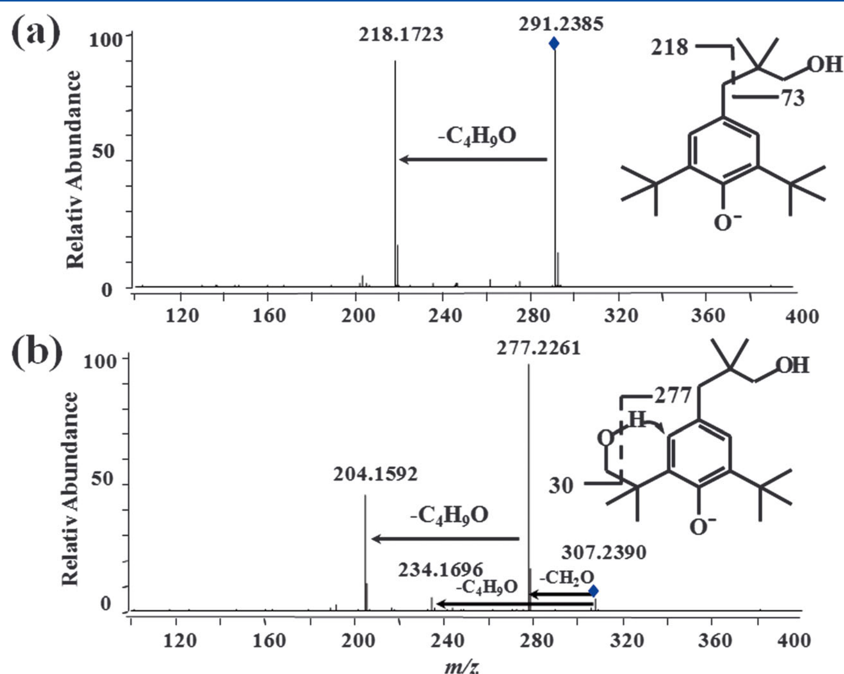

Figure 2. ESI-MS/MS spectra of CGP7930 (a) and the metabolite (b) at 40 eV collision energy.

and stable structure of CGP7930, its MS/MS spectrum only showed two abundant ions: one was the precursor ion at *m/z* 291 (the exact mass *m/z* 291.2385 ± 5 ppm) and the other one was the fragment ion at *m/z* 218 (the exact mass *m/z* 218.1723 ± 5 ppm) after CID at 40 eV collision energy (Fig. 2). The *m/z* value of the fragment ion is 73 Da lower than that of the precursor ion. $[M–H]^-$ of CGP7930 could undergo a favoured α-cleavage at the benzylic position by a neutral loss of $-C(CH_3)_2CH_2OH$ (i.e., $-C_4H_9O$, 73 Da). The fragmentation of CGP7930 $[M–H]^-$ was also used as the reference for helping identify the fragment ions of the metabolite moiety. The MS/MS spectra of the metabolite gave prominent ions at *m/z* 307, 277, 234, 204 (the exact mass *m/z* were 307.2390, 277.2261, 234.1696, 204.1592 ± 10 ppm, see Fig. 1), respectively. The ion at *m/z* 307, 16 Da higher than $[M–H]^-$ of CGP7930, was the undissociated precursor ion $[M–H]^-$ of the metabolite. Very similar to CGP7930, the fragment ion with *m/z* 234 resulted from a neutral loss of 73 Da ($-C_4H_9O$) via an α-cleavage on the precursor ion, which was also 16 Da higher than the corresponding fragment ion of CGP7930 at *m/z* 218. Moreover, this demonstrated that the metabolized monohydroxylation of CGP7930 did not occur on this benzenepropanol substitution of CGP7930, which would otherwise have lost a neutral mass of 73 + 16 Da. Because CGP7930 has two symmetric 3,5-bis(1,1-dimethylethyl) substitutions on the benzene ring, the chance of monohydroxylation reaction on both butyl groups is equivalent. Monohydroxylation on any of the six methyl carbons results in indistinguishable molecules. This explained why only one main metabolite was detected by ESI-MS after CGP 7930 was incubated with liver microsomes. It was noted that under the same CID condition at 40 eV, the intensity of the metabolite precursor ion $[M–H]^-$ at *m/z* 307 was significantly lower

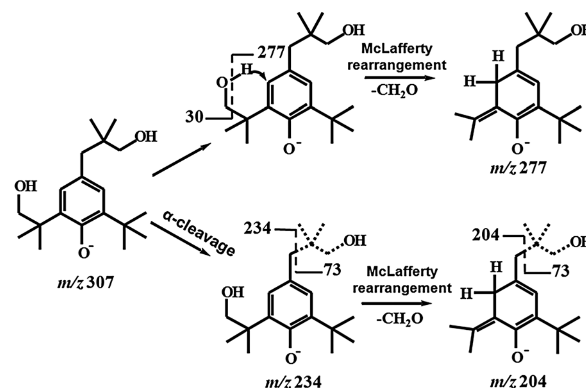

Figure 3. Proposed fragmentation mechanisms for the deprotonated monohydroxylation metabolite of CGP7930.

than that of CGP7930 at *m/z* 291 (see Fig. 2). Moreover, the MS/MS fragmentation of *m/z* 307 produced a base peak for the fragment ion at *m/z* 277 by the loss of 30 Da, which was ascribed to a neutral molecule loss via a rearrangement. The -OH group on the monohydroxylated site of the metabolite was an γ-H donor and the benzene ring was a typical H-acceptor. It readily underwent McLafferty rearrangement and lost a CH_2O with 30 Da to produce the fragment ion of *m/z* 277. This characteristic ion also confirmed our assumption of the metabolite structure that the monohydroxylation occurred at the *ortho*-butyl group of CGP 7930. This fragment ion at *m/z* 277 could further undergo an α-cleavage and yielded a fragment ion at *m/z* 204 by the loss of 73 Da. The structure of the monohydroxylation metabolite of CGP7930 and the fragmentation mechanisms are illustrated in Fig. 3.

Copyright © 2016 John Wiley & Sons, Ltd.

CONCLUSIONS

This work employed a LC/ESI-QTOF-MS/MS method to explore the *in vitro* metabolite of the positive allosteric modulator of the $GABA_B$ receptor: CGP7930. The metabolite of CGP7930 was obtained from *in vitro* incubation with liver microsomes of SD female rats. MS and MS/MS analyses revealed that the major metabolism of CGP7930 was the monohydroxylation and the molecular structure of the metabolite was identified for the first time. In addition, the fragmentation pathways of CGP7930 and of the monohydroxylation metabolite were elucidated to assist the determination of the metabolite structures. Fragmentation mechanisms involving a McLafferty rearrangement were proposed for the production of major fragment ions in the MS/MS of the CGP7930 metabolite. Experimental evidence demonstrated that the location of monohydroxylation was on one of the terminal carbons of the 1,1-dimethylethyl groups in CGP7930. This present study contributes to the understanding of the *in vitro* metabolism of CGP7930 and is useful for the development and validation of potential drug candidates based on positive allosteric modulators targeting $GABA_B$ receptors for treatment of depression.

Acknowledgements

The authors thank the National Natural Science Foundation of China (Grant Nos. 21275167 and 21275166), the National Natural Science Foundation of Hubei Province (Grant No. 2014CFA025), and the Preffered Research Foundation for the Returned Overseas Scholars from Ministry of Human Resources and Social Security of the People's Republic of China for financial support.

REFERENCES

[1] R. Agabio, G. Colombo. $GABA_B$ receptor ligands for the treatment of alcohol use disorder: preclinical and clinical evidence. *Front. Neurosci.* **2014**, *8*, 1.

[2] B. M. T. Keegan, T. J. R. Beveridge, J. J. Pezor, R. Y. Xiao, T. Sexton, S. R. Childers, A. C. Howlett. Chronic baclofen desensitizes $GABA_B$-mediated G-protein activation and stimulates phosphorylation of kinases in mesocorticolimbic rat brain. *Neuropharm.* **2015**, *95*, 492.

[3] K. Kumar, S. Sharma, P. Kumar, R. Deshmukh. Therapeutic potential of GABA(B) receptor ligands in drug addiction, anxiety, depression and other CNS disorders. *Pharmacol. Biochem. Behav.* **2013**, *110*, 174.

[4] X. Li, V. B. Risbrough, C. Cates-Gatto, K. Kaczanowska, M. G. Finn, A. J. Roberts, A. Markou. Comparison of the effects of the $GABA_B$ receptor positive modulator BHF177 and the $GABA_B$ receptor agonist baclofen on anxiety-like behavior, learning, and memory in mice. *Neuropharm.* **2013**, *70*, 156.

[5] M. Brusberg, A. Ravnefjord, R. Martinsson, H. Larsson, V. Martinez, E. Lindstrom. The $GABA_B$ receptor agonist, baclofen, and the positive allosteric modulator, CGP7930, inhibit visceral pain-related responses to colorectal distension in rats. *Neuropharm.* **2009**, *56*, 362.

[6] T. Gjoni, S. Urwyler. Receptor activation involving positive allosteric modulation, unlike full agonism, does not result in $GABA_B$ receptor desensitization. *Neuropharm.* **2008**, *55*, 1293.

[7] L. N. Cedillo, F. Miranda. Effects of co-administration of the $GABA_B$ receptor agonist baclofen and a positive allosteric modulator of the $GABA_B$ receptor, CGP7930, on the development and expression of amphetamine-induced locomotor sensitization in rats. *Pharmacol. Rep.* **2013**, *65*, 1132.

[8] I. S. Ebenezer. Effects of intraperitoneal administration of the $GABA_B$ receptor positive allosteric modulator 2,6-di-tert-butyl-4-(2-hydroxy-2,2-dimethylpropyl)phenol (CGP7930) on food intake in non-deprived rats. *Eur. J. Pharmacol.* **2012**, *690*, 115.

[9] P. Mares. Anticonvulsant action of $GABA_B$ receptor positive modulator CGP7930 in immature rats. *Epilepsy. Res.* **2012**, *100*, 49.

[10] M. A. Carai, G. Colombo, W. Froestl, G. L. Gessa. In vivo effectiveness of CGP7930, a positive allosteric modulator of the GABAB receptor. *Eur. J. Pharmacol.* **2004**, *504*, 213.

[11] L. H. Jacobson, J. F. Cryan. Evaluation of the anxiolytic-like profile of the $GABA_B$ receptor positive modulator CGP7930 in rodents. *Neuropharm.* **2008**, *54*, *854*.

[12] J. F. Cryan, K. Kaupmann. Don't worry 'B' happy!: a role for $GABA_B$ receptors in anxiety and depression. *Trends Pharmacol. Sci.* **2005**, *26*, 36.

[13] U. Jurva, L. Weidolf. Electrochemical generation of drug metabolites with applications in drug discovery and development. *Trac-Trends Anal. Chem.* **2015**, *70*, 92.

[14] L. Men, Y. L. Zhao, H. L. Lin, M. J. Yang, H. Liu, X. Tang, Z. G. Yu. Characterization of in vitro metabolites of TM-2, a potential antitumor drug, in rat, dog and human liver microsomes using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 2162.

[15] J. Spinner, M. Klima, J. Kempf, L. M. Huppertz, V. Auwarter, M. J. Altenburger, M. A. Neukamm. Determination of drugs of abuse in bovine dentin using liquid chromatography-electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2014**, *49*, 1306.

[16] R. Bandu, H. S. Ahn, J. W. Lee, Y. W. Kim, S. H. Choi, H. J. Kim, K. P. Kim. Liquid chromatography electrospray ionization tandem mass spectrometric (LC/ESI-MS/MS) study for the identification and characterization of in vivo metabolites of cisplatin in rat kidney cancer tissues: Online hydrogen/deuterium (H/D) exchange study. *PloS One* **2015**, *10*, e0134027.

[17] Q. Qian, S. L. Li, E. Sun, K. R. Zhang, X. B. Tan, Y. J. Wei, H. W. Fan, L. Cui, X. B. Jia. Metabolite profiles of icariin in rat plasma by ultra-fast liquid chromatography coupled to triple-quadrupole/time-of-flight mass spectrometry. *J. Pharm. Biomed. Anal.* **2012**, *66*, 392.

[18] K. S. Hakala, R. Kostiainen, R. A. Ketola. Feasibility of different mass spectrometric techniques and programs for automated metabolite profiling of tramadol in human urine. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2081.

[19] G. Cao, H. Cai, Y. Zhang, X. Cong, C. Zhang, B. Cai. Identification of metabolites of crude and processed Fructus Corni in rats by microdialysis sampling coupled with electrospray ionization linear quadrupole ion trap mass spectrometry. *J. Pharm. Biomed. Anal.* **2011**, *56*, 118.

[20] C. Soler, B. Hamilton, A. Furey, K. J. James, J. Manes, Y. Pico. Comparison of four mass analyzers for determining carbosulfan and its metabolites in citrus by liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 2151.

[21] J. Yang, D. Qian, S. Jiang, E. X. Shang, J. Guo, J. A. Duan. Identification of rutin deglycosylated metabolites produced by human intestinal bacteria using UPLC-Q-TOF/MS. *J. Chromatogr. B* **2012**, *898*, 95.

Copyright © 2016 John Wiley & Sons, Ltd.

[22] J. Z. Shen, C. Y. Yang, C. M. Wu, P. S. Feng, Z. H. Wang, Y. Li, Y. S. Li, S. X. Zhang. Identification of the major metabolites of quinocetone in swine urine using ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 375.
[23] S. Ma, M. Zhu. Recent advances in applications of liquid chromatography-tandem mass spectrometry to the analysis of reactive drug metabolites. *Chem. Biol. Interact.* **2009**, *179*, 25.
[24] P. Li, M. G. Bartlett. A review of sample preparation methods for quantitation of small-molecule analytes in brain tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Anal. Methods* **2014**, *6*, 6183.
[25] M. Fantini, M. Benvenuto, L. Masuelli, G. V. Frajese, I. Tresoldi, A. Modesti, R. Bei. In vitro and in vivo antitumoral effects of combinations of polyphenols, or polyphenols and anticancer drugs: Perspectives on cancer treatment. *Int. J. Mol. Sci.* **2015**, *16*, 9236.
[26] E. Kim, H. Kim, K. Suh, S. Kwon, G. Lee, N. H. Park, J. Hong. Metabolite identification of a new tyrosine kinase inhibitor, HM781-36B, and a pharmacokinetic study by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 1183.
[27] T. B. Stage, K. Brosen, M. M. H. Christensen. A comprehensive review of drug-drug interactions with metformin. *Clin. Pharmacokinet.* **2015**, *54*, 811.
[28] J. Joo, B. Lee, T. Lee, K. H. Liu. Screening of six UGT enzyme activities in human liver microsomes using liquid chromatography/triple quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 2405.
[29] W. Chan, L. Cui, G. W. Xu, Z. W. Cai. Study of the phase I and phase II metabolism of nephrotoxin aristolochic acid by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1755.
[30] M. O. Magnusson, R. Sandstrom. Quantitative analysis of eight testosterone metabolites using column switching and liquid chromatography tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1089.
[31] D. D. Wang, M. L. Zhang. Rapid quantitation of testosterone hydroxyl metabolites by ultra-performance liquid chromatography and mass spectrometry. *J. Chromatogr. B* **2007**, *855*, 290.
[32] S. Tachibana, M. Tanaka. Simultaneous determination of testosterone metabolites in liver microsomes using column-switching semi-microcolumn high-performance liquid chromatography. *Anal. Biochem.* **2001**, *295*, 248.
[33] M. Shimizu, T. Uno, H. O. Tamura, H. Kanazawa, I. Murakami, K. Sugawara, T. Tateishi. A developed determination of midazolam and 1′-hydroxymidazolam in plasma by liquid chromatography-mass spectrometry: Application of human pharmacokinetic study for measurement of CYP3A activity. *J. Chromatogr. B* **2007**, *847*, 275.
[34] J. A. Carrillo, S. I. Ramos, J. A. G. Agundez, C. Martinez, J. Benitez. Analysis of midazolam and metabolites in plasma by high-performance liquid chromatography: Probe of CYP3A. *Ther. Drug Monit.* **1998**, *20*, 319.
[35] H. Z. Bu, L. Magis, K. Knuth, P. Teitelbaum. High-throughput cytochrome P450 (CYP) inhibition screening via a cassette probe-dosing strategy. VI. Simultaneous evaluation of inhibition potential of drugs on human hepatic isozymes CYP2A6, 3A4, 2C9, 2D6 and 2E1. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 741.
[36] B. Kunic, T. Makovec, K. Breskvar. Comparison of two monooxygenase systems with cytochrome P450 in filamentous fungus *Rhizopus nigricans*. *Pflugers. Arch.* **2000**, *439*, R107.
[37] E. H. Oliw, J. Bylund, C. Herman. Bisallylic hydroxylation and epoxidation of polyunsaturated fatty acids by cytochrome P450. *Lipids* **1996**, *31*, 1003.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

 Copyright © 2016 John Wiley & Sons, Ltd.