RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2010; **24**: 2109–2121
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4621

A rapid and specific derivatization procedure to identify acyl-glucuronides by mass spectrometry

Alfin D. N. Vaz*, Wei Wei Wang, Andrew J. Bessire, Raman Sharma and Anne E. Hagen

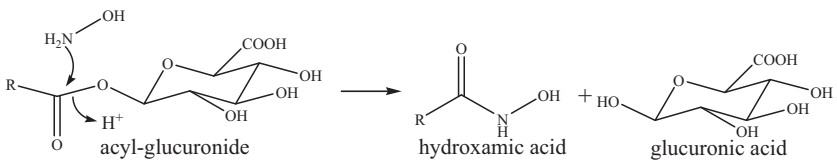
Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Global Research and Development, Groton, CT 06340, USA

Received 4 February 2010; Revised 10 May 2010; Accepted 11 May 2010

A simple procedure is described to identify acyl-glucuronides by coupled liquid chromatography/mass spectrometry after derivatization to a hydroxamic acid with hydroxylamine. The reaction specificity obviates the need for isolation of the acyl-glucuronide from an extract. Glucuronides derived from carbamic acids, and alkyl- and aromatic amines, are inert to the derivatization reaction conditions, making the hydroxamic acid derivative a fingerprint for acyl-glucuronides. Copyright © 2010 John Wiley & Sons, Ltd.

The glucuronidation of drugs, xenobiotics, their metabolites, and endobiotics is a common biotransformation process which increases their solubility for excretion via urine or bile by passive or active export mechanisms.[1–3] The oxygen atom of phenols, alcohols, and carboxylic acids and the nitrogen of alkylamines and aromatic nitrogen heterocycles are the most common sites for glucuronidation. Less commonly observed is the glucuronidation of amines that are carbamylated yielding stable carbamoyl-glucuronides.[4–6] Glucuronides of carboxylic acids, commonly termed acyl-glucuronides,[7,8] are of concern from a toxicity point of view as they have been implicated in drug-induced hypersensitivity, and hepatic failure. This toxicity has been attributed to the ability of acyl-glucuronides, or their intramolecular rearrangement products, to covalently modify cellular macromolecules.[9–14] Thus, identifying an acyl-glucuronide derived from a drug or its metabolite is of interest early in a drug's discovery phase.[15–18] Glucuronides are commonly identified in soft ionization mass spectrometry by the facile loss of 176 or 175 Da in their tandem mass (MS/MS) spectra (positive or negative ion modes), obtained on triple quadrupole or ion trap mass spectrometers. The loss of glucuronic acid limits the ability to assign the site of glucuronidation from the fragmentation pattern. Accordingly, in molecules where multiple possibilities exist for conjugation with glucuronic acid, secondary methods such as hydrolysis with β-glucuronidase or dilute sodium hydroxide, chemical derivatization to distinguish between phenolic, alcoholic, and *N*-glucuronides, and, more recently, nuclear magnetic resonance (NMR), are used to identify the location of glucuronidation.[19–22]

In this study we demonstrate the convenience of a single-step derivatization technique for fingerprinting acyl-glucuronides by mass spectrometry in molecules with multiple sites for glucuronidation. As shown in Scheme 1, the technique involves the *in situ* aminolysis of the acyl-glucuronide with hydroxylamine to yield the hydroxamic acid. The method was originally described by Schachter[7] for the colorimetric analysis of acyl-glucuronides and applied to their quantitation in urine.[7,8] In addition to the stability of ether-glucuronides from alcohols and phenols originally demonstrated towards this reagent,[7] we have established that glucuronides derived from aliphatic and aromatic amines, carbamic acids, and hydroxamic acids are inert to this reagent, making this technique highly specific for acyl-glucuronides.



Scheme 1. Aminolysis reaction showing nucleophilic displacement of glucuronic acid by the amine nucleophile of hydroxylamine to give the hydroxamic acid and free glucuronic acid.

EXPERIMENTAL

All chemical reagents used in this study were commercially obtained and of the highest purity. Hepatic microsomes and

*Correspondence to: A. D. N. Vaz, Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Global Research and Development, Groton, CT 06340, USA.
E-mail: alfin.vaz@pfizer.com

Copyright © 2010 John Wiley & Sons, Ltd.

cryopreserved hepatocytes were obtained commercially from Gentest Laboratories and Celsis In Vitro Technologies. For proprietary compounds examined in this study, only the functional components relevant to the study are shown, with an 'R' representing the rest of the molecule.

Lasofoxifene glucuronides

In the development of lasofoxifene, definitive metabolic studies using radiolabeled drug showed several urinary phenolic glucuronides.[23] Mouse urine from a ^{14}C-labeled lasofoxifene mass balance study was used to examine the reactivity with hydroxylamine.

N-Carbamoyl-glucuronides of desipramine and duloxetine

The carbamoyl-glucuronides were synthesized as follows. Desipramine (200 μM) or duloxetine (200 μM) was incubated with dog liver microsomes (2 mg/mL) in 100 mM sodium bicarbonate buffer adjusted to pH 7.5 with hydrochloric acid and 0.2% bovine serum albumin, supplemented with uridine diphosphoglucuronic acid (UDPGA, 5 mM) and alamethicin (50 μg/mL). The reactions were maintained at 37°C and flushed continuously with carbon dioxide. After 120 min the incubation was supplemented with additional UDPGA (5 mM), and after 240 min a 4× volume of acetonitrile was added to the reaction to precipitate protein. The supernatant, after centrifugation at 1100 *g* for 5 min, was evaporated to dryness in a vacuum centrifuge. The residue was re-dissolved in 0.1% formic acid/acetonitrile (90:10) (3 mL) and the supernatant used to conduct the derivatization reaction without purification.

N-Glucuronide of compound Y

This metabolite was identified in rat bile from a study with ^{14}C-compound Y in bile-duct cannulated rats. Its identity as an *N*-glucuronide at the indole nitrogen was established from its fragmentation pattern and exact mass on a ThermoFinnigan Orbitrap mass spectrometer.

Biochemical synthesis of diclofenac acyl-glucuronides

Diclofenac (100 μM) was incubated at 37°C with rat liver microsomes (1 mg/mL) in 50 mM potassium phosphate buffer pH 7.4, supplemented with UDPGA (1 mg/mL) and alamethicin (50 μg/mL). After 60 min a 5× volume of acetonitrile was added to the reaction to precipitate protein. The supernatant, after centrifugation at 1100 *g* for 15 min, was evaporated to dryness in a vacuum centrifuge. The residue was re-dissolved in DMSO/acetonitrile/water (20:10:70, 300 μL), centrifuged to remove insoluble matter, and the supernatant used to either purify the acyl-glucuronide(s) or conduct derivatization reactions without purification.

Glucuronides of carbazeran and compound X

Carbazeran or compound X (10 μM) was individually incubated at 37°C with cryo-preserved human hepatocytes (750 000 cells/mL) suspended in Williams E media and gassed with 5% CO_2/95% O_2. After 4 h the reactions were stopped with a 5× volume of acetonitrile and processed in the same manner as the diclofenac reaction.

Reaction with hydroxylamine

An aliquot (50–100 μL) of the re-dissolved extracts from microsomal or hepatocyte reactions, urine, or plasma was treated with an equal volume of a 55% aqueous hydroxylamine solution. After standing at room temperature for 1 to 12 h an aliquot was analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS). With the diclofenac acyl-glucuronides, the reaction was judged to be complete after 2 h at room temperature based on the absence of glucuronides in the reaction mixture as determined by mass spectrometry.

LC/MS/MS

Multiple ThermoFinnigan mass spectrometric platforms integrated with either Surveyor or Agilent liquid chromatographic systems were used for analysis, and include TSQ Quantum triple quadrupole, LCQ-Deca and LTQ ion trap instruments. In each case the mass spectrometer was optimized for the parent of the particular compound under study. Chromatographic separation was generally done on C-18 reversed-phase columns using gradients of acetonitrile with ammonium formate/acetate in the pH range from 3.0 to 6.8. The exact condition used in each analysis is indicated in the related figure legends. As multiple systems and analytical columns were used in this report, the identity of substrates, metabolites, and derivatives is based entirely on their mass spectral fragmentation patterns and not on their chromatographic retention times.

RESULTS AND DISCUSSION

Synthesis and reactivity of diclofenac-glucuronides

Figure 1(a) shows the UV and the ion current chromatograms at *m/z* 296 and 472 for the reaction of diclofenac with rat liver microsomes supplemented with UDPGA and alamethicin. Diclofenac eluted at 36.9 min (UV). The extracted ion chromatogram for the glucuronides (*m/z* 472) of diclofenac shows four chromatographically resolved peaks eluting at 29.8, 31.0, 31.7 and 32.0 min (UV). The MS^1 profile for the peak at 29.8 min shows an ion current at *m/z* 296, which is also the most intense ion current in the MS^2 spectrum (Fig. 1(b)) and corresponds to a loss of 176 Da, which is characteristic of glucuronides. This suggests considerable in-source fragmentation for this glucuronide of diclofenac. The fragmentation patterns of the peaks at 31.0, 31.7 and 32.0 min are similar, with no apparent in-source fragmentation (*m/z* 296 in the MS^1 chromatograms of Figs. 1(c), 1(d), and 1(e)). Their MS^2 spectra show losses of 18 and 194 Da as most intense (Figs. 1(c), 1(d), and 1(e)) and loss of 176 Da being significantly less intense, suggesting these glucuronides are likely to be rearrangement products of conjugated diclofenac due to intramolecular acyl group migration. Similar observations have been made for the ion source stability of the acyl-glucuronides of muraglitazar.[24] After treatment of the reaction extract with hydroxylamine the *m/z* 472

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

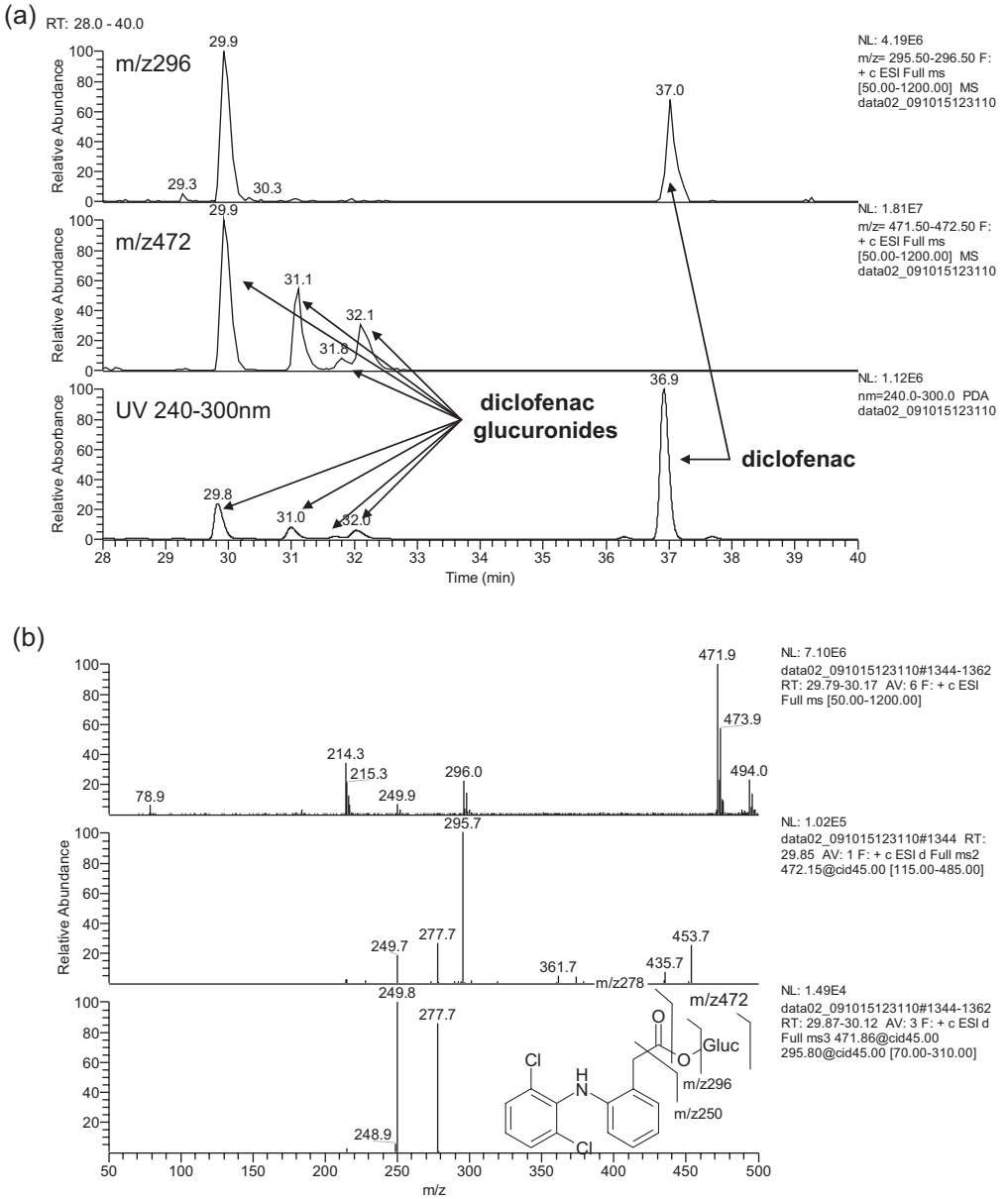


Figure 1. (a) Selective ion (*m/z* 296 and 472) and UV (240-300 nm) chromatograms of the reaction extract of diclofenac with cryopreserved rat hepatocytes showing multiple acyl-glucuronides. (b–e) Ion trap MS^n spectra of the respective *m/z* 472 chromatographic peaks at 29.8, 31.0, 31.4, and 32.2 min (UV trace). Panel b inset: fragmentation pattern for diclofenac acyl-glucuronide. LC/MS^n analysis was conducted on an integrated platform with separation achieved using a Phenomenex Luna C-18 3 μm, 4.6 × 150 mm column with a linear gradient of acetonitrile in 0.1% formic acid from 10 to 90% at a rate of 2%/min. MS^n analysis was conducted on a Finnigan LCQ Deca ion trap mass spectrometer in a data-dependent manner.

chromatographic peaks at 29.8, 31.0, 31.7, and 32.0 min (UV, Fig. 2(a)) disappear, indicating susceptibility to hydroxylamine treatment. A single new peak appears at 32.2 min (UV) with a protonated molecular mass of *m/z* 311 (Fig. 2(a)). The fragmentation pattern of this peak corresponds to the hydroxamic acid of diclofenac (Fig. 2(b)). Accordingly, all the chromatographically distinct *m/z* 472 peaks of diclofenac are acyl-glucuronides, consistent with the known intramolecular acyl migration to the alcohol groups of carbons 2, 3 and 4 of glucuronic acid. The aminolysis reaction with hydroxylamine does not distinguish between the 1-*O*-β-acyl-glucuronide and the rearranged esters of glucuronic acid.

Reactivity of other known glucuronides with hydroxylamine

To test the specificity of the hydroxylamine reaction towards acyl-glucuronides, other classes of glucuronides, whose structures had been independently established, were tested under conditions identical to those used for the acyl-glucuronides of diclofenac. As shown in Fig. 3, the phenol glucuronides of lasofoxifene (Fig. 3(a), glucuronides M3, M4, M7, and M24), the *N*-glucuronide of compound Y (Fig. 3(b)), the carbamoyl-glucuronides from desipramine (Fig. 3(c)) and duloxetine (Fig. 3(d)), the quarternary *N*-glucuronide of carbazeran (Fig. 3(e)), and the *O*-glucuronide of a

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

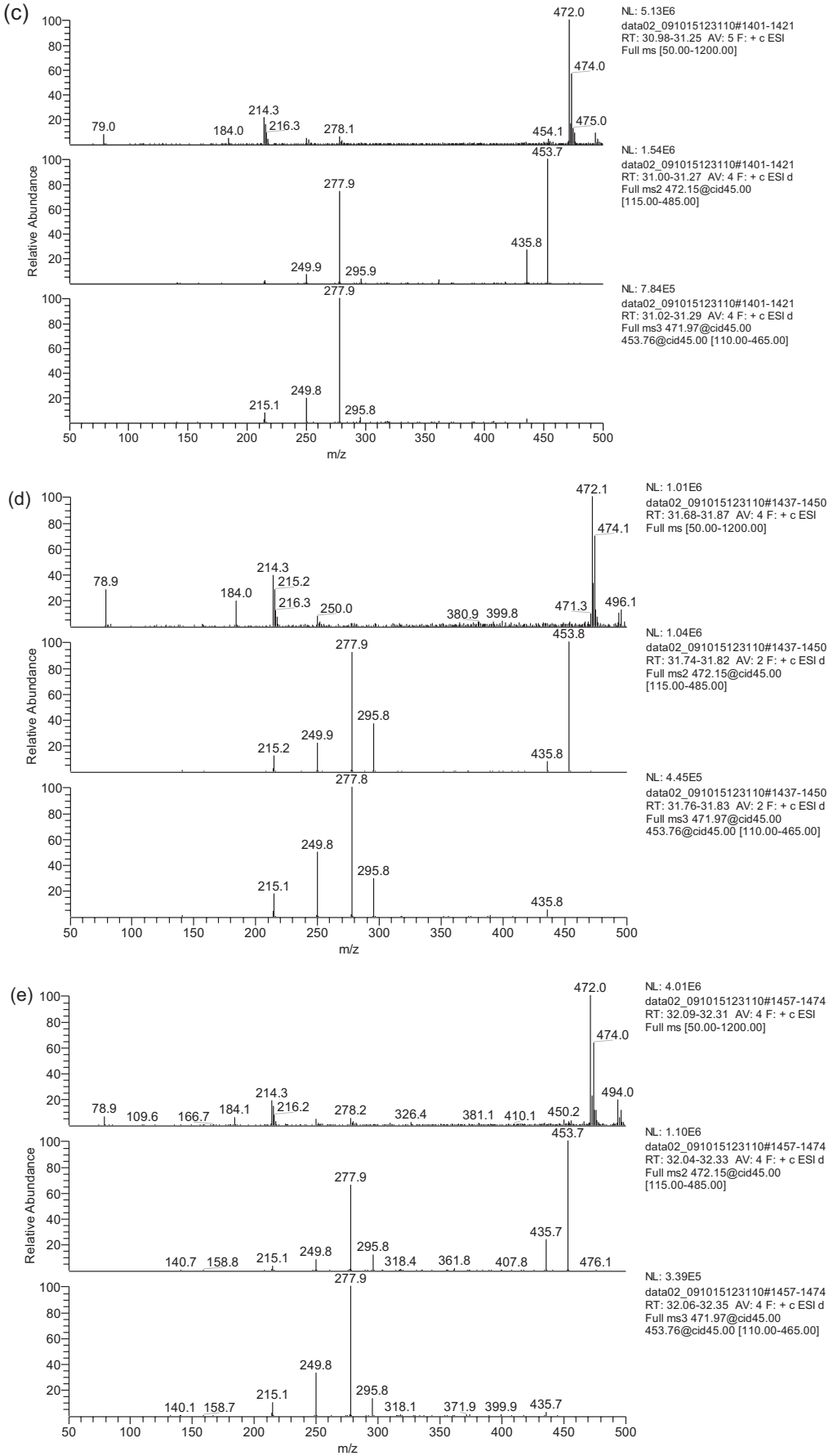


Figure 1. (*Continued*)

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

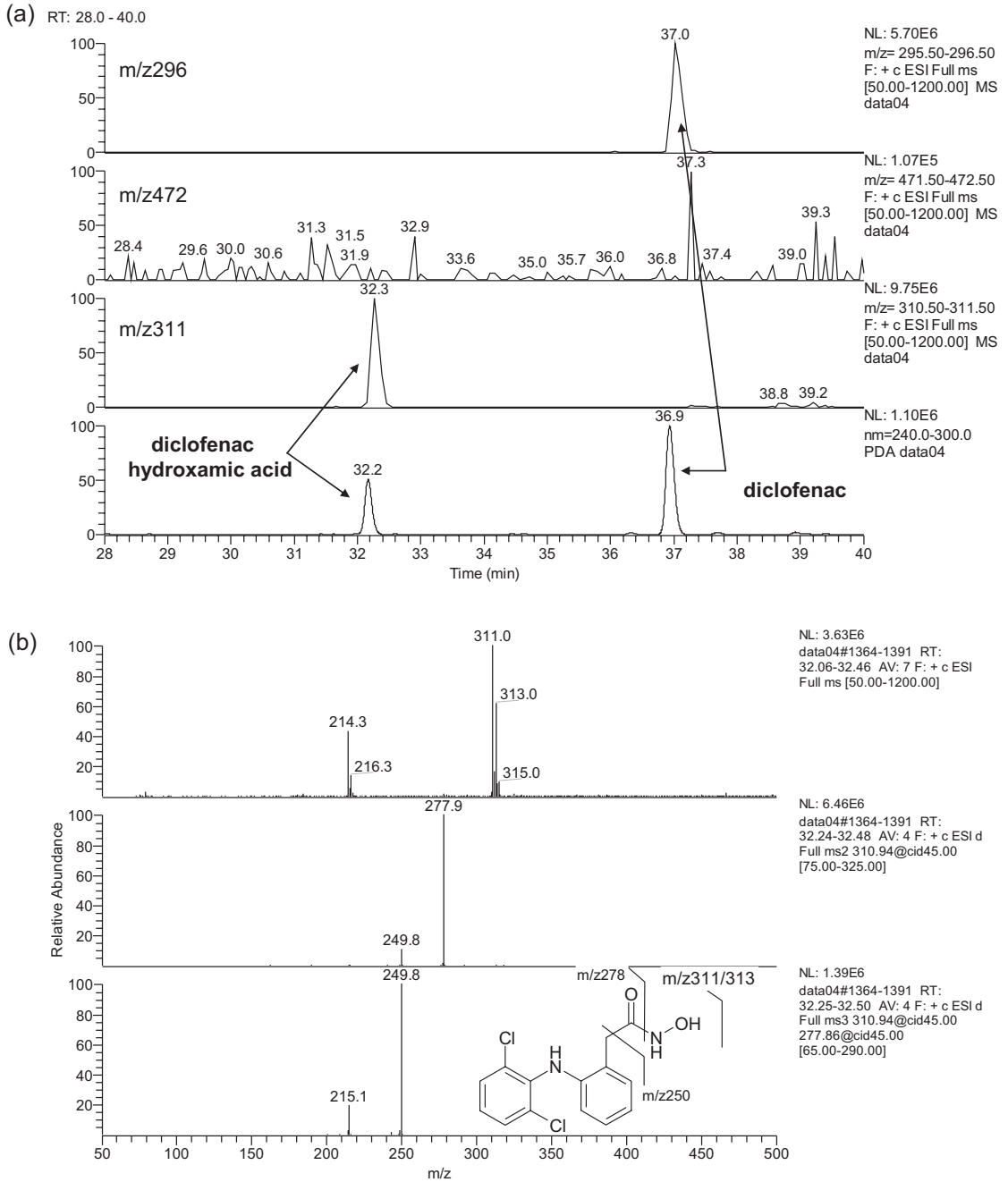


Figure 2. (a) Selective ion (*m/z* 296, 472, and 311) and UV (240–300 nm) chromatograms of the reaction extract of diclofenac treated with hydroxylamine for 12 h at room temperature showing disappearance of the *m/z* 472 chromatographic peaks and corresponding UV peaks from Fig. 1, and the appearance of the *m/z* 311 peak at 32.3 min with a corresponding UV trace at 32.2 min. LC/MS^n conditions were the same as in Fig. 1. (b) Ion trap MS^n spectrum of the hydroxamic acid derived from diclofenac acyl-glucuronides after treatment with hydroxylamine.

hydroxamic acid (Fig. 3(f)) were not aminolyzed with hydroxylamine under these conditions, showing the high degree of specificity of the hydroxylamine reaction with acyl-glucuronides.

Application to a discovery program

A discovery-stage new chemical entity, compound X, that lacked *in vitro* to *in vivo* correlation between liver microsomal stability and *in vivo* pharmacokinetics, was examined with rat hepatocytes to identify metabolic pathways. The major metabolic pathway in human hepatocytes was through glucuronidation. Figure 4(a) shows the ion current chromatograms for *m/z* 499 and 675 corresponding to the $[M+H]^+$ ion for compound X and its glucuronide(s), respectively. Four glucuronides (*m/z* 675) were identified at 21.2, 23.0, 23.3, and 23.5 min (UV) with the fragmentation patterns shown in Figs. 4(b)–4(e). Compound X has two aromatic amines, two heteroaromatic nitrogens, and one carboxyl group, all of

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

RCM

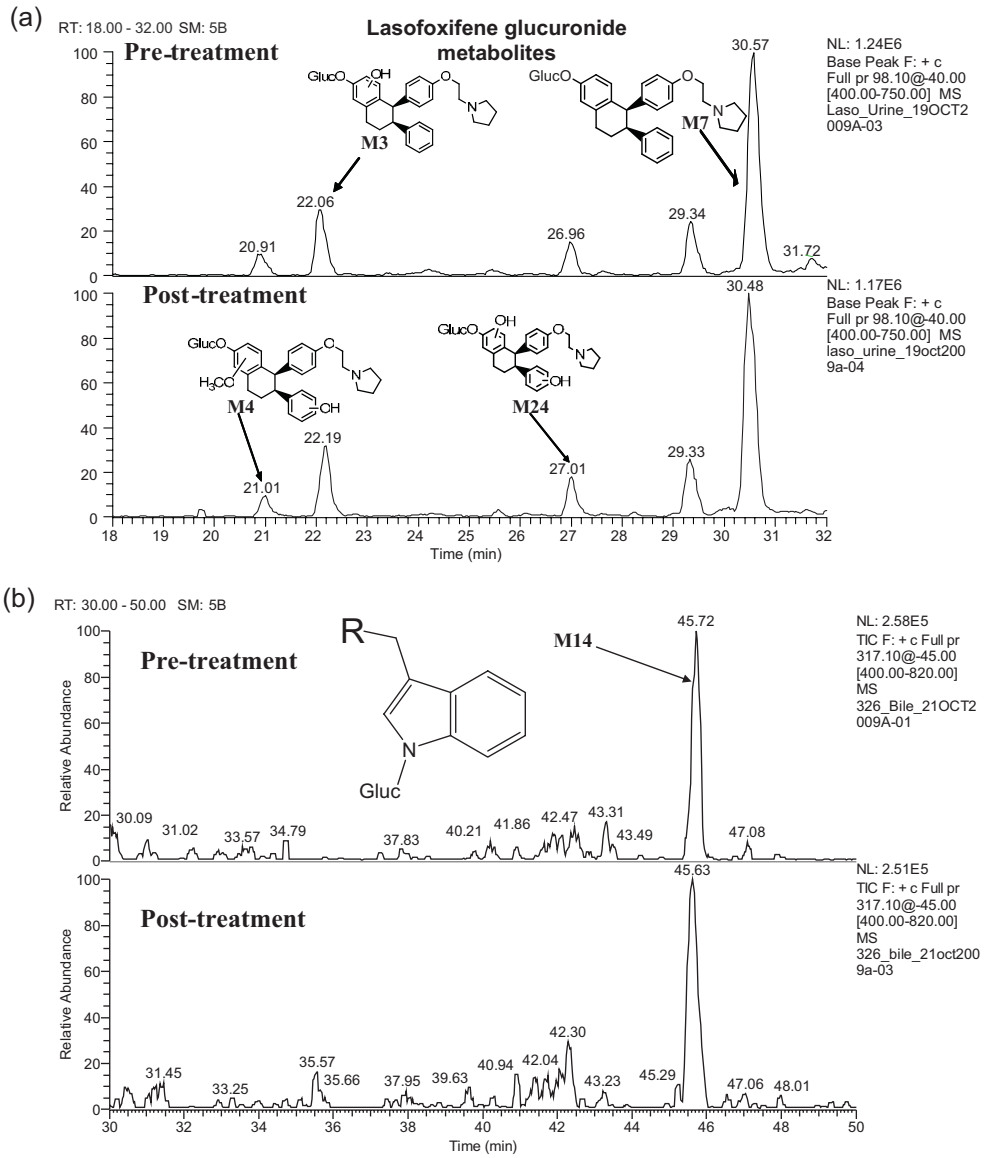


Figure 3. Ion chromatograms of independently characterized glucuronides before and after treatment with aqueous hydroxylamine as described. (a) Mouse urine extract showing the phenolic glucuronides (M3, M4, M7, and M24) of lasofoxifene.[23] (b) Ion chromatogram (*m/z* 317) of the glucuronide of compound Y. The *N*-glucuronide was assigned based on the exact mass of a fragment ion that contained only the indole ring with the glucuronic acid. (c, d) Ion (*m/z* 487 and 540) chromatograms of the *N*-carbamoyl-glucuronide of desipramine (c) and duloxetine (d). (e) Ion (*m/z* 536) chromatogram of the aromatic *N*-glucuronide of carbazeran. (f) Ion (*m/z* 419 and 595) chromatograms of a hydroxamic acid and its *O*-glucuronide. The 0.5 min shift in the retention time of the hydroxamic acid-*O*-glucuronide in the treated sample is likely due to a rise in pH to alkaline conditions caused by the hydroxylamine in the reaction.

which are potential sites for glucuronidation. The MS^1 spectra of the glucuronide peaks at 21.2, 23.0, and 23.3 min (Figs. 4(b)–4(d)) show a high degree of in-source fragmentation as evidenced by the presence of a prominent *m/z* 499 ion, and their MS^2 fragmentation patterns are essentially identical, with the loss of 176 Da to give the parent mass (*m/z* 499) as the primary ion. By contrast, the peak at 23.5 min shows little in-source fragmentation back to the parent (*m/z* 499) in the MS^1 spectrum (Fig. 4(e)), and in the MS^2 spectrum the major fragment ion is *m/z* 453, corresponding to the loss of both the carboxyl and glucuronic acid groups (46 + 176 Da). Additionally, the *m/z* 499 ion (parent carboxylic acid) is significantly less intense when compared to the corresponding spectra of the other glucuronide conjugates (Figs. 4(b)–4(d)). This is in contrast to the glucuronide peaks of diclofenac, where in-source fragmentation was observed for only one of the glucuronide peaks (Fig. 1(b)). Thus, the assignment of a 1β-*O*-acyl-glucuronide structure based on in-source fragmentation with the loss of 176 Da as described for muraglitazar,[24] and also as observed for diclofenac in this study, may not be universally applicable. The MS^2 fragmentation patterns for the glucuronide con-

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

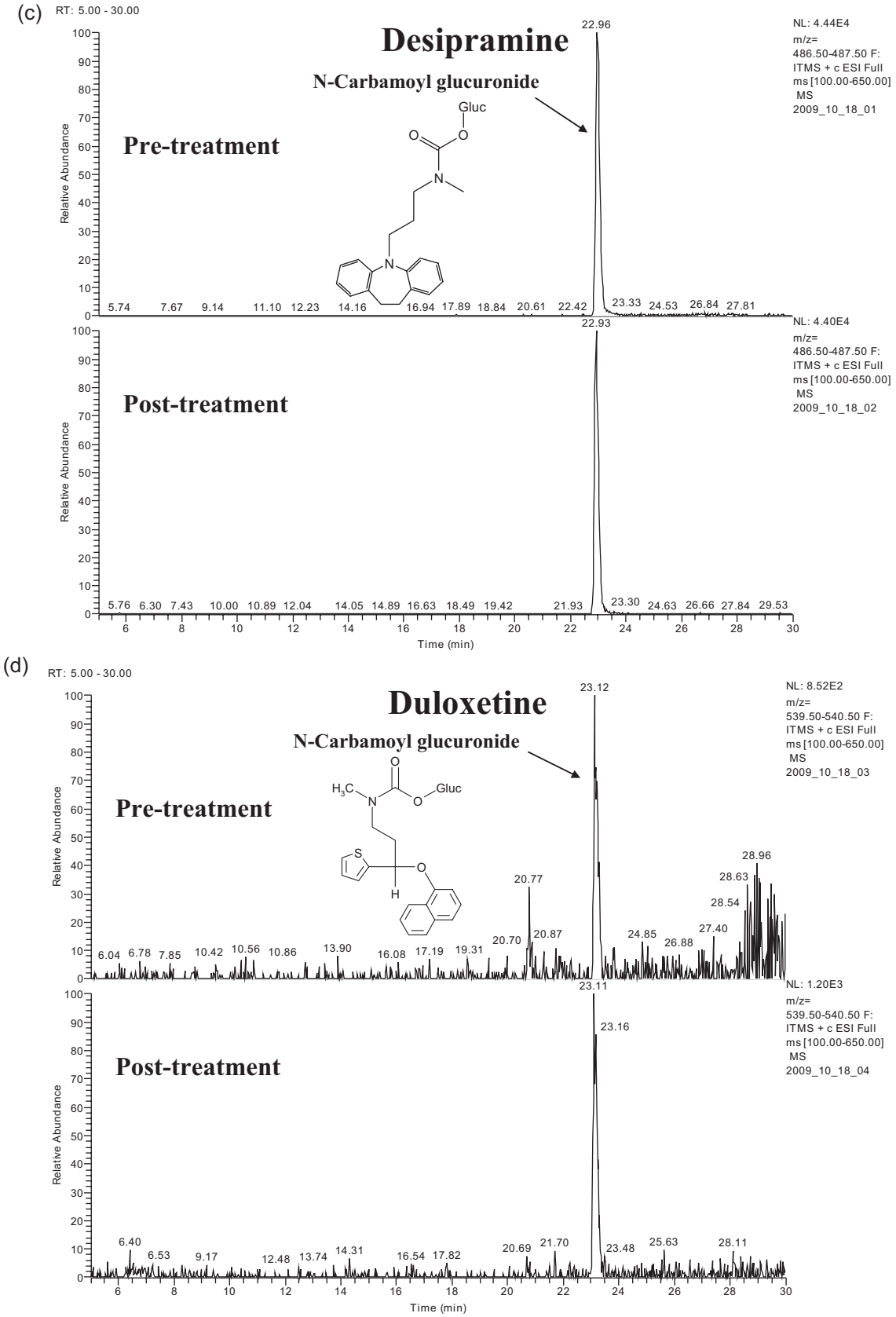


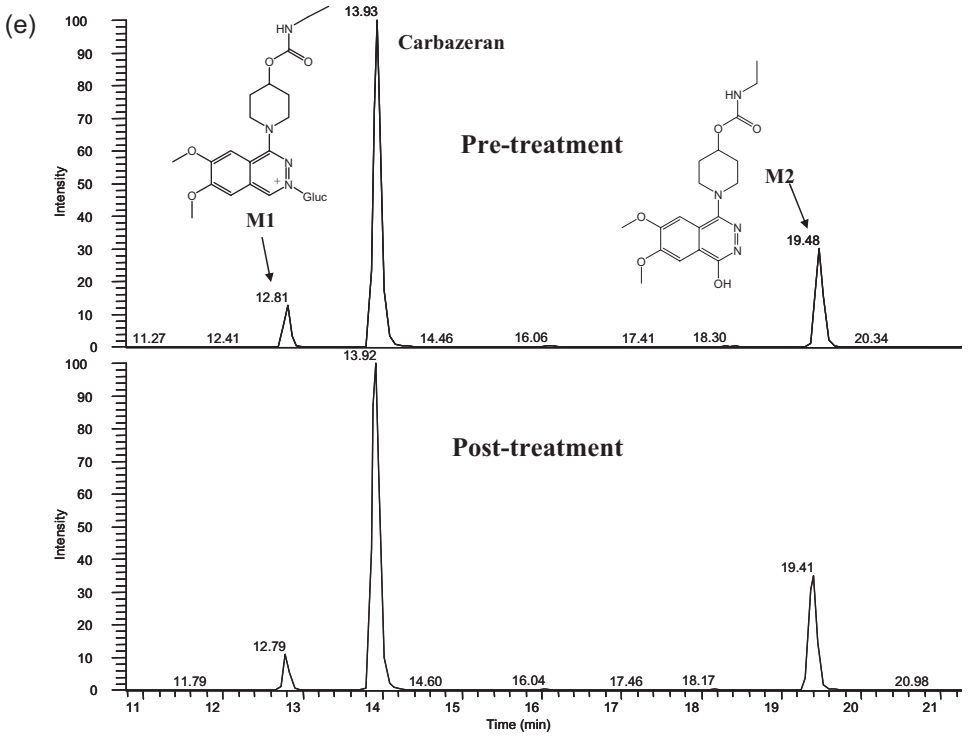
Figure 3. (*Continued*)

jugates of compound X do not reveal whether the glucuronide(s) are *N*- or acyl-derivatives. As shown in Fig. 5(a), the hepatocyte reaction extract of compound X after treatment with hydroxylamine resulted in the loss of all the *m*/*z* 675 glucuronide peaks, and yielded a single new peak with *m*/*z* 514 corresponding to the hydroxamic acid of compound X (Fig. 5(b)). Accordingly, the multiple acyl-glucuronide peaks seen in Fig. 4(a) must be due to acyl migration to the C-2, C-3 and C-4 hydroxyl groups of glucuronic acid.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

RCM



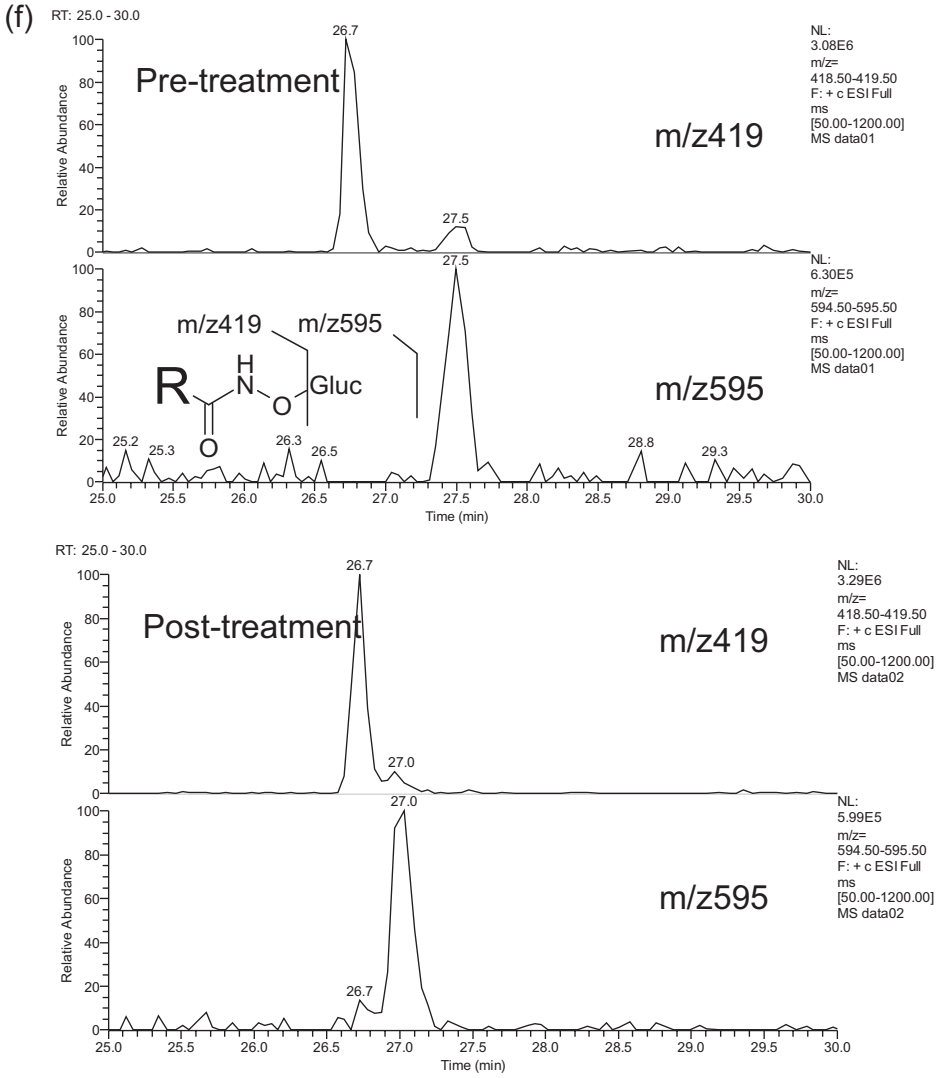


Figure 3. (*Continued*)

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

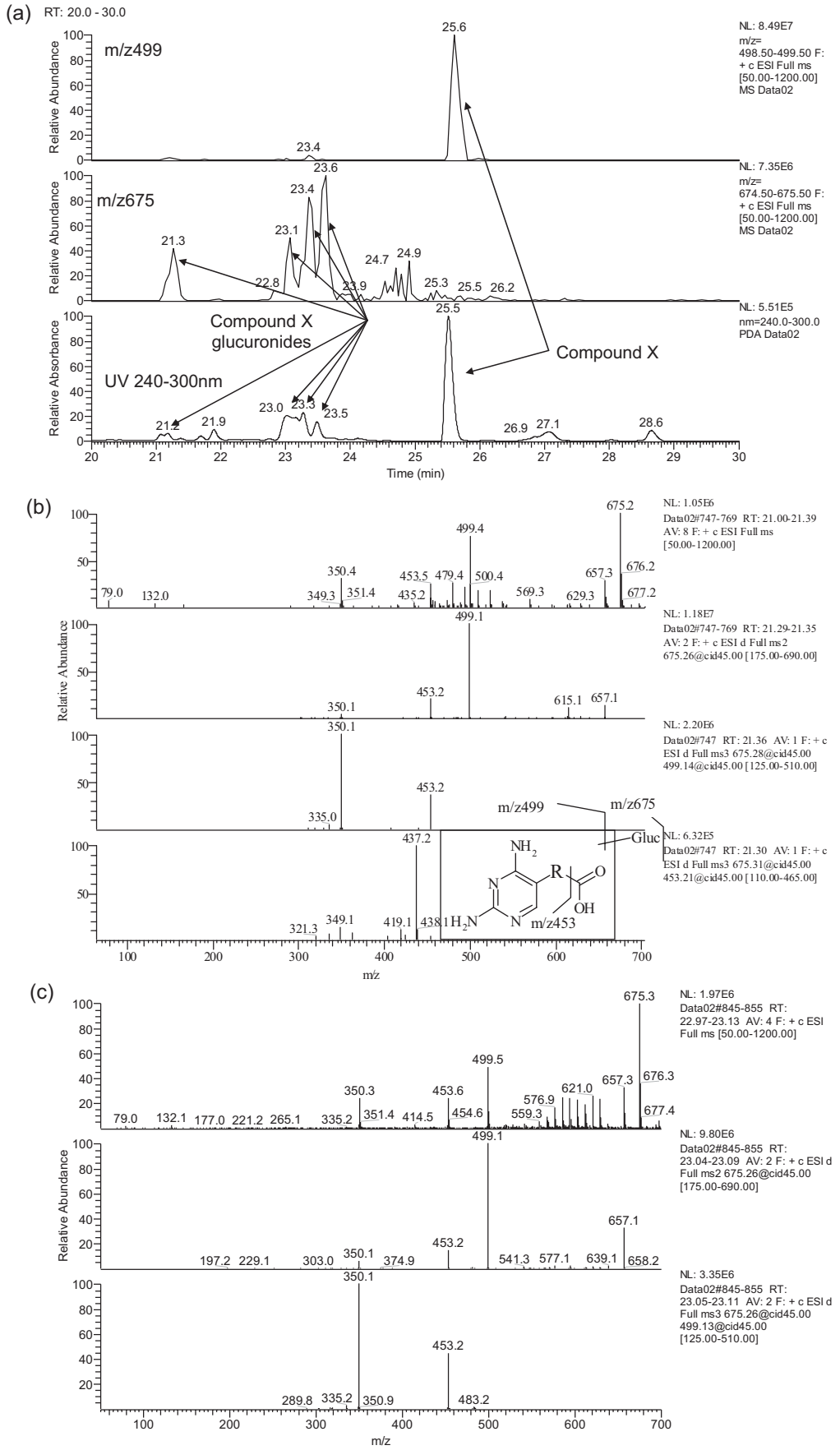


Figure 4. (a) Selective ion (*m/z* 499 and 675) and UV (240-300 nm) chromatograms of the reaction extract of compound X with cryo-preserved rat hepatocytes showing multiple acyl-glucuronides. (b–e) Ion trap MS^n spectra of the respective *m/z* 675 chromatographic peaks at 21.3, 23.1, 23.4, and 23.6 min. LC/MS^n conditions were the same as in Fig. 1.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

RCM

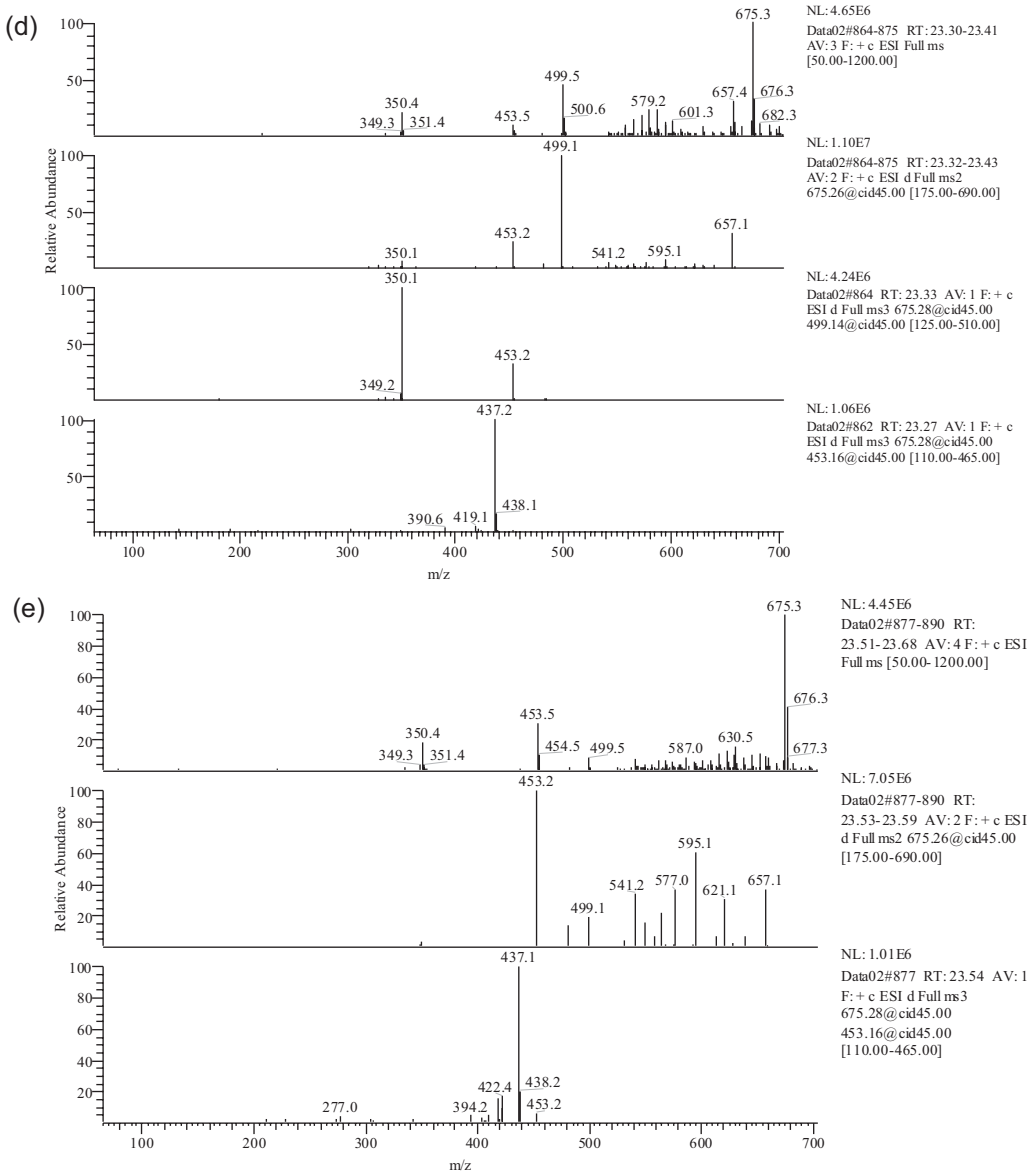


Figure 4. (*Continued*)

To determine if the aminolysis reaction with hydroxylamine results in hydrolysis of the glucuronides to the parent acid due to the high pH of the hydroxylamine solution, the acyl-glucuronides of diclofenac were chromatographically purified at pH 3.0. A fraction essentially free of parent carboxylic acid was treated with hydroxylamine and analyzed by LC/MS. Figure 6(a) shows that the purified acyl-glucuronide is essentially free of parent carboxylic acid. After treatment with hydroxylamine (Fig. 6(b)) the glucuronide peak is lost, with the quantitative appearance of the hydroxamic acid derivative (based on integration of the corresponding UV peak areas) and essentially no hydrolysis of the glucuronide to the parent carboxylic acid. This result shows that hydrolysis of the acyl-glucuronides under basic conditions in the presence of hydroxylamine is negligible relative to the extent of derivatization to the hydroxamic acid.

Potential applications to quantitative analysis of carboxylic acids and acyl-glucuronide metabolites

In the quantitative analysis of carboxylic acids, where steep gradients are frequently used to decrease the analysis cycle time, chromatographic separation of acyl-glucuronide metabolites from the parent carboxylic acid may not be achieved. This would result in artificially higher apparent levels of the carboxylic acid due to in-source fragmentation of the acyl-glucuronide(s). The derivatization with hydroxylamine as described above may provide a resolution to this problem, since hydroxylamine does not react with carboxylic acids, and its reaction with acyl-glucuronide(s) does not result in a product which hydrolyzes back to the parent carboxylic acid. This would allow for accurate quantitation of the carboxylic acid containing drug without interference from the acyl-glucuronide metabolite. Furthermore, this stability, coupled with the 14 Da mass difference

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

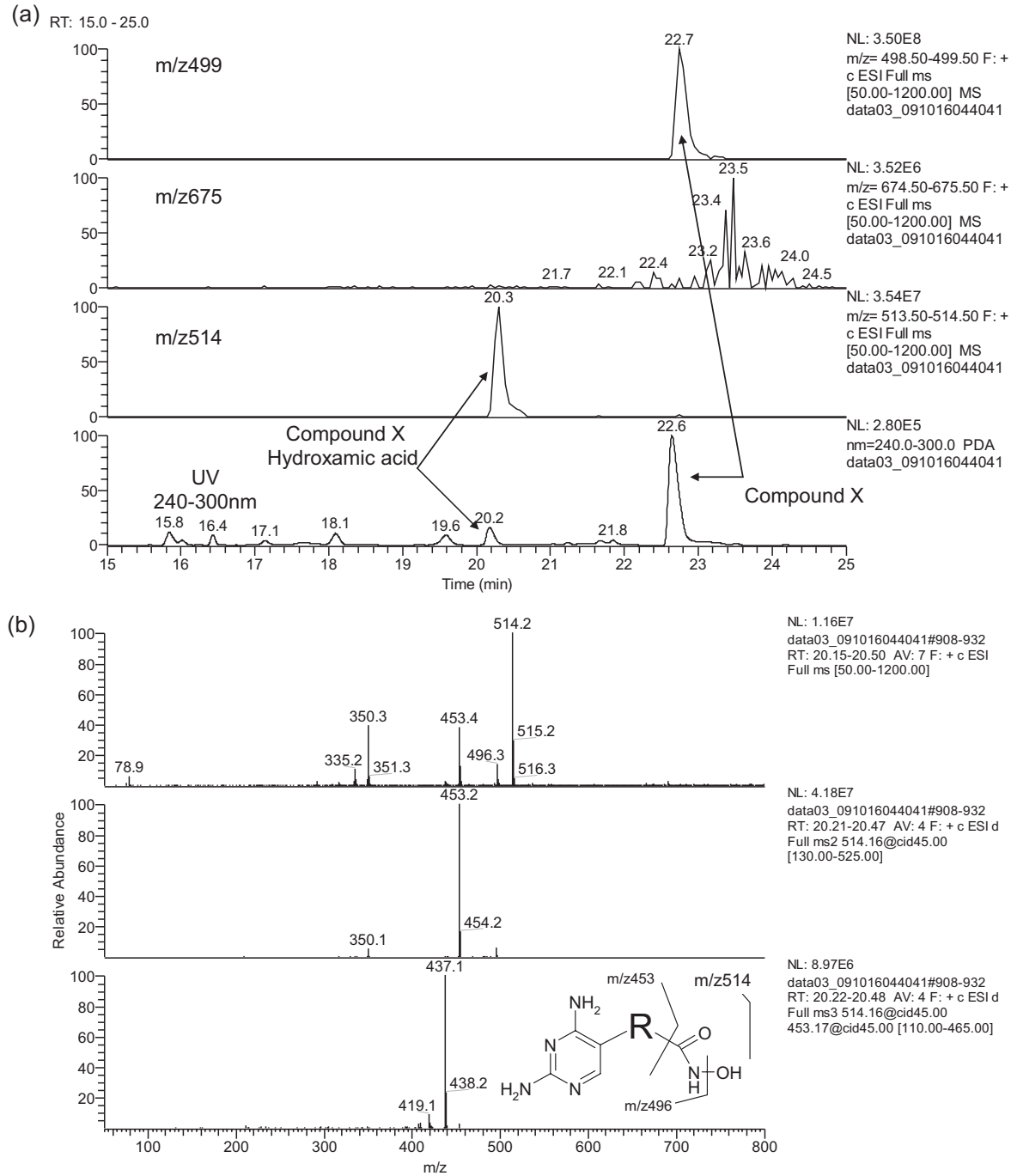


Figure 5. (a) Selective ion (*m/z* 499 675, and 514) and UV (240–300 nm) chromatograms of the hepatocyte reaction extract of compound X treated with hydroxylamine for 12 h at room temperature. The disappearance of the *m/z* 675 ion chromatographic peaks and appearance of the *m/z* 514 peak corresponding to the hydroxamic acid can be observed. LC/MS^n conditions were the same as in Fig. 1. (b) Ion trap MS^n spectrum of the hydroxamic acid derivative of compound X.

between the hydroxamic acid derivative and the parent carboxylic acid, may allow for the *simultaneous* quantitative analysis of the acyl-glucuronide(s) and its parent carboxylic acid.

In summary, this derivatization procedure provides a key advantage for the qualitative analysis of acyl-glucuronide metabolites in a drug discovery setting. Due to its simplicity, the technique allows for the rapid and positive identification of the acyl-glucuronide component of a mixture containing multiple glucuronide metabolites. This procedure also has a potential use in the bioanalysis of carboxylic acids and acyl-glucuronides which warrants further investigation. A limitation of the technique is that it cannot be used to determine the rearrangement rate of 1-β-*O*-acyl-glucuronides, which is a surrogate measure of their potential toxicity.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

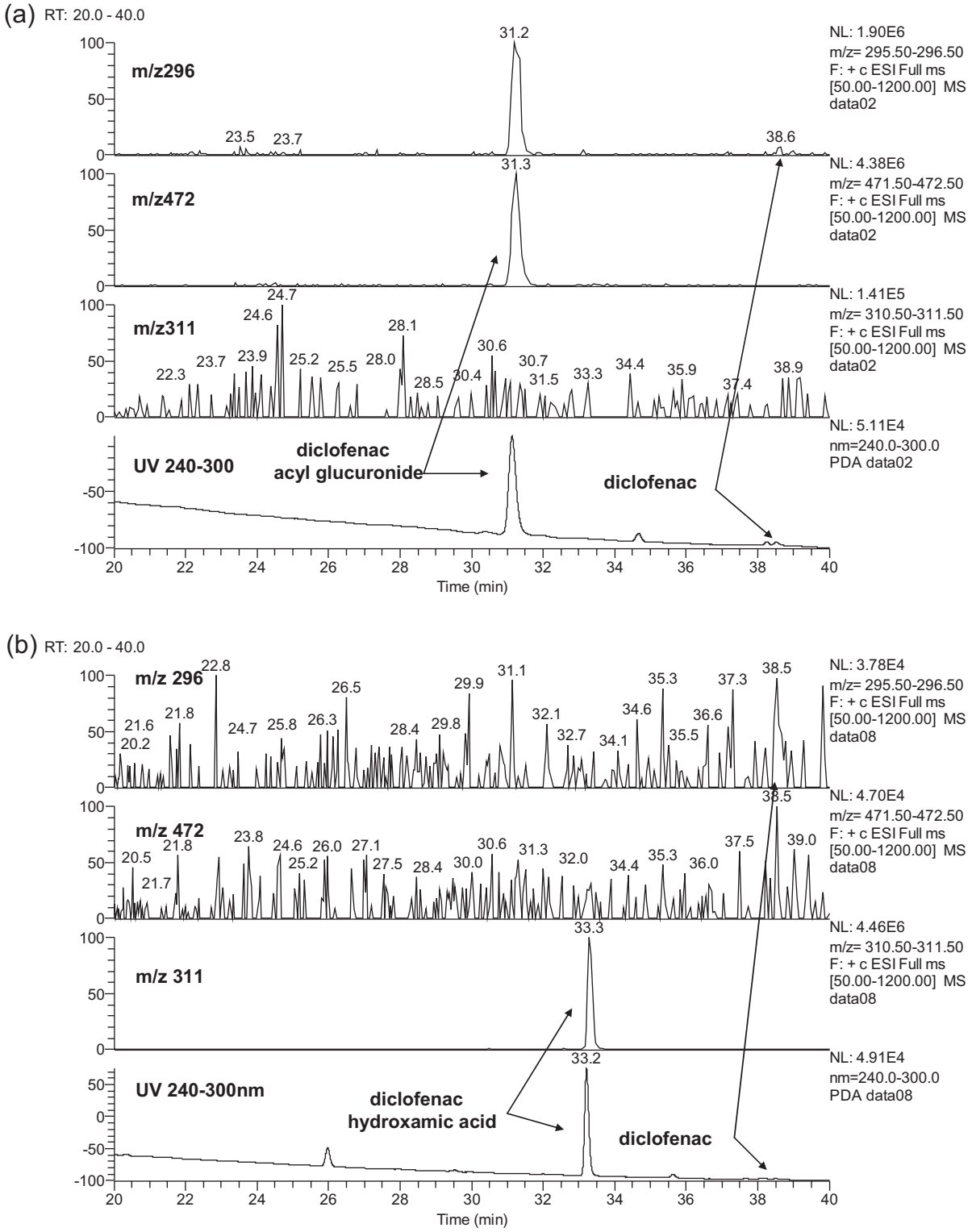


Figure 6. (a) Selective ion (*m/z* 296, 472, and 311) and UV (240–300 nm) chromatograms of a purified acyl-glucuronide of diclofenac showing in-source fragmentation and the extent of contamination by diclofenac present in the purified sample after 2 h at room temperature in an aqueous medium. (b) Selective ion (*m/z* 296, 472, and 311) and UV (240–300 nm) chromatograms of the reaction product from the purified acyl-glucuronide and hydroxylamine showing conversion of the acyl-glucuronide into the hydroxamic acid (*m/z* 311), the absence of unreacted acyl-glucuronide (*m/z* 472), and the extent of diclofenac (*m/z* 296) present in the sample after treatment with aqueous hydroxylamine for 2 h at room temperature. LC/MS^n conditions were the same as in Fig. 1.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

REFERENCES

1. Maier-Salamon A, Trauner G, Hiltscher R, Reznicek G, Kopp B, Thalhammer T, Jaeger W. *J. Pharm. Sci.* 2009; **98**: 3839.
2. Feng WY. *Curr. Drug Metab.* 2006; **7**: 755.
3. Zamek-Gliszczynski MJ, Hoffmaster KA, Nezasa K, Tallman MN, Brouwer KLR. *Eur. J. Pharm. Sci.* 2006; **27**: 447.
4. Elvin AT, Keenaghan JB, Byrnes EW, Tenthorey PA, McMaster PD, Takman BH, Lalka D, Manion CV, Baer DT. *J. Pharm. Sci.* 1980; **69**: 47.
5. Shaffer CL, Ryder TF, Venkatakrishnan K, Henne IK, O'Connell TN. *Drug Metab. Dispos.* 2009; **37**: 1480.
6. Li C, Kuchimanchi M, Hickman D, Poppe L, Hayashi M, Zhou Y, Subramanian R, Kumar G, Surapaneni S. *Drug Metab. Dispos.* 2009; **37**: 1378.
7. Schachter D. *J. Clin. Invest.* 1957; **36**: 297.
8. Shetty B, Melethil S. *Anal. Lett.* 1988; **21**: 395.
9. Treinen-Moslen M, Kanz MF. *Pharmacol. Therap.* 2006; **112**: 649.
10. Boelsterli UA. *Toxicol. Appl. Pharmacol.* 2003; **192**: 307.
11. Tang W. *Curr. Drug Metab.* 2003; **4**: 319.
12. Bailey MJ, Dickinson RG. *Chemico-Biological Interactions* 2003; **145**: 117.
13. Li C, Benet LZ. *Drug-Induced Liver Disease* 2003; 151.
14. Yang X-X, Hu Z-P, Boelsterli UA, Zhou S-F. *Curr. Pharm. Anal.* 2006; **2**: 259.
15. Walker GS, Atherton J, Bauman J, Kohl C, Lam W, Reily M, Lou Z, Mutlib A. *Chem. Res. Toxicol.* 2007; **20**: 876.
16. Anderson PD, Wang WW. Abstracts of Papers, 238th ACS National Meeting, Washington, DC, August 16–20, 2009.
17. Bolze S. In *Optimization in Drug Discovery*, Yan Z, Caldwell GW (eds). Humana Press Inc.: Totowa, New Jersey, 2004; 385.
18. Baba A. *Chem. Res. Toxicol* 2009; **22**: 1559.
19. Salomonsson ML, Bondesson U, Hedeland M. *Rapid Commun. Mass Spectrom.* 2008; **22**: 2685.
20. Frandsen H. *Food Chem. Toxicol.* 2007; **45**: 863.
21. Mullangi R, Bhamidipati RK, Srinivas NR. *Curr. Pharm. Anal.* 2005; **1**: 251.
22. Schaefer WH, Goalwin A, Dixon F, Hwang B, Killmer L, Kuo G. *Biol. Mass Spectrom.* 1992; **21**: 179.
23. Prakash C, Johnson KA, Gardner MJ. *Drug Metab. Dispos.* 2008; **36**: 1753.
24. Xue Y-J, Akinsanya JB, Raghavan N, Zhang D. *Rapid Commun. Mass Spectrom.* 2008; **22**: 109.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm