Journal of Pharmaceutical and Biomedical Analysis 88 (2014) 221–224

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Identification of combined conjugation of nabumetone phase I metabolites with glucuronic acid and glycine in minipig biotransformation using coupling high-performance liquid chromatography with electrospray ionization mass spectrometry

Lenka Češlová [a,*], Michal Holčapek [a], Milan Nobilis [b]

[a] Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, CZ-53210 Pardubice, Czech Republic
[b] Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy, Charles University, Heyrovského 1203, CZ-500 05 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 7 August 2013
Received in revised form 27 August 2013
Accepted 28 August 2013
Available online xxx

Keywords:
Drug metabolites
Conjugation with glucuronide acid
Conjugation with glycine
HPLC/MS
Tandem mass spectrometry

ABSTRACT

High-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) was applied for the analysis of nabumetone metabolites during the biotransformation in minipigs. In addition to known phase I metabolites, the identification of phase II metabolites was achieved on the basis of their full-scan mass spectra and subsequent MS^n analysis using both positive-ion and negative-ion ESI mode. Some phase I metabolites are conjugated with both glucuronide acid and glycine, which is quite unusual type of phase II metabolite not presented so far for nabumetone. These metabolites were found in small intestine content, but they were absent in minipigs urine.

1. Introduction

Nabumetone (4-(6-methoxy-2-naphthyl)-2-butanone) is a nonacidic, nonsteroidal, anti-inflammatory prodrug, used for the treatment of rheumatic and inflammatory conditions. An actual overview of the knowledge of nabumetone pharmacokinetics and biotransformation, as well as the methods for determination of phase I and phase II nabumetone metabolites in various biomatrices has been presented in our previous papers [1,2].

The formation of phase II conjugates increases the polarity of metabolites and strongly affects the chromatographic retention, ionization and fragmentation behavior. A major phase II metabolic pathway is glucuronidation [3]. The site of glucuronidation is generally an electron-rich nucleophilic heteroatom (O, N, S). The conjugation of the carboxyl functional group via a peptidic bond with amino acids is less common, but some reports can be found for glycine [3–6], taurine [3,6–8] and glutamine [6,9]. This conjugation results in a further increase of solubility of metabolites in water and thus improves their excretion.

The goal of this work is the identification of unusual phase II nabumetone metabolites, subsequent conjugates of glucuronide with glycine. To the best of our knowledge, this phase II conjugation reaction has not been reported so far.

2. Materials and methods

The liquid chromatograph used consisted of a Model 616 pump with a quaternary gradient system, a Model 996 diode-array UV detector, a Model 717+ autosampler, a thermostated column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, USA). The outlet of the UV detector of the liquid chromatograph was connected to the ion trap analyzer Esquire3000 (Bruker Daltonics, Bremen, Germany) with electrospray ionization. The data were acquired in the mass range *m/z* 50–800 in both positive-ion and negative-ion mode. The pressure of the nebulizing gas was 70 psi, the flow rate and the temperature of the drying gas was 12 l/min and 365 °C, respectively. The ion trap analyzer was tuned to give an optimum response for *m/z* 400. The isolation width for MS/MS experiments was $\Delta m/z$ 4, and the collision amplitude was 1 V. The parameter "compound stability" was set to 20%.

The column LiChroCART® 125 × 4 mm packed with LiChrospher 100 RP-C18, 5 μm, precolumn LiChroCART® 4 × 4 mm with the

* Corresponding author. Tel.: +420 46 6037397; fax: +420 46 6037068.
E-mail address: lenka.ceslova@upce.cz (L. Češlová).

0731-7085/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2013.08.053

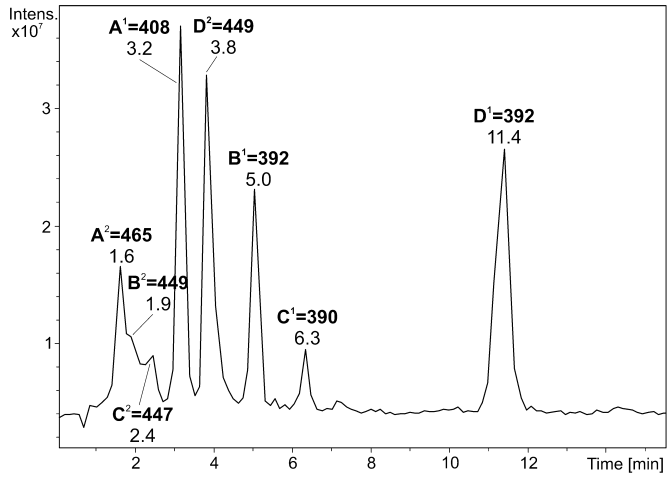

Fig. 1. The total ion current chromatogram of glucuronide metabolites of nabumetone (A^1, B^1, C^1, D^1) and their conjugates with glycine (A^2, B^2, C^2, D^2) recorded in negative-ion HPLC/ESI-MS.

same stationary phase (Merck, Darmstadt, Germany) were used for the analyses. The mobile phase composition was acetonitrile–water (10:90, v/v) with the addition of 0.1% acetic acid. The flow rate 1 ml/min, the column temperature 30 °C, the injection volume 100:l and the UV detection at 265 nm were used in all experiments. Analyzed samples and their preparation have been presented in our previous paper [2].

3. Results and discussion

The indirect determination of phase II nabumetone metabolites was reported in our previous paper [2]. Optimal conditions for the quantitative enzymatic deconjugation of phase II metabolites were found for the samples of minipig bile, small intestine contents and urine. Three principal phase II nabumetone metabolites (glucuronides) were discovered in minipig body fluids and their structures were confirmed using HPLC/ESI-MS analyses [2].

In this work, the HPLC coupled with ESI-MS was used for the direct identification of minor phase II nabumetone metabolites. The separation of phase II nabumetone metabolites using mobile phase with low content of acetonitrile is shown in Fig. 1, where

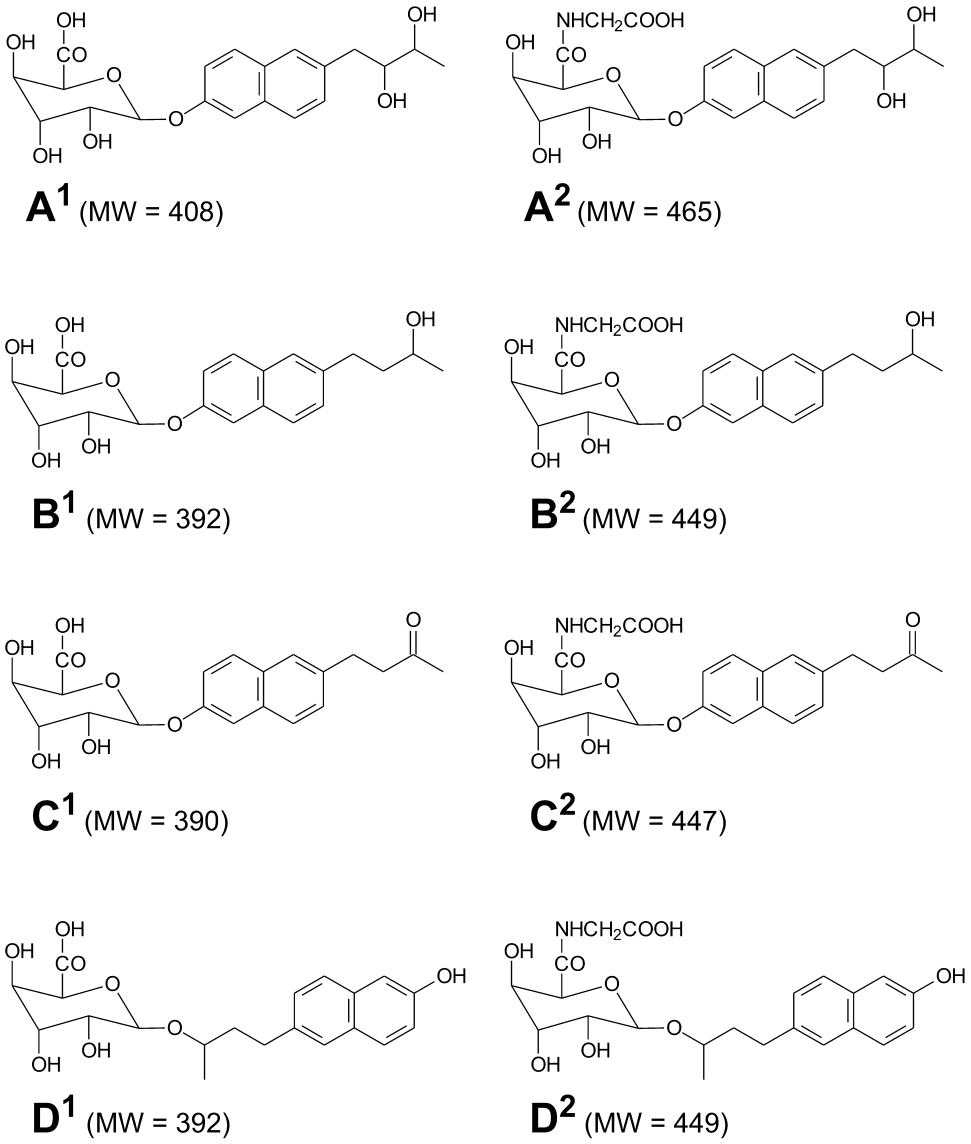

Fig. 2. The structures of newly identified phase II nabumetone metabolites. Four conjugates with glucuronic acid (A^1, B^1, C^1, D^1) and corresponding conjugates with glycine (A^2, B^2, C^2, D^2) were found in sample of small intestine content.

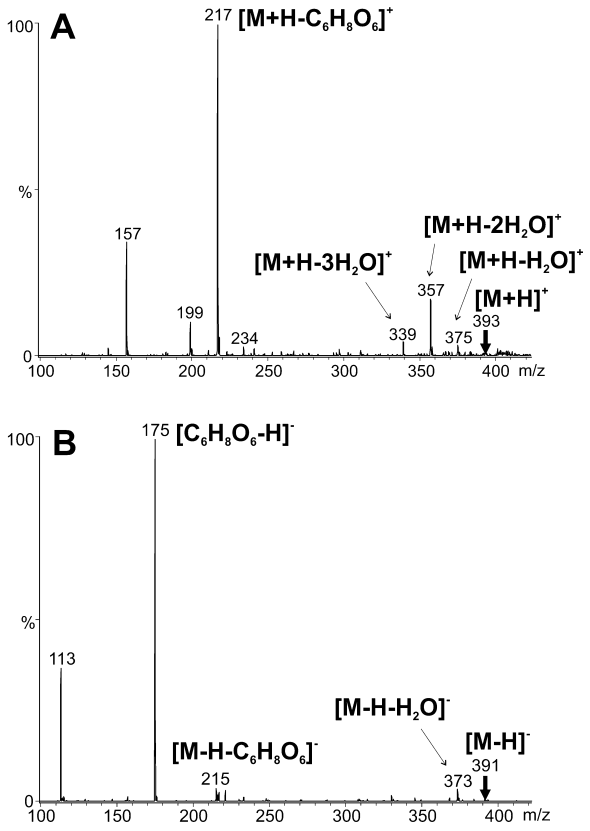

Fig. 3. ESI-MS/MS spectra of glucuronide metabolite (D^1) with the retention time t_R = 11.4 min and MW = 392: A/MS/MS spectrum of *m/z* 393 in the positive-ion mode, B/MS/MS spectrum of *m/z* 391 in the negative-ion mode.

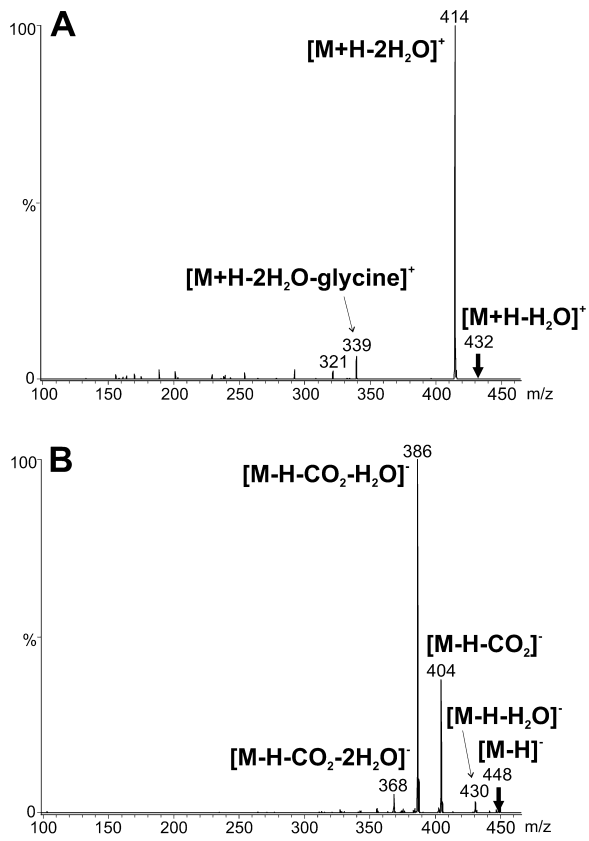

Fig. 4. ESI-MS/MS spectra of conjugate with glucuronide acid and glycine (D^2) with the retention time t_R = 3.8 min and MW = 449: A/MS/MS spectrum of *m/z* 432 in the positive-ion mode, B/MS/MS spectrum of *m/z* 448 in the negative-ion mode.

four glucuronides (A^1, B^1, C^1, D^1) and their corresponding conjugates with glycine (A^2, B^2, C^2, D^2) were identified in the samples of small intestine content. Mass spectrometric identification of these new metabolites (Fig. 2) employed two basic steps. First, the molecular weights of individual metabolites were determined using both positive-ion and negative-ion ESI mass spectra. In the negative-ion mode, the deprotonated molecules, $[M-H]^-$, are the base peaks in the spectra. In contrast to phase I metabolites, the peaks of protonated molecules are missing in the positive-ion ESI-MS spectra of phase II nabumetone metabolites, because the water molecules are lost immediately. Therefore, the series of $[M+H-xH_2O]^+$ ions were observed in the positive-ion spectra together with the adducts with sodium or potassium. In the second step, the identification of phase II nabumetone metabolites was performed mainly on the base of the interpretation of positive- and negative-ion tandem mass spectra (MS/MS). The consecutive neutral losses of water in the positive-ion MS/MS spectra does not bring additional information about the structure, but the neutral loss of the glucuronide moiety ($\Delta m/z$ 176, $C_6H_8O_6$) in the positive-ion MS/MS spectra, the fragment ions at *m/z* 175 (negatively charged glucuronide residue) and *m/z* 113 (*m/z* 175 minus glycol) in the negative-ion mass spectra is an excellent tool for the positive identification of glucuronide (Fig. 3). In addition to glucuronides, glycine conjugates were found. The identification of conjugates of the carboxyl functional group with amino acids is based on characteristic shifts of nominal and exact masses [3] and characteristic neutral losses in MS/MS spectra. The identification of glycine conjugates was performed based on the neutral loss of glycine ($\Delta m/z$ 75) in positive-ion MS/MS spectra (Fig. 4). In accordance with the nitrogen rule, the clear sign of metabolites containing glycine or other amino acids is the change of even/odd character of the molecular weights and/or ions observed in the spectra.

The metabolites A^1 and A^2 were assigned to the hydroxylated derivatives of metabolites B^1 and B^2. The position of hydroxyl group in the metabolite molecule is not possible to distinguish only on the base of mass spectra, but according to the lately presented results in metabolism of nabumetone [10], the hydroxyl group was attached to the side chain.

4. Conclusions

The complex mass spectrometric approach using the full-scan and MS/MS spectra in both positive-ion and negative-ion ESI mode was used for the identification of new metabolites in minipigs biotransformation. The molecular weights of metabolites can be determined based on the deprotonated molecules $[M-H]^-$ in the negative-ion ESI-MS and confirmed in the positive-ion mode using $[M+Na]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ ions. MS/MS and in some cases also MS^3 spectra yield the characteristic fragment ions with the typical neutral losses of glucuronide and water, which confirms the initial expectation about the presence of conjugates with glucuronide acid and glycine.

References

[1] M. Nobilis, J. Kopecký, J. Květina, Z. Svoboda, M. Pour, J. Kuneš, M. Holčapek, L. Kolářová, Comparative biotransformation and disposition studies of nabumetone in humans and minipigs using high-performance liquid chromatography with ultraviolet, fluorescence and mass spectrometric detection, J. Pharm. Biomed. Anal. 32 (2003) 641–656.
[2] M. Nobilis, M. Holčapek, L. Kolářová, J. Kopecký, M. Kuneš, Z. Svoboda, J. Květina, Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection, J. Chromatogr. A 1031 (2004) 229–236.
[3] M. Holčapek, L. Kolářová, M. Nobilis, High-performance liquid chromatography-tandem mass spectrometry in the identification and

determination of phase I and phase II drug metabolites, Anal. Bioanal. Chem. 391 (2008) 59–78.
[4] M.H. Liu, W. Zou, C.P. Yang, W. Peng, W.W. Su, Metabolism and excretion studies of oral administered naringin, a putative antitussive, in rats and dogs, Biopharm. Drug Dispos. 33 (2012) 123–134.
[5] C.X. Shan, W. Li, H.M. Wen, X.Z. Wang, Y.H. Zhu, X.B. Cui, Identification of liguzinediol metabolites in rats by ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry, J. Pharm. Biomed. Anal. 62 (2012) 187–190.
[6] C.H. Johson, O. Slanař, K.W. Krausz, D.W. Kang, A.D. Patterson, J.H. Kim, H. Luecke, F.J. Gonzalez, J.R. Idle, Novel metabolites and roles for a-tocopherol in humans and mice discovered by mass spectrometry-based metabolomics, Am. J. Clin. Nutr. 96 (2012) 818–830.
[7] J.C.L. Erve, S.C. Vashishtha, O. Ojewoye, A. Adedoyin, R. Espina, W. Demaio, R.E. Talaat, Metabolism of prazosin in rat and characterization of metabolites in plasma, urine, faeces, brain and bile using liquid chromatography/mass spectrometry, Xenobiotica 38 (2008) 540–558.
[8] A.M. Liu, A.D. Patterson, Z.T. Yang, X.Y. Zhang, W. Liu, F.Y. Qiu, H. Sun, K.W. Krausz, J.R. Idle, F.J. Gonzalez, R.K. Dai, Fenofibrate metabolism in the Cynomolgus monkey using ultraperformance liquid chromatography-quadroupole time-of-flight mass spectrometry-based metabolomics, Drug Metab. Dispos. 37 (2009) 1157–1163.
[9] K. Levsen, H.M. Schiebel, B. Behnke, R. Dötzer, W. Dreher, M. Elend, H. Thiele, Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview, J. Chromatogr. A 1067 (2005) 55–72.
[10] M. Nobilis, J. Mikušek, B. Szotáková, R. Jirásko, M. Holčapek, C. Chamseddin, T. Jira, R. Kučera, J. Kuneš, M. Pour, Analytical power of LLE-HPLC-PDA-MS/MS in drug metabolism studies: Identification of new nabumetone metabolites, J. Pharm. Biomed. Anal. 80 (2013) 164–172.