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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1312–1316
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Profiling urinary metabolites of naproxen by liquid chromatography–electrospray mass spectrometry

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Received 14 December 2005; received in revised form 21 February 2006; accepted 21 February 2006
Available online 3 April 2006

Abstract

Glucuronidation, an important metabolic process for the biotransformation of drugs into easily eliminable water-soluble detoxification products, can also lead to biologically active or toxic glucuronide conjugates.

The present work describes a liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) approach for the characterization of naproxen and *O*-6-desmethylnaproxen glucuronides. The method is fast and efficient and permitted to individuate α and β isomers of both naproxen and *O*-6-desmethylnaproxen glucuronides. The procedure could be potentially extended to the characterization of other drug metabolites.

Keywords: Naproxen; Glucuronides characterization; Metabolism; Urine

1. Introduction

Glucuronidation is an important metabolic process for the biotransformation of drugs into more water-soluble compounds that can be readily excreted from the body [1]. The reaction occurs mainly in the liver and is catalyzed by the multigenic family of enzymes, UDP-glucuronosyltransferases (UGTs). These enzymes covalently bind glucuronic acid from UDP-glucuronic acid (UDPGA) to molecules with an hydroxyl, carboxyl, amine or thiol group. The corresponding hydrophilic glucuronides are excreted into bile or urine. To date, over 46 different UGT isoforms from a number of species have been identified and at least two UGT gene families are described that conjugate drug and endobiotics in mammalian species: UGT1 and UGT2 [2]. Acyl-glucuronides have been implicated in a wide range of adverse drug effects (e.g. drug hypersensitivity reactions). They are formed by the esterification of carboxylic acids with glucuronic acid, and several human UGTs have been identified that catalyze the glucuronidation of carboxylic acid moieties. Human UGT1A3, 1A9, and 2B7 also catalyze [3–7] the glucuronidation of many non-steroidal anti-inflammatory drugs (NSAIDs), including ketoprofen, ibuprofen, diclofenac, and naproxen.

Naproxen [8] (6-methoxy-α-methyl-2-naphthyl-acetic acid) is commonly employed to reduce ongoing inflammation, pain, and fever; it is able to block [9] the cyclooxygenase (Cox) enzymes (Cox-1 and Cox-2), that both produce prostaglandins, a class of compounds that have several important functions, as the promotion of inflammation, pain and fever. However, naproxen is associated with serious side effects such as ulcers in the stomach, bleeding promotion after an injury or surgery, kidney failure, and with a number of minor side effects, such as nausea, vomiting, diarrhoea, constipation, decreased appetite, rash, dizziness, headache, and drowsiness. Finally, it interacts with other drugs; in particular, it reduces the action of diuretics and antagonizes the action of drugs used to treat hypertension.

After oral administration, naproxen is partially metabolized to its 6-*O*-desmethylated metabolite (DM-naproxen); then, both compounds are excreted in urine unchanged or conjugated [10–12] with glucuronic acid (naproxen and DM-naproxen) or sulphate (DM-naproxen).

Some analytical methods, based on liquid chromatography (LC), dealing with the determination of naproxen [13–16], naproxen and naproxen glucuronide [17], and naproxen, DM-naproxen and some conjugates [18], in biological fluids and/or pharmaceuticals have been reported. On the contrary, only

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doi:10.1016/j.jpba.2006.02.041

one LC-NMR study [19] has been reported dealing with the structural characterization of naproxen and DM-naproxen glucuronides. However, an expensive instrumentation was used and a tedious stop-flow chromatography was required. The essential lack of papers on this topic was not surprising, since the elucidation of the structure of the "free" (non-conjugated) metabolites has been given priority so far. The major reason for this was that intact glucuronide conjugates were regarded as "detoxified" drug metabolites. However, several examples of biologically active or toxic glucuronide conjugates of drugs came to light [20], and it is clearly an oversimplification to regard all glucuronide conjugates as detoxification products.

Thus, in the present work a fast and efficient way to characterize naproxen and DM-naproxen glucuronides, using liquid chromatography coupled with UV and electrospray mass spectrometry with an ion trap mass analyzer (LC-ESI-IT), is presented. The present approach permitted to individuate possible isomers of both naproxen and DM-naproxen glucuronides and could be potentially extended to the characterization of other drug metabolites.

2. Experimental

2.1. Chemicals

Naproxen was purchased from Sigma (St. Louis, MO). Stock solutions (1 mg/ml) of naproxen were prepared in methanol and stored at 4 °C in the dark. Dilute solutions were prepared just before use in triply distilled water. Organic solvents (Carlo Erba, Milan, Italy) were HPLC grade. Mobile phase was filtered through a 0.45 μm membrane (Whatman Limited, Maidstone, UK) before use. β-Glucuronidase from bovine liver was obtained from Sigma.

2.2. Apparatus

The LC system includes a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA) equipped with a Rheodyne injector with a 30 μl loop and a Supelcosil LC-18-DB column (250 mm × 4.6 mm i.d., 5 μm particle, Supelco, Bellefonte, PA, USA). Mobile phase was degassed by an SCM 1000 Vacuum membrane degasser (Thermo Separation Products). The detector were a photodiode-array (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer connected in series to a LCQ (Finnigan MAT, San Jose, CA, USA) ion trap mass spectrometer, equipped with an electrospray ionization (ESI) source, an ion trap (IT) mass analyzer and an electronically controlled, integrated dual syringe pump that delivers sample solution and/or sheath liquid from syringes into the ESI source, controlled by a Xcalibur software running on a personal computer.

2.3. Chromatographic and detection conditions

Preliminary measurements were performed on naproxen in order to optimize the operating parameters for ESI-MS acquisition. With this aim naproxen dissolved (5 ng/μl) in an acetonitrile/methanol/ammonium acetate buffer (10 mM, pH 5) mixture (6:40:54, v/v/v) was infused into the spectrometer through its syringe pump at a flow rate of 20 μl/min.

A linear binary gradient composed of acetonitrile/methanol/ammonium acetate buffer (10 mM, pH 5) (6:20:74, v/v/v) (solvent A) and acetonitrile/methanol/ammonium acetate buffer (10 mM, pH 5) (6:68:26, v/v/v) (solvent B) was used. The gradient programme was: 35 min linear from 100% A to 100% B; 20 min isocratic at 100% B; 5 min linear to 100% A; equilibration time 20 min. The flow rate was 0.2 ml min^{-1} and temperature was ambient.

The UV detection wavelength was 230 nm (10 Hz frequency, 5 nm band-width). Spectra were acquired in the 220–380 nm range (2 Hz frequency, 5 nm band-width).

The LCQ spectrometer was operated in the negative ion mode and the ESI-MS parameters were as follows: spray voltage 5.50 kV; sheath gas (nitrogen) 0.9 ml/min; capillary voltage −47.0 V; capillary temperature 190 °C; tube lens offset voltage −20.0 V; octapole 1 offset voltage 5.50 V; octapole 2 offset voltage 7.50 V; lens voltage 26.0 V; octapole RF amplitude 400 V; trap dc offset 10.0 V.

Mass spectra were recorded using two different acquisition modes: full scan (50–500 *m/z*) and MS/MS full scan, as specified in the text and in the figure captions. Collision energy adopted in the MS/MS full scan mode was 15% of maximum.

2.4. Sample collection and pre-treatment

Post dose urine samples (0–12 h) were collected from healthy donors who received once orally 220 mg of naproxen. All urine samples were stored at −20 °C. Before analysis, they were diluted 1:3 with acetonitrile and centrifuged at 5000 × *g* for 10 min. Finally, 30 μl of the resulting mixture were injected.

Enzymatic hydrolysis were performed on post dose urine samples (0–12 h); briefly, 0.5 ml of acetate buffer (pH 5.0, 1 M) containing 900 U of β-glucuronidase were added to 1.0 ml of urine, and incubated at 37 °C for 90 min. The resulting mixture was subjected to the centrifugation step and 30 μl were injected.

3. Results and discussion

Preliminary experiments were performed by direct infusion of naproxen standard solutions (5 ng/μl) into the mass spectrometer in order to optimize the instrumental parameters. A full-scan ESI mass spectrum of naproxen (negative ions) is reported in Fig. 1. The $[M-H]^-$ (*m/z* 229.0) ion was clearly observed in the spectrum, along with the corresponding isotope peak. Fragmentation of deprotonated naproxen in the ion source leads to the formation of two main product ions, arising from the loss of CO_2 (*m/z* 185.1) and of the consecutive losses of CO_2 and CH_3 (*m/z* 170.3).

A previously published LC-UV method from our group for the simultaneous determination of naproxen and naproxen glucuronide [17] was used as starting point to optimize the chromatographic conditions. The mobile phase used in that occasion, i.e. acetonitrile/methanol/ammonium acetate buffer (10 mM, pH 5) (6:40:54, v/v/v), was not suitable for the separation of at least

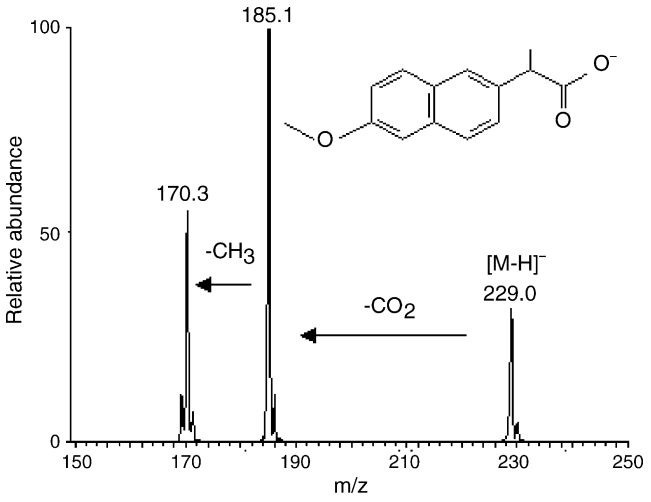

Fig. 1. Full-scan ESI mass spectrum of naproxen (negative ions) obtained during direct infusion experiments.

the known naproxen metabolites. Thus, to reach the proposed aim the linear binary gradient described in Section 2 was developed; Fig. 2 reports the LC-UV chromatograms relevant to a drug-free urine (dotted line) and a post dose urine (solid line) samples (0–12 h), obtained with the gradient elution. As apparent, in addition to the naproxen peak, some additional peaks (in particular 1–5) not detectable in the blank were clearly observable and could be attributed to naproxen metabolites (see later). The same results were obtained for all the analyzed samples.

The instrumental design described in Section 2 (in series coupling of UV and ESI detectors) permitted the simultaneous acquisition LC–MS chromatograms (obviously similar to the LC-UV chromatograms) and to obtain structural information on the most abundant chromatographic peaks. By acquiring the sample in full-scan mode, the mass spectrum of peak 3 was characterized by the *m/z* ion 215 as quasi-molecular ion, that was then easily attributed to *O*-6-desmethylnaproxen; mass spectra relevant to peaks 1 and 2 were both characterized by a quasi-molecular ion at *m/z* 391; both the mass spectra of peaks 4 and 5 presented a quasi-molecular ion at *m/z* 405. An *m/z* value of 391 could be due to the $[M-H]^-$ ions of *O*-6-desmethylnaproxen acyl-glucuronides (or *O*-6-desmethylnaproxen alkyl-glucuronides), while an *m/z* value of 405 could be attributed to naproxen acyl-glucuronides (*m/z* 405).

Then, in order to induce fragmentation of the quasi-molecular ions and consequently to obtain more structural information on the glucuronic acid conjugates (peaks 1, 2, 4 and 5) the sample was acquired using the MS/MS full scan mode, by isolating and fragmenting the *m/z* ions 391 of peak 1 and 2, and the *m/z* ions 405 of peak 4 and 5, respectively. The MS^2 full scan ESI

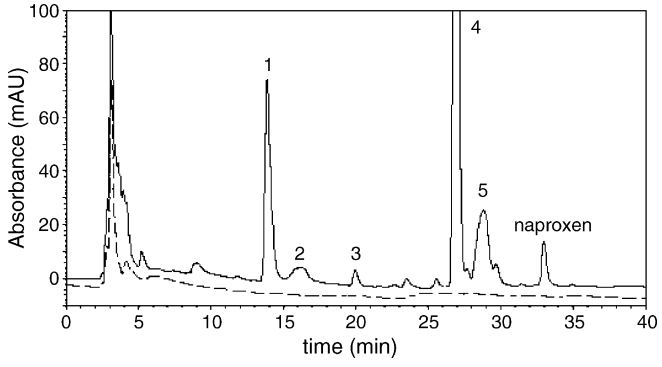

Fig. 2. LC-UV chromatograms relevant to a drug-free urine (dotted line) and a post dose urine (solid line) samples (0–12 h).

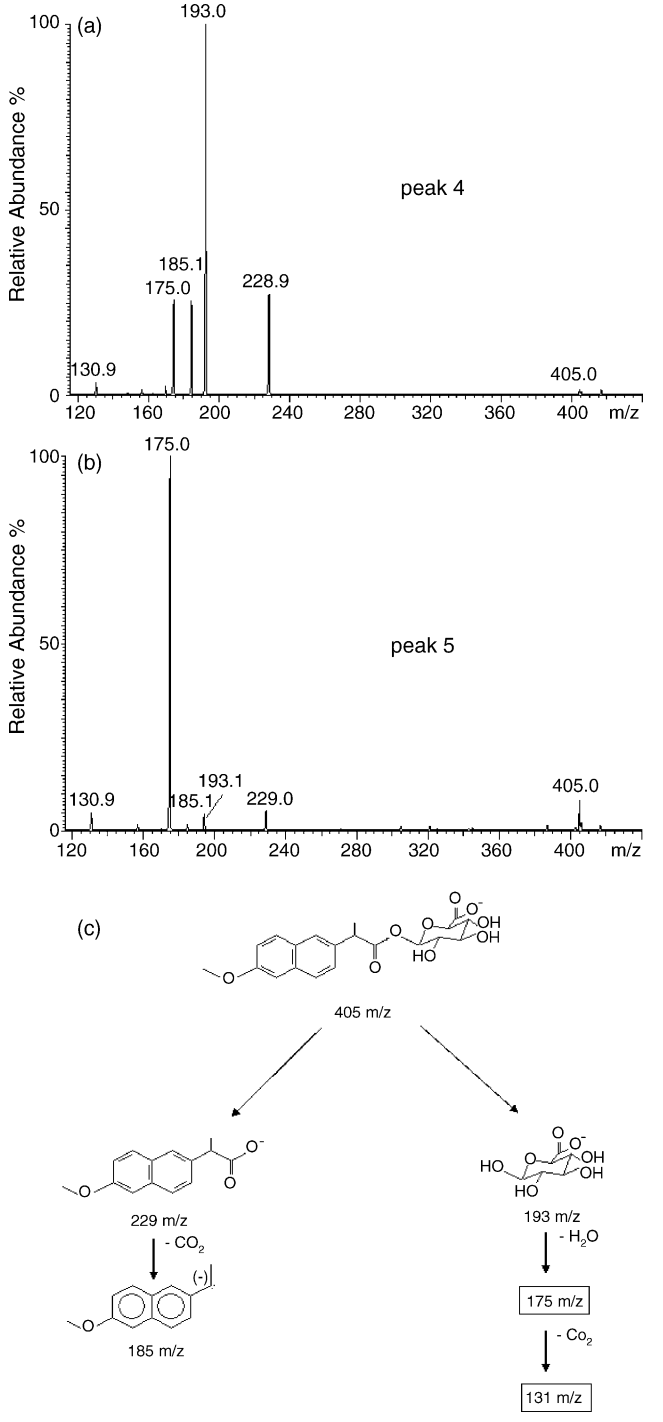

Fig. 3. MS^2 full scan ESI mass spectra obtained from the fragmentation of the *m/z* ion 405 of: (a) peak 4 and (b) peak 5. (c) Attribution of the main fragments observable in the spectra.

mass spectra obtained from the fragmentation of the *m/z* ion 405 of peaks 4 and 5 are shown in Fig. 3a and b, respectively, while Fig. 3c reports the attribution of the main fragments. In the case of naproxen glucuronidation, only the formation of acyl-glucuronides was possible; thus, peaks 4 and 5 could be due to two different isomeric forms, i.e. naproxen-1-β-acyl-glucuronide and naproxen-1-α-acyl-glucuronide, as suggested by evident differences in the relative abundance of the *m/z* ions present in the spectra. This hypothesis is partially supported by the observation that the α anomer of the glucuronic acid moiety (*m/z* ion 193) can undergo a more facile water elimination (giving a more intense *m/z* ion 175) due to stereochemical considerations; assuming the occurrence of an E2-type elimination (involving the C1 and C2 carbon atoms), the anti-periplanar arrangement (180) of the hydroxyl group and hydrogen in the α-anomer (both in the axial position on C1 and C2, respectively), would facilitate dehydration respects to the analogous process occurring on the β-anomer, where hydroxyl group and hydrogen are in reciprocal gauche position (60) [21].

A similar situation was observed in the case of the MS^2 full scan ESI mass spectra obtained from the fragmentation of the *m/z* ion 391 of peaks 1 and 2. In Fig. 4a and b are shown the relevant results, while Fig. 4c reports the attribution of the main fragments. As apparent, also in this case the same fragments were observable in the two spectra; again, the differences in the relative abundances clearly indicates the occurrence of two different isomers of *O*-6-desmethylnaproxen glucuronic conjugates, namely *O*-6-desmethylnaproxen-1-β-acyl-glucuronide (or *O*-6-desmethylnaproxen-1-β-alkyl-glucuronide) and *O*-6-desmethylnaproxen-1-α-acyl-glucuronide (or *O*-6-desmethylnaproxen-1-α-alkyl-glucuronide). However, as a very similar fragmentation was obtained in the case of naproxen glucuronides (compare Figs. 3c and 4c), the formation of *O*-6-desmethylnaproxen acyl-glucuronides was hypothesized.

Unfortunately, it was not possible to univocally attribute a chromatographic peak to a specific isomeric form on the basis of the ESI mass spectra. Thus, urine samples were subjected to an enzymatic hydrolysis as described in Section 2 in order to indirectly obtain further information on the identity of naproxen and DM-naproxen glucuronides. Fig. 5 reports an LC-UV chromatogram relevant to a post dose urine sample (0–12 h) subjected to enzymatic hydrolysis. By comparing the present chromatogram with that reported in Fig. 1, it is evident how peaks 1 and 4 disappeared from the chromatogram while peaks 2 and 5 remained unchanged; simultaneously, the naproxen and DM-naproxen peaks had a consistent increase. This experimental evidence clearly demonstrated that

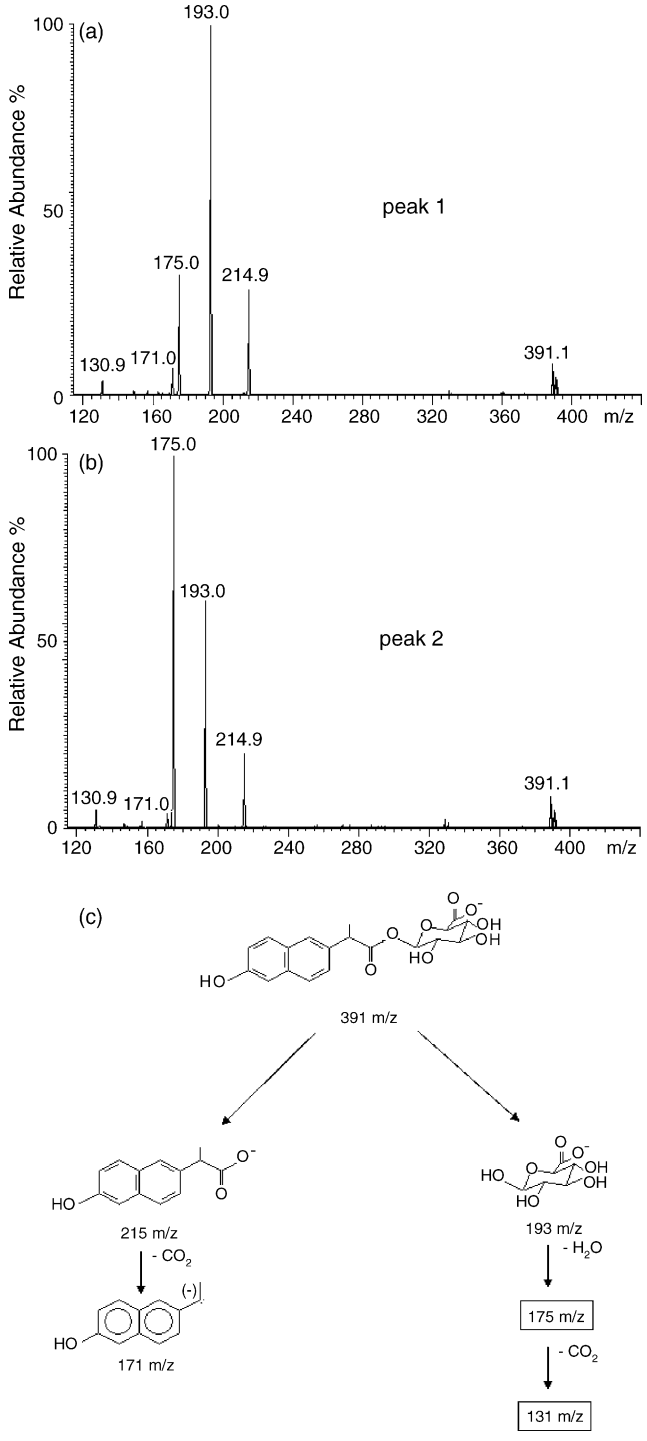

Fig. 4. MS^2 full scan ESI mass spectra obtained from the fragmentation of the *m/z* ion 391 of: (a) peak 1 and (b) peak 2. (c) Attribution of the main fragments observable in the spectra.

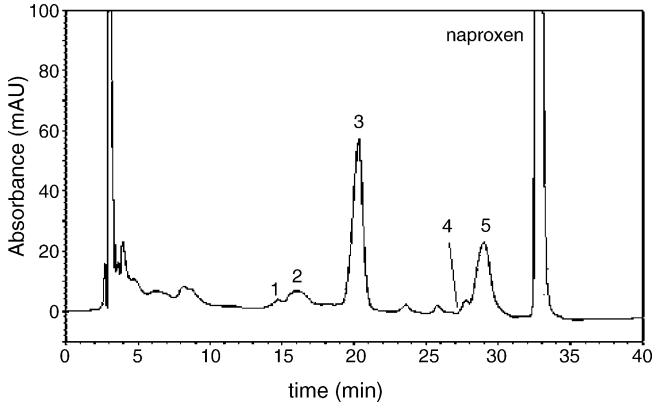

Fig. 5. LC-UV chromatogram relevant to a post dose urine sample (0–12 h) subjected to enzymatic hydrolysis.

the glucuronic acid conjugates represented by peak 1 and 4 were substrates for β-glucuronidase, and could be attributed to the *O*-6-desmethylnaproxen-1-β-acyl-glucuronide and naproxen-1-β-acyl-glucuronide, respectively; on the contrary, those eluting as peak 2 and 5 were not cleaved by the enzyme and could be attributed to the *O*-6-desmethylnaproxen-1-α-acyl-glucuronide and naproxen-1-α-acyl-glucuronide, respectively.

4. Conclusions

It is very common to consider intact glucuronide conjugates as "detoxified" easily eliminable water-soluble drug metabolites. However, it is also known that also biologically active or toxic glucuronide could be produced. In view of the above considerations, an LC–ESI-MS procedure for a rapid and efficient identification of naproxen and DM-naproxen glucuronides has been developed, permitting the characterization of two couples of α and β isomers of naproxen and DM-naproxen glucuronides, respectively. The method could be potentially extended to the characterization of other drug metabolites.

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