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Determination of naproxen in human urine by solid-phase microextraction coupled to liquid chromatography

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Abstract

An SPME–LC–UV method for the determination of the non-steroidal anti-inflammatory drug (NSAID) naproxen and, after hydrolysis, its glucuronide in human urine samples was developed for the first time using a carbowax/templated resin (CW/TPR-100)-coated fibre. The procedure required a very simple sample pre-treatment, an isocratic elution, and provides a highly selective extraction. All the aspects influencing adsorption (extraction time, temperature, pH and salt addition) and desorption (desorption and injection time and desorption solvent mixture composition) of the analyte on the fibre have been investigated. The linear range investigated in urine was 0.2–20 μg/ml (that covers the typical naproxen urinary concentration) and almost quantitative recoveries were obtained. Within-day and between-days R.S.D.% in urine were 4.5 and 6.0, respectively. The LOD and LOQ in spiked urine were 0.03 and 0.20 μg/ml, well below the usual naproxen urinary level.

Keywords: SPME–LC; Naproxen; Naproxen glucuronide; Metabolism; Urine

1. Introduction

The 6-methoxy-α-methyl-2-naphthyl-acetic acid (naproxen) belongs [1] to the class of non-steroidal anti-inflammatory drugs (NSAIDs). 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 [2–4] with glucuronic acid (naproxen and DM-naproxen) or sulphate (DM-naproxen). The metabolic pathways are shown in Fig. 1.

NSAIDs are commonly employed to reduce ongoing inflammation, pain and fever, since they are able to block [5] the cyclooxygenase (Cox) enzymes (Cox-1 and Cox-2), that both produce prostaglandins; these classes of compounds have several important functions, as the promotion of inflammation, pain and fever. However, prostaglandins produced by the Cox-1 enzyme, are also able to protect the stomach, support platelets and blood clotting. Thus, NSAIDs can cause ulcers in the stomach and promote bleeding after an injury or surgery. Moreover, they are associated with other serious side effects, i.e. kidney failure, and with a number of minor side effects, such as nausea vomiting, diarrhoea, constipation, decreased appetite, rash, dizziness, headache and drowsiness. Finally, they also interact with other drugs; in particular, they reduce the action of diuretics and antagonize the action of drugs used to treat hypertension.

In view of the above considerations, the development of a simple and reproducible method for the determination of naproxen and its glucuronide in biological fluids could be very useful for toxicological and pharmaceutical purposes. Several methods dealing with naproxen determination have been reported [6–12], mainly based on liquid chromatography following traditional extraction procedures.

Solid-phase microextraction (SPME) [13], is a solventless technique that has been mainly applied [14–20] in combination with GC; however, a growing interest for SPME coupled to LC was observed in the past few years as demonstrated by a number of recently published papers [21].

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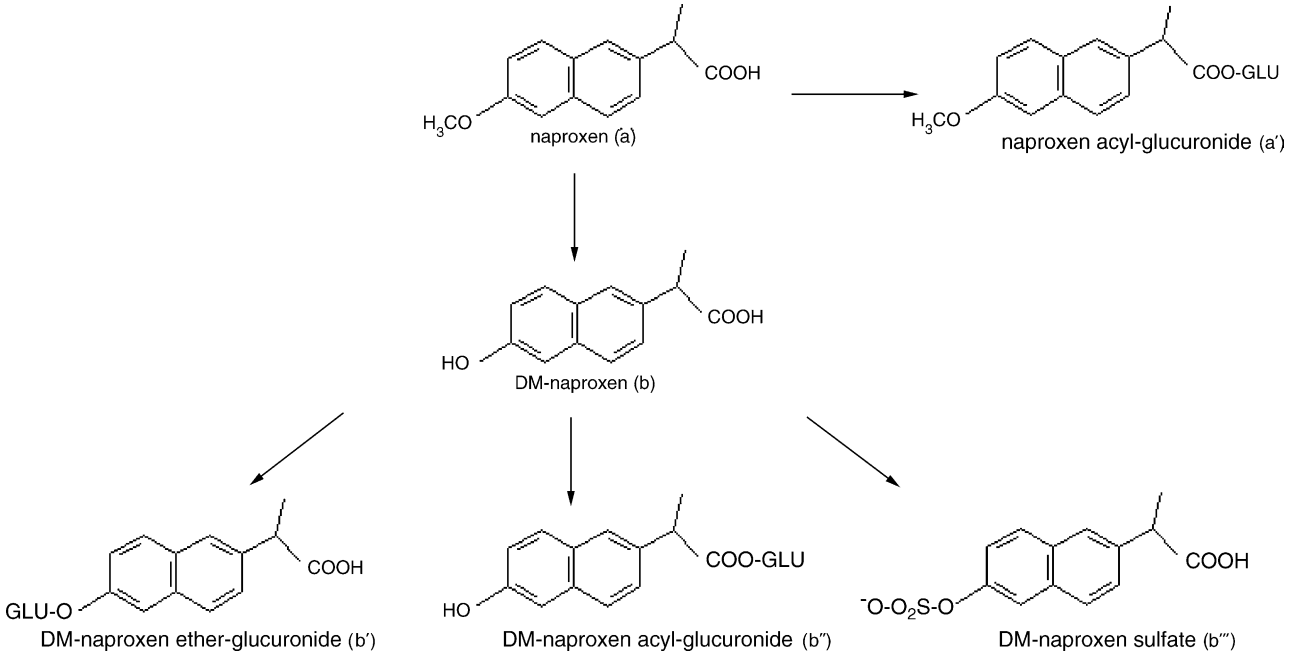

Fig. 1. The metabolic pathways of naproxen.

In the present paper, SPME of naproxen, was optimized and interfaced with LC–UV using a carbowax/templated resin (CW/TPR-100)-coated fibre. The developed procedure was then applied to the determination of naproxen in urine samples. The determination of naproxen glucuronide was also indirectly performed after chemical and enzymatic hydrolysis of the conjugate.

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 SPME interface (Supelco, Bellefonte, PA), consisted of a standard six-port Rheodyne valve equipped with a fibre desorption chamber (total volume: 60 μl), installed in place of the sample loop.

The LC system used in this study includes a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA) and a Supelcosil LC-18-DB column (250 mm × 4.6 mm i.d., particle 5 μm, Supelco, Bellefonte, PA, USA). Mobile phase was degassed by an SCM 1000 Vacuum membrane degasser (Thermo Separation Products). The detector was a photodiode-array (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer.

2.3. Chromatographic and detection conditions

The mobile phase consisted of an acetonitrile/methanol/ammonium acetate buffer (10 mM, pH5) mixture (6:40:54, v/v/v). The flow rate was 0.2 ml min^{-1} and temperature was ambient. The detection wavelength was 230 nm (10 Hz frequency, 5 nm band-width). Spectra were acquired in the 225–386 nm range (2 Hz frequency, 5 nm band-width).

2.4. Solid-phase microextraction

Fibers coated, respectively, with a 50 μm thick carbowax/templated resin film, a 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB) film and a 85 μM thick polyacrylate (PA) film (Supelco) were employed for comparative studies. A manual SPME device (Supelco) was used to hold the fiber. Working solutions were prepared by spiking 15 ml of an acetic acid (10 mM, pH 3) and NaCl (0.05 M) solution with different amounts of naproxen (0.1–200 ng/ml) into 15 ml clear vials (Supelco). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The extraction was carried out at 20 °C at pH 3 for 30 min under magnetic stirring. Naproxen desorption was performed in static desorption mode by soaking the fiber in mobile phase into the desorption chamber of the in-

terface for 10 min. Then, the valve was changed to the inject position and the fiber was exposed for 40 s to the mobile phase stream.

In order to evaluate percentages of desorption and carry-over, the fiber was left in the chamber after each experiment and a second chromatographic run was performed leaving the interface valve in the inject position (dynamic desorption); this operation mode ensured a total desorption of the analyte remained on the fiber.

2.5. Sample collection and pre-treatment

Drug-free urine samples (0–12 h) were collected from healthy donors. 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. Then, 0.15 ml of each sample were diluted with 15 ml of an acetic acid (10 mM, pH 3) and NaCl (0.05 M) solution and directly subjected to SPME.

Recoveries were calculated as peak area ratio of naproxen (standard solution)/naproxen (spiked urine samples). Urine samples were spiked with naproxen at 0.01–20 μg/ml concentration levels.

Acid and enzymatic hydrolysis were performed on post-dose urine samples (0–12 h).

Acid hydrolysis: A 0.5 ml of 37% HCl were added to 1.0 ml of urine, and incubated at 56 °C for 90 min. Then, the solution was diluted 1:1 with NaOH 2N. Finally, 50 μl of the resulting mixture were diluted and analyzed as previously described.

Enzymatic hydrolysis: A 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. Then, 25 μl of the resulting mixture were diluted and analyzed as previously described.

3. Results and discussion

3.1. Fibre-coating material

Preliminary experiments were performed in order to compare the extraction efficiency obtained using the CW/TPR-100, PA and PDMS/DVB-coated fibers, respectively. The CW/TPR-100 fiber was capable of the most efficient extraction and was then selected for further experiments.

3.2. Extraction time and temperature

The extraction time profiles were established by plotting the area counts versus the extraction time. Fig. 2 reports the results obtained at 20 °C. As apparent, after 60 min, the equilibrium was still not reached. In any case, since it is possible to obtain good extraction yields and reliable analysis also in non-equilibrium conditions, an extraction time of 30 min was chosen for further experiments. The extraction profile was also established at 50 °C; however, a response decrease was observed in this case (data not shown).

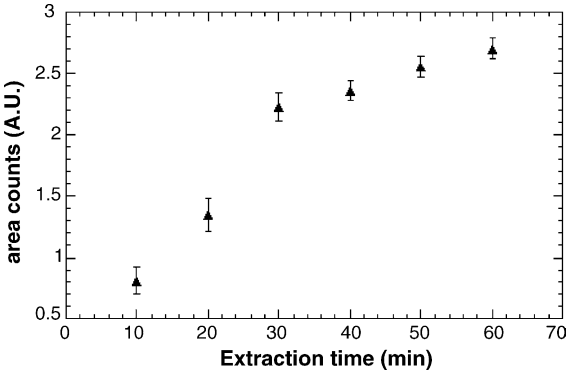

Fig. 2. Extraction time profiles obtained with the CW/TPR-100 fiber at 20 °C. The concentration is 0.5 μg/ml.

3.3. Ionic strength and pH

Generally speaking, salt addition improves the recovery, especially in the case of polar (hydrophilic) compounds that are difficult to extract. Thus, experiments were performed by increasing progressively the ionic strength of the extraction solutions. A signal enhancement of around 33% was obtained by the addition of 0.05 M sodium chloride, that was chosen as working concentration, since higher salt levels did not produce an additional signal increase.

Since analytes in the neutral forms are more efficiently extracted by the non-ionic polymeric coatings, the effect of the pH on the extraction efficiency was examined. Naproxen is in fact an acid compound and is present in its undissociated form at acid pH. As expected, a response increase was observed by decreasing pH from 9 to 3; the latter value was chosen for further experiments.

3.4. Desorption conditions and "carry-over"

The dynamic mode was first employed to desorb the analyte from the fiber in the SPME–LC interface; this approach produced quantitative recoveries but very broad chromatographic peaks. Thus, the static desorption technique was used for further experiments. The fiber was soaked in mobile phase for a variable period of time before injection into the LC column. The best conditions (recovery of 95 ± 2%) were reached after 10 min of static desorption; then, the fiber was exposed for 40 s to the mobile phase stream.

3.5. Linear range, detection limits and precision

The response of the developed SPME–LC procedure was linear in the range 2–200 ng/ml, with correlation coefficients

better than 0.999 and an intercept not significantly different from 0 at 95% confidence level.

The estimated LOD and LOQ obtained on standard solutions were 0.3 and 2.0 ng/ml, respectively, calculated according to IUPAC as 3- and 10-fold the standard deviation of the intercept of the calibration curve [22].

The within-day precision of the method was investigated on standard solutions at a concentration level of 10, 50 and 100 ng/ml by performing daily six replicates. The same solutions were analyzed six times each day for a period of 10 days for the day-to-day precision evaluation. The within-day and day-to-day R.S.D.% were 3.8 and 5.1, respectively, and were not concentration dependent.

3.6. Urine samples analysis

The developed procedure was then applied to urine samples. Recoveries were calculated as peak area ratio of naproxen (standard solution)/naproxen (spiked urine samples). Almost quantitative recoveries (94.5 ± 4.5%) were obtained and remained practically unchanged passing from 0.2 to 20 μg/ml level.

Calibration curve resulted linear in the range 0.2–20 μg/ml (that covers the typical naproxen urinary concentration) with correlation coefficients better than 0.999 and intercept not significantly different from 0 at 95% confidence level.

The estimated LOD and LOQ were 0.03 and 0.20 μg/ml, respectively (well below the usual naproxen urinary level) calculated as 3- and 10-fold the standard deviation of the intercept of the calibration curve [22].

The within-day and day-to-day R.S.D.% obtained in urine, in the concentration range 10–100 ng/ml, were 4.5 and 6.0, respectively.

Fig. 3 reports the SPME–LC–UV chromatograms obtained from a drug-free urine (dotted line) and a post-dose urine (solid line) samples (0–12 h). As apparent, the analyte was clearly detected and well resolved from matrix components. The estimated naproxen urinary concentration was 6.5 μg/ml.

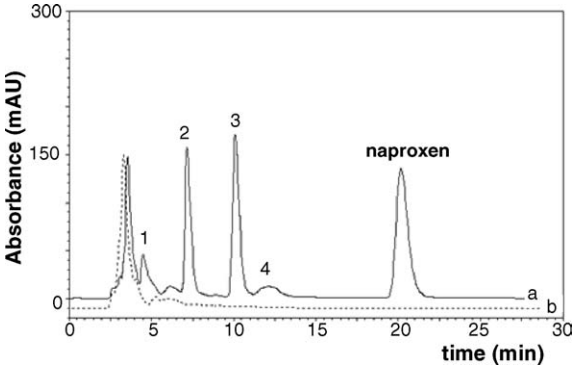

Fig. 3. SPME–LC–UV chromatograms relevant to urine samples (0–12 h), post-dose (—) and drug-free (- - -).

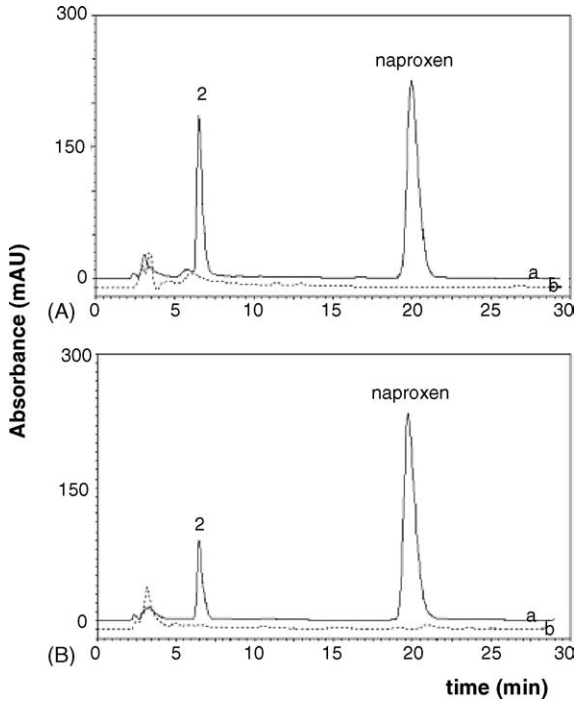

Fig. 4. SPME–LC–UV chromatograms relevant to post-dose urine samples (0–12 h), subjected to (A) acid and (B) enzymatic hydrolysis, respectively.

Furthermore, in addition to the analyte peak, four more peaks (1, 2, 3 and 4) not detectable in the blank having the same UV spectra of naproxen were observable. This was not unexpected, since it is known that the parent drug is excreted in urine also via phases I and II metabolites.

Acid and enzymatic hydrolysis were then independently performed as described in the experimental section in order to indirectly determinate the amount of naproxen glucuronide as a measurement of the difference for the parent drug before and after the hydrolysis. Fig. 4 reports the SPME–LC–UV chromatograms relevant to a post-dose urine sample (0–12 h), subjected to (A) acid and (B) enzymatic hydrolysis, respectively. As apparent, peaks 1, 3 and 4 disappeared from the chromatogram, in both cases, while the naproxen peak and peak 2, likely attributable to DM-naproxen, had a consistent increase. Moreover, while the naproxen peak increase was almost constant in both cases, the increase of peak 2 was smaller in the case of enzymatic hydrolysis. In fact, the acid hydrolysis was able to hydrolyze all the conjugates reported in Fig. 1. On the contrary, the enzymatic hydrolysis was specific for the glucuronic acid conjugates and the sulphate conjugate remained unchanged, thus explaining the smaller increase of peak 2 in this case.

The naproxen glucuronide concentration estimated after acid hydrolysis was 76.7 μg/ml, while 78.8 μg/ml were found after enzymatic hydrolysis (note that the variability is well comprised within the reproducibility of the method). Since very high concentration levels were estimated for naproxen

Table 1
Urinary excretion parameter of naproxen following the single oral administration of 220 mg

Parameter	Free	Deconjugation	
		Acid hydrolysis	Enzymatic hydrolysis
Concentration (μg/ml)	6.5	83.2	85.3
$Excretion_{0–12\,h}$ (mg)	2.2	28.3	29.0
$\%Excretion_{0–12\,h}$	1.0	12.8	13.2

glucuronide a very high peak relevant to this compound should be present in the chromatogram of Fig. 3 (before hydrolysis). The lack of such peak could be ascribed to the poor extraction efficiency of the fiber for naproxen glucuronide, as already observed [23] in the case of another glucuronated drug.

Table 1 resumes the urinary excretion parameters of naproxen following the single oral administration of 220 mg.

4. Conclusions

An SPME (CW/TPR-100 fiber)–LC–UV method for the determination of naproxen was developed for the first time. The procedure requires simple sample pre-treatment and allows an easy quantification of naproxen within its typical urinary concentration. The potential of the described procedure was demonstrated by the determination of naproxen and, indirectly after hydrolysis, naproxen glucuronide, in human urine samples of a patient under naproxen treatment.

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