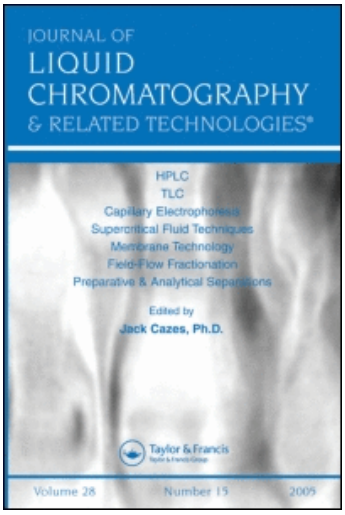

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Jana Sádecká[a]; Jana Netriová[b]
[a] Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Bratislava, Slovakia
[b] National Cancer Institute, Bratislava, Slovakia

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Determination of Naproxen and its Metabolite, 6-O-Desmethylnaproxen, in Human Urine by Capillary Isotachophoresis

Jana Sádecká
Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Bratislava, Slovakia

Jana Netriová
National Cancer Institute, Bratislava, Slovakia

Abstract: Analysis of naproxen (NP) and 6-O-desmethylnaproxen (DNP) in human urine samples was carried out using a column-coupling isotachophoretic analyzer equipped with a conductivity detector. The preseparation capillary (80 mm × 0.8 mm I.D.) was connected with an analytical capillary (160 mm × 0.3 mm I.D.). The preseparation capillary was filled with the leading electrolyte (LE): 20 mM hydrochloric acid adjusted with creatinine to pH 5.0; 0.1% methylhydroxypropylcellulose. The analytical capillary was filled with the LE: 10 mM hydrochloric acid adjusted with β-alanine to pH 4.0; 0.1% methylhydroxypropylcellulose. The terminating electrolyte was 10 mM 2-(N-morpholino)-ethanesulfonic acid adjusted with tris(hydroxymethyl) aminomethane to pH 6.9. Limit of quantitation was 1.4 μg/mL for NP and 0.5 μg/mL for DNP. The proposed method was successfully applied to the direct determination of free NP and DNP in urine samples. The total of free and conjugated NP and DNP was obtained by including an alkaline hydrolysis step.

Keywords: Naproxen, 6-O-desmethylnaproxen, Capillary isotachophoresis, Human urine

INTRODUCTION

Naproxen, (S)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid, is a non-steroidal anti-inflammatory drug used in the treatment of rheumatic and

Address correspondence to Jana Sádecká, Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Radlinského 9, 812 37 Bratislava, Slovakia. E-mail: jana.sadecka@stuba.sk

other inflammatory diseases. In human naproxen (NP) is metabolized to 6-O-desmethylnaproxen (DNP) and both compounds are excreted unchanged, as well as conjugated with glucuronic acid and sulfate.[1–3]

Among the techniques used for determining naproxen in biological fluids only a few allow the determination of naproxen in the presence of its 6-O-desmethylated metabolite. These methods include second-derivative UV spectrometry,[4] second-derivative synchronous fluorescence spectrometry,[5] liquid chromatography,[2,3,6–8] GC,[9,10] capillary zone electrophoresis,[11–17] as well as micellar electrokinetic capillary chromatography.[18] Chromatographic techniques like HPLC with mass spectrometry[3] and NMR[3,8] or more rarely GC with mass spectrometry[10] are the common choices for the investigation of the metabolic pattern of naproxen. However, the complexity of the matrix might raise problems, which can be solved by time-consuming sample preparation, using gradient eluent systems with a high consumption of organic solvents or increasing analysis time. Capillary electrophoresis has been established in biomedical analysis as a powerful alternative to HPLC or GC due to its excellent resolution, which enables direct analysis of body fluids.[11–14,18]

In our previous work,[19] a simple isotachophoretic (ITP) method has been developed for the separation of NP and DNP, which allowed the determination of naproxen in the presence of its metabolite DNP in human serum. This paper describes an ITP method, which can differentiate between NP and DNP and determine them simultaneously in human urine. The proposed method was successfully applied to the direct determination of free NP and DNP in urine samples. The total of free and conjugated NP and DNP was obtained by including an alkaline hydrolysis step.

EXPERIMENTAL

Chemicals and Solutions

Hydrochloric acid (p.a.), phosphoric acid (p.a.), and sodium hydroxide (p.a.) were obtained from Lachema (Czech Republic). β-alanine, creatinine, methylhydroxypropylcellulose, 2-(N-morpholino)-ethanesulfonic acid, and tris(hydroxymethyl)aminomethane were obtained from Sigma-Aldrich. Naproxen sodium salt (NP) (Sigma-Aldrich) was obtained as a gift from Dr. H. Shintani (National Institute of Health Sciences, Tokyo, Japan). 6-O-desmethylnaproxen (DNP) was synthesized by the method of Andersen.[6] The identity of DNP was confirmed using mass spectrometry. Stock solutions of NP and DNP containing 1 mg/mL were prepared by dissolution of the standards in water. Stock solutions were stored at 4°C and were stable at least 1 month. Standard solutions were obtained from the stock solutions by dilution with water. Calibration curves for NP and DNP were

made by spiking 1.0 mL of blank urine with standard solutions of NP and DNP to yield 2, 5, 10, 20, 50, 100, and 250 μg/mL.

Deionized, double distilled water was used in the preparation of the electrolyte systems and solutions of the model mixtures.

ITP Analysis

Isotachophoretic separations were performed using a Villa Labeco ZKI 02 (Slovakia) column-coupling isotachophoretic analyzer equipped with a conductivity detector. The preseparation capillary (80 mm × 0.8 mm I.D.) was connected with an analytical capillary (160 mm × 0.3 mm I.D.). The capillaries made of fluorinated ethylene-propylene copolymer were used.

The preseparation capillary was filled with the leading electrolyte (LE): 20 mM hydrochloric acid adjusted with creatinine to pH 5.0; 0.1% methylhydroxypropylcellulose. The analytical capillary was filled with the LE: 10 mM hydrochloric acid adjusted with β-alanine to pH 4.0; 0.1% methylhydroxypropylcellulose. The terminating electrolyte (TE) was 10 mM 2-(N-morpholino)-ethanesulfonic acid adjusted with tris(hydroxymethyl) aminomethane to pH 6.9. The driving current for the preseparation column was initially 350 μA. It was reduced to 150 μA prior to detection. The driving current for the analytical column was initially 30 μA, then it was reduced to 15 μA.

All pH measurements were made with a type OP-208/1 Precision digital pH-meter with a combined glass electrode type OP-8071-1/A (both Radelkis, Budapest).

Sampling and Sample Treatment

The healthy volunteers (weight 60–75 kg) were administrated a single oral dose of NP as a 500 mg tablet, following an overnight fast. Urine samples were collected immediately before administration and then 0–3, 3–6, 6–9, 9–12, 12–15, 15–24, 24–48, and 48–96 h post dose. The individual urine volumes were recorded and a pooled 96 h urine sample was prepared from the individual samples. To stabilize the acyl glucuronides[20] immediately after collection the urine was acidified to pH 5.0 with 83% H_3PO_4 and then stored at −20°C until analyzed. Urine samples were injected directly, using a micro syringe (5–7 μL). The urine assay described allows determination of free NP and DNP in urine. The total of free and conjugated NP and DNP could be obtained by including an alkaline hydrolysis step.[11,20] A 20 μL volume of NaOH (1 mol/L) was added to thw urine sample (100 μL) for the alkaline hydrolysis of the glucuronic acid conjugates. The hydrolysis reaction was left to proceed for 1 h at 37°C. Urine samples were diluted tenfold with deionized water and injected via a sampling valve (30 μL) or with a 10-μL micro syringe.

RESULTS AND DISCUSSION

One difficulty in the direct analysis of urine is the variable, but generally high concentration of electrolytes in urine because, in ITP, the ionic strength of the sample to be analyzed is limited. For instance, high concentrations of chloride result in a prolonged time of analysis. The excessive amounts of chloride (another anion) may be removed either by sample preparation or by using a column-coupling ITP instrument. In the commercial instrument used for our ITP experiments, the analytical capillary of 0.3 mm I.D. is combined with the preseparation capillary of 0.8 mm I.D., which increases the load capacity of the system without a considerable increase in the time of analysis. The matrix components were almost totally (98%) removed from the separation system prior to entering the analytical capillary, where further separation and detection take place. The detector in the preseparation capillary ensures that the time of current switching can be adjusted precisely with respect to the actual composition of the separated sample. Furthermore, a proper switching of the current leads to the ITP analysis (in the analytical capillary) of a very simplified part of the sample, which further eliminates interferences from the matrix.

In urine samples, NP and DNP occur along with a variety of other ionic components, the effective mobilities of some of them (e.g. citrate, lactate, and phosphate) are close to NP and DNP. The full ITP separation of NP and DNP from citrates, lactates, and phosphates was achieved at pH of the leading electrolyte, 5.0 in the preseparation capillary. At this pH all the components mentioned above created ITP zones migrating in front of the mixed zone of NP and DNP, and after the detection in the preseparation capillary they were removed from the separation compartment. The mixed zone of NP and DNP was then transferred quantitatively into the analytical capillary filled with the leading electrolyte pH 4.0. For the complete separation of NP, DNP, and other constituents of the sample, which could be transferred together with NP and DNP into the analytical capillary, lower pH of the LE was used in the analytical capillary. Further improvement in the resolution of NP and DNP was obtained using β-alanine as a counter-ion in the LE in the analytical capillary.[19] This composition of the LE was verified in a series of samples of blank urine spiked with NP and DNP, and there was proven to be good resolution of NP from DNP and from all other sample micro compounds.

Calibration curves were constructed using seven standard concentrations (2–250 μg/mL, 5 μL injected) in the blank urine. Curves were obtained by plotting the zone lengths of NP and DNP against the corresponding nominal concentration. Linear calibration curves were generated by unweighted linear regression analysis. The mean $\pm$ SD coefficients of determination (r^2), slopes, and intercepts for NP and DNP in urine were $r^2 = 0.997 \pm 0.003$ (slope $= 0.514 \pm 0.024$, intercept $= 0.14 \pm 0.06$) and $r^2 = 0.996 \pm 0.004$ (slope $= 0.532 \pm 0.019$, intercept $= 0.09 \pm 0.02$),

respectively. Linearity specifications for the urine assay, including the conjugate hydrolysis step, are almost identical to those shown for assay of the unconjugated material ($r^2 = 0.996 \pm 0.015$, slope $= 0.482 \pm 0.019$ and intercept $= 0.12 \pm 0.03$ for NP and $r^2 = 0.995 \pm 0.011$, slope $= 0.519 \pm 0.029$ and intercept $= 0.11 \pm 0.02$ for DNP).

For the limit of quantitation (LOQ), we used the value $(y + 10.SD)/b$, whereby the calculated intercept of the calibration line can be used as an estimate of y, SD is the standard deviation in the y-direction of the calibration line and b is the slope of the calibration line. For the limit of detection (LOD), the value $(y + 3.S)/b$ was calculated. LOQ and LOD were 1.4 μg/mL and 0.6 μg/mL for NP and 0.5 μg/mL and 0.3 μg/mL for DNP.

The recovery was determined by comparing the zone length from drug-free urine spiked with a mixture of NP and DNP (5, 50, and 200 μg/mL) versus zone length of the same concentrations prepared in water, injected directly into the capillary. Each sample was injected five times. Data on the recovery are given in Table 1. The recovery of NP averaged 101.5% (RSD, 2.2%). It is not statistically different over the range of concentrations studied. The recovery of DNP averaged 98.8% (RSD, 4.6%), it was found to be consistent over the evaluated concentration range.

The accuracy and precision of the method were evaluated by analyzing five-replicates of spiked urine at each of three concentrations (5, 50, and 200 μg/mL) against a calibration curve. Accuracy was evaluated as percent error [(mean of measured − mean of added)/mean of added] × 100, while the precision was given by the relative standard deviation. The results of the accuracy and precision of the method are given in Table 1 and are below 15%, which is an acceptable range for validation methods.[21]

The method was used to determine free NP and DNP, and the total of free and conjugated NP and DNP in urine samples collected at several time points,

Table 1. Accuracy, precision, and recovery of the analytical procedure for naproxen and 6-O-desmethylnaproxen

Amount added (μg/mL)	Amount found (μg/mL)	Accuracy (bias%)	Precision (RSD%)	Recovery (mean%)	Recovery (RSD%)
Naproxen					
5.0	5.2	+4.0	2.5	104.0	2.0
50.0	51.2	+2.4	0.8	102.4	0.9
200.0	196.5	−1.7	3.6	98.2	3.6
	Mean	+1.6	2.3	101.5	2.2
6-O-desmethylnaproxen					
5.0	4.8	−4.0	5.4	96.0	5.1
50.0	48.6	−2.8	3.6	97.2	3.2
200.0	206.3	+3.1	6.6	103.1	5.6
	Mean	−1.2	5.2	98.8	4.6

followed by a polled urine sample from 0 to 96 h. The highest urinary concentration of free NP and DNP was found in the 3 h sample. The maximum excretion rate (NP = 16.5 μg/min, DNP = 4.1 μg/min) was obtained after 3 h. The last sample, in which free NP and DNP could be detected, was collected 24 h after administration (excretion rate, NP = 0.4 μg/min and DNP = 0.3 μg/min). Figure 1 shows typical isotachopherogram of the human urine obtained on the analytical capillary. The total urinary excretion (0–96 h) of NP and DNP, both free and conjugated with glucuronic acid, was 45.3% (NP, free = 0.8%, conjugated = 44.5%) and 21.3% (DNP, free = 2.1%, conjugated = 19.2%). These results are in good agreement with previously published data.[2,6,22]

In conclusion, ITP offers same advantages over conventional chromatographic methods: (i) nonionic compounds do not interfere with the analysis of the ionic compounds, (ii) column-coupling ITP enables one to increase the injection volume of the sample and to eliminate the majority of interfering components present in urine, (iii) low running cost (two orders of magnitude compared with HPLC), decreased cost of capillaries, (iv) no organic solvents

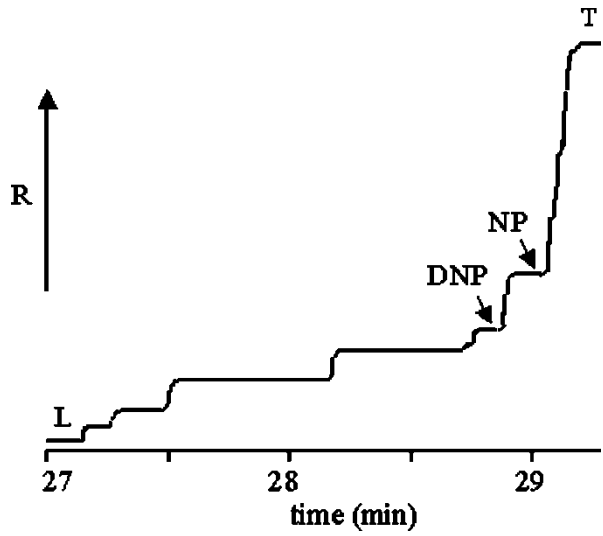

Figure 1. Isotachopherogram of the urine sample collected 24 h after administration of naproxen (500 mg). Injection volume: 5 μL. Record from the analytical capillary is shown. The analytical capillary was filled with the leading electrolyte: 10 mM hydrochloric acid adjusted with β-alanine to pH 4.0; 0.1% methylhydroxypropylcellulose. The terminating electrolyte was 10 mM 2-(N-morpholino)-ethanesulfonic acid adjusted with tris(hydroxymethyl) aminomethane to pH 6.9. The driving current was 15 μA. L-leading ion (chloride), T-terminating ion, DNP-6-O-desmethylnaproxen, NP-naproxen, R-increasing resistance.

are used in the preparation of LE. The largest drawback of ITP is lower resolution as compared with e.g., capillary zone electrophoresis.

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