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Isotachophoretic determination of naproxen in the presence of its metabolite in human serum

Miroslav Čakrt[a], Andrea Hercegová[a], Ján Leško[b], Jozef Polonský[a,*], Jana Sádecká[a], Ivan Skačáni[a]

[a]*Department of Analytical Chemistry, Faculty of Chemical Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic*

[b]*Central Laboratories, Faculty of Chemical Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic*

Abstract

An isotachophoretic method with conductivity detection was developed to determine naproxen in the presence of its metabolite 6-*O*-desmethylnaproxen in human serum. The leading electrolyte contained 10 m*M* hydrochloric acid, β-alanine, pH 4.0 and 0.1% methylhydroxypropylcellulose. The terminating electrolyte was 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid–tris(hydroxymethyl)aminomethane, pH 6.9, containing 20% (v/v) of ethanol. Naproxen was determined in serum supernatant after simple deproteination of the sample with ethanol. The isotachophoretic results were compared with those obtained by synchronous fluorescence spectrometry. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Isotachophoresis; Naproxen; Desmethylnaproxen

1. Introduction

Naproxen, (*S*)-6-methoxy-α-methyl-2-naphthaleneacetic acid, is widely used in the treatment of arthritis and for the relief of mild to moderate pain. It is given in daily dose rates of 500–1500 mg, mainly as sustained release tablets. Pharmacokinetic studies have shown that naproxen is metabolized by 6-*O*-desmethylation and more than 90% of the drug is excreted in urine as parent or as 6-*O*-desmethylnaproxen either unchanged or conjugated with sulfuric and glucuronic acid [1].

After a single oral dose of 500 mg, peak serum concentrations of naproxen are about 70 μg/ml whereas peak concentrations of 6-*O*-desmethylnaproxen are about 0.2 μg/ml. Owing to the presence of trace amounts of 6-desmethylnaproxen in serum, the method used to determine naproxen should be able to differentiate between naproxen and its metabolite. Among the techniques used for determining naproxen in biological fluids [2–15] only a few allow the determination of naproxen in the presence of its 6-desmethylated metabolite. These methods include liquid chromatography [2,7], GC [13], second-derivative UV spectrometry [14] and second-derivative synchronous fluorescence spectrometry [15]. The limit of quantitation of naproxen in human plasma obtained by liquid chromatography was 1.5 μg/ml with UV detection at 330 nm and 5 μg/ml with fluorescence detection (excitation at 330 nm, emission at >389 nm) [2]. A better limit of quantitation (0.1 μg/ml in human plasma) was

*Corresponding author. Tel.: +421-7-5932-5722; fax: +421-7-5292-6043.

E-mail address: herceg@chelin.chtf.stuba.sk (J. Polonský).

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obtained using either UV absorption at 232 nm or fluorescence at 330 or 355 nm [7]. The limit of detection obtained by second-derivative synchronous fluorescence spectrometry was 1.6 ng/ml and 1.9 ng/ml for naproxen and 6-*O*-desmethylnaproxen [15].

In this article, a isotachophoretic method for the determination of naproxen in the presence of its metabolite 6-*O*-desmethylnaproxen in human serum is reported. Naproxen was determined in serum supernatant after simple deproteination of the sample with ethanol. The isotachophoretic results were compared with those obtained by synchronous fluorescence spectrometry.

2. Experimental

2.1. Chemicals and standards

All chemicals were analytical reagent grade. Deionized, redistilled water was used in the preparation of the electrolyte systems and solutions of the model mixtures.

Naproxen sodium salt (NP) (Sigma) 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 [7]. The identity of DNP was confirmed using mass spectrometry. Control serum EXATEST was obtained from Imuna (Šarišské Micha'any, Slovak Republic). Stock solutions of NP and DNP containing 1000 μg/ml and 100 μg/ml, respectively, were prepared by dissolution of the standards in water and than stored at 4°C. Standard solutions were obtained from the stock solutions by dilution with purified water. Serum standards were prepared daily by adding known amount of the standard to drug-free control serum.

2.2. Isotachophoresis (ITP)

2.2.1. Instrumentation

Isotachophoretic separations were performed using a Villa Labeco ZKI 02 (Slovak Republic) column-coupling isotachophoretic analyzer equipped with a conductivity detector. The analytical capillary (160 mm×0.3 mm I.D.) was connected with a preseparation capillary (80 mm×0.8 mm I.D.). Capillaries made of fluorinated ethylene–propylene copolymer were used. The electrolyte systems are given in Table 1.

2.2.2. Pretreatment of serum samples

A 0.5 ml volume of serum was mixed with 0.5 ml volume of ethanol. The precipitate formed was removed by centrifugation at 3000 *g* for 5 min. The upper layer was transferred to a clear tube, than 30 μl was injected into the ITP analyzer. Care must be taken not to inject any precipitate.

2.3. Synchronous fluorescence spectrometry (SFS)

2.3.1. Instrumentation

All fluorescence measurements were done on a Perkin-Elmer LS 50 Luminescence spectrometer equipped with a xenon discharge lamp (20 kW) and 1×1 cm quartz cell. The LS 50 spectrometer was

Table 1
Electrolyte systems for ITP

Parameter	Conditions
Leading electrolyte[a]	
LE1	10 m*M* HCl adjusted with creatinine to pH 4.5
LE2	10 m*M* HCl adjusted with 6-aminocaproic acid to pH 4.25
LE3	10 m*M* HCl adjusted with creatinine to pH 4.0
LE4	10 (20) m*M* HCl adjusted with β-alanine to pH 4.0
Terminating electrolyte	
TE	10 m*M* MES[b] adjusted with Tris[c] to pH 6.9

[a] 0.1% methylhydroxypropylcellulose was added to all leading electrolytes in order to suppress the electroosmotic flow.
[b] MES=2-(*N*-morpholino)ethanesulfonic acid.
[c] Tris=tris(hydroxymethyl)aminomethane.

Table 2
Instrumental parameters for synchronous florescence spectrometry

Parameter	Compound	
	NP	DNP
Synchronous spectrum scanning range/λ_{ex} (nm)	200–375	200–375
Constant wavelength difference $\Delta\lambda$ (nm)	20	100
Scan speed (nm/min)	200	200
Slit width/ex/em (nm)	3.0/3.0	3.0/3.0
Savitzky-Golay filter size/points	11	11

interfaced with a Epson PC AX2 microcomputer supplied with FL data manager software (Perkin-Elmer) for spectral acquisition and subsequent manipulation of spectra. All instrumental parameters used are summarized in Table 2.

2.3.2. Pretreatment of serum samples

A 0.4 ml volume of serum containing 1.0–40.0 μg of NP and 0.1–2.0 μg of DNP was mixed with 0.8 ml of acetonitrile and centrifuged for 1 min at 3000 *g*. Then 0.1 ml of the supernatant was mixed with 4.0 ml of 0.05 mol/l NaOH.

3. Results and discussion

3.1. Method development

Considering the requirement that acids in question (Fig. 1) should be sufficiently dissociated and considering the data from Ref. [16], we selected the leading electrolytes in the pH range 4.0–4.5 for isotachophoretic experiments (Table 1). The studied pH range determined by these electrolyte systems was restricted as naproxen migrate in the zone of terminating ion at a high pH and the use of a low pH would be rather problematic, because even the short analytical capillary caused a high-voltage values when run with MES as the terminating electrolyte in the systems with a pH<4.0. ITP separabilities of NP and DNP at various pH values are illustrated by isotachopherograms in Fig. 2a–d. From these it follows that the pH had small influence on the separation. However, the choice of the counter-ion had a great impact on the resolution. It was found that creatinine as a counter-ion unenabled the resolution of NP and DNP regardless of the pH of leading electrolyte (Fig. 2a and 2c). On the contrary, when β-alanine was included as a counter-ion of the leading electrolyte pH 4.0, good separation was obtained (Fig. 2d). At pH 4.0, the relative step heights (RSHs) of NP and DNP are 0.45 and 0.25.

According to Ref. [16], the treatment of serum with ethanol almost eliminated proteins and allowed a large sample volume to be injected into the capillary which resulted in a better limit of quantitation. To overcome the loss of analyte due to unhomogeneity in density of sample and terminating electrolyte in vertically arrangement of the instrument capillaries, we further used the terminating electrolyte 10 m*M* MES–Tris (pH 6.9) containing 20% (v/v) of ethanol. To enhance the separation capacity of the preseparation capillary, the concen-

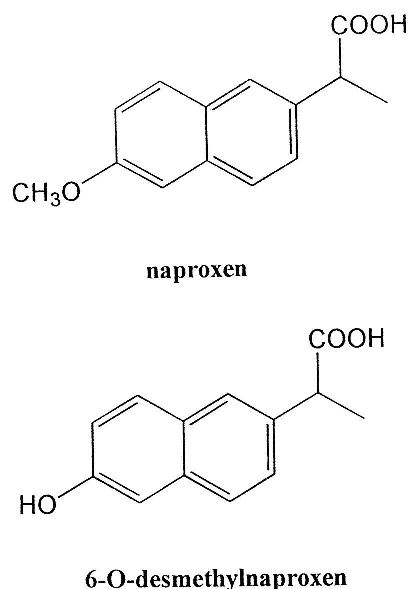

Fig. 1. Structures of naproxen and 6-*O*-desmethylnaproxen.

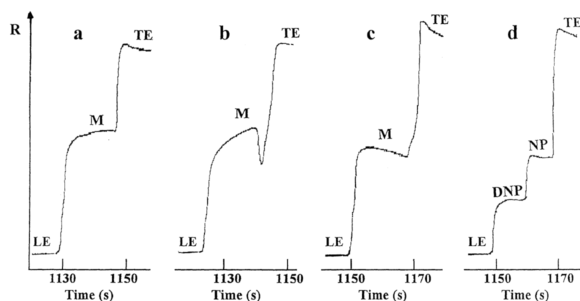

Fig. 2. Isotachophoretic separation of a model mixture of naproxen and 6-*O*-desmethylnaproxen (50 μg/ml each, 5 μl injected) in LE1 (a), LE2 (b), LE3 (c) and LE 4 (d). For further details see Table 1. Only records from the analytical capillary are shown. The driving current was 200 μA in the preseparation capillary. The driving current was initially 35 μA in the analytical capillary. It was reduced to 20 μA prior to detection. R=Increasing resistance, LE=Leading ion, DNP=6-*O*-desmethylnaproxen, NP=naproxen, TE=terminating ion.

tration of hydrochloric acid in system LE4 was increased to 20 m*M*. During the investigation different lengths of capillary were used. For routine purposes a preseparation capillary 80 mm long and an analytical capillary 160 mm long was satisfactory. The analysis time was 28–32 min. To reduce the separation time, the experiments were started at an elevated current 350 μA in the preseparation capillary. The driving current was initially 35 μA in the analytical capillary. It was reduced to 15 μA prior to detection. Typical isotachopherograms obtained from deproteinized serum samples are illustrated in Fig. 3a–c. Fig. 3a and 3b show a representative isotachopherogram of processed blank. The matrix components with higher mobility than analytes were removed from the preseparation capillary (see Fig. 3a) prior to entering the analytical capillary, where further separation and detection take place (Fig. 3b). Fig. 3c shows the isotachopherogram of a blank serum spiked with NP (20 μg/ml) and DNP (20 μg/ml).

3.2. Selectivity

Several human serum samples from different subjects and synthetic serum samples were tested for the absence of interfering compounds. The relative step heights of endogenous compounds in serum were compared with that of NP and DNP. As it can be seen in Fig. 3b, none of the endogenous compounds in serum interfered with analysis.

3.3. Instrument calibration

Calibration samples were prepared daily by spiking 100.0 μl of ten-fold concentrated aqueous solutions, containing both analytes, into 1.0 ml of control serum. The final concentrations in serum standards were 5.0/0.25, 10.0/0.5, 20.0/1.0, 30.0/2.0, 50.0/3.0, 75.0/5.0 and 100.0/10.0 μg/ml for NP/DNP. In addition, aqueous solutions at the same concentrations were prepared. Each concentration was measured three times. Unweighted least squares linear regression of the zone length as a function of the theoretical concentrations was applied to each standard curve (formula: $y=a+b.x$, where $x=$ concentration (μg/ml) and $y=$zone length (s)). In both, water and serum, the zone length of NP and DNP varied linearly with concentration over the range of assay (Table 3). The coefficients of determination (r^2) for the calibration curves were equal to or better than 0.998.

3.4. Recovery

The recovery was determined by comparing the zone length from drug-free serum spiked with mixture of NP/DNP (20.0/0.5, 50.0/1.0 and 10.0/5.0 μg/ml), processed according to the described method versus zone length of the same concentrations prepared in water injected directly onto the capillary. Each sample was injected five times. Data on the recovery are given in Table 4. The recovery of NP averaged 97.3% (RSD, 1.6%). It is not statistically different over the range of concentrations studied. The recovery of DNP averaged 93.9% (RSD, 5.1%), it was found to be consistent over the evaluated concentration range.

3.5. Accuracy and precision

The accuracy and precision of the method were evaluated by analyzing five-replicates of spiked serum at each of three concentrations (20.0/0.5, 50.0/1.0 and 10.0/5.0 μg/ml for NP/DNP) against a calibration curve. Accuracy was evaluated as percent error [(mean of measured-mean of added)/mean of

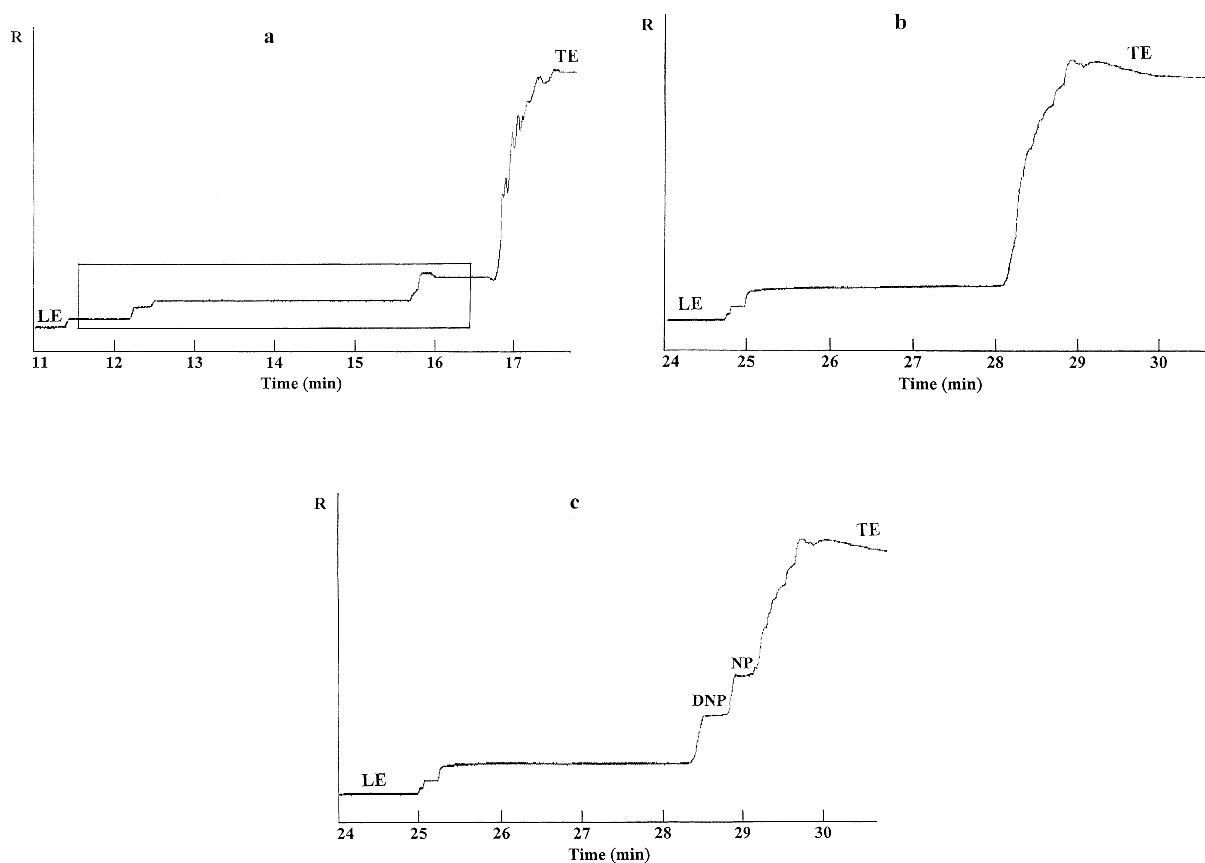

Fig. 3. Isotachopherogram of drug-free serum (a,b) and serum containing a mixture of NP and DNP at concentration of 20 μg/ml each after precipitation with ethanol (c). 30 μl injected. The preseparation capillary was filled with the leading electrolyte 20 m*M* HCl, β-alanine, pH 4.0, 0.1% methylhydroxypropylcellulose and the analytical capillary was filled with 10 m*M* HCl, β-alanine, pH 4.0, 0.1% methylhydroxypropylcellulose. Terminating electrolyte was 10 m*M* MES–Tris, pH 6.9 containing 20% (v/v) of ethanol. The driving current was 350 μA in the preseparation capillary. The driving current was initially 35 μA in the analytical capillary. It was reduced to 15 μA prior to detection. (a)=record from the preseparation capillary–rectangular enclosed the part of the sample that is transferred out from the separation compartment; (b,c)=records from the analytical capillary. LE=leading ion, DNP=6-*O*-desmethylnaproxen, NP=Naproxen, TE=Terminating ion.

Table 3
Results of the calibration using the zone length measured with the contact conductivity detector positioned in the analytical capillary[a]

Analyte	Calibration equation	r^2	Calibration range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
Water					
Naproxen	$y=0.02+1.020x$	0.9998	5.0–100.0	0.2	0.4
DNP	$y=-0.01+1.152x$	0.9998	0.25–10.0	0.2	0.4
Serum					
Naproxen	$y=0.06+1.007x$	0.9986	5.0–100.0	0.2	0.4
DNP	$y=0.08+1.038x$	0.9980	0.25–10.0	0.2	0.4

[a] The calibration relates the zone length in s (y) to the concentration of the ion (x) in μg/ml. Number of calibration points $n=7$, r^2coefficient of determination.

Table 4
Accuracy, precision and recovery data

Naproxen						6-*O*-desmethylnaproxen					
Added (μg/ml)	Found (μg/ml)	Accuracy, bias (%)	Precision, RSD (%)	Recovery		Added (μg/ml)	Found (μg/ml)	Accuracy, bias (%)	Precision, RSD (%)	Recovery	
				Mean (%)	RSD (%)					Mean (%)	RSD (%)
20.00	19.87	−0.6	1.2	98.1	1.2	0.50	0.43	−14.0	7.3	95.1	5.6
50.00	49.39	−1.2	1.9	97.5	1.9	1.00	0.92	−8.0	7.0	91.2	6.1
10.00	9.75	−2.5	1.7	96.2	1.7	5.00	5.19	3.8	3.9	95.4	3.7
	Mean	−1.4	1.6	97.3	1.6		*Mean*	−9.1	6.1	93.9	5.1

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 4 and are below 15% which is an acceptable range for validation methods [17].

3.6. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ was defined arbitrary as the sample concentration resulting in a zone length of 0.5 s. To determine accuracy and precision on the LOQ, spiked serum samples were used ($n=6$). The LOD was defined as the sample concentration resulting in a zone length of 0.3 s. The LOQ was 0.4 μg/ml for NP. At this level, the mean concentration found was 0.39±0.11 μg/ml (RSD, 15.3%; accuracy, −2.5%). The LOD was 0.2 μg/ml for NP. The LOQ was 0.4 μg/ml for DNP: At this level, the mean concentration found was 0.36±0.13 μg/ml (RSD, 19.7%; accuracy, −10.0%). The LOD was 0.2 μg/ml for DNP.

3.7. Stability

A stock solution of NP and DNP (100 μg/ml) stored at 4°C was stable for at least 3 months. For stability studies, control serum was spiked with mixture of NP/DNP (20.0/0.5, 50.0/1.0 and 10.0/5.0 μg/ml). The stability was conducted at the ambient temperature (22°C) and at 4°C. Analytes in serum samples at the ambient temperature were stable during 8 h, whatever the concentration; at 4°C they were stable for at least 3 days.

3.8. Determination in human serum. Comparison with synchronous fluorescence spectrometry

Since the therapeutic levels of DNP in serum are below the LOQ of the ITP method, only NP concentration levels were determined in the sera of twenty patients receiving a single 500-mg dose of NP (Fig. 4). Serum was treated as in procedure described above. The isotachophoretic results were compared with those obtained by synchronous fluorescence spectrometry.

The synchronous fluorescence spectra for NP was obtained in the similar manner as that described by Konstantianos [15]. Fig. 5 shows the synchronous fluorescence spectra obtained for NP and DNP at different wavelength differences $\Delta\lambda$ between excitation and emission monochromators. As can be seen from Fig. 5, spectral overlaps of NP and DNP may occur at $\Delta\lambda=100$ nm, while NP could be determined well at $\Delta\lambda=20$ nm. Calibration graph was constructed by plotting the peak height at 330.6 nm ($\Delta\lambda=20$ nm) versus serum NP concentration (μg/ml). The calibration plot was found to be linear in the range 2.5–100.0 μg/ml. The coefficient of determination (r^2), intercept (±standard deviation) and line slope (±standard deviation) were 0.9995, 2.2 (±0.1) and 2.15 (±0.03), respectively. For the LOQ, we used the value $(y+10\cdot S)/b$, whereby the calculated intercept of the calibration line can be used as an estimate of y, S is the standard deviation in the y-direction of the calibration line and b is the slope of the calibration line. For the LOD, the value $(y+3\cdot S)/b$ was calculated. LOQ and LOD were 1.6 μg/ml and 0.5 μg/ml, respectively. Using these data, NP concentration levels were determined in the sera of twenty patients receiving a single 500-mg

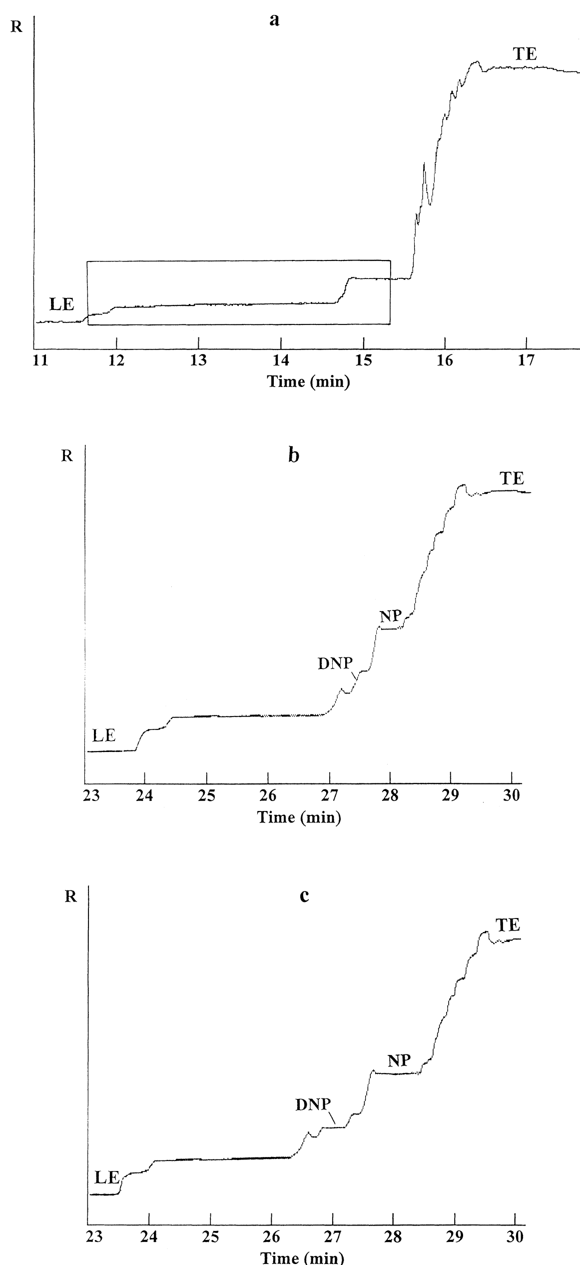

Fig. 4. Isotachopherogram of serum collected at 12 h after administration of 500 mg of naproxen (a,b) and the same serum after addition of 30 μg/ml of NP and 30 μg/ml of DNP (c). Precipitation with ethanol was used. (a) record from the preseparation capillary–rectangular enclosed the part of the sample that is transferred out from the separation compartment. (b,c) records from the analytical capillary. Conditions are the same as those given in Fig. 3. LE=Leading ion, DNP=6-*O*-desmethylnaproxen, NP=naproxen, TE=terminating ion.

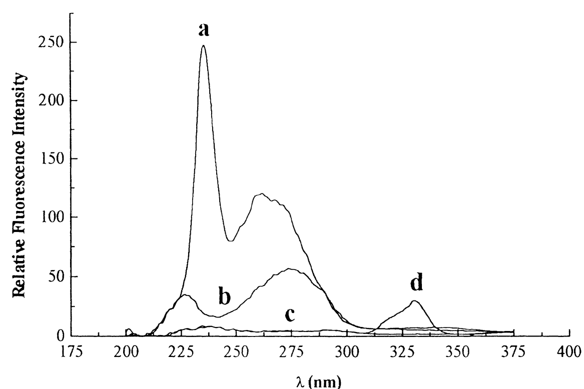

Fig. 5. Synchronous fluorescence spectra of serum containing (a,d) NP (12 μg/ml) and (b,c) DNP (0.75 μg/ml) after precipitation with acetonitrile. (a) NP, $\Delta\lambda=100$ nm, (b) DNP, $\Delta\lambda=100$ nm, (c) DNP, $\Delta\lambda=20$ nm, (d) NP, $\Delta\lambda=20$ nm.

dose of NP. Serum was treated as in procedure described above.

Correlation results for ITP versus SFS data are presented in Fig. 6. Linear regression analysis of data gave the following equation: $c(\mathrm{SFS})=(0.89\pm1.90)+(1.009\pm0.039)\cdot c(\mathrm{ITP})$, $r^2=0.975$, where c(SFS) and c(ITP) are the concentrations of naproxen determined by synchronous fluorescence spectrometry and isotachophoresis, respectively. A Student's t-test was performed to determine whether the experimental intercept of the above regression line differs significantly from the theoretical value of zero. The absolute value for t is 0.469 (it does not exceed the 95% criterion of $t_c=2.093$ for $n=20$ samples), thus the intercept is not significantly different from zero.

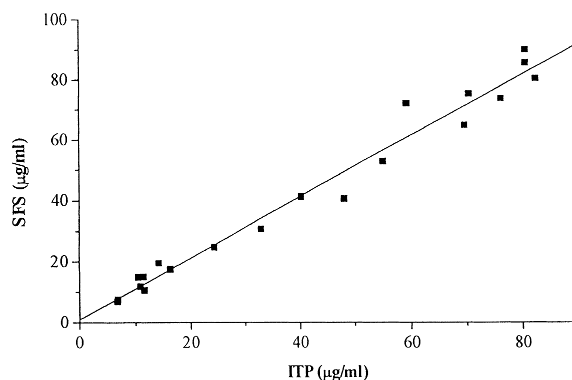

Fig. 6. Correlation of isotachophoresis naproxen concentrations with synchronous fluorescence determinations.

Another Student's t-test was performed in order to determine whether the slope differs significantly from unity. The absolute value calculated for t is 0.228 (it does not exceed the 95% criterion of $t_c = 2.093$ for $n = 20$ samples), so the slope is not significantly different from unity.

3.9. Interference studies

Other the available antirheumatics, acetylsalicylic acid, diclofenac, fenoprofen, flurbiprofen, ibuprofen and ketoprofen, were tested for their potential interference in the isotachophoretic and fluorescence spectrometric methods. No interferences was observed by using ITP and fluorescence spectrometry with any of the above drugs except for acetylsalicylic acid, which interfere with the determination of naproxen by using fluorescence spectrometry at mass ratio 100:1 (acetylsalicylic acid:naproxen).

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