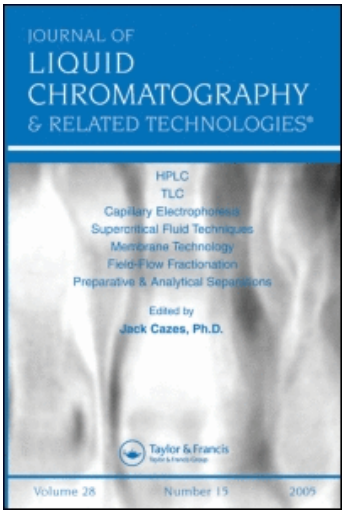

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Bioanalysis of Naproxen by High Performance Reversed Phase Liquid Chromatography with Photometric and Fluorimetric Detection

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BIOANALYSIS OF NAPROXEN BY HIGH PERFORMANCE REVERSED PHASE LIQUID CHROMATOGRAPHY WITH PHOTOMETRIC AND FLUORIMETRIC DETECTION

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ABSTRACT

Methods for the quantitative determination of NAPROXEN and its main metabolite in plasma and urine are described. The separation is based on reversed phase liquid chromatography with LiChrosorb RP 8 (5 μm) as the support and methanol/phosphate buffer pH 7 as mobile phase, in some cases with addition of tetrabutyl ammonium ion as ion-pairing agent to improve the chromatographic selectivity. With UV-detector and a simple filter fluorometer an extraction-evaporation procedure is used for both plasma and urine determinations, while the high selectivity and sensitivity of a sophisticated fluorescence detector permits the direct injection of diluted samples on to the column. Use of an internal standard improves the within-run precision ($s_{rel\%}$), which for plasma determinations of NAPROXEN are - with UV-detection, 0.2 - 1.7 % (range 10 - 40 μg/ml), with filter fluorometer, 2.4 - 5.9 % (range 12 - 58 μg/ml), and with fluorescence detector, 0.8 - 4.1 % (range 5 - 20 μg/ml).

INTRODUCTION

NAPROXEN ((+)-6-methoxy-α-methyl-2-naphthylacetic acid) is an antiphlogistic drug with analgetic and antipyretic properties[2], which is recommended for the treatment of

rheumatoid arthritis and osteoarthrosis. Pharmacokinetic studies have been performed in various laboratory animals and humans[3,4,5]. After normal dosage (2x250 mg daily) steady-state levels in man range from 25 to 50 µg per ml/plasma. The areas under the plasma concentration vs time curves increase linearly with dosage up to 500 mg twice a day, but with higher doses the plasma response will be non-linear probably because of saturation of plasma-binding sites and as a consequence an accelerated renal clearance[6,7]. The biological half-life, independent of the dose, is 10-17 h, and the capacity of the human body to conjugate and excrete the drug is maintained up to a single dose of 4 gms. The main metabolite in human urine is 6-hydroxy-α-methyl-2-naphthylacetic acid[8] that, like NAPROXEN, is excreted to a large extent in the form of glucuronide conjugate. A glycine conjugate of NAPROXEN has also been identified. A few other, minor metabolites, so far unidentified, are also formed. The metabolites are rather polar and they are excreted rapidly so that almost all drug present in human blood is the unchanged compound[3], and virtually all of the drug is bound to plasma proteins (>99 %) mainly to albumin[9]. High plasma levels of metabolites may however exist in patients with renal disorders[10].

The assays in the above-mentioned studies (except ref. 10) were performed by a GLC-method with flame-ionization detection and involving a derivatization with diazomethane described briefly in ref. 4. Recently a method based on fluorimetric

measurements for determination of NAPROXEN in serum was published[11].

In this paper, reversed phase HPLC methods are described for the determination of NAPROXEN and its main metabolite, 6-hydroxy-α-methyl-2-naphthylacetic acid (HNA), in plasma and urine. Both UV photometric and fluorimetric measurements are utilized. Data on the performance of the chromatographic system have been given earlier[12].

EXPERIMENTAL

Apparatus

The liquid chromatograph comprised the following components: a LDC 711-47 pump, Rheodyne 7120 and Altex model 905-23 equipped with a loop filler port model 905-29 injection valves, columns measuring 100 x 3.2, 100 x 4.0 and 150 x 4.0 mm (l x i.d.) of precision bore stainless steel (Handy & Harman) with modified (zero dead volume) Svagelok^{R} end fittings, a Waters model 440, UV-detector (254 nm), a Labotron FFM 32 fluorescence detector equipped with a flow cell (12 µl) cooled by running tap water during the measurements, and for determinations in plasma, a standard mercury lamp (4 watt, phosphorcoated with energy maximum at 360 nm), a primary filter (AGA, Sweden) with transmission between 300 and 350 nm (maximum at 336 nm), a secondary filter with maximum light transmission above 350 nm - and for urine determinations a short-wave UV

mercury lamp, two primary filters C7-57, with maximum transmission at 280 - 360 nm, and the AGA-filter mentioned above, the same secondary filter as for plasma determinations but complemented with an aperture plate (diam. 12 nm), a Schoeffel FS 970 fluorescence detector, excitation wave-length 230 nm and no filter on the emission side. Philips PM 8100 or Linear model 252, recorders.

The computing integrator LDC 304/50 was used for quantitations by peak height measurements.

Static fluorescence measurements were performed with a Perkin-Elmer MPF-3 spectrofluorimeter. pH-measurements were made on an Orion Ionalyzer model 801.

Chemicals

LiChrosorb RP 8 (5 and 10 μm), the chromatographic support, was obtained from Merck (Darmstadt, G,F,R.).

NAPROXEN (6-methoxy-α-methyl-2-naphthylacetic acid), 6-methoxy-2- naphthylacetic acid (MNA, internal standard) and 6-hydroxy-α-methyl-2- naphthylacetic acid (HNA, metabolite) were kindly supplied by Syntex (Maidenhead, Great Britain).

Tetrabutylammonium hydrogen sulphate (Labkemi, Sweden) was recrystallized from boiling methyl ethyl ketone (certified, Fisher), concentration about 1 g/ml, after cooling and standing in an ice-bath for about 2 h. The precipitate was washed with ice-cold methyl ethyl ketone and diethyl ether (anhydrous, May & Baker, Great Britain), and dried in air overnight. The final drying was performed in an oven at 75° C.

N-Hexane (für die Spektroskopie) used in extractions, and methanol (zur Analyse) used in the mobile phase were from Merck, (Darmstadt, G.F.R.) all other solvents and substances were of analytical grade.

Stock solutions of NAPROXEN, HNA and MNA are prepared in acetic acid (1 - 2 mg/ml) for reasons of solubility and stability; alkaline solutions (0.1 N NaOH) cannot be used since HNA is unstable under such conditions, only about 31 % remaining after storage at room temperature for 2 days, and degradation occurs even when solutions are frozen, 97 % and 88 % remaining after 2 and 30 days, respectively, after storing at -20^{o} C. NAPROXEN and MNA are stable under these conditions.

Determination of Partition Properties

Partition experiments were performed with equal volumes of aqueous and organic phases in centrifuge tubes at room temperature. The tubes were mechanically shaken for 30 minutes. After centrifugation the concentration of the acids was determined by fluorimetric measurements of the aqueous phase after alkalization (to pH=11) at excitation and emission maxima of 278 and 352 nm respectively.

Chromatographic Technique

Details of the column packing procedure by a balanced-density slurry technique have been given previously[12]. Columns have also been packed with methyl isobutyl ketone as the slurry medium by a modified upward slurry

packing technique as described by Bristow et al.[13]. After packing, the columns are tested at one flow rate by the procedure described by Bristow and Knox[14]. The chromatographic system is kept at ambient temperature.

Analytical Methods

Plasma

1) UV-detector at 254 nm (NAPROXEN)

Extraction

1. 500 µl plasma is mixed with 500 µl of internal standard solution (normally 10 - 20 µg/ml) and acidified with 200 µl 6 M H_2SO_4. The mixture is extracted for 20 minutes in a centrifuge tube with 4.00 ml of n-hexane + diethylether (1+1 v/v) at ambient temperature.
2. After centrifugation for 5-10 minutes at 2500 rpm the organic phase is transferred to another small centrifuge tube with a Pasteur pipette and evaporated at room temperature under a gentle stream of compressed air or nitrogen.
3. The sample is redissolved in 500 µl of the chromatographic mobile phase and 30 - 40 µl is injected on to the column.

Chromatography

Column:	100 x 3.2 mm (l x i.d.)
Mobile phase:	Phosphate buffer pH 7.0 (ionic strength = 0.1) + methanol (6+4 v/v)
Flow rate:	0.5 - 1.0 ml/min

2) Fluorescence detector, Labotron FFM 32 (NAPROXEN and metabolite)

Extraction

1. 500 µl plasma is mixed with 50 µl internal standard solution (normally 100 - 200 µg/ml) and acidified with 100 µl 6 M H_2SO_4. The mixture is extracted with 2.0 ml of n-hexane + diethyl ether (1+1 v/v) for 20 minutes in a centrifuge tube at ambient temperature.
2. Same procedure as with the UV-detector.
3. The residue is redissolved in 100 µl of mobile phase and 50 µl is injected on to the column.

Chromatography

The same system as with the UV-detector.

3) Fluorescence detector, Schoeffel FS 970 (NAPROXEN and metabolite)

Sample preparation

After the addition of internal standard solution the plasma is diluted 100 times with deionised water and 20 µl is injected directly on to the column.

Chromatography

The same system as above, but with column dimensions 100 x 4.0 mm.

Urine (Sum of free and conjugated NAPROXEN and metabolite, respectively).

1) UV-detector at 254 nm

Extraction

1. 500 µl urine is mixed with 100 µl internal standard solution (normally 100 - 300 µg/ml) and acidified with 100 µl 6 M H_2SO_4, the mixture is heated for 15 min in a boiling water bath to hydrolyze the conjugates. (When concentrations of free NAPROXEN and metabolite are desired the heating is omitted).
2. After cooling under tap water the sample is extracted with 2.0 ml of n-hexane + diethyl ether (1+1 v/v) for 20 minutes in a centrifuge tube at ambient temperature.
3. After centrifugation for 5 - 10 min at 2500 rpm the organic phase is transferred to a small glass tube with a Pasteur pipette and evaporated at ambient temperature under a gentle stream of compressed nitrogen.
4. The residue is redissolved in 500 µl chromatographic mobile phase and 50 µl is injected on the column.

Chromatography

Column:	150 x 4.0 mm (l x i.d.)
Mobile phase:	Phosphate buffer pH 7.0 (ionic strength=0.1) + methanol (7 + 3 v/v) containing 0.0035 M tetrabutylammonium hydrogen sulphate (neutralized with NaOH).
Flow rate:	0.5 - 1 ml/min.

2) Fluorescence detector, Labotron FFM 32

Extraction

The same procedure as with the UV-detector.

Chromatography

Column: 150 x 4.0 mm (1 x i.d.)

Mobile phase: Phosphate buffer pH 7.0 (ionic strength=0.1) + methanol (7 + 3 v/v) containing 0.001 M tetrabutylammonium hydrogen sulphate (neutralized with NaOH).

Flow rate: 0.5 - 1.0 ml/min.

3) Fluorescence detector, Schoeffel FS 970.

Sample preparation

1. After the addition of internal standard solution the urine is diluted 100 to 150 times with deionized water depending on expected concentration levels and 6 M H_2SO_4 is added and the sample is hydrolyzed as described above.
2. 20 µl of the hydrolyzed sample is injected on to the column.

Chromatography

Column: 100 x 4.0 mm (1 x i.d.)

Mobile phase: Phosphate buffer pH 7.0 (ionic strength=0.1) + methanol (6+4 v/v).

Flow rate: 0.5 - 1.0 ml/min.

Standard curves are obtained by analyzing plasma or urine samples spiked with NAPROXEN, metabolite (HNA) and internal standard (MNA) (50 to 100 µl of proper dilutions from the stock solutions to the body fluid) simultaneously with the unknown samples. The contents of NAPROXEN and HNA are determined by referring the peak height ratios of the unknowns to the standard curve.

RESULTS AND DISCUSSION

Extraction Studies

In extractions from biological material, it is essential to use an extraction medium that gives as little coextraction of endogenous compounds as possible. The nonpolar n-hexane was therefore chosen as the basic solvent for the extraction studies. Preliminary studies on the distribution of NAPROXEN gave log $k_{d(HX)}/ K_{HX} = 4.3$ in extractions to n-hexane with phosphate buffers (ionic strength=0.1) as the aqueous phase, which means that a quantitative extraction of the compound is achieved at pH < 2.3 if equal phase volumes are used. From literature data [e.g. ref. 14] it can be assumed that the distribution constant ($k_{d(HX)}$) for the internal standard (MNA) and the metabolite (HNA) are about four and ten times smaller, respectively, than the value for NAPROXEN.

Some results on extractions from plasma with pure n-hexane are given in Table 1. The plasma was acidified with sulphuric

TABLE 1

Recoveries in Plasma Extractions

Extraction solvent: n-hexane

Substance	µg/ml	equilibration time (min)	V_{org}/V_{aq} [1)]	yield (%)	s (%)	n
NAPROXEN	29.3	10	2.3	21.9	2.9	3
MNA	26.4	10	2.3	8.1	1.1	3
NAPROXEN	28.1	60	3.2	42.7	1.1	3
MNA	26.2	60	3.2	22.2	0.5	3
NAPROXEN	29.6	30	8.0	43.5	-	2
MNA	12.0	30	8.0	26.4	-	2

1) phase volume ratio.

acid to pH below 1 before the extractions; some different phase volume ratios and equilibration times were used. The quantitations were performed by peak area measurements utilizing the following relation[16]:

$$M = A \times u \times a \times \varepsilon^{-1} \qquad (1)$$

where M = amount of sample in mmoles, A = peak area in mm^2, u = flow rate in ml/mm chart paper, a = absorbance/mm and ε = molar absorptivity of the compound.

The molar absorptivities of NAPROXEN and MNA (= about 4×10^3) were given earlier[12]. The extraction degrees are rather low, indicating, as expected, association reactions in the aqueous phase, e.g. protein binding that is known to be very high (> 99 %) for NAPROXEN. An increase in the phase volume

ratio from 3.2 to 8 did surprisingly not affect the yield; the equilibration times were, however, different possibly indicating that the extraction process is slow.

Attempts to increase the degree of extraction were performed by using mixtures of n-hexane with some polar solvents. The hydrogen acceptors diethyl ether, ethyl acetate and methyl ethyl ketone were chosen in order to obtain a specific solvation of the acids through bonding to the carboxylic hydrogen atom. Mixtures with diethyl ether and methyl ethyl ketone gave acceptably high yields (Table 2), 98 and 93 % respectively, while with ethyl acetate a surprisingly low yield (32 %) was obtained in view of its high polarity - the

TABLE 2

Recoveries in Plasma Extractions With Mixed Solvents

Extraction solvent: n-hexane + hydrogen acceptor (1 + 1 v/v)
Extraction time: 30 min.
Phase volume ratio: $(V_{org}/V_{aq}) = 3.2$

Initial concentrations (µg/ml): NAPROXEN - 28.1
MNA - 26.2

Solvent	Substance	Yield[1] (%)
ethyl acetate	NAPROXEN	31.9
"-	MNA	15.9
methyl ethyl ketone	NAPROXEN	93.0
"-	MNA	94.7
diethyl ether	NAPROXEN	97.6
"-	MNA	96.0

1) mean of two determinations.

solubility parameter[17] is 8.6, cf. 7.4 for diethyl ether and 9.4 for acetone (value for methyl ethyl ketone is not available).

Blank chromatograms obtained with the three mixtures are shown in Figure 1. The cleanest chromatogram is obtained with ethyl acetate, but as mentioned above the recoveries of NAPROXEN are too low. Large disturbances in the chromatogram are obtained when using methyl ethyl ketone, which also assumes a yellow colour in the extraction and it gives furthermore a heavy proteinprecipitate at the interface between the two liquid

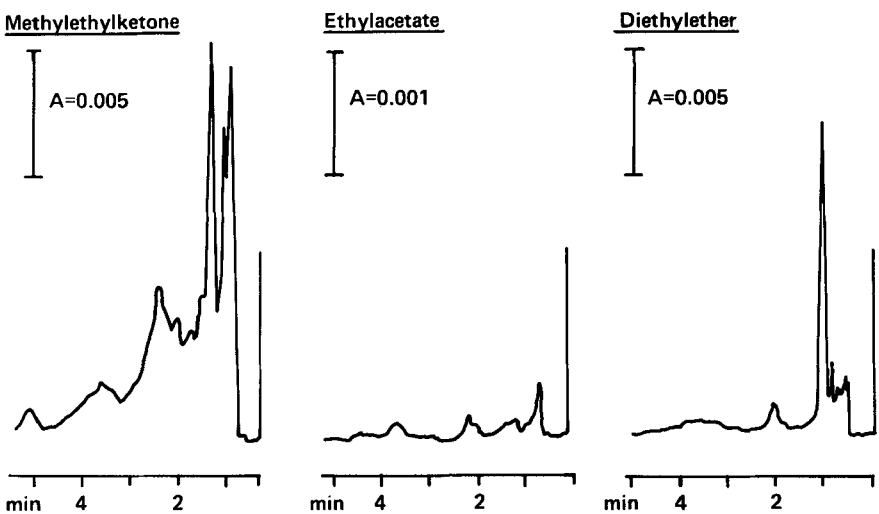

FIG. 1. Blank chromatograms after extraction with different media from plasma

Support: LiChrosorb RP 8
Mobile phase: phosphate buffer pH 7 + methanol (60+40 v/v %)
Extraction solvent: n-hexane + hydrogen acceptor (1+1 v/v)
Detector: Waters model 440

phases, while with the other solvents only light precipitates at the bottom of the tubes are obtained. The mixture diethyl ether - n-hexane gives acceptable results both regarding blank chromatogram and extraction yield and was consequently chosen for further studies, and it was also found to be suitable in extractions from urine; a blank chromatogram is shown in Fig. 2.

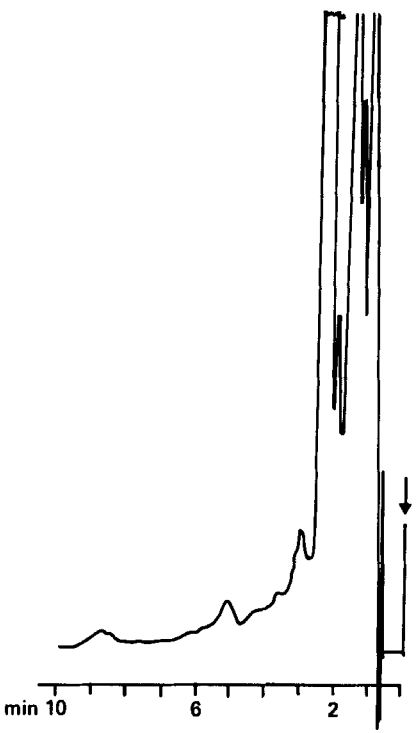

FIG. 2. Blank chromatogram from urine using UV-detection

Analysis performed according to "Analytical methods, no 1"

The developed method for urine permits the determination of either the free fractions (by omitting the hydrolysis), or the sum of free and conjugated forms of NAPROXEN and the metabolite. In the latter case a hydrolysis time of 15 min with 0.9 M H_2SO_4 was found to be adequate, yielding recoveries of over 90 and 96 % for added HNA and NAPROXEN respectively.

Quantitative Determinations

UV-Detector

The detection limit on injecting NAPROXEN in pure mobile phase is roughly 5 ng by UV-detection at 254 nm. Since the expected plasma levels of NAPROXEN after an oral administration of normal doses are rather high, 5 - 120 µg/ml, it was not necessary to optimize the method with regard to sensitivity - a single extraction followed by evaporation of the organic phase, redissolution in a comparatively large volume and injecting an aliquot (4 %) of this solution completely satisfies the needs also for most pharmacokinetic studies.

A plasma sample chromatogram for determination of NAPROXEN, that only takes about 3 minutes is shown in Fig. 3. Disturbances from endogenous compounds are mainly obtained at the front, but there is also a peak with a retention time similar to the internal standard; it is however so small that it normally does not interfere in the quantitations.

In studies on the repeatability of the method (see Table 3) it was shown that use of an internal standard improved the

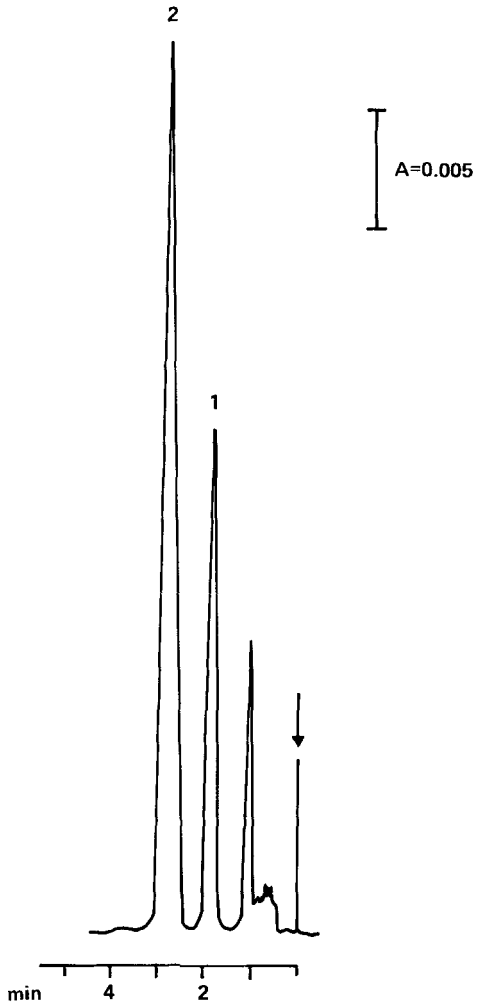

FIG. 3. Sample chromatogram of NAPROXEN in plasma using UV-detection

Analysis performed according to "Analytical methods, no 1"
Flow rate: 1 ml/min
1. Internal standard, MNA - 14.1 µg/ml
2. NAPROXEN - 34.7 µg/ml

TABLE 3

Quantitative Determinations of NAPROXEN in Plasma by UV-detection

The analysis performed according to "Analytical method, no. 1" after incubation of samples 2.5 h.

Range of standards a) 20 - 40 µg/ml
b) 5 - 80 µg/ml

Concentration of internal standard a) 14 µg/ml
b) 17 µg/ml

Conc. of NAPROXEN (µg/ml)	Quantitation by peak height recovery	s(%)	peak height ratio recovery	s(%)	n
10	-	-	98.3	0.17	5
24	109.3	2.13	104.0	1.46	6
34	104.2	2.70	103.6	1.00	6
40	-	-	101.0	1.72	5

results, mainly the precision, since the standard deviation decreased from 2 - 3 % to 1 - 1.5 %.

With the mobile phase used above for the determination of NAPROXEN in plasma, the metabolite (HNA) elutes too near the front. It was retained by decreasing the methanol-content by 10 % and furthermore by adding an ion - pairing counter ion, tetrabutylammonium (TBA). Blank and sample chromatograms, that now take about 12 minutes, are demonstrated in Fig. 4. However, not only HNA is retained but also some endogenous compounds; some slightly disturb the internal standard and another elutes

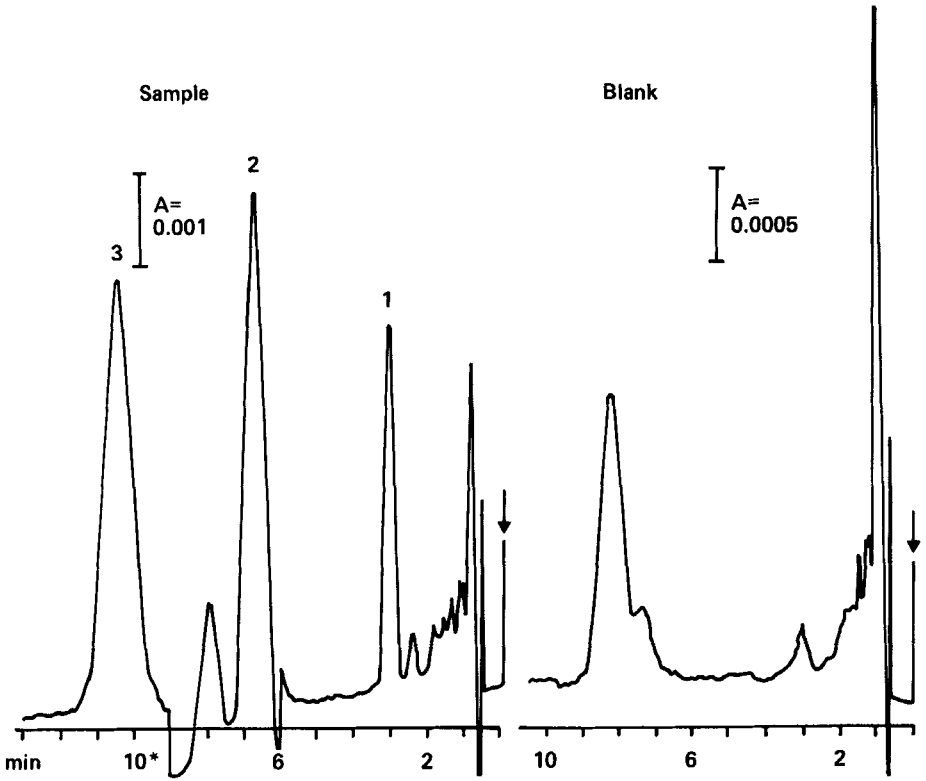

FIG. 4. Chromatograms of NAPROXEN and metabolite (HNA) in plasma using UV-detection

Analysis performed according to "Analytical methods, no 1", but with a different mobile phase: phosphate buffer pH 7 (ionic strength = 0.1)+ methanol (6+4) + 0.0035 M TBA
Flow rate: 1 ml/min
1. HNA 3.7 µg/ml
2. MNA 16.7 µg/ml
3. Naproxen 10.0 µg/ml

near the metabolite, bringing about an increase in the detection limit. This is important since the metabolite normally is present in plasma in rather low concentrations, typically below

0.4 μg/ml, and for quantitative determinations a fluorescence detector (see below) must be used. Raised levels of HNA in plasma are obtained in uremic patients, but then many endogenous compounds are also present in higher concentrations giving rise to problems concerning the blank.

As mentioned in the introduction, NAPROXEN and its metabolites are excreted into the urine to a large extent in the form of conjugates, and normally it is the total amount, the sum of free and conjugated drug, that is of interest. It is however also possible to determine only the free fractions of the compounds by this method if so desired.

The urinary concentrations of the compounds may be rather high in the early fractions (100 - 200 μg/ml), and a sample chromatogram containing comparatively low amounts is shown together with a blank in Fig. 5. Endogenous compounds elute mainly with the front and the metabolite is separated from them by ion-pairing with a small concentration of TBA, and the chromatogram takes about 12 minutes. Some quantitative results obtained by comparison of peak heights with aqueous standards are given in Table 4; the recoveries are in the range 91 - 95 % with a precision of 2 - 3 % (s) at rather low concentration levels, 20 - 40 μg/ml.

Fluorescence Detectors

Uncorrected excitation and emission spectra of NAPROXEN obtained in an alkaline aqueous buffer are illustrated in Fig. 6. The compound has three distinct excitation maxima at 238,

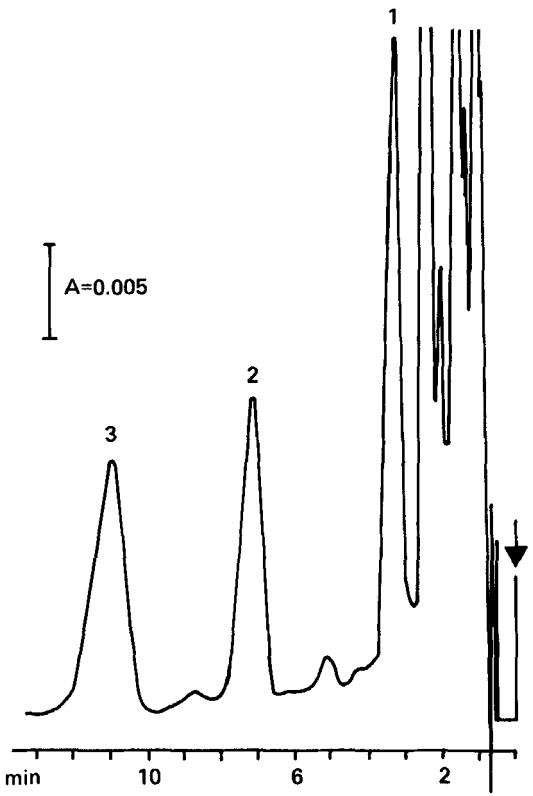

FIG. 5. Sample chromatogram of NAPROXEN and metabolite in urine using UV-detection

Analysis performed according to "Analytical methods, no 1"
Flow rate: 1 ml/min
1. HNA 48 µg/ml
2. MNA 42 µg/ml
3. NAPROXEN 52 µg/ml

278 and 330 nm respectively, and the emission maximum is at 354 nm. (Similar spectra were published by Anttila[10].) Two different fluorescence detectors were used for the liquid

TABLE 4

Quantitative Determinations in Urine by UV-Detection

The analysis performed according to "Analytical methods, no 1".

Compound	µg/ml	Recovery (%)	s (%)	n
NAPROXEN	21.6 - 43.4	95.5	2.10	10
MNA	19.1 - 34.3	94.8	2.65	10
HNP	32.4	90.9	3.18	4

chromatographic work, Labotron, FFM 32, which is a simple filter fluorometer equipped with a low energy standard mercury lamp for excitation, and Schoeffel FS 970, which is a more sophisticated instrument with a monochromator for choice of excitation wavelength and equipped with a high energy deuterium lamp. The most favourable conditions for analysis will depend on the performance of the apparatus, mainly regarding the energy distribution of the excitation lamp, the transmission of the monochromator and filters, and the response profile of the photomultiplier; the optimal excitation wavelength for Labotron was found to be around 330 nm, but for Schoeffel at 230 nm. Since the developed fluorescence intensity according to theory is directly proportional to the light intensity of the lamp, there is a large difference in obtained sensitivity between the two instruments. With Labotron the detection limits are about 10 times higher than with the UV-DETECTOR when injected in pure mobile phase, i.e. for NAPROXEN about 50 ng, MNA about 20 ng and

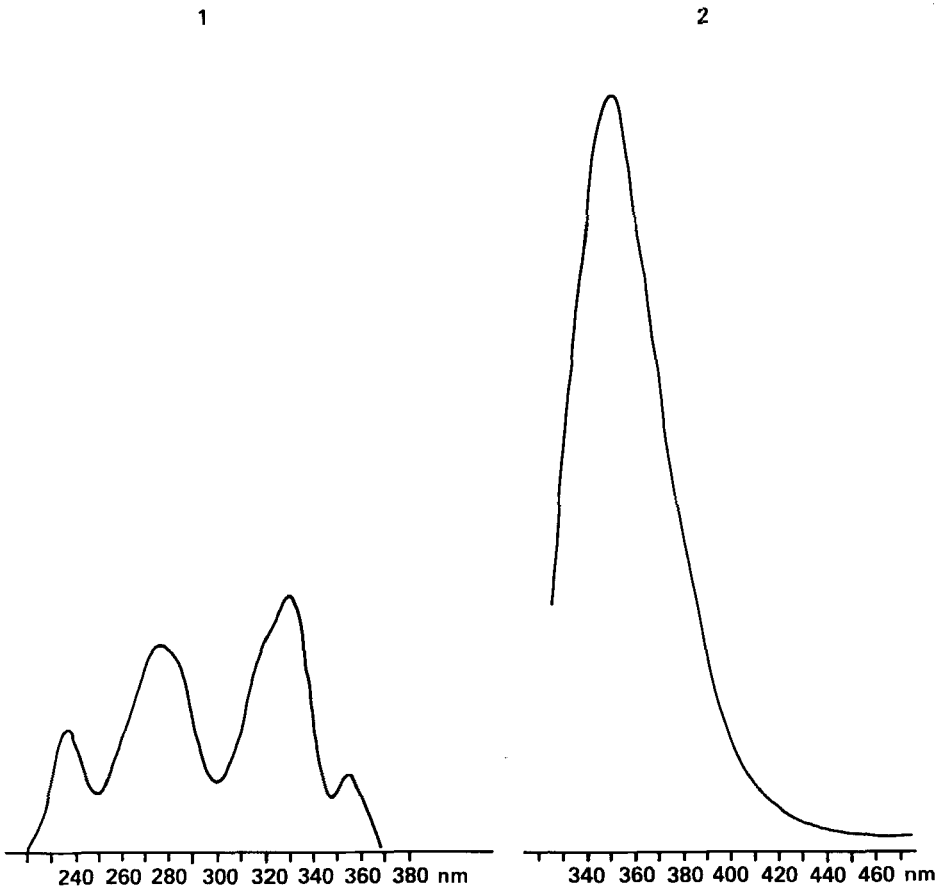

FIG. 6. Uncorrected excitation and emission spectra of NAPROXEN

Sample: NAPROXEN 3.56×10^{-6} M in phosphate buffer pH 10.81
1. Excitation spectrum obtained by emission at 354 nm
   Sensitivity: 0.1
2. Emission spectrum obtained by excitation at 331 nm
   Sensitivity: 0.3

for HNP about 15 ng, while with the Schoeffel the detection limit for NAPROXEN under optimal conditions is about 5 pg, i.e. 1000 times better than the UV-detector.

The low sensitivity of the filter fluorometer necessitated a slight modification in the sample work up procedure involving the injection of a larger aliquot of the sample. Chromatograms obtained from plasma and urine extractions (see Fig:s 7 and 8)

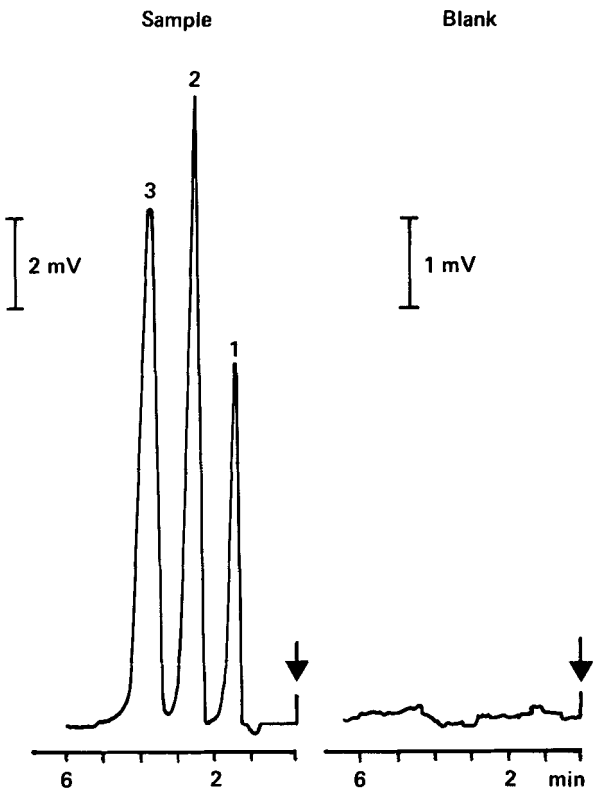

FIG. 7. Chromatograms of NAPROXEN and metabolite in plasma using a filter fluorometer

Analysis performed according to "Analytical methods, no 2".
Flow rate: 0.6 ml/min
1. HNA 7.2 µg/ml
2. MNA 16.7 µg/ml
3. Naproxen 20.0 µg/ml
Excitation: 300 - 340 nm
Emission: > 340 nm

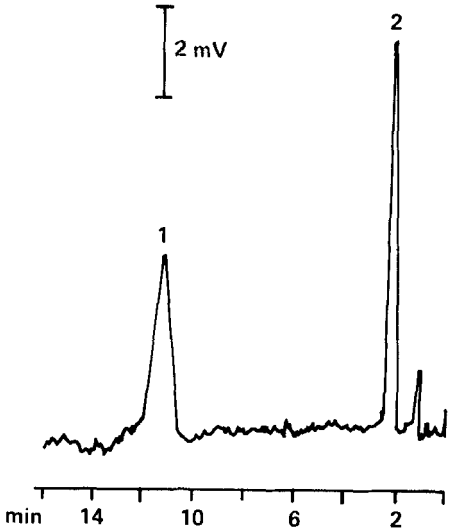

FIG. 8. Sample chromatogram of NAPROXEN and metabolite in urine using a filter fluorometer

Analysis performed according to "Analytical methods, no 2", but without internal standard.
Flow rate: 0.6 ml/min
1. NAPROXEN 100 µg/ml
2. HNA 100 µg/ml

illustrate that the most important feature of this detector is the eminent selectivity, blank chromatograms from plasma are perfectly clean, and in the urine blanks only one endogenous peak that elutes early appears in the chromatogram. Some quantitative results obtained on spiked plasma samples with this detector are shown in Table 5. Some plasma samples from uremic patients that were analyzed using this detector gave, like the UV-detector mentioned above, several peaks that were neither present in blanks nor samples from other patients or volunteers,

TABLE 5

Quantitative Determinations of NAPROXEN in Plasma Using a Filter Fluorometer

The analysis performed according to "Analytical methods, no 2".

Range of standards: 2 - 80 µg/ml, 8 points

Concentration of internal standard, MNA: 15 µg/ml
n = 3

µg/ml	Recovery (%)	s (%)
12	111.4	2.4
58	97.4	5.9

indicating that the method has to be modified before accurate results can be obtained from such samples.

The combination of the high selectivity obtained by fluorescence measurements with the high sensitivity of the more sophisticated detector, Schoeffel FS 970, made it possible to considerably simplify the sample treatment: after addition of internal standard, plasma or urine is simply diluted 100 times and an aliquot is injected directly on to the column. Examples of blank and sample chromatograms obtained from plasma (Fig. 9) show that fluorescent endogenous compounds elute early and the baseline is clean after about 3 minutes. The metabolite is slightly disturbed by an endogenous compound, but as shown below reliable quantitative determinations can still be performed. If necessary it is however possible to separate the compound completely from the disturbance by using less (32 %) methanol in

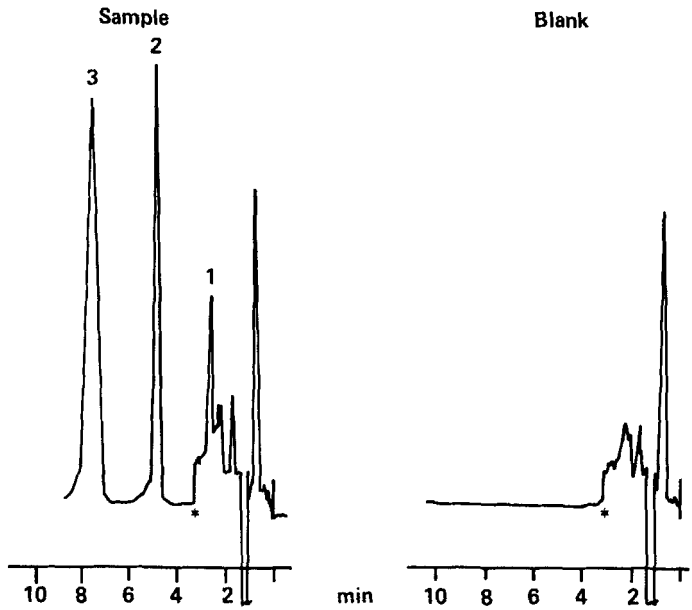

FIG. 9. Chromatograms of NAPROXEN and metabolite in plasma using a fluorescence detector

Analysis performed according to "Analytical methods, no 3".
Flow rate: 1 ml/min
1. HNA, 0.5 µg/ml
2. Internal standard
3. NAPROXEN, 20 µg/ml
Detector sensitivity: 1 A
Recorder amplification: 2 mV and after* 10 mV

the mobile phase as illustrated in Fig. 10. A drawback is then the separation time which is almost twice as long.

Since both NAPROXEN and the metabolite are excreted to a large extent in conjugated form in the urine, a blank chromatogram for hydrolyzed urine (Fig. 11) is shown illustrating the possibility of determining the total amounts of

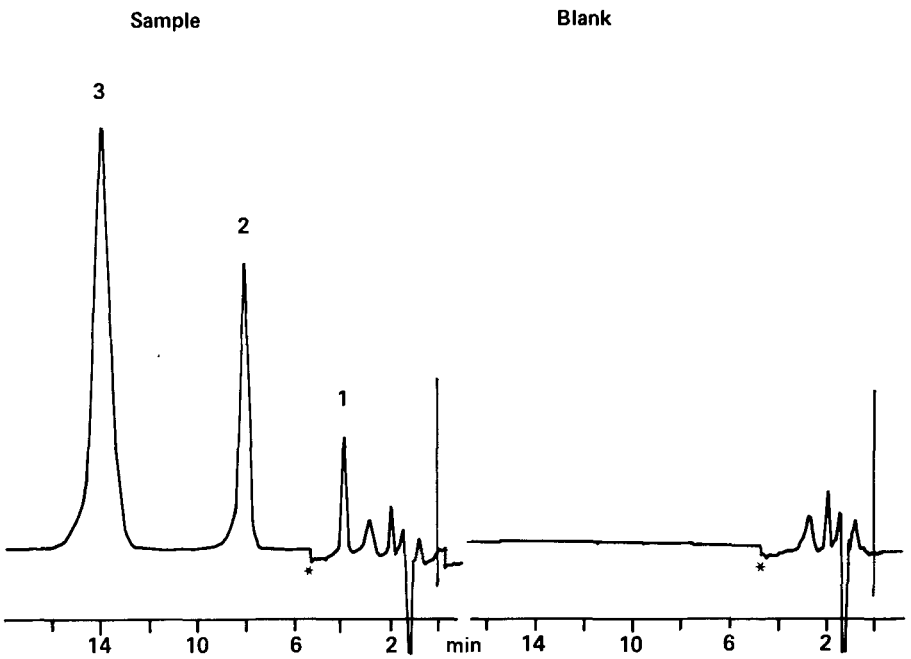

FIG. 10. Chromatograms of NAPROXEN and metabolite in plasma using a fluorescence detector

Analysis performed according to "Analytical methods, no 3", but with a different mobile phase - phosphate buffer pH 7/methanol (68+32 v/v)
Flow rate: 1 ml/min
1. HNA 0.5 µg/ml
2. Internal standard, 15 µg/ml
3. NAPROXEN, 30 µg/ml
Detector sensitivity: 1 A
Recorder amplification: 2 mV and after * 10 mV

the excreted compounds. As in the case of plasma most of the fluorescent endogenous compounds elute early, but there are some minor peaks with higher retention times; they do however not disturb the quantitations.

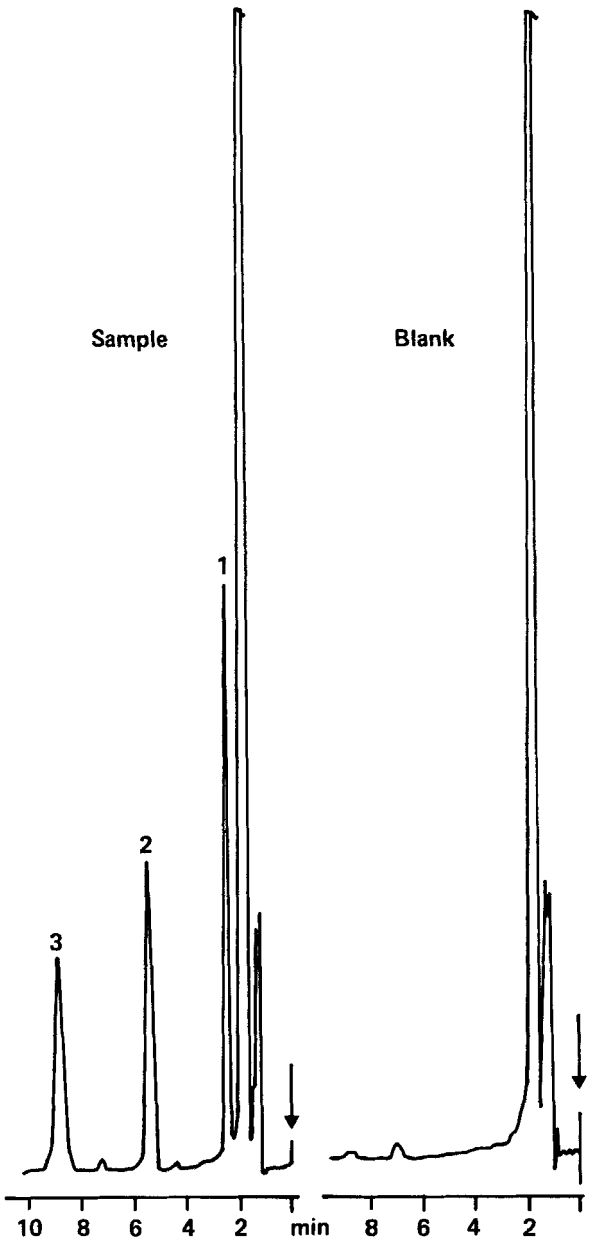

Fig. 11. Chromatograms of NAPROXEN and metabolite in urine using a fluorescence detector. Analysis performed according to "Analytical methods no 3;" flow rate: 1 ml/min; 1. HNA 10 µg/ml, 2. Internal standard (MNA), 15 µg/ml, 3. NAPROXEN, 10 µg/ml

In quantitative studies performed with the UV-detector mentioned above, it was found to be an advantage regarding the precision to include an internal standard in the procedure. Some results (Table 6) obtained on a series of peak height measurements after application of the simple methods for both

TABLE 6

Determinations in Plasma and Urine with and without Internal Standard using a Fluorescence Detector

Analysis performed according to "Analytical methods, no 3".

Concentration of internal standard, MNA: Plasma - 10 µg/ml
Urine - 15 µg/ml

A. Plasma

NAPROXEN (µg/ml)	HNA	s (%) peak height	s (%) peak height ratio	n
5		8.81	1.44	6
	0.1	6.03	11.7	6
10		3.41	2.46	5
	3	1.56	1.33	6
20		2.64	0.81	6
	5	2.86	2.64	6

B. Urine (n = 6)

10		5.74	0.98
	1	4.39	2.82
25		1.43	1.58
	10	1.13	1.00
50		1.86	2.40
	25	3.92	2.26

plasma and urine in which the fluorescence detector was used indicate with some exceptions that also in this case the use of an internal standard improves the precision of the results.

Some data on quantitative determinations in plasma and urine are summarized in Table 7. Very good figures on the precision ($s_{rel(\%)}$) in the range 0.7 - 3.4 % for HNA, and 1.2 - 4.1 % for NAPROXEN were obtained, and the accuracy seems

TABLE 7

Quantitative Determinations of NAPROXEN and Metabolite in Plasma and Urine using a Fluorescence Detector

Analysis performed according to "Analytical methods, no. 3".

Range of standard curve: HNA - 0.10 - 3.10 µg/ml
NAPROXEN - 2.02 - 30.24 µg/ml

Concentration of internal standard: 10.30 µg/ml
n = 5

A. Plasma

NAPROXEN (µg/ml)	HNA	Found (µg/ml)	s (%)
5.04		5.41	4.09
	0.207	0.204	3.42
20.16		20.46	2.99
	2.07	2.05	1.46

B. Urine

25.20		24.20	1.15
	2.07	1.93	0.69

to be acceptable, recoveries vary within 93 - 107 % for the two compounds.

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APPENDIX

The studies described above were performed on pooled plasma and urine samples. When the method was applied to determinations in urine samples from healthy volunteers to whom 250 mg of NAPROXEN had been administered as a single oral dose, several peaks that were not present in pooled urine appeared in chromatograms (see Fig. 12) run with the fluorescence detector. Since the method involves the direct injection of urine after hydrolysis and dilution, all these peaks are probably NAPROXEN-metabolites[8]. The chromatogram illustrates that there seems to be at least nine such peaks of which four elutes later than NAPROXEN. Their presence do, however, not interfere with the quantitative determinations since all three compounds (NAPROXEN, HNA and MNA) are baseline-separated.

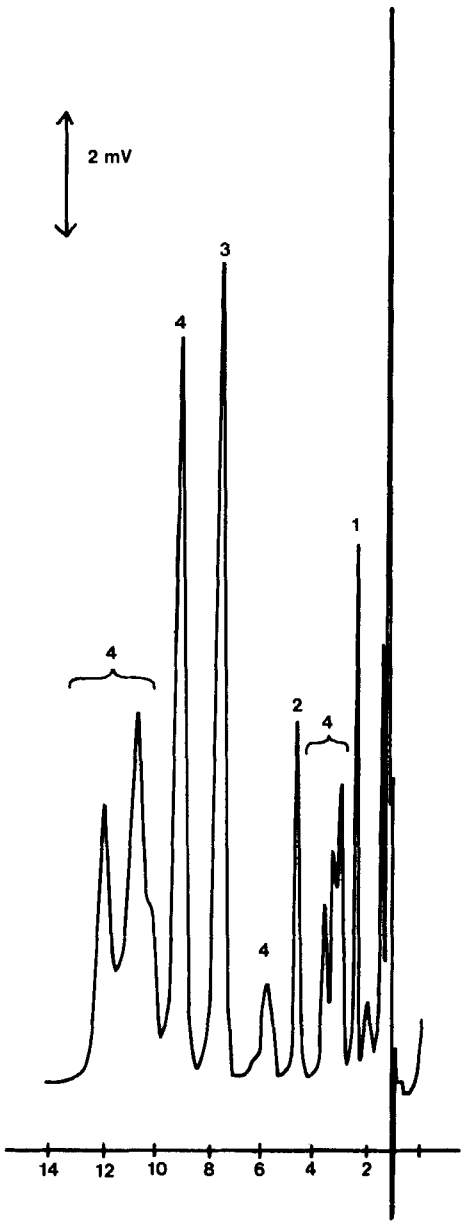

Fig. 12. Chromatogram on a urine sample from a healthy volunteer using a fluorescence detector. Dose: 250 mg of NAPROXEN; Sample: 60 ml urine collected during 0-2 h, analyzed according to "Analytical methods nr 3;" Flow rate: 1 ml/min; 1. HNA 8 µg/ml, 2. MNA 15 µg/ml, 3. NAPROXEN 33 µg/ml, 4. Probable metabolites.

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