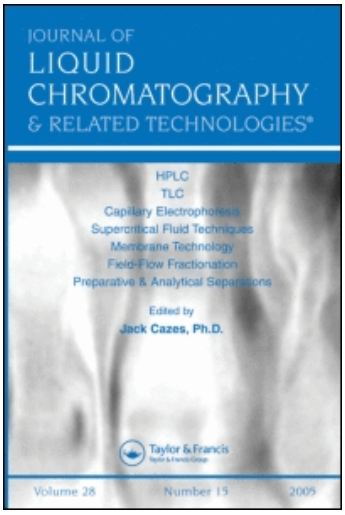

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A High-Performance Liquid Chromatographic Method for the Quantitative Determination of Naproxen and Des-Methyl-Naproxen in Biological Samples

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NAPROXEN AND DES-METHYL-NAPROXEN IN BIOLOGICAL SAMPLES

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ABSTRACT

A HPLC method for the quantitative analysis of naproxen and its major metabolite des-methyl-naproxen in biological fluid samples is described.

Two methods of detection are compared: U.V. spectrophometry and spectrophotofluorometry. In both procedures an internal standard is used: diflunisal in the U.V. procedure and the ethoxy-analog of naproxen during fluorometry. The sensitivity of the fluorometric detection is higher than that of the U.V. detection; the limit being respectively 0.1 µg and 2.0 µg per milliliter sample. The fluorescence detection procedure can also be applied to very small samples (0.05 ml) in the therapeutic concentration rang range. Both procedures have been applied to clinical and laboratory studies in which they appear to be very satisfactory because of their ease of handling and their suitability for routinely performed analysis.

INTRODUCTION

Naproxen ((+)-6-methoxy-α-methyl-2-naphtalene-acetic acid; Fig. 1.1) is a nonsteroidal anti-inflammatory drug which is used

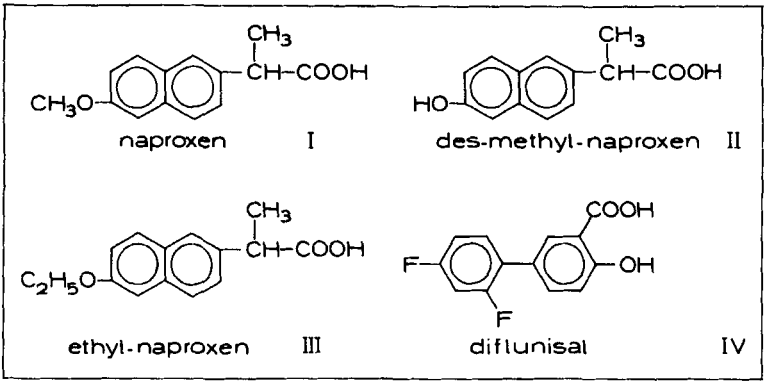

FIGURE 1

Structural formulas of naproxen (I), 6-0-des-methyl-naproxen (II), ethoxy-naproxen (III) and diflunisal (IV).

extensively for the treatment of arthritic diseases (1). In the course of a study on the clinical efficacy of several anti-inflammatory compounds, we are investigating also the pharmacokinetic profile of naproxen as related to its therapeutic effectiveness. For these studies we needed a rapid and accurate procedure for the quantitative analysis of naproxen in plasma and urine. Since it is known from literature data (2) that naproxen in man undergoes two major biotransformations, viz. demethylation to 6-0-des-methyl-naproxen (II) and conjugation with glucuronic acid, the procedure should differentiate between these compounds. The analysis of naproxen in biological samples has been the subject of various reports. Gas-liquid chromatography has been used (3, 4, 5) but this requires a derivatization step for estrification of the carboxylic acid function of naproxen. We have experience with gas-liquid chromatography of naproxen after methylation with diazomethane. Although this procedure gives excellent results in general, it has a major drawback in the fact that the des-methyl-metabolite (II) yields naproxen itself. Of course it is possible

to obtain a better specificity by using other derivatization techniques, e.g. butylation (3). An interesting and relatively sensitive analytical method for naproxen makes use of the strong fluorescent properties of naproxen (6, 7, 8). Direct spectrophotofluorometry however requires rather extensive procedures for extraction and sample preparation and purification. And even then, application of direct fluorometry to samples of patients, receiving sometimes comedication with a number of other drugs, carries a large risk of interferences.

Since we wanted to avoid complicated sample preparation and derivatization techniques we developed a specific high-performance liquid chromatographic method for the determination of naproxen and its des-methyl-metabolite in biological fluids. Although several methods have been described for the HPLC analysis of naproxen in biological samples (9, 10), in our hands the methods lack the sensitivity needed for thorough pharmacokinetic studies. In this paper we report the HPLC analysis of both naproxen and des-methyl with subsequent detection and quantification by spectrophotofluorometry. This method is compared with the standard U.V. detection and its advantages are discussed.

MATERIALS AND METHODS

Materials

Naproxen as well as 6-O-des-methyl-naproxen were kindly supplied by Syntex Research (Palo Alto, Calif., U.S.A.). Diflunisal (IV), used as an internal standard, was kindly supplied by Merck, Sharp and Dohme (Brussels, Belgium). The other internal standard, the ethoxy-analog of naproxen (III), was obtained from Syntex Research (Palo Alto, Calif., U.S.A.). The column packing material used was Lichrosorb RP 8 (5 μm) which was obtained from E. Merck

(Darmstadt, G.F.R.). All other chemicals were of analytical grade and also obtained from Merck.

Apparatus and Chromatography

A Hewlett-Packard 1081 B high-performance liquid chromatograph was used, equipped with an automatic sampling system and a HP fixed wave-length (254 nm) U.V. detector. The output of the detector was fed to the HP 3351 Lab Data System, which was operated in the internal standard mode. The fluorescence detector used was the Perkin Elmer model 3000 fluorescence spectrometer which was operated at an excitation wave-length of 235 nm (slit width 10 nm) and an emission wave-length of 350 nm (slit 5 nm). The column was stainless steel, 15 cm x 4.6 mm I.D. and was packed with Lichrosorb RP 8 (5 µm). The mobile phase was a mixture of methanol and citrate buffer (pH 6.5, 0.0 M). The methanol buffer volume ratio was 50 : 50 for plasma and 40 : 60 for urine. The flow rate was fixed at 1.5 ml/min and the column temperature at 30°C when U.V. detection was used. In case of fluorometric detection the flow rate was lower (1.0 ml/min) but the temperature higher (35°C). A major factor in this respect is the large injection peak in case of U.V. detection which is absent in fluorometric detection. These differences were desirable for obtaining optimal conditions in both cases. In the experiments with U.V. detection diflunisal was used as internal standard. The fluorescence detection necessitated the use of another standard, with fluorescence properties equal to those of naproxen; for that purpose the ethoxy-analog was used.

Sample Preparation

Plasma (0.5 ml) was pipetted into a stoppered test tube, containing the internal standard, along with hydrochloric acid (0.7 ml, 1.5 N). After homogenization by mechanical shaking for 30 min

the mixture was extracted with 5 ml of a mixture of diethyl-ether and n-hexane (1 : 1 volume ratio). After centrifugation for 15 min at 1500 g, the organic layer was transferred into another tube and evaporated at 30°C under a gentle stream of dry filtered air. The residu was reconstituted in 1 ml of the mobile phase and aliquots of 10 µl were injected into the column. When fluorometric detection was applied the reconstituted residu of samples containing over 10 µg/ml was diluted 20-fold prior to the injection of again 10 µl.

When urine samples (usually 0.25 ml) were analyzed the same procedure was followed, except for the acidification which was performed with acetic acid (4.2 N) instead of hydrochloric acid. The reason for this modification was the fact that des-methyl-naproxen, which is present in most urine samples but never in plasma samples in measurable amounts, appeared to be unstable during the sample preparation procedure when hydrochloric acid was used. For plasma samples we prefer acidification with hydrochloric acid because it results in a better precipitation of plasma proteins.

Calibration and Recovery

Calibration was performed by adding known amounts of naproxen to blanc plasma or blanc urine samples and by handling these according to the procedure outlined above. Since urine samples usually contain both naproxen and its metabolite des-methyl-naproxen also calibration for the latter had to be performed in the same way in case of urine samples. Calibration graphs were obtained by plotting the concentration of naproxen calculated by the data system against the concentration added. The data system was operated in the internal standard mode and calibrated with one of the known added concentrations, usually 50 µg/ml. For the calculations peak height ratios of naproxen to internal standard were

used. The overall recovery of naproxen and des-methyl-naproxen in the procedure was determined by comparing peak height ratios obtained after direct injection of standard solutions to peak height ratios of extracted naproxen solutions to standard solutions of the internal standard.

In order to estimate the overall precision of the method samples with various concentrations were analysed in ten times for calculation of the standard deviation of the measurement.

RESULTS AND DISCUSSION

Under the conditions described no interference from endogenous compounds in plasma or urine is encountered (Fig. 2a). Table I

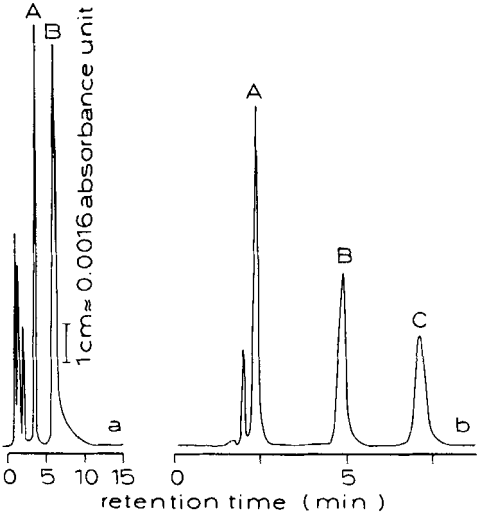

FIGURE 2a

HPLC chromatogram of naproxen (A) and diflunisal (B) after extraction from plasma, using ultraviolet detection.

FIGURE 2b

HPLC chromatogram of 6-O-des-methyl-naproxen (A), naproxen (B), ethoxy-naproxen (C) after extraction from urine, using fluorescence detection.

TABLE I

Retention Times of Naproxen and Some Other Anti-inflammatory Drugs in the Chromatographic System Described in the Text

	RT^a (min)	RT^b (min)
salicylic acid	1.65	
6-0-des-methyl-naproxen	1.70	2.30
oxyphenbutazon	2.55	
naproxen	3.60	4.80
ethoxy-naproxen	5.20	7.00
phenylbutazon	5.70	
diflunisal	6.25	
mefenamic acid	12.75	
flufenamic acid	17.60	

a: flow 1.5 ml/min, column temperature 30°C
b: flow 1.0 ml/min, column temperature 35°C

lists the retention times of naproxen, its des-methyl-metabolite and some other anti-inflammatory drugs in the chromatographic system employed. It is obvious that also these compounds are not likely to cause difficulties in the analysis of naproxen. It may be important to notice already here that in plasma samples we never were able to detect any des-methyl-naproxen. This metabolite hower is consistently found in urine samples. Probably the rate of excretion, augmented with the rate of further metabolism (conjugation to glucuronic acid) is so high that the metabolite is eliminated very shortly after it has been formed, so that no appreciable plasma levels are reached. The recovery of naproxen and des-methyl-naproxen taken through the whole procedure was 95% on the average,

without any systematic variations. The other chromatographic parameters are dependent upon the method of detection and therefore they are indicated separately in the following:

Ultraviolet detection

Calibration graphs were constructed for naproxen concentrations ranging from 1-100 µg/ml. A plot of naproxen concentration as calculated by the data system (y) versus the concentration of naproxen added (x) yielded a perfectly straight line, fitting the equation:

$$y = 0.949x + 1.511 \qquad (1)$$

(correlation coefficient = 0.9999, n = 6)

This equation results when calibration is performed with the 50 µg/ml sample. Since the data system neglects the intercepts of equation 1 it is obvious that optimal analytical results can only be obtained when the calibration point is as close as possible to the samples to be analysed.

The overall precision of the method was measured on the basis of samples with 3 different concentrations. The results of Table II clearly are illustrating the fact that accuracy is best for concentrations near to the point of calibration of the data system (50 µg/ml).

The sensitivity of the procedure is comparable to that of common G.C. procedures: when using 0.5 ml samples concentrations of 1 µg/ml can be determined.

Fluorometric detection

Under the circumstances of optimal fluorescence of naproxen the original internal standard diflunisal did not show any fluorescence at all. The fluorescence of diflunisal is optimal at an excitation wave-length of 235 nm and an emission wave-length of

TABLE II

Overall precision of the method with U.V. detection as illustrated by 10-fold analysis of three different concentrations of naproxen added to plasma samples

added (μg/ml)	found (μg/ml)	standard deviation
5.08	5.85	0.140 (2.40%)
50.80	50.49	0.361 (0.71%)
101.60	100.50	3.14 (3.13%)

410 nm. The second standard, the ethoxy-analog of naproxen, appeared to be suitable (Fig. 2b). Calibration in this case was performed with the use of two different amounts of internal standard: for the naproxen range from 0.1-10 μg/ml sample the amount of standard was 1.25 μg and for the naproxen range from 5-100 μg/ml sample an amount of 25 μg was employed. In both cases linear calibration graphs were obtained by plotting the amount of naproxen calculated (y) against the amount added (x): for the naproxen range 0.1-10 μg/ml sample (calibration 5 μg/ml)

$$y = 1.029x - 0.012 \qquad (2)$$

(correlation coefficient = 0.9993, n = 5)

and for the naproxen range 5-100 μg/ml sample (calibration 50 μg/ml)

$$y = 1.1013x + 0.215 \qquad (3)$$

(correlation coefficient = 0.9995, n = 5)

As compared with equation 1 it is obvious that the value of the intercepts in equations 2 and 3 are substantially smaller. This

indicates a higher sensitivity and better accuracy in the low concentration range. It is obvious that the slopes of equations 2 and 3 are very similar. In fact a very satisfactory calibration graph for the whole concentration range can be obtained by combining all data in one equation:

$$y = 1.017x + 0.537 \qquad (4)$$

(correlation coefficient = 0.9997, n = 10)

Although this equation confirms the overall linearity it is clear that accurate measurements in the lower concentration range can only be performed by using equation 2 based on addition of the lowest amount of internal standard.

The precision of the fluorometric method again was measured by repeated analysis of samples with different concentrations and appears to be at least as good as that of the U.V. procedure as can be seen in Table III. The sensitivity of this procedure is definitely higher than that of the U.V. procedure. When using 0.5 ml samples concentrations of 0.1 µg/ml can be determined.

TABLE III

Overall precision of the method with fluorometric detection of naproxen (cf. Table II)

added (µg/ml)	found (µg/ml)	standard deviation
0.154	0.149	0.006 (3.9%)
1.536	1.504	0.024 (1.6%)
10.24	10.14	0.14 (1.4%)
51.20	51.18	0.62 (1.2%)
102.40	103.87	1.24 (1.2%)

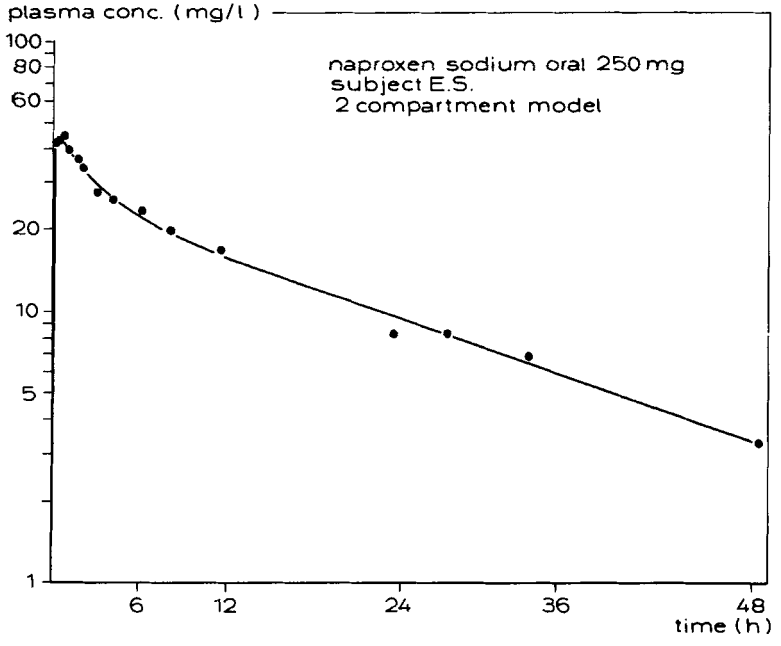

FIGURE 3

Computer fitting of a plasma curve of naproxen sodium, using a two-compartment model. The concentrations were assessed with the fluorometric detection method.

CONCLUSION

The procedures described combine ease of handling with accurate and sensitive analytical results. Furthermore they are suitable for routine measurements and can easily be adapted to automatic analysis. The procedure with U.V. detection in our experience is very suitable for the routine measurement of naproxen plasma levels of patients during chronic medication. Results of such measurements combined with their clinical and pharmacological significance will be reported elsewhere. When, however, studies of the detailed pharmacokinetics of naproxen after single dose are

TABLE IV

Comparative analysis of 9 plasma samples of a human volunteer after an oral dose of 250 mg of naproxen, by U.V. detection and fluorometric detection

time after administration (hr)	U.V. (g/ml)	fluorometry (g/ml)
0.5	65.62	67.16
1.0	60.45	60.96
2.0	49.97	50.59
3.1	42.89	44.28
4.2	37.04	38.47
6.3	30.74	31.97
8.0	28.58	28.78
12.0	19.31	19.60
24.3	12.29	12.22

concerned or in other cases where the accurate measurement of low concentrations of naproxen is required, or when only very small samples are available the fluorometric detection method becomes necessary when high-performance liquid chromatography is the analytical method of choice. As an example of such a situation Fig. 3 shows the plasma curve of naproxen after oral administration of a 250 mg dose to a human volunteer. The plasma curve appears to obey two-compartment kinetics, although in previous studies a kinetic analysis according to a single compartment model is proposed (11). Details of this pharmacokinetic behaviour will be published elsewhere. Finally, Table IV shows a set of experimental data, viz. plasma concentrations of naproxen in a volunteer, that have been calculated by high pressure liquid chromatography with

U.V. as well as fluorometric detection. These data indicate that both methods are equally reliable in the concentration range in which the U.V. procedure can be adequately applied.

REFERENCES

1. Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, G.S., Drugs 18, 241 (1979).
2. Segre, E.J., J. clin. Pharmacol. 15, 316 (1975).
3. Wan, S.H. and Shaikh, M.B., J. Chromatogr. 170, 473 (1979).
4. Amos, B., Marple, L., Smithers, J. and Martin, S.B., J. Chromatogr. 206, 151 (1981).
5. Desager, J.P., Vanderlist, M. and Harvengt, C., J. clin. Pharmacol. 16, 189 (1976).
6. Antilla, M., J. Pharm. Sci. 66, 433 (1977).
7. Mortensen, A., Jensen, E.B., Petersen, P.B., Husted, S. and Andreasen, F., Acta Pharmacol. Toxicol. 44, 277 (1977).
8. Von Held, H., J. Clin. Chem. Clin. Biochem. 16, 579 (1978).
9. Slattery, J.T. and Levy, G., Clin. Biochem. 12, 100 (1980).
10. Nielsen-Hudsk, F., Acta Pharmacol. Toxicol. 47, 267 (1980).
11. Runkel, R.A., Kraft, K.S., Boost, G., Sevelius, H., Forchielli, E., Hill, R., Mogoun, R., Szakacs, J.B. and Segre, E., Chem. Pharmac. Bull. 20, 1457 (1972).