CHROM. 12.485

CONVENIENT AND SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR KETOPROFEN, NAPROXEN AND OTHER ALLIED DRUGS IN PLASMA OR URINE

ROBERT A. UPTON, JEAN N. BUSKIN, THEODOR W. GUENTERT, ROGER L. WILLIAMS and SIDNEY RIEGELMAN

Drug Studies Unit, Department of Pharmacy, University of California, San Francisco, Calif. 94143 (U.S.A.)

SUMMARY

A new high-performance liquid chromatography technique enables convenient and rapid assay of ketoprofen and naproxen in biological samples at a sensitivity (10 and 2 ng/ml, respectively in plasma; 20 and 50 ng/ml in urine) far greater than previously available. Superior sensitivity is attributable to the buffered neutral eluent employed, which yields improved separation from material of biological origin. There is no interference from the major ketoprofen and naproxen metabolites tested and excellent reproducibility and accuracy can be maintained. Moreover, the same system can be used to assay probenecid and also shows promise of applicability to ibuprofen, fenoprofen and other members of the aryl-alkanoic acid class of non-steroidal anti-inflammatory agents.

INTRODUCTION

Ketoprofen and naproxen are two of several aryl-alkanoic acid derivatives currently being marketed or undergoing clinical evaluation as non-steroidal anti-inflammatory agents. Available assays for these drugs in biological fluids include thin-layer or paper chromatography[1–4], gas–liquid chromatography after derivatization[3,5–7], spectrophotometry[1,2], radio-isotope tracing[4], colorimetry or polarography[5] and combinations of these techniques. The methods are tedious and/or for ketoprofen assay, possess inadequate sensitivity for pharmacokinetic determinations.

A high-performance liquid chromatography (HPLC) assay for ketoprofen reported recently[8] represents an improvement in convenience but offers assay sensitivity no lower than 1.5 µg/ml, about one third the peak plasma concentration arising from a six hourly 50-mg dose. As a consequence, plasma concentrations could be traced by this method for no longer than about half of a 6-h dosing interval nor for more than about 2 h after a single dose. Two other recently reported HPLC techniques[9,10] are more complex and time-consuming, both involving multiple extraction steps, but any improvement in sensitivity appears to be marginal.

Because of the advantage to certain research or treatment facilities, this

laboratory is evaluating the possibility of the use of one system for assay of a number of non-steroidal anti-inflammatory agents and other drugs associated with treatment of arthritic conditions. The HPLC assay described below is simple and rapid but, nevertheless, provides a sensitivity of 10 ng/ml of ketoprofen in plasma and 50 ng/ml in urine. It thus allows quantitation of ketoprofen in these fluids for 24 h after taking even a single 50-mg dose. The same system further permits precise and accurate quantitation of naproxen at a sensitivity (2 ng/ml) greater than otherwise[6,7,11] available (1 to 2 μg/ml) and without the complication of derivatization as required for gas chromatography[6,7]. Assay of ibuprofen and fenoprofen similarly appears possible and some preliminary specifications for determination of probenecid in plasma or urine are also presented.

MATERIALS AND METHODS

Solutions

Acetonitrile is HPLC grade (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). Water is deionized and then distilled from an all-glass still. All other solvents and reagents are of analytical grade. In the preparation of HPLC eluent, buffers are adjusted to pH and passed through a 0.45-μm membrane filter (Type HA, Millipore), prior to the addition of acetonitrile. Ketoprofen was obtained from Ives Labs. (New York, N.Y., U.S.A.), naproxen from Syntex Labs. (Palo Alto, Calif., U.S.A.) and probenecid from Merck Sharp & Dohme (West Point, Pa., U.S.A.).

Ketoprofen standards are solutions of 100, 10 and 1.0 μg/ml in 0.01 M phosphate buffer (Na_2HPO_4–KH_2PO_4) at pH 6.0, containing 1.0, 0.1 and 0.01% of methanol, respectively. The ketoprofen for the 100-μg/ml solution is first dissolved in the small amount of methanol. The other two solutions are obtained by aliquot dilution. Naproxen standard solutions of 200, 20 and 2 μg/ml in 0.01 M phosphate buffer (pH, 6.0) containing 5, 0.5 and 0.05% methanol, are prepared similarly. Standard solutions are stored at 4°.

Assay set-up

The assay design is outlined in Fig. 1. Naproxen is used as the internal standard for the assay of ketoprofen, and ketoprofen as the internal standard for naproxen assay. For analysis of ketoprofen in plasma, 350 μl of naproxen solution (2 μg/ml)* is pipetted into 20-ml screw-cap centrifuge tubes to be used for samples and standard curve alike. For ketoprofen analysis in urine, 350 μl of the 20 μg/ml naproxen solution is taken. For naproxen assays either in plasma or in urine, 350 μl of the 10 μg/ml ketoprofen solution is employed.

In those tubes to be used for a ketoprofen standard curve, ketoprofen solution is added to provide standards of 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2, 5, 10 and 20 μg for plasma assay and 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2, 5, 10, 20, 50, 75, 100 and 150 μg standards for urine assay. A naproxen standard curve includes 0, 0.02, 0.04, 0.1, 0.2, 0.4, 1.0, 2, 4, 10, 20, 40 and 100 μg naproxen standards for plasma assay and 0, 0.2, 0.4,

---

* Similar assay specifications result from use of a 20-μg/ml solution (as for urine), but a dual pen recorder or integrator is then needed for low-level ketoprofen quantitation.
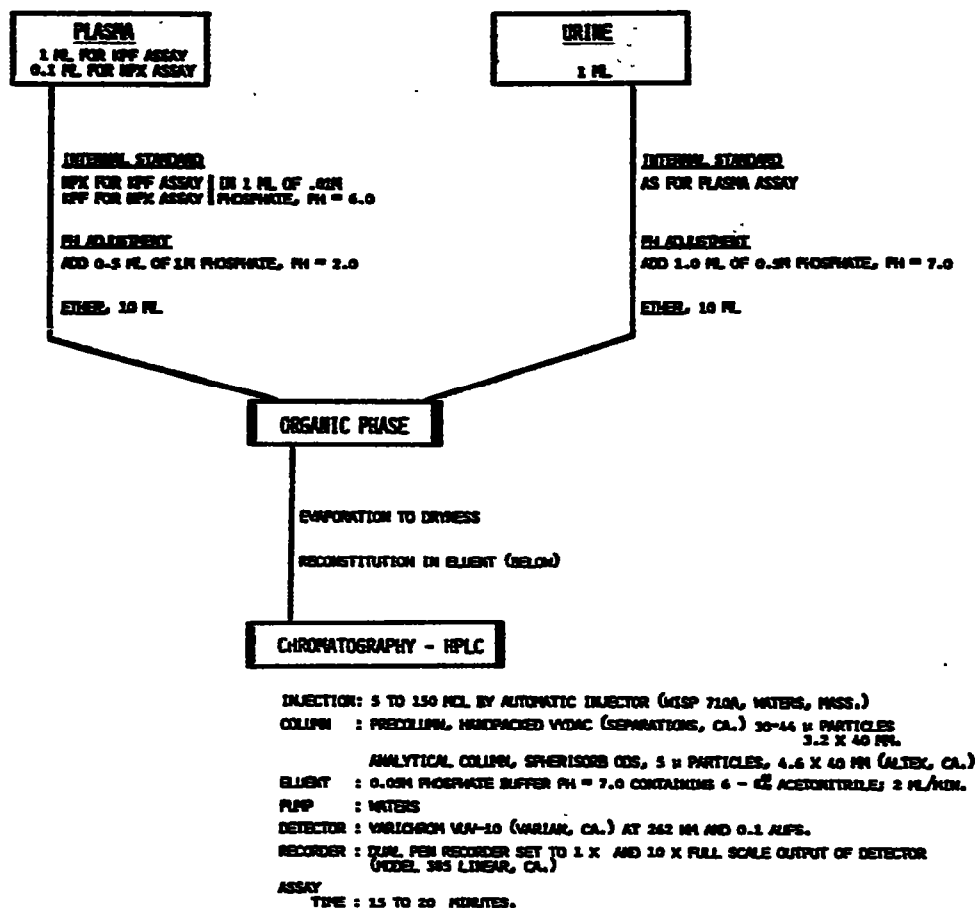
Fig. 1. Ketoprofen (KPF) and naproxen (NPX) assays in plasma and urine.

1.0, 2, 4, 10, 20, 40, 100, 150, 200 and 250 μg standards for urine assay. Additionally, all standard curves are run with a tube containing neither ketoprofen nor naproxen.

The content of all tubes, whether for samples or calibrators is adjusted to 1.0 ml total volume with 0.01 M phosphate buffer at pH, 6.0. At this stage 1.0 ml of the plasma or urine to be assayed is added. For naproxen assays, however, 0.1 ml of plasma is sufficient except when extraordinary sensitivity might be required. For standard curves the plasma and urine derive from drug-free donors.

Extraction

The pH of each sample is adjusted by addition of 0.5 ml of 1.0 M phosphate buffer at pH 2.0 for plasma assays or, for urine assays, with 1.0 ml of 0.5 M phosphate buffer at pH 7.0. After 10 ml of diethyl ether is added, tubes are vortexed for 1 min and centrifuged at about 2000 g for 3 min. The upper organic phase is transferred by Pasteur pipette to a disposable 15-ml culture tube and evaporated to dryness at 40° either under vacuum or under a stream of dry nitrogen. The extract is reconstituted

TABLE I

SPECIFICATIONS FOR KETOPROFEN AND NAPROXEN ASSAY IN PLASMA AND URINE

Parameter	Ketoprofen Plasma	Ketoprofen Urine	Naproxen Plasma	Naproxen Urine
Range (ng/ml)	10–20,000	50–150,000	200–1,000,000	50–200,000
Linearity	7.9 ± 3.1 at 10–200 ng/ml	3.2 ± 1.6 at 50–2000 ng/ml	9.9 ± 2.6 at 200–10,000 ng/ml	3.0 ± 0.5 at 50–4000 ng/ml
Coefficient of variation (C.V.) of concentration-normalized peak height ratios (%)	4.4 ± 1.8 at 100–2000 ng/ml	3.5 ± 1.3 at 1000–20,000 ng/ml	4.4 ± 3.0 at 4000–100,000 ng/ml	2.8 ± 1.1 at 2000–40,000 ng/ml
	3.0 ± 1.0 at 1000–20,000 ng/ml	2.1 ± 1.0 at 10,000–150,000 ng/ml	2.7 ± 1.7 at 40,000–1,000,000 ng/ml	2.4 ± 0.8 at 20,000–200,000 ng/ml
	(20 curves each with 5 points/range)	(11 curves each with 5 points/range)	(3 curves each with 5 points/range)	(5 curves each with 5 points/range)

Precision C.V. (%)

	Ketoprofen Plasma				Ketoprofen Urine		Naproxen Plasma		Naproxen Urine	
	At 10* ng/ml	At 20* ng/ml	At 350 ng/ml	At 1500 ng/ml	At 3500 ng/ml	At 15,000 ng/ml	At 70,000 ng/ml	At 300,000 ng/ml	At 7000 ng/ml	At 30,000 ng/ml
Intraday (6 samples)	14.7	4.1	2.3	0.5	0.4	0.5	0.5	2.0	0.9	1.2
Interday (6 days)			0.8		2.3		2.3		1.5	
Bias Deviation of mean from amount spiked (%)										
6 samples on 1 day	−8.0	−4.5	3.4	0.2	4.1	−0.4	−0.4	−1.9	1.7	−2.1
1 sample/day for 6 days			3.3		1.6		+0.6		0.8	

Parameter	Ketoprofen Plasma	Ketoprofen Urine	Naproxen Plasma	Naproxen Urine
Extraction recovery (%)	90	80	90	80

* 3 samples only.

in 250 µl of HPLC eluent, vortexed for 15 sec and transferred to a disposable poly-
propylene micro centrifuge tube (29 mm, Brinkmann).

Hydrolysis of conjugates

The urine assay described allows quantitation of unchanged ketoprofen or
naproxen in urine. The total of free and conjugated drug can be easily obtained by
including an alkaline hydrolysis step. Because of the greater drug concentration
anticipated after hydrolysis only 0.1 ml of urine need be taken for the assay. To this
volume of urine, 1.0 ml of the buffer containing internal standard is added as for the
other assays, plus 0.1 ml of 1 M NaOH. After allowing 15 min hydrolysis at room
temperature, 2 ml of 1 M phosphate buffer at a pH of 2, is added together with
0.1 ml of 1 M hydrochloric acid. The mixture is extracted with ether and processed
further as described above.

Chromatography

Samples are loaded onto the HPLC column by an automatic sampler (WISP
710A; Waters Assoc.) with facility to set the injection volume of each sample inde-
pendently. Chromatography is performed using a Spherisorb 5-µm octadecyl reversed-
phase column, 40 × 4.6 mm (Altex), protected by a 40 × 3.2 mm pre-column dry-
packed by hand with Vydac reversed-phase material, 30–44 µm (Separations Group).
The columns are eluted with 0.05 M phosphate (Na_2HPO_4–KH_2PO_4) buffer at pH 7.0,
containing between 6 and 8% of acetonitrile depending on the condition of the
Spherisorb column. Eluent is pumped (Model 6000A HPLC pump, Waters Assoc.)
through the columns at 2.0 ml/min (pressure about 1000 p.s.i.) and monitored at
262 nm (Varichrom VUV-10 detector; Varian). By employing a dual pen recorder
(Model 385; Linear) with a 10-fold discrepancy in setting of the pens (i.e. with the
pens effectively at 0.1 and 0.01 a.u.f.s., respectively) and utilizing the variable injection
volume capability of the automated sampler, a 5000-fold range of peak heights ratios
can comfortably be accommodated by the system without involvement of the operator.
Most effective use of the automatic sampler in this way does presume some prior
knowledge of those samples generating the highest chromatogram peaks. Injection
volumes are between 5 and 200 µl.

Quantitation

Standard curves are run daily. Quantitation is based on peak height ratios,
i.e. the height of the peak representing the compound being assayed divided by the
height of the peak representing the internal standard. Since assays cover a several
thousand fold spread in concentrations, each standard curve is split into a high,
medium and low range to avoid excessive weight being given to more concentrated
standards. An unweighted least-squares regression is fitted to each range inde-
pendently. The ranges are defined in Table I (under linearity specifications) where it
can be noted that they are set with an overlap of two calibrators. Samples in the
overlap region are always quantitated using the lower range.

RESULTS

Range, linearity, precision, bias and extraction recovery specifications for the

assay of ketoprofen and naproxen in plasma and in urine are shown in Table I. Linearity specifications for the urine assay including the conjugate hydrolysis step are almost identical to those shown for assay of the unconjugated material. The naproxen/plasma assay specifications given in Table I are for 20 to 100,000 ng in a 0.1 ml sample with detection at 262 nm (uncorrected). Greater sensitivity (down to 2 ng/ml) can be achieved with detection at 229 nm and/or by taking 1.0 ml of plasma. Ketoprofen sensitivity, usually more critical than naproxen sensitivity, is, however, compromised if both agents must be assayed at 229 nm, *e.g.* with unattended automatic sampling. When taking 1.0 ml of plasma and assaying for naproxen at 262 nm the coefficient of variation for concentration-normalized peak height ratios was 10.3 % in the range 20 to 400 ng/ml (5 points). Assaying 1.0 ml of plasma at 229 nm the coefficient of variation in the range 2 to 20 ng/ml was 4.7 % (5 points).

Typical plasma concentration-time profiles and urinary excretion rate plots obtained with this assay can been seen in Fig. 2 for ketoprofen and in Fig. 3 for naproxen. Profiles of the type shown were completely redetermined on separate days, thus using separate standard curves. For all points within a profile, the mean ratio of the second determination to the first was 0.993 (S.D. = 0.065; $n = 12$) for ketoprofen in plasma, 1.008 (S.D. = 0.042; $n = 8$) for ketoprofen in urine, 1.005 (S.D. = 0.036; $n = 15$) for naproxen in plasma and 1.007 (S.D. = 0.041; $n = 6$) for naproxen in urine. While the assay itself therefore appears to have excellent reproducibility characteristics, over longer periods of time such consistency may not be obtainable since evidence to be presented in a subsequent publication indicates that urine samples containing ketoprofen or naproxen can change upon storage.

Retention times are approximately 10 and 16 min for naproxen and ketoprofen. The metabolites, 3′-hydroxy- and 4′-hydroxyketoprofen[12] and desmethylnaproxen[13], are completely resolved from their parent compounds, being eluted
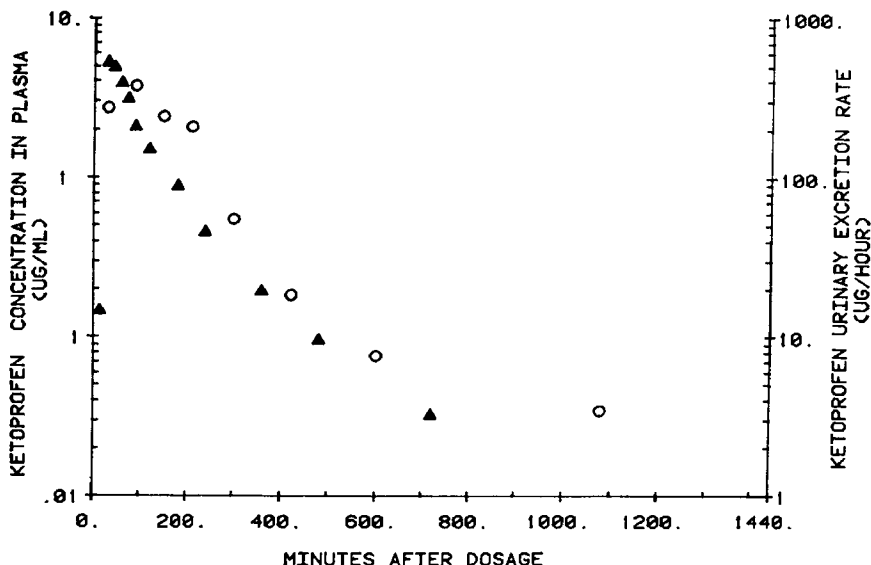

Fig. 2. Plasma concentration–time profile (▲, left axis) and urinary excretion rate plot (○, right axis) in one individual after a single 50-mg ketoprofen dose.
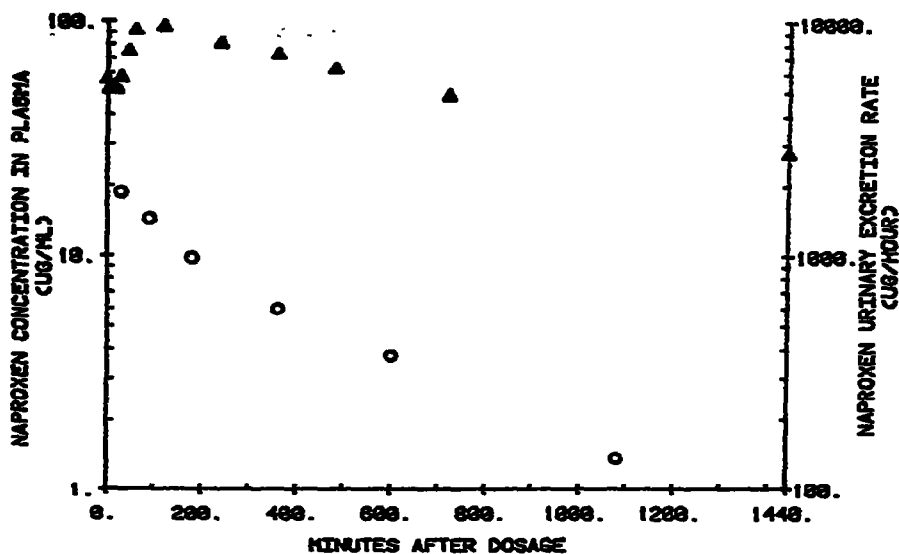
Fig. 3. Plasma concentration–time profile (▲, left axis) and urinary excretion rate plot (○, right axis) after the last of a series of twice daily 375 mg naproxen doses (different individuals).

much earlier. Ibuprofen, fenoprofen and probenecid can be chromatographed on the same system with retention times of 24, 20 and 18 min. Salicylate elutes much earlier than even naproxen and thus does not interfere. A large peak from the plasma of most individuals, even those drug-free, is apparent about 40 min after sample injection. By adjusting the sample injection interval it is possible to cause this peak to emerge at the end of the subsequent chromatogram. It is this schedule which governs the maximal frequency of injection of plasma extracts; for ketoprofen/naproxen assays usually one per 18 to 20 min.

Spherisorb column life is about 300 ketoprofen plasma samples but longer for urine samples or for those plasma samples where only 0.1 ml is extracted. If a 300-sample column life appears short, the potential user should consider that the analytical column employed for this assay is actually marketed as a pre-column (guard column) and is therefore offered at about one-third to one-half the cost of conventional analytical columns. Since column packing generally seems to lose efficiency with increasing length of columns, the 4.0-cm Spherisorb column has a relatively high plate count per cm length. When new it characteristically gives a 1700–2000-plate performance. When running ketoprofen plasma assays, the Vydac precolumn is repacked (handpacked, dry) with every Spherisorb column change.

Probenecid plasma concentration and urinary excretion rate profiles for one individual have also been determined by the above method (Fig. 4). The volume of plasma or urine used was 0.1 ml and naproxen was the internal standard. Plasma calibrators were 0, 0.2, 0.4, 1, 2, 4, 10, 20, 40, 100, 200, 400, 1000 and 2000 μg/ml. Calibrators at 0.2 and 0.4 μg/ml were omitted for urine. The plasma standard curve had a coefficient of variation for concentration-normalized peak height ratios of 7.3% between 0.2 and 20 μg/ml, 7.2% between 10 and 200 μg/ml and 4.3% between 100 and 2000 μg/ml. For urine, the coefficient of variation was 4.2% between 1 and 20 μg/ml, 4.4% between 10 and 200 μg/ml and 6.7% between 100 and 2000 μg/ml.
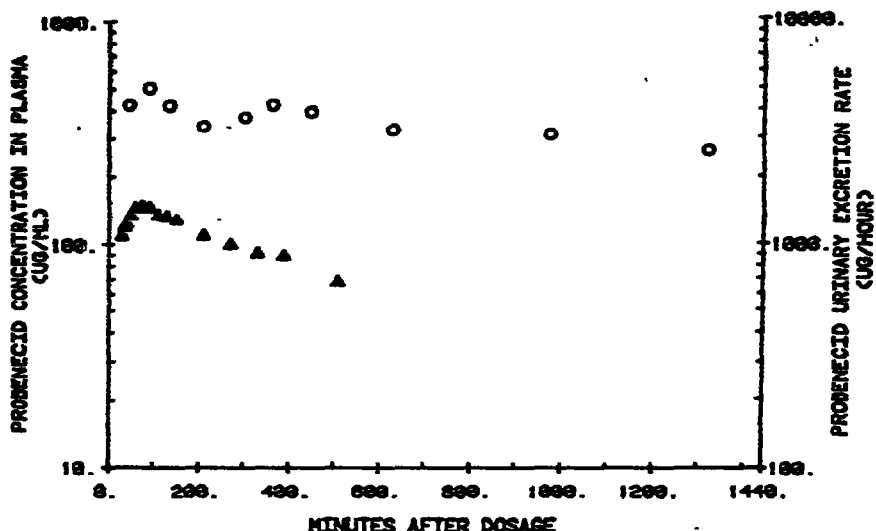
Fig. 4. Plasma concentration-time profile (▲, left axis) and urinary excretion-rate plot (○, right axis) in one individual after the last of a series of twice daily 1 g probenecid doses.

While probenecid and ketoprofen are well separated chromatographically at similar concentrations, probenecid levels can be many thousand fold those of ketoprofen leading to some merging of the peaks. If the need should arise to simultaneously measure these compounds at such divergent concentrations, this can be easily accomplished by joining two Spherisorb columns in series, increasing the acetonitrile content in the eluent to 12.5% and decreasing the flow-rate to 1.5 ml/h. Retention times for naproxen, ketoprofen and probenecid of about 6, 8.5 and 11 min result.

DISCUSSION

The sensitivity of the plasma ketoprofen assay described above (10 ng/ml) is 150 times that of the assay reported by Jefferies et al.[8]. In the chromatograms presented by Jefferies et al., some interference from extraneous material is evident. When using acidic eluent systems such as theirs in this laboratory, difficulty is experienced in separating ketoprofen from plasma material typically giving rise to a triplet of peaks with similar retention time to that of ketoprofen. With such systems, the limit of sensitivity has been confined by the relative contribution of these interfering peaks. Using the currently presented method, however, with undetectable contribution from such peaks (Fig. 5), the limits of sensitivity are determined by noise limitations of the machinery and are therefore much lower.

Farinotti and Mahuzier[9], too, employed an octadecyl reversed-phase column but used an unbuffered 45% aqueous methanol eluent. They quoted a sensitivity of 0.1 μg/ml for their ketoprofen assay in plasma even though their minimum standard curve calibrator was 0.5 μg/ml. Despite considerably more complex sample work-up than by the Jefferies method (or the method reported in this paper), the blank plasma chromatogram presented by Farinotti and Mahuzier still exhibited a peak of bio-
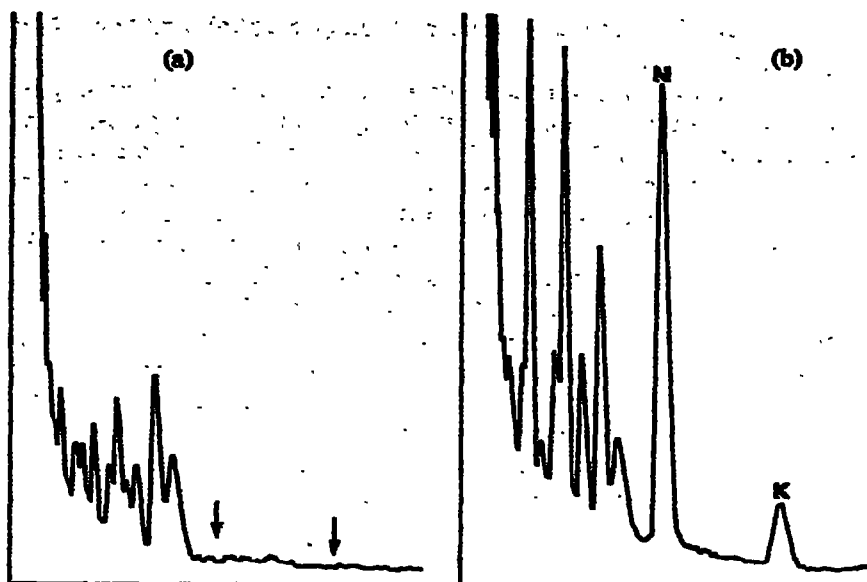
Fig. 5. Chromatograms from plasma taken before (a) and 12 h after (b) a single 50-mg ketoprofen dose. Both chromatograms were obtained at 0.01 a.u.f.s. N = naproxen internal standard; K = ketoprofen at a concentration of 42 ng/ml of plasma.

logical origin, for all practical purposes in the position of the ketoprofen peak. Since this extraneous peak reached about 4% of the height of a peak representing 5 μg/ml of ketoprofen, it is probable that biogenic material contributes 30% or more to the peak arising from a plasma sample which contains only 0.5 μg/ml of ketoprofen. Thus both previously reported HPLC techniques give results of doubtful accuracy for plasma samples with less than about 1.5 μg/ml of ketoprofen.

Farinotti and Mahuzier[9] reported the peak height ratios they obtained from plasma standards containing between 0.5 and 15 μg/ml of ketoprofen. The concentration-normalized peak height ratio had a coefficient of variation of 19%, indicating marked deviation from linearity for their plasma assay (in contrast, 3.0 to 4.4% for this concentration range with the current assay). Indeed, marked trending can be seen in the standard plot for calibrators less than 5 μg/ml. If the peak height ratio–concentration relationship reported by Farinotti and Mahuzier[9] is used to convert the peak height ratio shown for their 0.5 μg/ml calibrator, then the predicted concentration is 0.28 μg/ml, a 44% underestimate. The prediction for the 1.0 μg/ml calibrator is 0.84 μg/ml (16% underestimate) but more accurate for the 2.5- to 15-μg/ml standards. In contrast, with 20 standard curves prepared by the method described in the current paper, the prediction for the 10 ng/ml point was 10.9 $\pm$ 1.9 ng/ml (mean $\pm$ S.D.) and for the 20 ng/ml point was 20.6 $\pm$ 1.3 ng/ml.

Compared to their plasma assay, the urine assay of Farinotti and Mahuzier has better linearity with a coefficient of variation of normalized peak height ratios of 9% (3.2% with the current assay, even with concentrations 200 times less). Extraneous peaks are not apparent in the blank urine chromatograms they show, but the minimum urine calibrator used is not less than 10 μg/ml. This does not compare

favorably with the sensitivity of either the Jefferies method (1.5 μg/ml) or the method reported here (50 ng/ml for urine).

In developing assays on reversed-phase columns the analyst not using ion-pair techniques often adjusts eluent conditions for virtually complete suppression of ionization, judging that retention will be effected according to pH/partition concepts. Many compounds, however, might be so lipophilic that their partition into a non-polar phase is significant even at pH's where little in the aqueous phase is un-ionized. It is, for example, possible to extract ketoprofen and naproxen into ether at a pH approaching 7 (extraction for the urine assay) where possibly less than 1% in the aqueous phase is un-ionized. Less partition into the C_{18} reversed-phase material is probably achieved at pH 7.0 than at 3.5 (cf. eluents of 8% acetonitrile used here with 35% methanol used by Jefferies et al.) but, nevertheless, partition appears to be still sufficient to give satisfactory chromatography and a basis for separation of ketoprofen and naproxen from compounds interfering at lower pH.

It is thus possible, by utilizing a buffered neutral eluent, to exploit the convenience of HPLC for assay of both ketoprofen and naproxen at a sensitivity much greater than previously available. Moreover, the system described, or very simple variants of this system, show promise of general application to a series of similar compounds all associated with anti-inflammatory therapy.

ACKNOWLEDGEMENTS

REFERENCES

1 B. Lotti, Boll. Chim. Farm., 114 (1975) 351–354.
2 R. Ballerini, A. Cambi and P. Del Soldato, J. Pharm. Sci., 66 (1977) 281–282.
3 W. S. Mitchell, P. Scott, A. C. Kennedy, P. M. Brooks, R. Templeton and M. G. Jeffries, Curr. Med. Res. Op., 3 (1975) 423–430).
4 F. Delbarre, J. C. Roucayrol, B. Amor, J. Ingrand, G. Bourat, J. Fournel and J. Courjaret, Scand. J. Rheumatol., Suppl., 14 (1976) 45–52.
5 P. Populaire, B. Terlain, S. Pascal, B. Decouvelaere, G. Lebreton, A. Renard and J.-P. Thomas, Ann. Pharm. Fr., 31 (1973) 679–690.
6 S. H. Wan and S. B. Matin, J. Chromatogr., 170 (1979) 473–478.
7 J. P. Desager, M. Vanderbist and C. Harvengt, J. Clin. Pharmacol., 16 (1976) 189–193.
8 T. M. Jefferies, W. O. A. Thomas and R. T. Parfitt, J. Chromatogr., 162 (1979) 122–124.
9 R. Farinotti and G. Mahuzier, J. Pharm. Sci., 68 (1979) 484–485.
10 A. Bannier, J. L. Brazier and B. Ribon, J. Chromatogr., 155 (1978) 371–378.
11 L. J. Dusci and L. P. Hackett, J. Chromatogr., 172 (1979) 516–519.
12 P. Populaire, B. Terlain, S. Pascal, B. Decouvelaere, A. Renard and J.-P. Thomas, Ann. Pharm. Fr., 31 (1973) 735–749.
13 R. Runkel, M. Chaplin, G. Boost, E. Segre and E. Forchielli, J. Pharm. Sci., 61 (1972) 703–708.