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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE SIMULTANEOUS ANALYSIS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN PLASMA

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SUMMARY

A high-performance liquid chromatographic assay has been developed for the determination of a number of non-steroidal anti-inflammatory drugs in plasma. The samples were prepared by adding acetonitrile and perchloric acid to 200 μl of plasma. Diclofenac, fenoprofen, ketoprofen, naproxen, phenylbutazone, piroxicam and sulindac were quantified in the supernatant produced using a mobile phase of phosphoric acid 0.03% (pH 2.5)-acetonitrile and a detecting wavelength of 254 nm. The reproducibility, linearity, precision and specificity of the assay were determined and found to be satisfactory. Alteration of the detection wavelength to 229 nm also permitted accurate determination of ibuprofen concentration in plasma. While reduction of the organic solvent content of the mobile phase and alteration of wavelength to 313 nm produced a system capable of quantifying salicylate and its metabolites in plasma and by further reducing the detecting wavelength to 237 nm, aspirin also was quantifiable. These methods have been applied in a cross-sectional study of medication compliance among rheumatoid arthritis patients treated with non-steroidal anti-inflammatory drugs.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the mainstay of drug therapy for the inflammation and pain associated with various forms of arthritis. The measurement of NSAID concentrations in plasma is required in pharmacokinetic studies and in the clinical situation for therapeutic monitoring and assessment drug toxicity. Although most NSAIDs can be quantified using spectrophotometric or spectrofluorometric techniques, these methods are not specific and are liable to interference from the drug's own metabolites and other commonly used analgesics. Gas chromatographic methods are time-consuming

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and complex and have been largely replaced by high-performance liquid chromatography (HPLC) methods. A number of HPLC methods for detection of individual NSAIDs especially aspirin/salicylate in plasma or urine appear frequently in the literature [1–8]. The majority of these methods for plasma are similar in approach involving an initial acidification step, an extraction with an organic solvent, evaporation to dryness followed by reconstitution in either methanol or the mobile phase. The acid, organic solvent, mobile phase and wavelength in each assay differ, making it difficult to routinely use these methods for screening plasma levels for compliance or toxicity in a clinical setting. The similarity of the various assays suggests that a single assay procedure capable of detecting several NSAIDs in plasma should be possible, however few have been reported. Dusci and Hackett [9] described an assay that detects four commonly used NSAIDs, Shimek et al. [10] reported an assay suitable for indomethacin, sulindac and tolmetin while Wåhlin-Boll et al. [11] reported a method suitable for aspirin, salicylate, diflunisal, indoprofen and indomethacin. In each of these assays there is some modification of the wavelength and/or the mobile phase to enable detection of all the drugs named. A more recent method reported by Levine and Caplan [12], suitable for fenoprofen, ibuprofen, indomethacin, naproxen and tolmetin, uses the same wavelength and mobile phase. The present paper reports a simple but reliable HPLC method, which is capable of measuring many of the commonly available NSAIDs in plasma. The assay detects therapeutic concentrations of diclofenac, fenoprofen, ketoprofen, naproxen, piroxicam, sulindac and phenylbutazone using the same wavelength and mobile phase, and with modifications it can also be used to measure ibuprofen, aspirin and salicylate.

EXPERIMENTAL

Reagents and sample preparation

All drugs and reagents used in the assay procedure are listed in Table I. To a 200-μl sample of plasma in a 1.5-ml polypropylene Eppendorf® centrifuge tube were added 20 μl of 30% perchloric acid and 400 μl of acetonitrile containing a known concentration of the internal standard, *p*-toluic acid. The tube was vortexed for 2 min, then centrifuged for 3 min at 11 300 *g*. The resultant supernatant was transferred to a 2-ml high grade borosilicate glass automatic injector vial for HPLC analysis.

Reversed-phase HPLC

Samples were injected using an HPLC automatic injector (Kortec K65, Melbourne, Australia) equipped with a 20-μl injection loop. A Waters Assoc. M-45 solvent delivery system delivered the eluent at a flow-rate of 1 ml/min to a Waters C_{18} μBondapak column (300×3.9 mm I.D., 10 μm average particle size) fitted with a guard column (23×3.9 mm I.D.) packed with μBondapak C_{18}/Corasil® (Waters). The mobile phase, acetonitrile–0.03% phosphoric acid pH 2.5±0.2 (45:55, v/v) was filtered and degassed under pressure before use. All chromatography was carried out at controlled ambient temperature (18±2°C).

The absorbance was measured at 254 nm using a Waters 441 fixed-wavelength

TABLE I

DRUGS AND REAGENTS USED IN ASSAY

Drug/reagent	Source
Drugs	
Acetylsalicylic acid	Sigma (St. Louis, MO, U.S.A.)
Diclofenac sodium	Ciba Geigy (Sydney, Australia)
Fenoprofen calcium	Eli Lilly (Sydney, Australia)
Gentisic acid	Sigma
Ibuprofen sodium dihydrate	Boots (Nottingham, U.K.)
Indomethacin	Merck Sharp & Dohme (Sydney, Australia)
Ketoprofen	May & Baker (Melbourne, Australia)
Naproxen	Syntex Pharmaceuticals (Sydney, australia)
Phenylbutazone	Ciba Geigy
Piroxicam	Pfizer (Sydney, Australia)
Salicylic acid	British Drug Houses (Poole, U.K.)
Salicyluric acid	Sigma
Sulindac	Merck Sharp & Dohme
Reagents	
Acetonitrile, liquid chromatography grade	Waters Assoc. (Melbourne, Australia)
Methanol, liquid chromatography grade	Waters Assoc.
Perchloric acid, analytical grade	British Drug Houses
Phosphoric acid, analytical grade	Drug Houses of Australia (Sydney, Australia)
p-Toluic acid, analytical grade	Sigma

absorbance detector and recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). Range settings of 0.01 to 0.2 a.u.f.s. were used, depending on the drug being assayed.

Quantification

Calibration curves were prepared for each drug separately in both water and plasma by spiking water and blank plasma with a known amount of the drug in question to produce the required concentration. The concentration range chosen for each drug included plasma levels normally found after oral administration of the recommended therapeutic doses (Table II). The standard curves were prepared by plotting the ratio of peak height of the drug to the peak height of the internal standard versus concentration of the drug. Standards were prepared in methanol each week and stored at 4 °C.

RESULTS AND DISCUSSION

A wavelength of 254 nm was selected for the assay, since most NSAIDs were found to absorb adequately at this wavelength (Fig. 1). The extinction coefficients for seven NSAIDs dissolved in mobile phase are given in Table II. Fig. 2c and d show HPLC profiles of therapeutic concentrations of these seven NSAIDs in plasma. Fig. 2d is a composite chromatogram, as ketoprofen and naproxen were not sufficiently separated in the chosen solvent system. This was not considered

TABLE II

DETAILS OF BACKGROUND INFORMATION AND THE CONDITIONS USED FOR EACH NSAID

Drug	Background details			Conditions used	
	Reported clinical plasma levels (μg/ml)	Ref.	$E^{1\%}_{1\,cm}$ in mobile phase	Concentration range (μg/ml)	Absorbance range (a.u.f.s.)
Diclofenac	$\geqslant 1$	13	168.8	0.5–10	0.00–0.01
Fenoprofen	40–70	14	62.5	1 –100	0.00–0.02
Ketoprofen	5–25	14	712.5	16 –80	0.00–0.20
Naproxen	30–80	14	187.5	16 –80	0.00–0.20
Piroxicam	3–8	14	200.0	1 –10	0.00–0.02
Phenylbutazone	40–95	14	168.8	10 –100	0.00–0.20
Sulindac	1–4	15	456.3	0.7–4.8	0.00–0.02

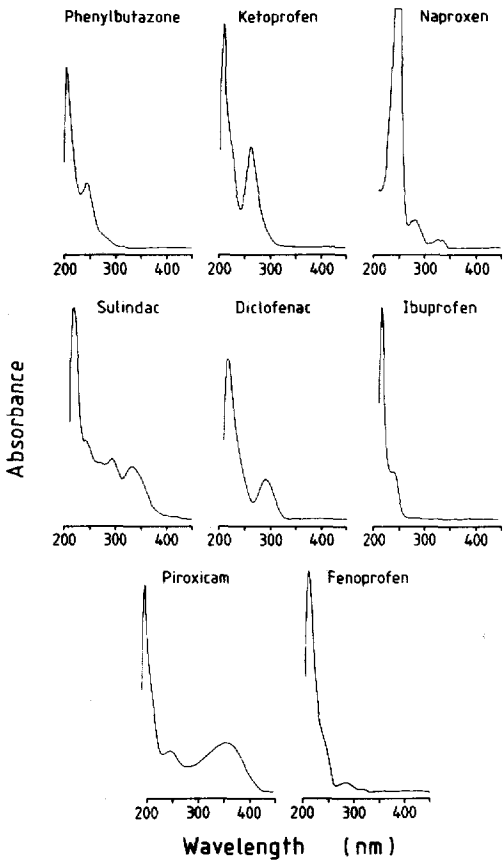

Fig. 1. Absorption spectra for some NSAIDs dissolved in mobile phase, acetonitrile–0.03% phosphoric acid (45:55, v/v). Drug concentrations were: diclofenac, ibuprofen, ketoprofen, naproxen, phenylbutazone and sulindac, 10 μg/ml; fenoprofen, 2.5 μg/ml; piroxicam, 1 μg/ml.

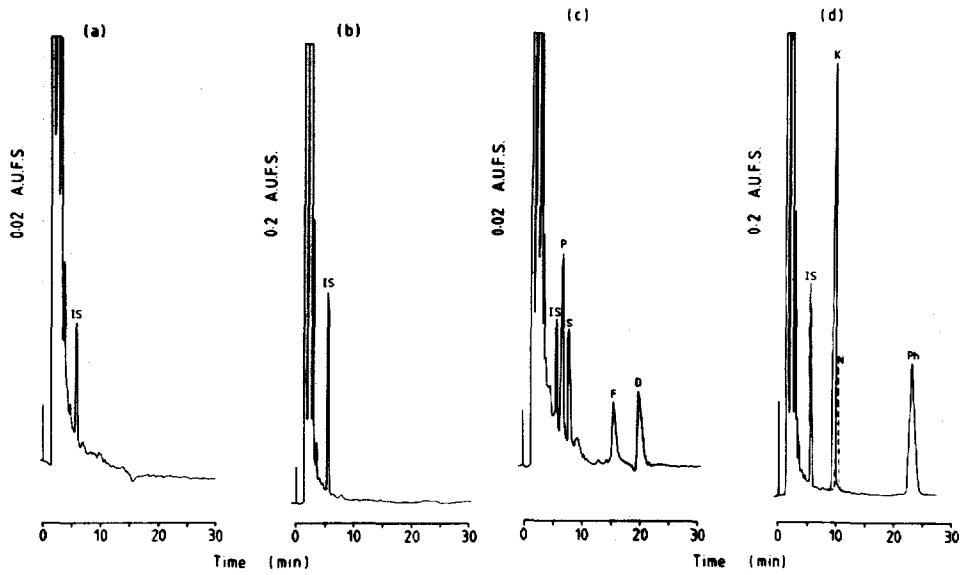

Fig. 2. HPLC profiles of 200 μl of extracted plasma. (a, b) Blank plasma at 0.02 and 0.2 a.u.f.s., respectively; IS = internal standard, *p*-toluic acid. (c) Spiked plasma containing diclofenac (D, 6.5 ng on-column ≡ 1 μg/ml of plasma), fenoprofen (F, 129 ng on-column ≡ 20 μg/ml of plasma), piroxicam (P, 13 ng on-column ≡ 2 μg/ml of plasma) and sulindac (S, 6.5 ng on-column ≡ 1 μg/ml of plasma) at 0.02 a.u.f.s. (d) Spiked plasma containing ketoprofen (K, 310 ng on-column ≡ 48 μg/ml of plasma), naproxen (N, 310 ng on-column ≡ 48 μg/ml of plasma) and phenylbutazone (Ph, 258 ng on-column ≡ 40 μg/ml of plasma) at 0.2 a.u.f.s.

a major problem, since the use of naproxen and ketoprofen together is unlikely, these drugs being from the same class of NSAIDs, i.e. propionic acid derivatives. Under the chromatographic conditions described the drugs eluted with retention times ranging from 6 min for piroxicam to 22 min for phenylbutazone (Table III). The assay was sufficiently sensitive for use in pharmacokinetic studies of the various NSAIDs, with limits of detection well below the plasma concentrations normally observed in a therapeutic situation (Table III). The method was

TABLE III

RETENTION TIMES, LIMITS OF DETECTION AND ABSOLUTE RECOVERIES OF THE CONCENTRATION RANGES USED FOR EACH OF THE NSAIDs

Drug	Retention time (min)	Limit of detection (μg/ml)	Recovery (%)	
			Mean	Range
Diclofenac	19.0	0.4	96.7	92–104
Fenoprofen	15.0	2.0	99.8	90–106
Ketoprofen	8.5	0.05	101.8	99–106
Naproxen	9.5	0.2	104.9	99–111
Phenylbutazone	22.0	0.3	114.4	106–126
Piroxicam	6.0	0.2	99.8	90–105
Sulindac	7.5	0.1	97.3	87–107

TABLE IV

REGRESSION LINES FOR THE CALIBRATION CURVES OF EACH NSAID IN WATER AND PLASMA

Equation = slope ± intercept; r = Pearson's correlation coefficient.

Drug	Water		Plasma	
	Equation	r	Equation	r
Diclofenac	$0.109C+0.043$	0.996	$0.110C+0.033$	0.997
Fenoprofen	$0.033C-0.001$	0.999	$0.035C-0.074$	0.999
Ketoprofen	$0.052C+0.037$	0.996	$0.052C+0.084$	0.996
Naproxen	$0.012C+0.022$	0.984	$0.014C-0.005$	0.999
Penylbutazone	$0.014C-0.020$	0.991	$0.016C+0.023$	0.999
Piroxicam	$0.367C+0.036$	0.996	$0.363C+0.045$	0.998
Sulindac	$0.782C-0.126$	0.998	$0.681C+0.021$	0.991

also efficient with the absolute recoveries for the seven NSAIDs ranging between 96.7 and 114.4% (Table III).

Over the concentration ranges examined, linear relationships were observed between peak-height ratios and drug concentrations in both plasma and water with correlation coefficients greater than 0.99 (Table IV). The precision of the

TABLE V

PRECISION OF THE ASSAY

Drug	Theoretical concentration (μg/ml)	n	Experimental concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Diclofenac	1.0	10	0.90 ± 0.06	6.7
	10.0	9	10.39 ± 0.36	3.5
Fenoprofen	10.0	10	11.01 ± 0.48	4.3
	70.0	10	68.20 ± 2.33	3.4
Ketoprofen	16.0	10	15.29 ± 0.48	3.1
	80.0	9	73.87 ± 2.59	3.5
Naproxen	16.0	10	15.10 ± 0.54	3.6
	80.0	10	77.28 ± 3.15	4.1
Phenylbutazone	20.0	10	20.43 ± 0.59	2.9
	100.0	9	103.44 ± 3.52	3.4
Piroxicam	1.0	10	0.93 ± 0.04	4.3
	5.0	11	4.88 ± 0.20	4.1
Sulindac	1.6	10	1.68 ± 0.60	3.6
	4.8	10	4.20 ± 0.10	2.4

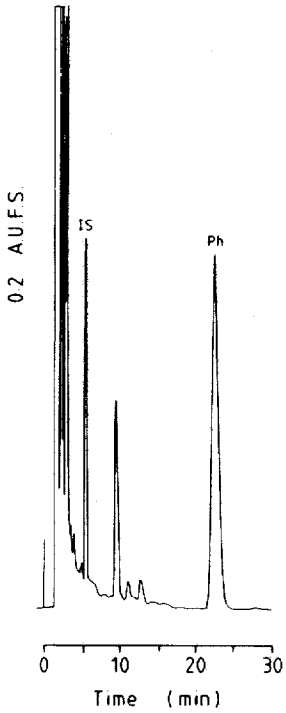

Fig. 3. HPLC profile of plasma from a patient with rheumatoid arthritis prescribed 300 mg of phenylbutazone daily. Peaks: Ph = phenylbutazone; IS = internal standard, *p*-toluic acid.

assay for each drug at a high and low concentration is shown in Table V. In all but one instance the coefficient of variation (C.V.) was less than 5%.

In all normal plasma samples examined the chromatograms were free of interfering plasma peaks; however, there was some interference with the diclofenac peak in haemolysed plasma samples of patients taking diclofenac. There was also some interference from the metabolites of phenylbutazone in plasma of patients regularly taking phenylbutazone. Fig. 3 shows a chromatogram of a patient taking phenylbutazone, in which three metabolic products of the drug are eluting at 9, 10.5 and 12 min. The peak at 9 min would most likely interfere with ketoprofen or naproxen peaks in the unlikely circumstance of a patient taking a combination of these drugs. No interfering metabolite peaks were observed in the plasma of patients prescribed the other NSAIDs. Interference by other drugs was examined by analysing plasma samples from patients taking various drugs. Table VI lists the medication being taken by these subjects. None of these patients' plasma showed peaks which would interfere with the NSAIDs being analysed in this study.

A number of HPLC assays for NSAIDs in plasma involve time-consuming extraction and evaporation procedures [1–12]. Preparation of the sample in the current assay involved a simple protein precipitation using acetonitrile and perchloric acid. After centrifugation, the resultant supernatant could be injected straight into the detection system. Dusci and Hackett [2, 9] used acetonitrile

TABLE VI

DRUGS WHICH WERE FOUND NOT TO INTERFERE WITH THE ASSAY

Allopurinol	Digoxin	Nicotinic acid
Amiloride	Doxepin	Nitrazepam
Amitriptyline	Doxylamine succinate	Northisterone
Atenolol	Ethinyloestradiol	Oxazepam
Atropine sulphate	Frusemide	Oxprenolol
Aurothiomalate	Glyceryl trinitrate	Paracetamol
Azathioprine	Hydrochlorothiazide	Pindolol
Cimetidine	Hydroxychloroquine	Prednisolone
Chlorothalidone	Hyoscine hydrobromide	Propantheline bromide
Chlorothiazide	Hyoscyamine	Propranolol
Codeine phosphate	Imipramine	Quinine
Cyclopenthiazide	Insulin	Salbutamol
D-Penicillamine	Isocarboxazide	Salazopyrin
Debrisoquine sulphate	Isosorbide nitrate	Tetracycline
Dexamethasone	Methyclothiazide	Theophylline
Dextropropoxyphene	Methyldopa	Thyroxine
Diazepam	Metoprolol tartrate	Tolazoline hydrochloride
Diflunisal	Mianserin	Verapamil

alone to precipitate and extract NSAIDs from plasma; however, the volume of solvent required in their procedure necessitated an evaporation-to-dryness step prior to injection onto the column. Shah and Jung [16] used a lower acetonitrile-to-plasma ratio of 2:1 in their assay for ibuprofen. However, this ratio in our system resulted in extremely variable recovery. By using perchloric acid, which has been used previously for protein precipitation in other HPLC assays [17], a 2:1 ratio of acetonitrile to plasma yielded low background noise and good recoveries. Rumble et al. [18] used methanol to improve perchloric acid extraction of salicylate from plasma; however, this procedure was unsuitable for the automatic injector employed in the current study, due to gelling of the supernatant produced by this extraction after periods of standing. More recently Lalande et al. [19] used phosphoric acid to improve the recovery with acetonitrile.

The mobile phase and detection wavelength conditions described proved to be satisfactory for the seven NSAIDs given in Table II. Previously published multi-purpose NSAIDs assays have involved a change in the detecting wavelength [9], the mobile phase [10] or both the wavelength and mobile phase [11] in order to measure all the cited drugs. Furthermore many of the multi-drug assays provide insufficient detail of the reproducibility and efficiency of the methods in the detection of each of the NSAIDs mentioned [9, 11,12].

The assay was not sufficiently sensitive to measure therapeutic concentrations of ibuprofen and indomethacin at 254 nm. However, changing the wavelength to 229 nm allowed reliable determination of ibuprofen plasma concentrations using the same sample preparation procedure and mobile phase. In this system ibuprofen eluted at 20 min and therapeutic levels (16–80 μg/ml) could be reliably measured over a sensitivity range of 0–0.02 a.u.f.s. with 97% absolute recovery over

TABLE VII

COMPARISON OF DRUG PLASMA CONCENTRATIONS OF COMPLIANT AND NON-COMPLIANT SUBJECTS IN A SURVEY STUDY OF COMPLIANCE [20]

Drug concentrations were quantified using the current assay.

Drug	Non-compliant			Compliant		
	n	Concentration (μg/ml)		*n*	Concentration (μg/ml)	
		Median	Interquartile range		Median	Interquartile range
Ibuprofen	15	6	0–18	7	13	5–30
Naproxen	16	26	0–42	46	37	27–50
Phenylbutazone	5	34	4–64	5	78	72–92
Salicylate	26	0	0–24	47	75	31–132

this concentration range and coefficients of variation of 4.5 and 3.6% at the lowest and highest concentration. Lowering the percentage of acetonitrile in the mobile phase to 25% and changing the detecting wavelength to 313 nm produced a system suitable for measuring therapeutic levels of salicylic acid and its metabolite salicyluric acid; aspirin and gentisic acid could also be measured in this system at a wavelength of 237 nm.

Detection of the metabolites of sulindac, including the active sulphide, was not investigated during the development of the assay due to material availability problems; however, comparison of the current assay with the sulindac assay published by Dusci and Hackett [2] suggests that the current detection system has the potential to measure the sulphone and sulphide metabolites of sulindac. Dusci and Hackett used the same detection wavelength (254 nm) and a similar mobile phase (acetonitrile–phosphate buffer adjusted to pH 3) in their quantification of sulindac and its metabolites.

The assay has been used during a cross-sectional study of medication compliance among rheumatoid arthritis patients in the community [20]. Table VII shows plasma concentrations of NSAIDs measured in subjects who were classified by interview as being either compliant or non-compliant with their prescribers' medication instructions. The observed differences in plasma concentration of compliant and non-compliant patients was statistically significant ($p < 0.05$, Mann Whitney U test). The assay has been subsequently used for both single-dose and chronic pharmacokinetic studies of salicylate and its major metabolite salicylurate.

CONCLUSIONS

The method outlined was found to be a rapid and simple assay for seven commonly used NSAIDs in plasma. It proved to be reliable and sufficiently sensitive for routine clinical use in monitoring therapeutic plasma levels of diclofenac, fenoprofen, ketoprofen, naproxen, piroxicam, sulindac and phenylbutazone. With modifications in detection wavelength and/or the organic solvent ratio in the

mobile phase, ibuprofen and salicylate and its metabolites could also be quantified. Most of the previously documented assays for NSAIDs, measured only one drug and involved more complicated extraction methods.

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