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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF OVERDOSE CONCENTRATIONS OF SOME NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN PLASMA OR SERUM

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SUMMARY

Separate methods are described for the determination of the non-steroidal anti-inflammatory drugs diflunisal, indomethacin, fenoprofen, ibuprofen, ketoprofen, naproxen, mefenamic acid and piroxicam at overdose concentrations in human plasma or serum, using high-performance liquid chromatography and ultraviolet detection A common extraction, involving protein precipitation with acetonitrile, is employed for all methods A 25 cm Hypersil ODS (5 μ particle size) analytical column is used for all chromatographic separations, with a mobile phase of acetonitrile–acetate buffer (pH 4 2 or 4 8) The methods are all reproducible and can also determine concentrations that fall within the normal therapeutic range for each drug

INTRODUCTION

In the year January to December 1986, there were an estimated 19 99 million prescriptions dispensed for non-steroidal anti-inflammatory drugs (NSAIDs) in Great Britain [1], some 74% of these being for the drugs diflunisal, indomethacin, fenoprofen, ibuprofen, ketoprofen, naproxen, mefenamic acid and piroxicam During the same period, the National Poisons Information Service, London, received 1187 telephone enquiries relating to the drugs named above [2], this constituting 2.3% of the total number of telephone enquiries in 1986 Reported overdoses with NSAIDs are few and are mostly included in review articles [3,4] In cases involving the eight drugs named above, published accounts always report symptoms, but rarely give drug concentrations or any

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analytical confirmation that the symptoms reported were directly attributable to the drug suspected [5–31] Numerous chromatographic methods are available to measure these NSAIDs at therapeutic concentrations in blood However, only a few of these employ rapid chromatographic separation and simple extraction and do not use another currently prescribed NSAID as an internal standard [32–42] Accordingly, rapid and simple methods, using high-performance liquid chromatography, have been developed for the determination of overdose concentrations of each of the drugs of interest in plasma or serum Although the extraction, chromatographic and detection systems are common to all methods, different internal standards, detection wavelengths and variations in the mobile phase (buffer/acetonitrile ratio, buffer pH and flow-rate) were required for each drug

EXPERIMENTAL

Materials and reagents

Drugs These were supplied as follows· ibuprofen by Boots (Nottingham, U.K), phenylbutazone by Ciba Labs (Horsham, U K), fenoprofen calcium and benoxaprofen by Eli Lilly (Basingstoke, U K), ketoprofen, pentobarbitone and sodium thiopentone by May & Baker (Dagenham, U K), diflunisal and indomethacin by Merck, Sharp & Dohme (Hoddesdon, U K), zomepirac sodium dihydrate by Ortho Pharmaceuticals (High Wycombe, U K), mefenamic acid by Parke-Davis (Eastleigh, U K.), piroxicam by Pfizer (Sandwich, U K), colchicine by Sigma (Poole, U K), naproxen by Syntex Pharmaceuticals (Maidenhead, U K), feprazone by WB Pharmaceuticals (Bracknell, U K).

Chemicals Sodium hydroxide pellets, acetic acid and water (all AnalaR grade) were supplied by BDH (Dagenham, U.K.); acetonitrile (HPLC grade S) and methanol (HPLC grade) were supplied by Rathburn (Walkerburn, U K); industrial methylated spirit was supplied by Alcohols Ltd (London, U K)

Biological media Horse serum was supplied by Gibco (Uxbridge, U K), human plasma was supplied by drug-free volunteers from within the Poisons Unit

Chromatography

The HPLC system consisted of a 750/03 reciprocating pump (Applied Chromatography Systems, Macclesfield, U K.), a Knauer 87 00 variable-wavelength UV monitor (Roth Scientific, Farnborough, U K), a WISP 710B injection system (Millipore U.K., Harrow, U K), a Sampler/Event control module No 19400A and an integrator No 3390A (Hewlett-Packard, Wokingham, U K), a 7×0.2 cm I D Co Pell ODS (37–53 μm particle size) guard column (Whatman, Maidstone, U.K) and a 25×0 4 cm I D Hypersil ODS (5 μm

TABLE I

SUMMARY OF CHROMATOGRAPHIC CONDITIONS FOR EACH METHOD

Method	Mobile phase		Flow-rate (ml/min)	Wave-length (nm)	Integrator sensitivity (mV FSD[a])
Diflunisal	Acetonitrile–pH 4 2 buffer[b]	(50 50, v/v)	1 0	254	256
Indomethacin	Acetonitrile–pH 4 2 buffer	(65 35, v/v)	1 6	240	128
Fenoprofen	Acetonitrile–pH 4 2 buffer	(55 45, v/v)	1 8	280	64
Ibuprofen	Acetonitrile–pH 4 8 buffer	(60 40, v/v)	1 9	220	256
Ketoprofen	Acetonitrile–pH 4 8 buffer	(60 40, v/v)	1 5	260	128
Naproxen	Acetonitrile–pH 4 2 buffer	(55 45, v/v)	1 5	240	128
Mefenamic acid	Acetonitrile–pH 4 2 buffer	(60 40, v/v)	2 5	280	128
Piroxicam	Acetonitrile–pH 4 2 buffer	(50 50, v/v)	1 5	360	128

[a]FSD = full scale deflection
[b]Buffer = sodium acetate

particle size) analytical column (Hichrom, Reading, U K) The UV detector signal was always monitored via its 1 V/absorbance unit integrator output, and the integrator sensitivity was set to give a suitable deflection for the lowest standard used in each method

The mobile phases were mixtures of acetonitrile and either pH 4 2 or pH 4 8 sodium acetate buffers The pH 4 2 buffer was prepared by mixing 200 ml of 1 *M* acetic acid with 50 ml of 1 *M* sodium hydroxide and diluting to 1000 ml with AnalaR water The pH 4 8 buffer was prepared by mixing 88 ml of 1 *M* acetic acid with 50 ml of 1 *M* sodium hydroxide and diluting to 1000 ml with AnalaR water The pH values were finely adjusted with either 1 *M* acetic acid or 1 *M* sodium hydroxide

The mobile phase, the flow-rate, the detection wavelength and the integrator sensitivity used in each method are shown in Table I

Standard preparation

Stock solutions of each drug were prepared in either methanol or industrial methylated spirit (IMS), these being interchangeable IMS was preferentially used when available, owing to its lower cost Standard ranges were produced from these stocks in either horse serum or human plasma Drug-free horse serum was more easily obtained than drug-free human plasma and was used in all cases but one, human plasma only being used when absolutely necessary (see Discussion) The standards were prepared in volumetric flasks (5 ml for naproxen, 10 ml for all other methods) using a Hamilton PB600 repeating dispenser (Phase Separations, Queensferry, U K) to dispense the stocks The concentrations for each standard range were designed to encompass concentrations from high therapeutic to high overdose After preparation, aliquots of

TABLE II

SUMMARY OF STANDARD PREPARATION PROCEDURES FOR EACH METHOD

Compound	Standard concentrations (mg/l)	Stock prepared in	Standard prepared in	Standard preparation procedure
Diflunisal	100,200,300,400,600	Methanol	Horse serum	Direct addition of stock to horse serum
Indomethacin	5,10,20,30,40	IMS	Human plasma	Stock dispensed, evaporated and reconstituted in human plasma
Fenoprofen	50,100,200,300,400	IMS	Horse serum	Direct addition of stock to horse serum
Ibuprofen	50,100,200,300,400	Methanol	Horse serum	Direct addition of stock to horse serum
Ketoprofen	10,30,50,70,100	IMS	Horse serum	Direct addition of stock to horse serum
Naproxen	100,200,300,400,500	IMS	Horse serum	Stock dispensed, evaporated and reconstituted in horse serum
Mefenamic acid	20,40,80,120,160	IMS	Horse serum	Direct addition of stock to horse serum
Piroxicam	5,10,20,30,50	Methanol	Horse serum	Stock dispensed, evaporated and reconstituted in horse serum

standards were stored at $-20°C$ Low- and high-concentration quality control specimens (controls) were prepared in the same fashion, using separate stocks to those used in standard preparation Table II summarises standard preparation procedures

Extraction procedures

The extraction procedures for all methods involved the protein precipitation of plasma or serum using acetonitrile Extraction of standards, controls and samples was carried out in duplicate The standard, control, or sample volume was transferred to a 60×7 mm I D glass Dreyer tube (S Murray, Old Woking, U K) using an Eppendorf pipette (Anderman, Kingston-upon-Thames, U K) Internal standards were added using Hamilton repeating dispensers, and any miscellaneous additions were made using either a Hamilton dispenser or an Eppendorf pipette The tubes were mixed on a vortex (Rotamixer, Hook & Tucker Instruments, Croydon, U K) for 30 s and then centrifuged (room temperature, 9950 *g*) for 2 min (Eppendorf 5412 microcentrifuge, Anderman) The supernatant was decanted into an autosampler vial (type 07-CPV[A], Chromacol, London, U K) and a volume was injected onto the HPLC system Extraction volumes, internal standard used, miscellaneous additions, and volumes injected are shown in Table III

Method of obtaining samples

Samples were obtained from hospitals referring to the National Poisons Information Service, London, U K , for information relating to the drugs of in-

TABLE III

EXTRACTION VOLUMES, CONSTITUENTS AND HPLC INJECTION VOLUMES FOR EACH DRUG

Drug	Sample volume (μl)	Internal standard	Internal standard volume (μl)	Miscellaneous additions	HPLC injection volume (μl)
Diflunisal	50	Zomepirac sodium dihydrate in water (200 mg/l)	50	100 μl Acetonitrile	40
Indomethacin	100	Feprazone in acetonitrile (25 mg/l)	100	-	40
Fenoprofen	50	Sodium thiopentone in acetonitrile (10 mg/l)	50	-	35
Ibuprofen	50	Benoxaprofen in acetonitrile (50 mg/l)	200	80 μl pH 4 8 buffer	100
Ketoprofen	100	Phenylbutazone in acetonitrile (100 mg/l)	100	-	50
Naproxen	10	Pentobarbitone in acetonitrile (500 mg/l)	200	-	50
Mefenamic acid	70	Sodium thiopentone in acetonitrile (13 mg/l)	100	-	80
Piroxicam	100	Colchicine in acetonitrile (20 mg/l)	100	-	100

terest If overdose was suspected in a patient, a 10-ml sample of lithium-heparinised blood and a 50-ml sample of urine were requested for analysis

RESULTS AND DISCUSSION

Figs 1–8 show drug-free, standard and overdose sample chromatograms for the drugs of interest The arrows on the drug-free sample chromatograms indicate the positions of the drugs of interest Table IV gives the retention times of each drug and internal standard in the individual methods

For all methods, standard curves were constructed by calculating peak-height ratios between drug and internal standard and plotting them against concentration For each standard curve, the correlation coefficient, slope and y-axis intercept were calculated, and the latter two were used to calculate the concentration in controls and samples In all cases, the correlation was found to be reproducible and good. Minimal variation in slope was seen between standard curves within a method, except in the case of piroxicam. For this method, the slope increased slightly between days If chromatograms of a piroxicam standard from successive days were compared, the piroxicam peak height remained approximately constant, but the colchicine peak height was slightly reduced This would indicate a lack of stability of the internal standard when stored cold in acetonitrile, although colchicine is reported to be stable in neutral and slightly alkaline solutions [43] However, this did not affect the precision or accuracy of the method, as no change in the colchicine peak height was ob-

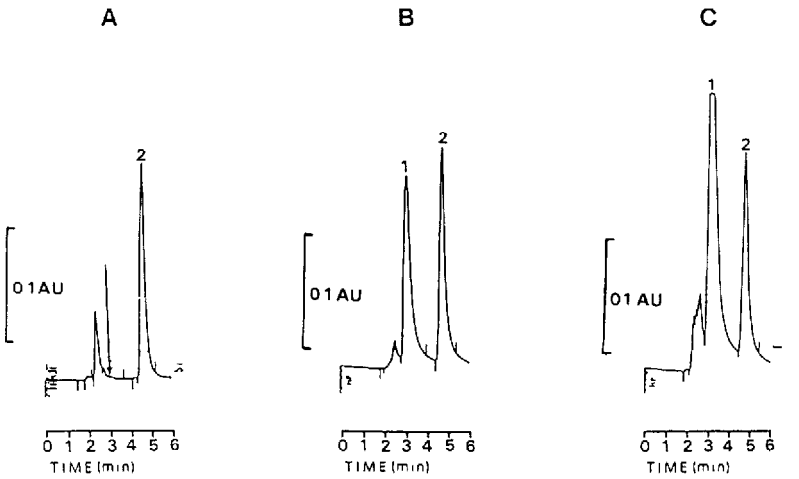

Fig 1 Chromatograms from diflunisal extracts (A) drug-free plasma plus internal standard, (B) 200 mg/l diflunisal standard, (C) sample containing 448 mg/l diflunisal Peaks 1 = diflunisal, 2 = zomepirac

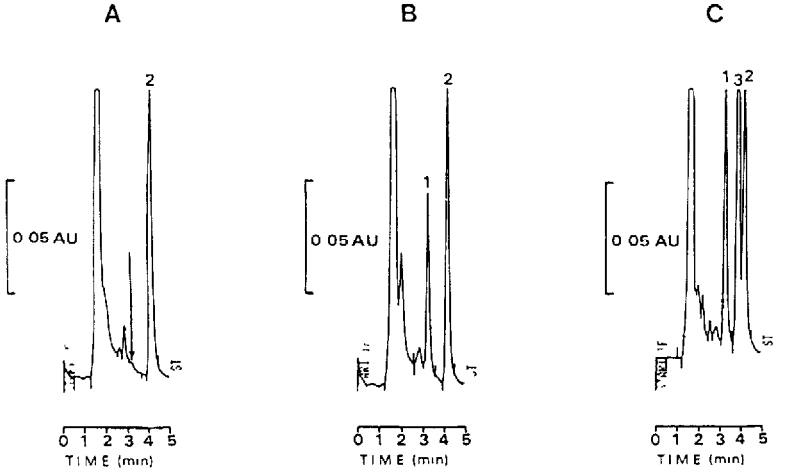

Fig 2 Chromatograms from indomethacin extracts (A) drug-free plasma plus internal standard, (B) 20 mg/l indomethacin standard, (C) sample (1 in 2 dilution) containing 80 mg/l indomethacin and phenylbutazone Peaks 1 = indomethacin, 2 = feprazone, 3 = phenylbutazone

served within an assay Freshly prepared internal standard solution had a useful life (determined by peak height) of ca 6 months

Table V shows the mean correlation coefficient, slope and y-axis intercept for fifteen successive standard curves produced for each method Intra- and inter-assay variations were determined for each method, the low- and high-concentration controls being analysed fifteen times within a single assay and

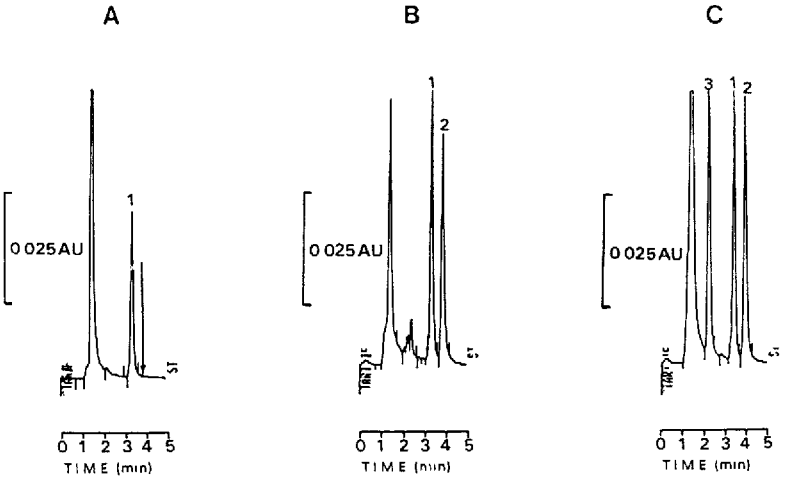

Fig 3 Chromatograms from fenoprofen extracts (A) drug-free plasma plus internal standard, (B) 200 mg/l fenoprofen standard, (C) sample containing 230 mg/l fenoprofen and an unknown compound Peaks 1 = thiopentone, 2 = fenoprofen, 3 = unknown

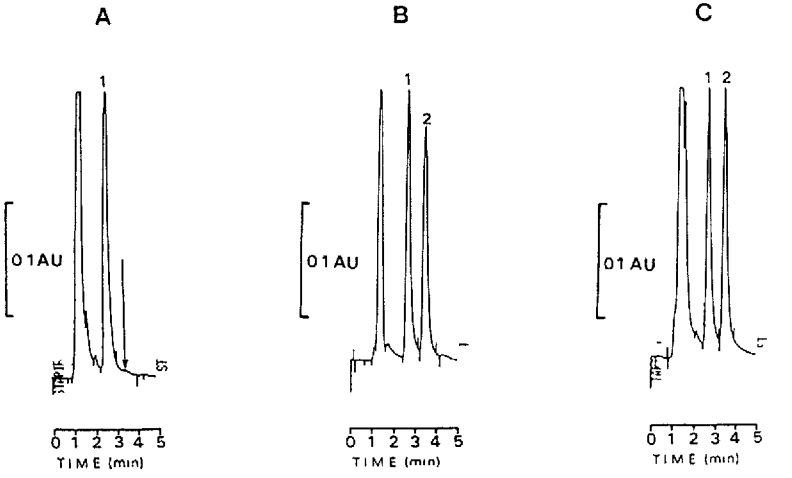

Fig 4 Chromatograms from ibuprofen extracts (A) drug-free plasma plus internal standard, (B) 200 mg/l ibuprofen standard, (C) sample containing 273 mg/l ibuprofen Peaks 1 = benoxaprofen, 2 = ibuprofen

in fifteen separate assays. The errors between the assigned and determined concentrations were also calculated, each being shown as a positive value The mean error was estimated by dividing the average error from the fifteen deter-

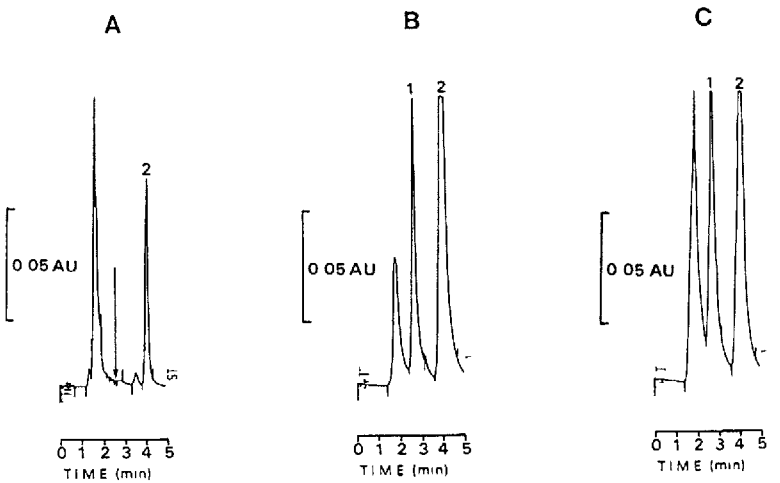

Fig 5 Chromatograms from ketoprofen extracts (A) drug-free plasma plus internal standard, (B) 30 mg/l ketoprofen standard, (C) sample (1 in 2 dilution) containing 114 mg/l ketoprofen Peaks 1 = ketoprofen, 2 = phenylbutazone

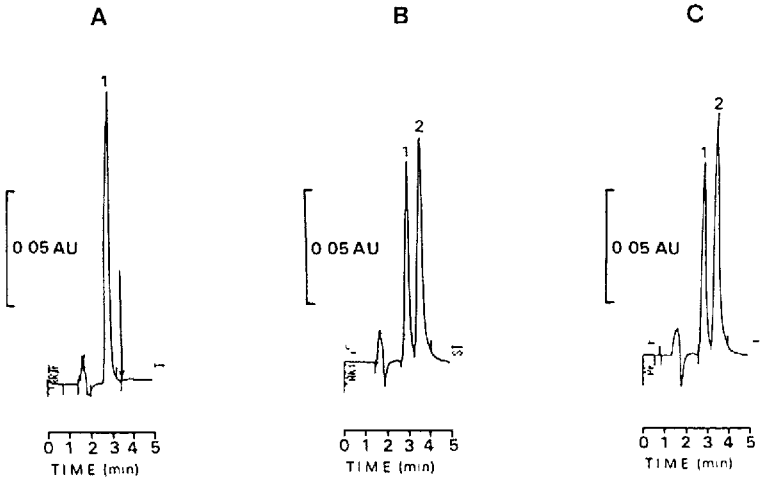

Fig 6 Chromatograms from naproxen extracts (A) drug-free plasma plus internal standard, (B) 300 mg/l naproxen standard, (C) sample (1 in 2 dilution) containing 667 mg/l naproxen Peaks 1 = pentobarbitone, 2 = naproxen

minations by the assigned concentration and expressing it as a percentage. Table VI gives details of intra- and inter-assay coefficients of variation (C V.), mean concentration and mean error for each method. For all methods, intra- and inter-assay C V values were excellent The calculated mean errors were acceptable, except, perhaps, in the case of indomethacin For this method, a

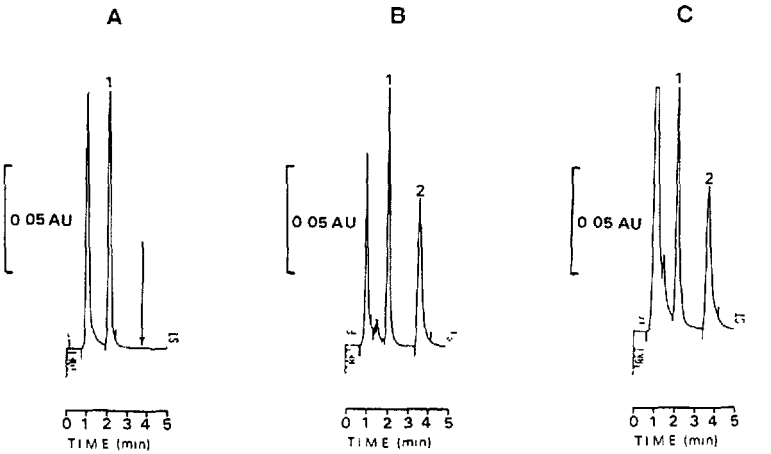

Fig 7 Chromatograms from mefenamic acid extracts (A) drug-free plasma plus internal standard, (B) 40 mg/l mefenamic acid standard, (C) sample containing 50 mg/l mefenamic acid Peaks 1 = thiopentone, 2 = mefenamic acid

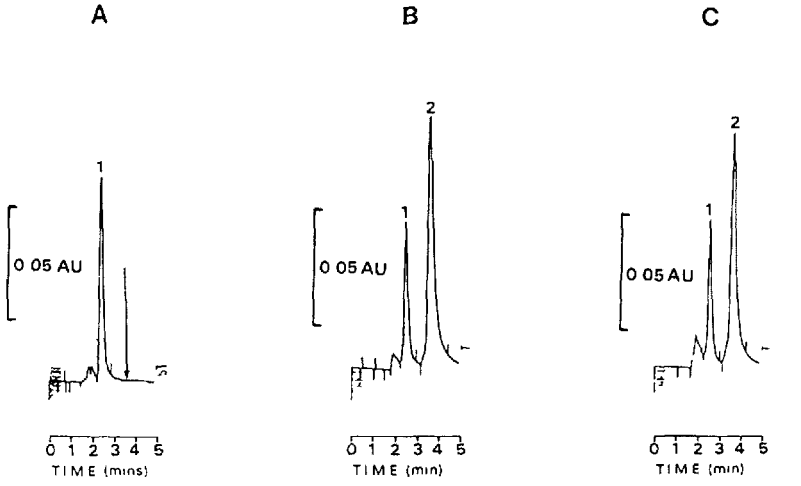

Fig 8 Chromatograms from piroxicam extracts (A) drug-free plasma plus internal standard, (B) 30 mg/l piroxicam standard, (C) sample containing 29 mg/l piroxicam Peaks 1 = colchicine, 2 = piroxicam

discrepancy between standards and controls was found. This has since been corrected by preparing new controls

The initial aim of this project was to produce methods that were both rapid and simple, with extraction procedures that were based on protein precipitation. However, certain modifications of some methods from their simplest form were necessary to improve chromatography In the case of diflunisal, the ex-

TABLE IV

RETENTION TIMES OF DRUG AND INTERNAL STANDARD IN THE INDIVIDUAL METHODS

Drug	Internal standard	Retention time (min)
Diflunisal		3 0
	Zomepirac	4 6
Indomethacin		3 2
	Feprazone	4 1
Fenoprofen		3 8
	Thiopentone	3 3
Ibuprofen		3 4
	Benoxaprofen	2 6
Ketoprofen		2 6
	Phenylbutazone	4 0
Naproxen		3 5
	Pentobarbitone	2 9
Mefenamic acid		3 7
	Thiopentone	2 1
Piroxicam		3 6
	Colchicine	2 5

TABLE V

MEAN CORRELATION COEFFICIENT, SLOPE AND *y*-AXIS INTERCEPT DETERMINED FROM FIFTEEN STANDARD CURVES FOR EACH METHOD

Drug	Mean correlation coefficient	Mean slope	Mean *y*-axis intercept
Diflunisal	0 9995	0 0044	−0 0505
Indomethacin	0 9998	0 0323	0 0022
Fenoprofen	0 9995	0 0038	−0 0108
Ibuprofen	0 9998	0 0042	−0 0095
Ketoprofen	0 9995	0 0198	−0 0036
Naproxen	0 9987	0 0034	0 0120
Mefenamic acid	0 9996	0 0116	−0 0240
Piroxicam	0 9998	0 0522	−0 0638

traction volumes had to be altered so that the same proportion of acetonitrile was present in both the mobile phase and the extraction supernatant. If the proportions differed, the diflunisal peak shape deteriorated, eventually resulting in a double peak This was through to be some form of solvent effect Similarly, the ibuprofen and mefenamic acid standard curve intercepts moved fur-

TABLE VI

INTRA- AND INTER-ASSAY IMPRECISION AND INACCURACY

Method	Quality control concentration (mg/l)	Intra-assay			Inter-assay		
		Mean concentration found (mg/l)	C V (%)	Mean error (%)	Mean concentration found (mg/l)	C V (%)	Mean error (%)
Diflunisal	150	146 9	0 9	2 0	149 7	2 1	1 6
	450	449 6	1 2	1 0	447 8	1 3	1 1
Indomethacin	7 5	8 1	1 7	8 3	8 1	2 6	7 6
	37 5	39 8	0 6	6 2	38 3	1 9	2 6
Fenoprofen	75	77 1	1 7	2 8	76 2	1 9	2 0
	375	383 6	1 1	2 3	372 9	1 5	1 2
Ibuprofen	150	149 8	1 1	0 8	152 6	1 0	1 8
	375	377 7	0 9	0 8	386 3	1 0	3 0
Ketoprofen	15	15 4	1 7	2 9	15 2	1 8	2 1
	75	73 0	1 2	2 7	73 4	1 2	2 2
Naproxen	150	153 9	2 0	2 7	148 9	2 1	1 5
	450	443 6	1 3	1 5	452 4	1 6	1 4
Mefenamic acid	25	24 8	0 5	0 8	25 0	2 6	1 9
	100	99 5	0 8	0 8	98 5	1 0	1 6
Piroxicam	8	7 7	1 5	3 1	8 3	2 9	3 9
	24	22 8	1 2	5 2	23 5	1 5	2 3

ther away from zero if the acetonitrile proportions in the mobile phase and extraction supernatant became dissimilar It was found to be necessary to evaporate the IMS when preparing standards for the indomethacin, naproxen and piroxicam methods. Removal of the IMS resulted in an improvement in standard curve linearity, previously poor linearity being attributed to solvent effects caused by the relatively high concentration of IMS in the higher standards All standards were prepared in horse serum, except those used in the indomethacin method With this method, late-eluting peaks were found to be present in two separate batches of horse serum and these interfered with subsequent injections onto the HPLC system These peaks were never identified but were not present in drug-free human plasma Ideally, all standards should have been prepared in drug-free human plasma, but with only a limited supply available, it was necessary to restrict its use to where it was absolutely essential For the methods using horse serum, a comparison for each drug between a single standard concentration prepared in both horse serum and drug-free human plasma showed no difference in peak-height ratio Consequently, horse serum was accepted as a suitable substitute for human plasma

Using the methods described, 239 suspected overdose samples have been analysed, 73% of these involving either ibuprofen or mefenamic acid Table

TABLE VII

NUMBERS OF SAMPLES ANALYSED AND RANGE OF CONCENTRATIONS DETERMINED FOR EACH METHOD

Drug name	Number of samples analysed	Determined concentration range (mg/l)
Diflunisal	8	10–448[a]
Indomethacin	10	1– 80[a]
Fenoprofen	17	31–828
Ibuprofen	85	5–950[a]
Ketoprofen	9	3–114[a]
Naproxen	13	52–700[a]
Mefenamic acid	89	2–151[a]
Piroxicam	8	4– 37
Total	239	

[a]Samples were analysed where no drug was detected, but these are not included in the concentration range

TABLE VIII

COMPOUNDS DETECTED IN SAMPLES BY DRUG-SCREENING PROCEDURE AND CHECKED FOR INTERFERENCE IN THE RELEVANT METHOD

Method	Compounds not interfering within the method, but detected by the screening procedure
Diflunisal	Diazepam, ethanol, flurazepam (as desalkyl metabolite), salicylate
Indomethacin	Diazepam, ethanol, mefenamic acid, phenylbutazone, temazepam
Fenoprofen	Ethanol, mefenamic acid
Ibuprofen	Amitriptyline, cimetidine, codeine, diazepam, dothiepin, lorazepam, nortriptyline, phenobarbitone, phenytoin, salicylate, temazepam, trichloroethanol, trimipramine
Ketoprofen	Ethanol
Naproxen	Dihydrocodeine, dothiepin, mefenamic acid, propoxyphene
Mefenamic acid	Carbamazepine, codeine, diazepam, ethanol, paracetamol, phenobarbitone, phenytoin, primidone, quinine, temazepam, trichloroethanol
Piroxicam	Diazepam, ethanol, temazepam

VII gives details of the number of samples analysed and the range of concentrations determined using each method

A proportion of samples analysed yielded concentrations that were in excess of the highest standards These samples were subsequently diluted to bring them within the appropriate standard range and reanalysed All samples analysed also underwent a comprehensive drug-screening procedure [44], the screen utilising both blood and urine, if urine was available Compounds that

were detected in samples using the screening procedure and did not interfere in the relevant method are shown in Table VIII Only one compound was identified that would interfere in any of the methods This was sulphamethoxazole in the ketoprofen assay Sulphamethoxazole had a retention time of 2 5 min, compared with 2 6 min for ketoprofen. However, its isolation by the screening procedure prevented misidentification in the ketoprofen assay.

Few metabolites of the drugs of interest were available for testing for possible interference in the respective assays Two indomethacin metabolites, desmethylindomethacin and deschlorobenzoylindomethacin, were found to elute early on the indomethacin system Similarly, 3-hydroxymefenamic acid and 3-carboxymethylmefenamic acid ran early on the mefenamic acid system Metabolites of the drugs of interest are all more polar than their parent compounds, and would be expected to elute earlier on a reversed-phase HPLC system Published analytical methods corroborate this [37,40,45–47] In the methods described where the internal standard elutes earlier than the drug, a number of samples were prepared without internal standard to determine if any metabolites ran in the same position No instances of this were found and consequently drug metabolites were not expected to be an interference problem.

Using the methods as described, the limits of detection would be at most the concentrations listed in Table IX

For diflunisal, indomethacin, ibuprofen, naproxen and mefenamic acid, the methods have been adapted to enable the determination of low therapeutic concentrations. This required minimal modification of the methods described (reduction of internal standard concentration and an increase in integrator sensitivity) and the use of standards to encompass the therapeutic ranges It may be possible to incorporate all of the methods into a single procedure and thereby produce a screen for the drugs of interest. However, this would entail a much longer chromatographic elution time. Moreover, unless a UV diode

TABLE IX

LIMITS OF DETECTION FOR EACH OF THE METHODS DESCRIBED

Method	Limit of detection (mg/l)
Diflunisal	5
Indomethacin	1
Fenoprofen	5
Ibuprofen	5
Ketoprofen	1
Naproxen	5
Mefenamic acid	2
Piroxicam	1

array detector were available, the selection of a single wavelength to detect all of the drugs, with minimal interference, would prove difficult

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