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IDENTIFICATION AND SIMULTANEOUS DETERMINATION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

F LAPICQUE, P NETTER*, B BANNWARTH, P TRECHOT, P GILLET, H LAMBERT and R J ROYER

Département de Pharmacologie Clinique et URA CNRS 1288, Faculté de Médecine, B P 184, 54505 Vandoeuvre les Nancy Cedex (France)

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SUMMARY

An isocratic high-performance liquid chromatographic procedure is presented for the screening of plasma samples for the presence of sixteen non-steroidal anti-inflammatory drugs Detection was achieved simultaneously at two wavelengths (254 and 370 nm) and the purity of the eluted peaks was tested using absorbance ratios at the two wavelengths, identification could thus be effective without interferences from substances of other pharmacological classes The drugs were extracted simultaneously with diethyl ether after acidification and separated from each other on an octadecyl reversed-phase column using only one eluent, acetonitrile–0 3% acetic acid–tetrahydrofuran (36 63 1 0 9, v/v) The recovery, precision and reproducibility of the method were satisfactory as it allowed the determination of the drugs from infra- to supratherapeutic concentrations

INTRODUCTION

An increasing number of non-steroidal anti-inflammatory drugs (NSAIDs) have become available since the introduction of acetylsalicylic acid (aspirin) Their widespread usage has enhanced interest in determining the nature and concentration of NSAIDs implicated in various therapeutic situations, such as compliance measurement, toxicological analysis, relationship with side-effects that occurred with a known or unknown treatment and wash-out testing.

High-performance liquid chromatography (HPLC) allows the measurement of low concentrations of most drugs in biological fluids with great selec-

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TABLE I

SEPARATION OF NSAIDs BY HPLC REVIEW OF LITERATURE

Drug	Extraction	Column	Separation	Ref
Ibuprofen Indomethacin Naproxen Oxyphenbutazone Phenylbutazone	Precipitation, acetonitrile	μBondapak C_{18}, 10 μm, 300 mm × 3 9 mm (Waters)	Acetonitrile–45 m*M* phosphate + H_3PO_4 (pH3) (60 40, v/v), various wavelengths	1
Indomethacin Naproxen Sulindac and metabolites	Precipitation or diethyl ether extract	Spherisorb C_{18}, 5 μm, 500 mm × 4 6 mm	Methanol–water (pH 2 5) (various proportions), 245, 254 and 288 nm	2
Acetylacetic acid Diclofenac Fenoprofen Ibuprofen Indomethacin Ketoprofen Mofebutazone Naproxen Paracetamol Phenacetin Phenylbutazone Probenecid Salicylic acid Sulphinpyrazone Tolfenamic acid	Precipitation, acetonitrile	μBondapak C_{18}, 10 μm, 300 mm × 3 9 mm (Waters)	Methanol–phosphate (pH 4) (55 45, v/v) and variations, 254 (280–220) nm	3
Indomethacin Sulindac Tolmetin and metabolites	Acidification, methylene chloride	ODS C_{18}, 5 μm, 500 mm × 4 6 mm	Gradients, methanol–sodium acetate buffer, 254 nm	4
Acetylsalicylic acid Diflunisal Indobufen Indomethacin Indoprofen Salicylic acid	Acidification, hexane–diethyl ether or diethyl ether	μBondapak C_{18}, 10 μm, 300 mm × 3 9 mm	Methanol–0 01–0 03 *M* phosphate (40 60 to 70 30, v/v), 254 or 280 nm	5
Aspirin Carprofen Diclofenac Diflunisal Fenbufen Fenoprofen Fluriprofen Flufenamic acid Ibuprofen	HCl, diethyl ether	Hibar C_{18}, 5 μm, 250 mm × 4 mm (Merck)	Acetonitrile–0 05 *M* sodium acetate gradient (25 to 55% in acetonitrile), 254 nm	6

TABLE I (continued)

Drug	Extraction	Column	Separation	Ref
Indomethacin Indoprofen Ketoprofen Lonazolac Mefenamic acid Naproxen Niflumic acid Pirprofen Sulindac Tiaprofenic acid Zomepirac				
Acetylsalicylic acid Diflunisal Fenoprofen Flurbiprofen Ibuprofen Indomethacin Ketoprofen Mefenamic acid Naproxen Salicylic acid	Chloroform–acetonitrile or diethyl ether–hexane	Spherisorb C_{18}, 5 μm, 250 mm × 4 5 mm	Acetonitrile–H_3PO_4 (pH 3 2)–methanol (52 35 13, v/v), 250 nm	7
Diclofenac Diflunisal Fenbufen Fenoprofen Ibuprofen Indomethacin Ketoprofen Naproxen Piroxicam Salicylic acid Sulindac	Precipitation, perchloric acid	ODS-Hypersil C_{18}, 5 μm, 160 mm × 5 mm (Shandon)	Isopropanol–0 1 *M* phosphate–formic acid (17 1000 1, 176 1000 1 or 540 1000 1, v/v), 240 nm	8
Diclofenac Fenoprofen Ketoprofen Naproxen Piroxicam Phenylbutazone Sulindac	Precipitation, perchloric acid	μBondapak C_{18}, 10 μm, 300 mm × 3 9 mm (Waters)	Acetonitrile–0 03% H_3PO_4 (45 55, v/v), 254 nm	9
Fenoprofen Ibuprofen Indomethacin Ketoprofen Paracetamol Salicylic acid	Bond-Elut C_8	Spherisorb C_{18}, 5 μm, 500 mm × 4 6 mm	Acetonitrile–H_3PO_4 (pH 2 3)–methanol (52 32 13, v/v), 250 nm	10

TABLE II

NSAIDs INVESTIGATED

No	Substance	Trade name[a]	Gift from
1	Diclofenac (sodium)	Voltarène	Ciba-Geigy
2	Diflunisal	Dolobis	Merck Sharp & Dohme
3	Etodolac	Lodine	Wyeth France
4	Fenbufen	Cinopal	Lederle
5	Fenoprofen (calcium)	Nalgesic	Eli Lilly France
6	Flurbiprofen	Cebutid	Boots Dacour
7	Ibuprofen	Brufen	Boots Dacour
8	Indomethacin	Indocid	Merck Sharp & Dohme
9	Ketoprofen	Profénid	Specia
10	Naproxen	Naprosyne	Cassenne
10	Naproxen (sodium)	Apranax	Syntex
11	Niflumic acid	Nifluril	UPSA
12	Piroxicam	Feldène	Pfizer
13	Pirprofen	Rangasil	Ciba-Geigy
14	Salicylate	—	—
15	Sulindac	Arthrocine	Merck Sharp & Dohme
16	Tenoxicam	Tilcotil	Roche
17	Tiaprofenic acid	Surgam	Roussel Uclaf

[a]In France

tivity and sensitivity; numerous methods have been described for the individual determination of NSAIDs, but only a few techniques allow the determination of some of them together (Table I) [1–10]. None of the cited methods makes it possible to separate in a satisfactory manner the various NSAIDs contained in one isocratic elution, but require a gradient or variations of the eluent composition, or several operating wavelengths, in order to determine some NSAIDs. Moreover, none of these papers reported any data relevant to the usual technical characteristics such as sensitivity, selectivity, precision and reproducibility, except for the technique described by Owen et al [9]; however, the suggested procedure permitted the detection of only seven NSAIDs and in addition ketoprofen and naproxen could not be separated.

The aim of this work was to obtain a method suitable for the simultaneous identification of the most often used NSAIDs (Table II) in a single sample even in toxicological cases, i.e., together with drugs of other pharmacological classes, such as benzodiazepines, phenothiazines and barbiturates, and allowing the measurement of a wide range of concentrations from infra- to supratherapeutic levels.

EXPERIMENTAL

Chemicals and reagents

The drugs involved in this study were given to us by the manufacturing laboratories; see Table II for NSAIDs and Table VI for drugs analysed for inter-

ference purposes. Biphenylacetic acid (BPAA), the main metabolite of fenbufen, was a gift from Lederle (Rungis, France), desoxysulindac from Merck Sharp & Dohme Chibret (Paris, France) and (benzoyl-4-phenyl)-2-butyric acid (BPBA), used as internal standard (I.S.), from Specia (Paris, France). Acetonitrile of chromatographic purity was obtained from SDS (Valdonne, France). All other chemicals used were analytical-reagent grade and water was doubly distilled.

Solutions

Standard solutions of each NSAID investigated at a concentration of 1 mg/ml were prepared in methanol, except for ibuprofen (10 mg/ml); piroxicam, salicylate and tenoxicam were dissolved in toluene, water and 0 01 *M* sodium hydroxide solution (1 mg/ml), respectively. All solutions were stored at 4°C in the dark for one month.

For eluent optimization, working solutions were obtained by diluting each drug alone in the tested eluents and a solution "M" containing all the drugs was used for the simultaneous assay. Working solutions were prepared in methanol (except for tenoxicam, in water) every day for calibration purposes, with all the NSAIDs at concentrations corresponding to chromatographic peaks of comparable height.

UV characteristics

The UV spectra of the NSAIDs were obtained from data measured in 1-cm quartz cuvettes on a Perkin-Elmer (Bois d'Arcy, France) Lambda 1 UV–visible spectrophotometer; the compounds were dissolved separately in the selected eluent, acetonitrile–0.3% acetic acid–tetrahydrofuran (THF) (36·63.1:0.9, v/v), at a concentration yielding absorbances (A) of about 1.0 at the wavelength of maximum absorption. For each compound the maximum absorbance (A_{max}) and the corresponding wavelength, λ_{max}, were obtained, from which the maximum molar absorptivity, ϵ_{max}, was deduced; ϵ_{254} and ϵ_{370} were calculated in the same manner using the absorbances at 254 and 370 nm.

Plasma samples

Plasma from patients undergoing no treatment was used and stored at −20°C. For analysis, aliquots of drug working solutions were placed in a haemolysis tube and methanol was evaporated under a stream of nitrogen; the plasma samples (200 μl) were thereafter incubated for 20 min with the dry residue.

In addition, samples of blood were collected in oxalate tubes from patients hospitalized for taking NSAIDs or on a regular regimen of some of the drugs investigated; these samples were centrifuged and stored at −20°C until assay.

Extraction

Volumes of 100–500 μl (usually 200 μl) of plasma from patients or of blank plasma spiked for calibration were placed in a haemolysis tube and 0.1–5.0 μg of internal standard and 0 1 ml of 1 *M* hydrochloric acid were added. The drugs and internal standard were extracted by mechanical agitation for 10 min; four extraction procedures were tested (a) 3 ml of diethyl ether; (b) two successive extractions with 3 ml of diethyl ether; (c) 5 ml of chloroform; and (d) 3 ml of ethyl acetate

The organic phase was separated by centrifugation (10 000 *g* for 5 min), transferred into another tube and evaporated under a stream of nitrogen. The dry residue was dissolved in 200 μl of the chromatographic eluent and injected at the top of the column

Chromatography

HPLC was performed using a Waters (Saint-Quentin en Yvelines, France) apparatus consisting of a Model 510 pump, a thermostatic oven, a Model 490E variable-wavelength UV spectrophotometer with a four-channel detection device Acquisition and calculations were made using Maxima software (Waters) installed on an IBM-AT3 microcomputer. The separation was obtained on a Waters Nova Pak octadecyl reversed-phase end-capped column (300 mm × 3.9 mm I D) after injection with a Rheodyne Model 7125 injector equipped with a 50-μl loop.

The tested eluents were prepared by varying the proportions of organic solvents (acetonitrile and THF) and of aqueous solutions of acetic acid and were degassed with nitrogen before use A flow-rate of the eluent through the column of 0.9 or 1.0 ml/min was used, giving a pressure of about 15 MPa, and the column was allowed to equilibrate for 1 h with eluent before injections.

For eluent optimization studies, each drug was injected alone in order to permit its identification in the solution "M" containing all the NSAIDs tested and detection was fixed at 254 nm, which seemed to be an acceptable value deduced from literature data (Table I). Validation of the retention time was obtained by injecting plasma extracts from patients on a regular regimen of the drugs investigated.

Calibration graphs were obtained with blank plasma spiked with all the investigated drugs together. Their respective concentrations in each assay were chosen in order to yield the same order of magnitude for the chromatographic peak heights, and for each drug the eight calibration concentrations ranged from infra- to supratherapeutic levels. The correlations between peak-area ratios of drug to I S. versus drug concentration were determined simultaneously for the NSAID by means of the Maxima software.

The dead time, t_0, of the chromatographic system was determined after injection of sodium nitrite and the number of plates, N, was calculated from the base width of the peaks of some of the NSAIDs investigated. These values were

used for the determination of the capacity factor, k', the selectivity factor, α, and the resolution, R_s

RESULTS AND DISCUSSION

Optimization of the separation

Preliminary experiments showed the importance of the presence in the eluent of an acidic compound such as acetic acid used to balance the ionization of the acidic NSAIDs. The organic component was acetonitrile with the addition of THF as a modifier of the elution power (enhanced for some compounds and diminished for others) [11] We therefore investigated the influence of the relative proportions of these three components.

Variation of the acetic acid concentration (Fig. 1) produced little change in the retention times for most of the drugs, except for diflunisal, for which the order of elution with adjacent chromatographic peaks differed greatly. Diflunisal was found to be eluted in a similar manner to fenbufen and before the I.S. at low acetic acid concentrations; the amount of acetic acid allowed the retention time of diflunisal to be increased, as it was detected after the I.S. using 0 75% acetic acid (pH 2.95), and even after pirprofen at 1.5% (pH 2.92) We therefore chose 0.3% of acetic acid as the lowest concentration producing the separation of most of the drugs at an apparent pH of 3.0, which represents satisfactory conditions for an ocatadecylsilica column.

For all the NSAIDs tested, the retention time decreased with increase in the amount of acetonitrile in the mobile phase. The order of elution remained unchanged, except for piroxicam–sulindac, etodolac–flurbiprofen and diclofenac–indomethacin, for which inversion of the elution order appeared at 33, 40 and 45% of acetonitrile, respectively (Fig. 2). The plots of log k' for each drug against acetonitrile or water concentration could be approximated as

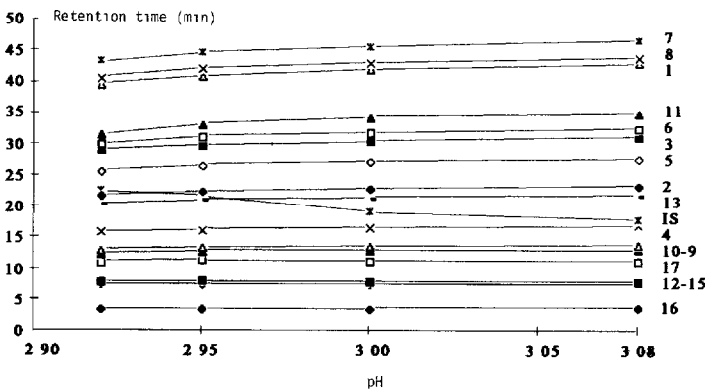

Fig 1 Retention times of NSAIDs as a function of pH of the eluent (variation of proportion of acetic acid) Acetonitrile–x% acetic acid–THF (36 63 1 0 9, v/v), column temperature, 40°C, flow-rate, 1 ml/min

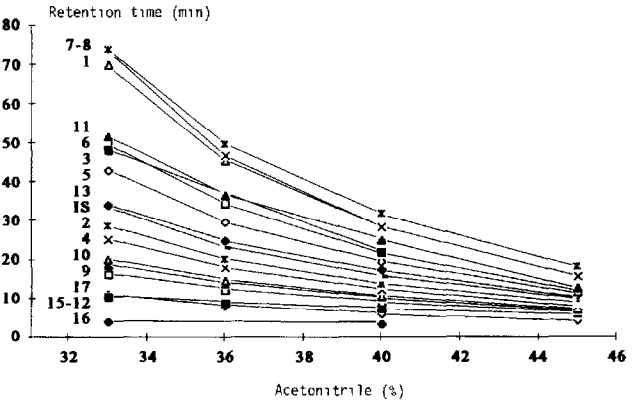

Fig 2 Retention times of NSAIDs as a function of proportion of acetonitrile (x) in the eluent Acetonitrile-0 3% acetic acid-THF [x $(99-x)$ 1, v/v], column temperature, 35 °C, flow-rate, 1 ml/min

TABLE III

CORRELATION BETWEEN CAPACITY FACTOR (k') FOR NSAIDs AND WATER CONCENTRATION IN THE ELUENT (w)

$\log k' = aw + b$, r = correlation coefficient, p = probability for regression analysis Acetonitrile-0 3% acetic acid-THF [x $(99-x)$ 1, v/v] with x = 0 33, 0 36, 0 40, 0 45, column temperature, 35 °C, flow-rate, 1 ml/min

NSAID	a	$-b$	r	p
16	1 939	1 189	1 00	0 000
15	4 812	2 505	0 9992	0 001
12	2 829	1 198	0 9996	0 000
17	4 352	1 998	0 9997	0 000
9	4 468	2 003	0 9996	0 000
10	4 479	1 983	0 9997	0 000
4	4 884	2 146	0 9996	0 000
2	4 888	2 092	0 9992	0 001
I S	5 070	2 139	0 9996	0 000
13	4 869	1 987	0 9990	0 001
5	5 404	2 244	0 9996	0 000
3	5 170	2 035	1 00	0 000
6	5 758	2 410	0 9997	0 000
11	5 437	2 171	0 9956	0 004
1	5 686	2 223	0 9996	0 000
8	5 019	2 360	0 9994	0 001
7	5 371	1 985	0 9998	0 000

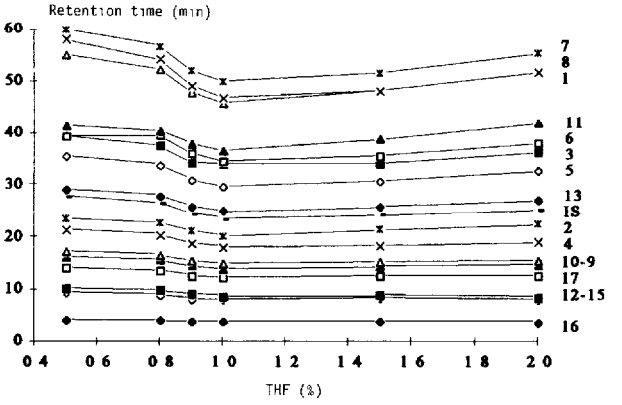

Fig 3 Retention times of NSAIDs as a function of proportion of THF in the eluent Acetonitrile–0 3% acetic acid–THF (a b c, v/v), with $a+b+c=100$ and $a/b=1$ 75, column temperature, 35 °C, flow-rate, 1 ml/min

TABLE IV

RESOLUTION OF THE SEPARATION OF THE NSAIDs AS A FUNCTION OF THE PROPORTION OF THF IN THE ELUENT

Acetonitrile–0 3% acetic acid–THF (a b x, v/v), with $a+b+x=100$ and $a/b=1$ 75, column temperature, 35 °C, flow-rate, 0 9 ml/min

NSAIDs resolved	Resolution					
	0 5% THF	0 8% THF	0 9% THF	1 0% THF	1 5% THF	2 0% THF
16–15	19 60	18 95	17 98	18 20	18 34	18 18
15–12	2 08	2 72	3 33	2 55	2 18	2 29
12–17	9 29	9 14	9 12	9 41	9 56	10 85
17–9	4 37	4 33	4 26	4 23	4 16	4 47
9–10	1 79	1 76	1 82	1 85	1 80	1 94
10–4	6 50	6 32	6 01	5 89	5 81	6 06
4–2	3 02	3 38	3 63	3 62	4 56	5 15
2–I S	5 39	5 03	4 68	4 62	3 91	3 85
I S –13	1 29	1 62	1 85	1 97	2 10	2 01
13–5	6 08	5 80	5 55	5 39	5 46	5 89
5–3	3 30	3 31	3 24	3 25	3 23	3 29
3–6	0 00	1 74	1 59	1 60	1 56	1 64
6–11	1 74	0 729	1 685	1 95	2 70	3 00
11–1	8 29	7 60	6 91	6 70	6 50	6 33
1–8	1 67	1 17	0 959	0 748	0 00	0 00
8–7	1 07	1 47	1 76	2 03	2 21	2 21

straight lines (Table III), as expected [12]. A relative proportion of 36% acetonitrile seemed to be a good compromise, as it allowed sufficient separation for every compound within 50 min. For larger amounts of acetonitrile the peaks became less well separated.

Increasing the proportion of THF in the eluent generally decreased the retention times for THF concentrations up to 1% and increased them at higher concentrations, as shown in Fig 3. Its influence on resolution, however, was not uniform (Table IV) and was significant mainly for compounds showing adjacent peaks (Fig. 4), an increase in the proportion of THF enhanced the separation of I.S.–pirprofen and of indomethacin–ibuprofen but yielded a lower resolution for diclofenac–indomethacin, indeed, no separation was observed with 1 5% THF and above. At 0.8% THF the best separation between etodolac and flurbiprofen was obtained, but the separation of flurbiprofen from niflumic

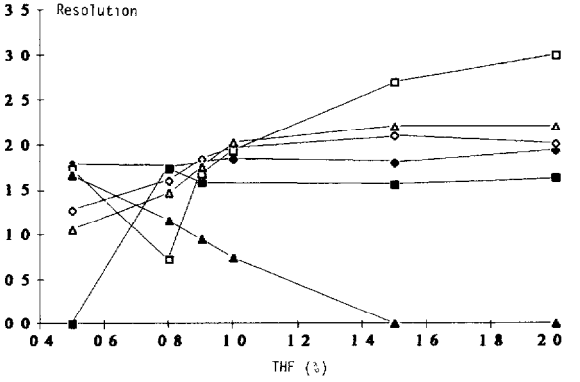

Fig 4 Resolution of the separation between NSAIDs 9 and 10 (♦), I S and 13 (◇), 3 and 6 (■), 6 and 11 (□), 1 and 8 (▲) and 8 and 7 (△) as a function of the proportion of THF in the eluent Acetonitrile–0 3% acetic acid–THF (*a b c*, v/v), with $a+b+c=100$ and $a/b=1\ 75$, column temperature, 35 °C, flow-rate, 1 ml/min

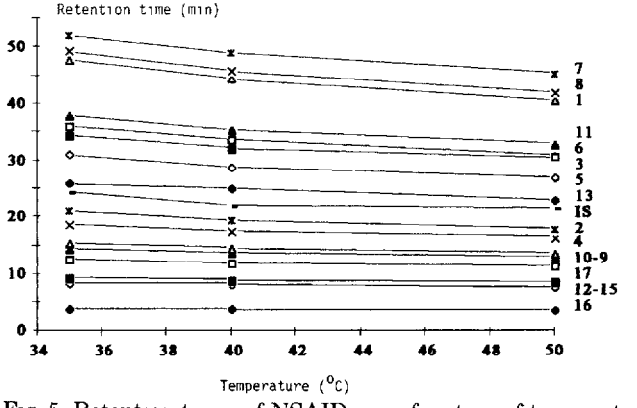

Fig 5 Retention times of NSAIDs as a function of temperature Acetonitrile–0 3% acetic acid–THF (36 63 1 0 9, v/v), flow rate, 0 9 ml/min

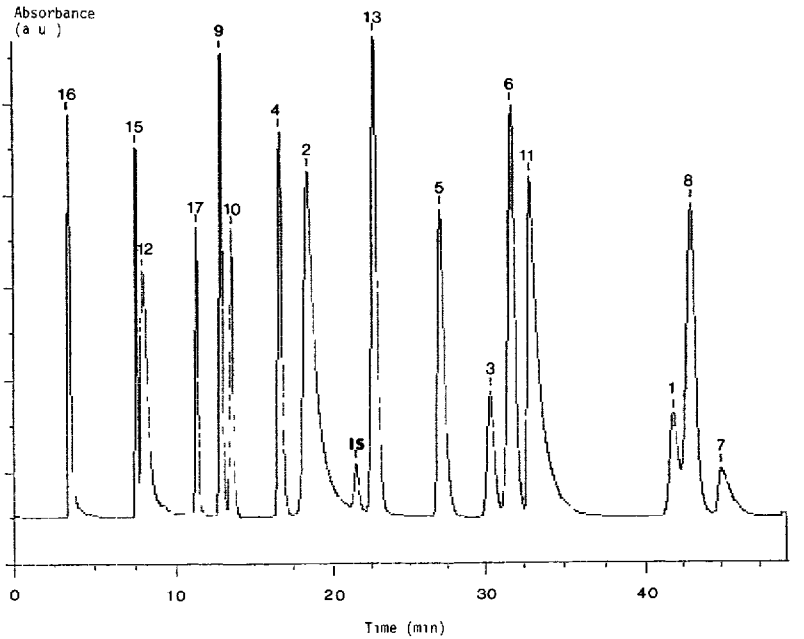

Fig 6 Separation of NSAIDs Acetonitrile–0 3% acetic acid–THF (36 63 1 0 9, v/v), column temperature, 40 °C, flow-rate, 1 ml/min

acid was poor ($R_s < 0.8$). A concentration of 0.9% THF seemed to be an acceptable compromise, as it allowed a resolution of greater than 1 for almost all the compounds, except between diclofenac and indomethacin, for which R_s was 0.959.

An increase in the oven temperature decreased all the retention times (Fig 5) and also the resolution of some adjacent peaks, such as for etodolac and flurbiprofen. A similar effect was observed by varying the flow-rate. A temperature of 40 °C and a flow-rate of 1 ml/min were adopted for subsequent work. The selected eluent, acetonitrile–0.3% acetic acid–THF (36 63 1 0.9, v/v), allowed the effective separation of all the NSAIDs and the I.S., as shown in Fig. 6.

Choice of wavelengths

The UV spectra of the NSAIDs were obtained as solutions in the eluent; all the compounds exhibited absorption maxima between 224 and 379 nm (Table V). We chose first the value of 254 nm as a wavelength allowing high values of the molar absorptivity ϵ_{254} compared with the maximum ϵ_{max} for most of the drugs; only ibuprofen exhibited a low absorption over the whole wavelength range investigated.

Extraction of plasma samples led to intrinsic peaks eluting in the first 5 min,

TABLE V

UV CHARACTERISTICS OF THE NSAIDs IN THE ELUENT

Acetonitrile-0 3% acetic acid-THF (36 63 1 0 9, v/v), column temperature 40°C, flow-rate, 1 ml/min, N D = not detected

NSAID	λ_{max} (nm)	ϵ_{max} ($mol^{-1}\,cm^{-1}$)	ϵ_{254} ($mol^{-1}\,cm^{-1}$)	ϵ_{370} ($mol^{-1}\,cm^{-1}$)
1	276	15720	7410	N D
2	224	28840	15310	N D
3	227	37290	4180	N D
4	286	23710	6190	N D
5	272	1930	1040	N D
6	247	19050	18690	N D
7	264	293	189	N D
8	262	17840	17690	1106
9	258	17680	17050	N D
10	273	4770	3760	N D
11	286	25120	8060	3924
12	361	19720	13470	19093
13	265	11530	9160	N D
14	300	3960	1060	N D
15	286	15650	14845	4269
16	379	20240	10450	19800
17	306	15360	7980	N D

and one near piroxicam for few samples; these peaks were detected at 254 nm and could therefore interfere with oxicams and even with salicylate; they disappeared at higher wavelengths. For these reasons, 370 nm was chosen for the second detector channel; this wavelength permitted optimum detection of the oxicams and allowed the interference peaks, due to plasma or other drugs, to be no longer detected. However, salicylate could not be determined using only this general method; the presence of one peak (254 nm) at a retention time corresponding to salicylate needs to be confirmed by another technique. For this reason, salicylate was not included in this study

Interferences

Three other NSAIDs were analysed using this technique, but were not included in this investigation: mefenamic acid and phenylbutazone gave too long retention times (51 and 77 min, respectively), but could be assayed using a higher proportion of acetonitrile in the eluent; oxyphenbutazone could not be detected.

The principal metabolites of the investigated NSAIDs were also analysed if available as pure substances: biphenylacetic acid (metabolite of fenbufen) eluted together with its parent drug, desoxysulindac, was not detected and the

5-hydroxy metabolite of tenoxicam eluted just after tenoxicam and was separated from it.

Interferences by drugs of other pharmacological classes were examined by injecting them as solutions in the eluent (Table VI), in order to establish whether this method is also applicable to toxicological cases Some were found to elute near the NSAIDs or the I.S : prednisone and prednisolone near tenoxicam, and clonazepam, nitrazepam, secobarbital and triazolam near sulindac and piroxicam. However, none of these interfering drugs was detected at 370 nm, in contrast to sulindac and oxicams; this emphasized the need for the

TABLE VI

SUBSTANCES TESTED FOR INTERFERENCES

Acepromazine	Desoxysulindac[g]	Nordazepam[a]
Aceprometazine	Dextropropoxyphen[a]	Oxazepam
Alimemazine	Diazepam[a]	Oxyphenbutazone
Amidopyrine	Disopyramide	Paracetamol[a]
Amiodarone	Doxepine	Phenobarbital[a]
Amitriptyline	Floctafenine	Phenylbutazone
Amobarbital	Flunitrazepam	Phenytoin
Barbital	Glafenic acid[a]	Prazepam[a]
Bromazepam[a]	Haloperidol	Prednisolone[h]
Biphenylacetic acid[b]	Hydroxyquinidine	Prednisone[h]
Butalbital[a]	Hydroxyglafenic acid[a]	Primidone
Butobarbital	Imipramine	Progabide[a,d]
Caffeine	Isoniazide	Promethazine
Carbamazepine[a]	Levomepromazine	Propericiazine[a]
Chlorazepate[a,c]	Loprazolam	Propranolol[a]
Chlordiazepoxide	Lorazepam[a]	Quinidine[a]
Chlormezanone	Maprotiline	Secobarbital[e]
Chloroquine	Medazepam	Sulphamethoxazole
Chlorpromazine	Mefenamic acid[a]	Sulpiride[a]
Clobazam[a]	Meprobamate[a]	Theophylline
Clometacine[d]	Metapramine[a]	Thiopental
Clomipramine	Methotrexate	Tiapride
Clonazepam[a,e]	Metoclopramide[a]	Triazolam[a,e]
Clotiazepam[a,f]	Metopimazine	Trifluoperazine
Colchicine	Nefopam	Valproate[a]
Desipramine	Nitrazepam[e]	Vinylbital

[a]Also tested in plasma sample
[b]Fenbufen metabolite, eluted together with parent drug
[c]Interference with ketoprofen at 254 nm, identified using absorbance ratio 254/307 nm
[d]Interference with I S at 254 nm, difference at 370 nm
[e]Interference with sulindac/piroxicam at 254 nm, resolved at 370 nm
[f]Interference with ibuprofen at 254 nm, identified using absorbance ratio 254/307 nm
[g]Sulindac metabolite, not detected
[h]Interference with tenoxicam at 254 nm, resolved at 370 nm

second detection channel at 370 nm However, sulindac gave a low value of ϵ_{370} and we decided to test the purity of the eluting peaks by monitoring on the third channel the ratio between the absorbances at two wavelengths (A_{330}/A_{307}). Clometacine and progabide, eluting together with the I S., were detected at 370 nm; the I.S. was not detected at this wavelength and could therefore be distinguished from these drugs, but another substance should then be used as the I.S. The only serious problem was encountered with chlorazepate, eluting with ketoprofen, and with clotiazepam, having a similar retention time to ibuprofen. They could be differentiated using another absorbance ratio to monitor the purity of the eluting peaks, e.g., A_{254}/A_{307}, for these two occasional associations, a second assay must be carried out at another wavelength.

With the above method, the identification of an NSAID could be achieved in an unambiguous manner, using its relative retention time and taking into account possible detection at 370 nm or the use of absorbance ratios (Table VII); the first two channels were used for detection and determination of drug concentrations and the last two only for testing the purity of the detected peak. Thus none of the tested compounds produced major interferences

TABLE VII

IDENTIFICATION OF NSAIDs USING A FOUR-CHANNEL DETECTOR

Acetonitrile–0 3% acetic acid–THF (36 63 1 0 9, v/v), column temperature, 40 °C, flow-rate, 1 ml/min, N D = not detected

NSAID	Retention time (min)		Absorbance ratio value	
	254 nm	370 nm	254/307 nm	330/307 nm
16	3 40	3 40	2 65	1 06
14	3 62	N D	1 45	0 34
15	7 42	7 42	2 65	1 60
12	7 97	7 97	N D	1 54
17	10 98	N D	1 75	0 59
9	12 62	N D	27 15	0 68
10	13 28	N D	5 90	2 12
4	16 62	N D	1 85	0 30
2	17 83	N D	6 00	0 72
I S	21 27	N D	N D	N D
13	22 48	N D	6 00	0 60
5	26 85	N D	N D	N D
3	30 03	N D	N D	N D
6	31 28	N D	N D	N D
11	32 67	32 67	2 40	1 15
1	41 42	N D	N D	N D
8	42 83	42 83	4 10	1 18
7	45 5	N D	N D	N D

Extraction of plasma samples

The extraction efficiency was determined by comparing the peak-area responses from known amounts of drugs and I.S. injected directly with processed plasma samples with NSAIDs added The results for the four procedures tested are reported in Table VIII After one operation the best extraction was obtained with ethyl acetate for most compounds, but many plasma peaks were generated; these peaks were also observed after extraction with chloroform but were shown to disappear when diethyl ether was used. This last extraction solvent was therefore chosen. The extraction efficiency was greater than 95% for most drugs, except for pirprofen (60%) and tenoxicam (71%), their efficiencies could not be improved through additional extraction.

The extraction of blank plasma samples generally did not produce any interfering peaks (Fig. 7), except for some of them in the vicinity of piroxicam These peaks were observed at 254 nm but were no longer detected at 370 nm, as explained above.

Over the concentration range investigated, the regression analysis of peak-area ratios between the drugs and the I.S. as a function of drug concentration yielded regression coefficients greater than 0.998 for all drugs (Table IX), and allowed the determination of concentrations over a wider range than those currently encountered in therapeutic conditions. It should be noted that for

TABLE VIII

EXTRACTION EFFICIENCY WITH FOUR EXTRACTION PROCEDURES

a, Once with diethyl ether, b, twice with diethyl ether, c, chloroform, d, ethyl acetate

NSAID	Extraction efficiency (%)			
	a	b	c	d
1	98	98	98	98
2	98	98	98	98
3	98	—	—	—
4	95	98	80	90
5	98	98	96	98
6	98	98	90	98
7	98	98	96	98
8	98	98	98	98
9	98	98	90	98
10	98	98	98	98
11	98	98	80	98
12	98	98	98	98
13	60	60	87	90
14	68	70	90	98
15	98	98	82	98
16	71	—	—	—
17	95	98	81	98

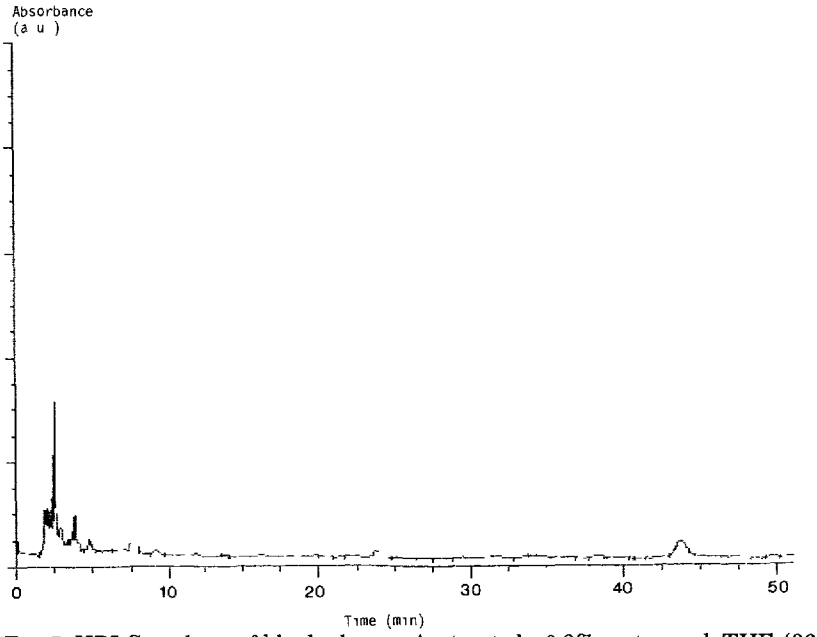

Fig 7 HPLC analysis of blank plasma Acetonitrile–0 3% acetic acid–THF (36 63 1 0 9, v/v), column temperature, 40 °C, flow-rate, 1 ml/min

TABLE IX

CALIBRATION GRAPHS AND LIMITS OF DETECTION FOR NSAIDs

$C = a + bR$ where C = amount of drug, R = peak-area ratio between drug and I S , a and b = regression parameters and r = correlation coefficient ($n = 8$)

NSAID	Therapeutic level (μg/ml)	Concentration range studied (μg/ml)	Limit of detection (μg/ml)	a	b	r
1	0 5–2	0 1–80	0 05	0 0530	4 06	0 9995
2		0 08–120	0 08	0 1833	1 34	0 9959
3		0 2–100	0 1	0 0459	5 21	0 9996
4	9–12	0 1–80	0 025	0 0210	2 26	0 9999
5	20–50	1–800	0 04	0 5335	16 06	0 9995
6	2–12	0 04–60	0 02	0 0185	0 924	0 9998
7	25–50	1–800	1	0 0063	73 07	0 9991
8	0 3–3	0 1–80	0 03	0 0357	1 21	0 9997
9	0 5–6	0 02–30	0 01	0 0107	0 883	0 9993
10	25–70	0 2–100	0 05	0 0687	4 44	0 9996
11		0 3–240	0 1	−0 0526	3 26	0 9997
12	5–7	0 1–80	0 03	0 1210	2 49	0 9986
13	20–30	0 08–120	0 05	0 0950	1 94	0 9980
15	2–6	0 02–30	0 01	0 0120	1 64	0 9996
16		0 1–80	0 02	0 0860	3 26	0 9993
17		0 05–40	0 02	0 0237	2 07	0 9997

TABLE X

PRECISION AND REPRODUCIBILITY OF THE ASSAY

S D = standard deviation and C V = coefficient of variation for eight measurements

NSAID	Expected concentration (μg/ml)	Precision			Reproducibility		
		Mean (μg/ml)	S D (μg/ml)	C V (%)	Mean (μg/ml)	S D (μg/ml)	C V (%)
1	4 0	3 99	0 17	4 3	3 94	0 23	5 8
2	2 0	2 14	0 20	9 4	2 05	0 20	9 8
3	6 0	6 12	0 18	2 9	6 04	0 36	6 0
4	4 0	4 12	0 11	2 7	4 01	0 18	4 5
5	40 0	39 33	1 15	2 9	39 46	1 79	4 5
6	1 0	0 978	0 037	3 8	0 988	0 048	4 9
7	40 0	39 92	0 91	2 3	38 48	1 88	4 9
8	4 0	4 07	0 10	2 5	3 87	0 23	5 9
9	0 5	0 488	0 015	3 1	0 489	0 029	5 9
10	6 0	6 11	0 12	2 0	6 24	0 32	5 1
11	12 0	11 61	0 36	3 1	11 51	0 56	4 9
12[a]	4 0	4 03	0 16	4 0	3 80	0 24	6 3
13	2 0	2 00	0 06	3 0	1 95	0 10	5 1
15	0 5	0 470	0 022	4 7	0 493	0 027	5 4
16[a]	4 0	4 13	0 12	2 9	4 11	0 16	3 9
17	2 0	2 01	0 06	3 0	1 99	0 09	4 5

[a]Determined at 370 nm

TABLE XI

VARIATION OF RETENTION TIMES IN DIFFERENT ANALYSES

S D = standard deviation and C V = coefficient of variation for eight measurements on the same day and sixteen measurements on subsequent days

NSAID	Same day			Subsequent days		
	Mean (min)	S D (min)	C V (%)	Mean (min)	S D (min)	C V (%)
16	3 384	0 039	1 16	3 106	0 093	2 74
15	7 564	0 085	1 12	7 468	0 261	3 50
12	8 055	0 093	1 16	7 850	0 271	3 45
17	11 42	0 11	0 95	11 00	0 41	3 76
9	13 07	0 12	0 94	12 52	0 48	3 86
10	13 76	0 12	0 90	13 20	0 51	3 87
4	16 97	0 16	0 93	16 15	0 67	4 15
2	20 03	0 13	0 66	18 00	0 75	4 16
I S	21 96	0 21	0 95	20 79	0 90	4 32
13	23 24	0 22	0 97	21 96	0 89	4 05
5	27 85	0 25	0 90	26 19	1 16	4 41
3	31 14	0 26	0 83	29 32	1 34	4 55
6	32 50	0 26	0 81	30 56	1 39	4 55
11	34 75	0 23	0 67	32 24	1 31	4 07
1	43 07	0 29	0 68	40 36	1 89	4 67
8	44 20	0 32	0 72	41 58	2 33	5 61
7	46 84	0 32	0 68	43 76	2 04	4 65

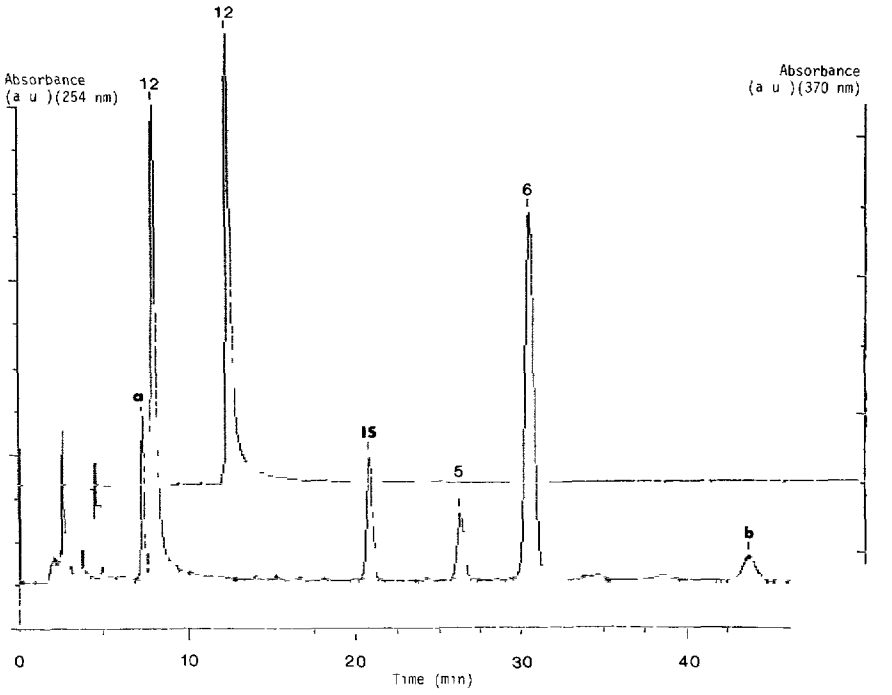

Fig 8 HPLC analysis at 254 and 370 nm of plasma sample from a self-poisoned patient showing the presence of piroxicam (12), fenoprofen (5), flurbiprofen (6) and two unknowns (a and b) Acetonitrile–0 3% acetic acid–THF (36 63 1 0 9, v/v), column temperature, 40°C, flow-rate, 1 ml/min

some compounds, such as ibuprofen, the sensitivity of the assay might be improved by changing the wavelength to another value, 266 nm in this instance.

Precision and reproducibility were determined at medium concentrations for each drug (Table X) The coefficients of variation ranged from 2.0 to 6.0%, except for diflunisal, for which precision and reproducibility were 9 4 and 9.8%, respectively; in fact, diflunisal exhibited a tailing peak, the integration of which can be less accurate. An increase in the acetic acid concentration in the eluent decreased its base width and could be a solution to overcoming this problem if diflunisal is the only drug to be determined, as selectivity is also affected by the modification of the eluent

Validation of the method

The first criterion for the identification of compounds relies on the accuracy required for the determination of their retention times In this study (Table XI), the variation of retention times was always less than 1.2% in one day and 5 6% for subsequent days.

The method was applied to a blind analysis of plasma samples from patients selected for regular treatment with NSAIDs or other drugs tested for chromatographic interference purposes (see Table VI); the presence or the absence

of NSAIDs was shown without any ambiguity in each specimen. Plasma samples from patients hospitalized as a result of self-poisoning and suspected of NSAID uptake were analysed. An example of an uptake of three NSAIDs is shown in Fig. 8. Piroxicam, fenoprofen and flurbiprofen were identified together; one of the two unknown peaks (b) was attributed to clotiazepam and this fact was corroborated by subsequent anamnesis.

CONCLUSION

The proposed method allowed accurate identification of NSAIDs for screening purposes, based on three main advantages: effective separation between all the drugs investigated, stability of the retention times due to isocratic elution and easy differentiation of most inherent interfering substances, using two simultaneous wavelengths of detection and the absorbance ratios at two different wavelengths, which allowed the purity of the eluting peaks to be checked. The recoveries, precision and reproducibilities are satisfactory and render the method suitable for the measurement, in small plasma sample volumes, of various ranges of concentrations therapeutic or overdose levels, or even low concentrations for testing a wash-out before a clinical study.

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