CHROMBIO. 2765

SEPARATION AND IDENTIFICATION OF NON-STEROIDAL ANTIRHEUMATIC DRUGS CONTAINING A FREE CARBOXYL FUNCTION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Different methods for the separation and identification of eighteen non-steroidal antirheumatics (non-steroidal, anti-inflammatory drugs) and the application of these methods for systematic toxicological analysis are reviewed. A method for separating these compounds by high-performance liquid chromatography using an RP-18 column and mixtures of acetonitrile and acetate buffers as eluents is presented and the detectability of these substances with respect to their different UV maxima is discussed.

INTRODUCTION

Since acetylsalicylic acid (aspirin) was introduced at the end of the 19th century, more than twenty so-called non-steroidal, antirheumatic substances (henceforth called NSAIDs, which stands for non-steroidal, anti-inflammatory drugs), apart from the standard substances such as indomethacin and phenylbutazone, have been made commercially available.

Although these substances are mentioned in current textbooks on pharmacology, until recently not much has been said about their therapeutic usefulness or their effects or adverse effects. One might conclude that the research results are too controversial to afford a thorough discussion. However, these drugs are used in ever-increasing quantities and we decided it would be worthwhile to investigate the analytical properties of this group of substances as far as systematic toxicological analysis is concerned.

All of the substances belonging to this group show analgesic, antiphlogistic and antipyretic effects and interact with intracellular enzymes and biological

membranes. They are also strongly bound to protein. As metabolites, we mainly encounter glucuronides, most of the substances and their metabolites being excreted via the kidneys.

Although there is still a great deal of controversy about the mechanism of their effects, there is no doubt that prostaglandin synthetase is inhibited. Although the toxicological relevance of the substances per se is not very important, combinatorial effects with other, toxicologically more relevant, drugs should be taken into account if the contribution of different substances in a case of poisoning is to be evaluated.

Numerous papers [1—21] deal with the quantitative determination of a single or very few compounds of this group but, except for the work of Chalmers et al. [20] and the paper by Giachetti et al. [21], we have found no publication dealing with the group as a whole or especially with the
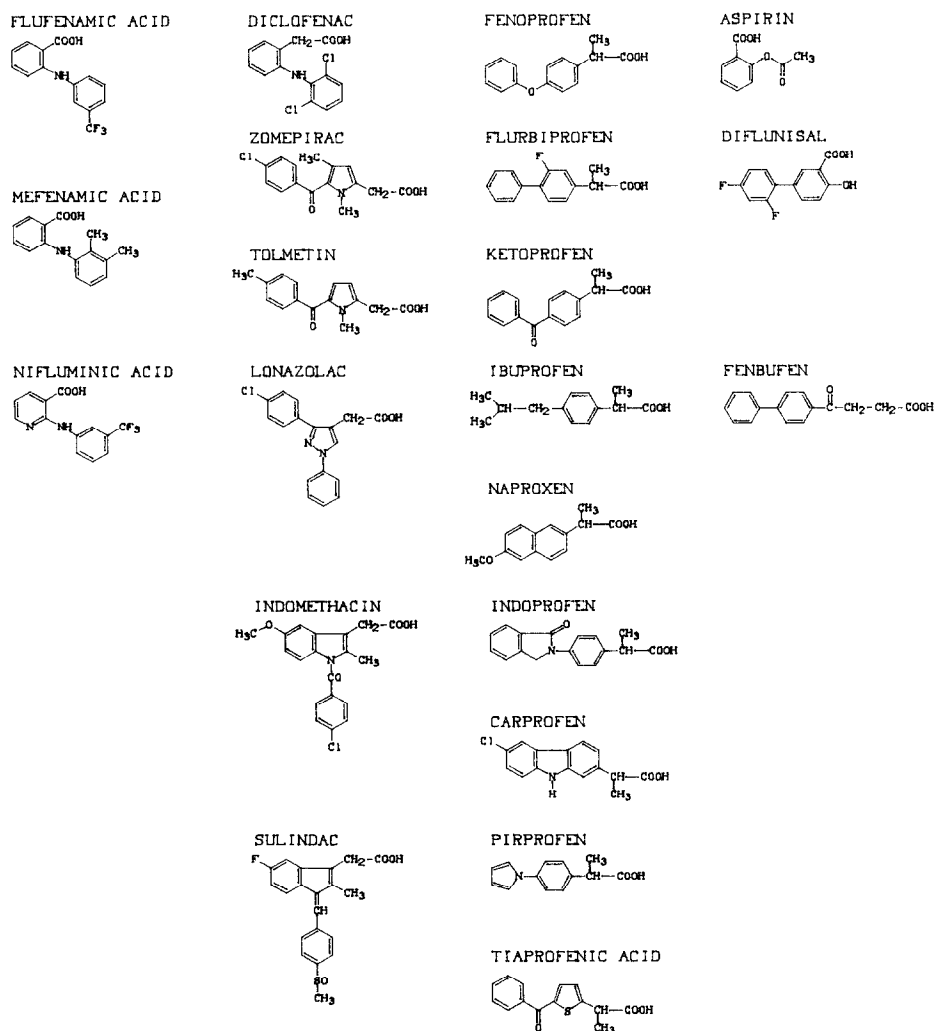

Fig. 1. Formulae of NSAIDs containing a free carboxyl group. Column 1: fenamates; column 2: fenac compounds; column 3: profens; column 4: salicylates and fenbufen.

members of the group containing a free carboxyl function. As a result of having this function, these substances are difficult, if not virtually impossible, to determine underivatized by gas chromatography (GC); although a thin-layer chromatographic (TLC) system could be found to separate them, there are no useful colour reagents for detection on the plates. High-performance liquid chromatography (HPLC) therefore seems to be the method of choice for the identification and determination of the substances of this group. In the relevant literature, most of the authors use reversed phases, especially RP-18 [2–5, 7, 9, 11—18], few use C_8 and C_2 and therefore mainly acetonitrile [3, 7, 11—15, 16] or methanol (4, 5, 17, 18], together with acetic acid [2, 3, 7, 11—15], phosphoric acid [3, 16—18], citric acid or aqueous solutions thereof or buffer solutions of these acids with different pH values.

For detection, most authors use UV detectors [2–4, 6–17, 19] and a few use fluorimetric methods [5]. Every such system affords a solution to the problem of determining one or more members of the group but cannot be used as a general screening method for biological material, especially in a "general-unknown" case.

Only recently, systematic work on the analysis of NSAIDs in horse urine was presented using a combination of TLC, GC, HPLC and mass spectrometry for identification [20] or describing a method for the GC separation of the methylated compounds [21].

The NSAIDs can be divided into seven different groups according to their particular structure: (1) Salicylates: salicylic acid, diflunisal. (2) Pyrazolidines: phenylbutazone, feprazone, oxyphenbutazone, kebuzone, mofebutazone, phenazone. (3) Anthranilic acid derivatives: mefenamic acid, flufenamic acid, nifluminic acid. (4) Arylacetic acid derivatives: diclofenac, sulindac, indomethacin, zomepirac, tolmetin, lonazolac. (5) Arylpropionic acid derivatives (profens): carprofen, indoprofen, ibuprofen, ketoprofen, fenoprofen, naproxen, flurbiprofen, pirprofen, tiaprofenic acid. (6) Oxicams: piroxicam, sudoxicam, isoxicam. (7) Arylbutyric acid derivatives: fenbufen, indobufen.

In Fig. 1., the formulae of the NSAIDs containing a free carboxyl function are shown.

EXPERIMENTAL

Materials

All substances shown in Fig. 1 were included in this investigation (Table I). Some were given to us by the manufacturers, others were extracted from commercial preparations bought at the chemists. The extraction was made with chloroform or diethyl ether. The crude extracts were then recrystallized from mixtures of ethanol and water, washed with water and dried.

Standard solutions were made up by dissolving 10 mg of each drug in 100 ml of a mixture of acetonitrile—water (1:1). Ketoprofen decomposes slowly, with three different decomposition products eluting after the parent substance. Diclofenac decomposes slowly too. The standard solution of carprofen turns red. The other NSAIDs do not show alterations when using water as a solvent, but when using alkaline solutions, acetylsalicylic acid and indomethacin are hydrolysed: acetylsalicylic acid is not stable at pH values above 8 and

TABLE I

SUBSTANCES INVESTIGATED

Code	Substance	Trade name	Manufacturer
ASP	Aspirin		Bayer (Leverkusen)
CAR	Carprofen	Imadyl	Roche
DIC	Diclofenac	Voltaren	Ciba-Geigy
DIF	Diflunisal	Fluniget	MSD
FEN	Fenbufen	Lederfen	Lederle
FEP	Fenoprofen	Feprona	Lilly
FLP	Flurbiprofen	Froben	Boots
FLF	Flufenamic acid	Arlef	Parke Davis
IBU	Ibuprofen	Brufen	Boots
INM	Indomethacin	Indocid	MSD
INP	Indoprofen	Flosint	Carlo Erba
KET	Ketoprofen	Alrheumun	Bayer
LON	Lonazolac	Irritren	Tossa
MEF	Mefenamic acid	Parkemed	Parke Davis
NAP	Naproxen	Proxen	Grünenthal
NIF	Nifluminic acid	Actol	Squibb
PIP	Pirprofen	Rengasil	Ciba
SUL	Sulindac	Clinoril	MSD
TIP	Tiaprofenic acid	Surgam	Roussel
ZOM	Zomepirac	Zomax	Cilag

decomposes within a few minutes, while indomethacin decomposes at pH values above 10. The decomposition products of both substances are eluted immediately after the solvent peak in the chromatogram.

For quantitative determinations, N-phenylanthranilic acid (NPAA; Merck, Darmstadt, F.R.G.) was chosen as an internal standard.

Acetonitrile (p. chr.), sodium dihydrogen phosphate (p.a.), orthophosphoric acid (p.a.), acetic acid (p.a.) and sodium hydroxide (p.a.) were obtained from Merck.

Tap-water was first deionized and then doubly distilled in a quartz apparatus.

High-performance liquid chromatography

We used a Perkin-Elmer Series 3B liquid chromatograph, an LC 100 column oven fitted with a Rheodyne 7105 injection valve and an LC-75 UV—VIS variable-wavelength detector connected to an analogue recorder and a Sigma 10 chromatographic data processing unit via an interface module. The chromatographic separation was effected using a Hibar RP-18 column (5 μm; 250 × 4 mm I.D.) and a short pre-column filled with Pherisorb RP-8 (30—40 μm; 30 × 4 mm I.D.). The 0.05 M buffer solutions were filtered through a Millipore filter immediately before use. All solvents were degassed using helium before and during the runs.

Extraction of urine

Urine samples were obtained from outpatients of the Rheumatological Department of the Clinic of Internal Medicine of Innsbruck University who

were on a regular regime of some of the drugs investigated. The samples were stored in the refrigerator at 4°C prior to extraction.

To 10-ml aliquots of the urine samples, 1 ml of a solution of 100 μg/ml NPAA in 0.05 M boric acid buffer (used as an internal standard) was added and mixed well. The mixture was then acidified by adding 1 ml of 1 M hydrochloric acid and extracted three times with 20 ml of diethyl ether. The organic layers were combined, the diethyl ether evaporated to dryness and the residue reconstituted in 5 ml of a mixture of acetonitrile—water (1:1). Of these solutions 10—50 μl were injected.

The extracts were stored at room temperature. Control analyses carried out at different times showed no significant alterations during several months of storage; even extracts containing indomethacin, which is very sensitive to hydrolysis, showed no signs of deterioration.

RESULTS AND DISCUSSION

Choice of detection wavelength

Table II shows that the substances investigated do not exhibit common maxima or minima, so one has to compromise on the wavelength chosen for a screening run. We chose a wavelength of 254 nm, partly to make the method

TABLE II

UV MAXIMA AND MINIMA IN METHANOL

	Substance	Maxima	E_{max}*	Other maxima	Minima
Profens	FEP	270	1740		253
	FLP	245	14840		220
	KET	254	19230		230
	NAP	270	5440	260, 315, 330	320, 296, 265
	INP	281	15530	226	245
	CAR	299	22170	261	275, 256
	PIP	264	11700		233
	TIP	305	14320		260
Fenac compounds	DIC	279	11670		258
	ZOM	322	12460	251	228, 282
	TOL	312	20120	253	273
	LON	278	15340		336
	INM	318	6980		301
	SUL	284	9360	328	304
Fenamates	FLF	287	10100	339	255, 317
	MEF	280	8520	347	250, 211
	NIF	287	23790		245
	NPAA	285	14730	347	253, 315
Salicylate plus	ASP	279	1140		259
fenbufen	DIF	252	12870	310	244, 295
	FEN	280	19330		237

*E_{max} = Maximum extinction coefficient.

available for laboratories having only a fixed-wavelength detector and partly because ibuprofen shows a relatively high sensitivity at this wavelength whilst exhibiting the lowest coefficients of absorption over the whole range and no measurable absorption above 275 nm.

The absorption maxima and minima do not show any considerable deviation when using acetonitrile—acetate buffer, pH 4.5 (50:50) as solvent.

Mobile phase and influence of the buffer system

An acid buffer system was used to suppress ionization and to take advantage of the properties of reversed-phase systems. Literature on the pK_a values of NSAIDs is scarce and only very few useful values could be found. As we did
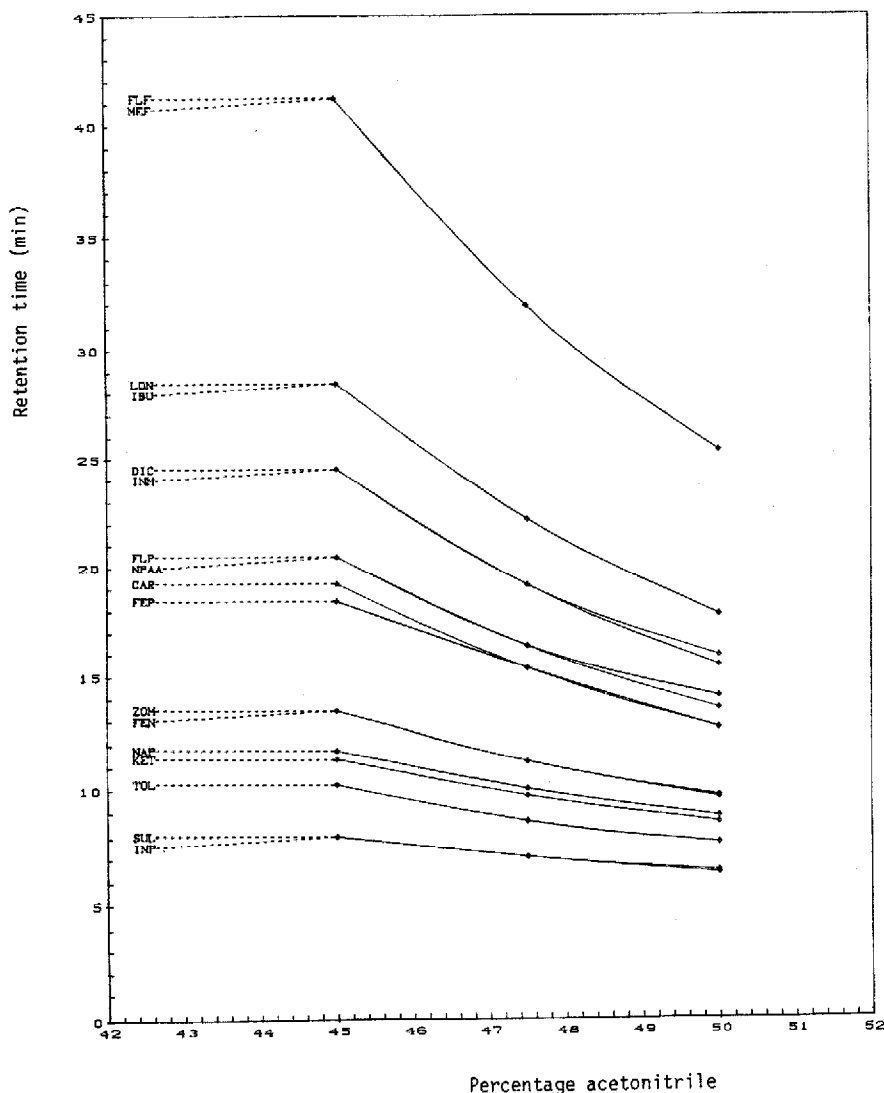

Fig. 2. Retention times as a function of the volume ratio of acetonitrile—0.05 M phosphate buffer (pH 2.5).

not intend to determine pK_a values during this investigation, we made the following assumptions.

Two of the substances investigated are donor-substituted derivatives of benzoic acid, so a pK_a of more than 4.22 is to be expected. Twelve substances are donor-substituted derivatives of acetic acid and, in this case, a pK_a of more than 4.75 is to be expected.

The following pK_a values were found in the literature: carprofen, 4.70; ibuprofen, 5.41; fenoprofen, 4.30; fenbufen, 4.00; diflunisal, 4—5; zomepirac, 4.37.

Increasing the percentages of acetonitrile decreased the retention times proportionally (Figs. 2 and 3). The retention times were halved when the acetonitrile content of the solvent mixture was increased by 10%.
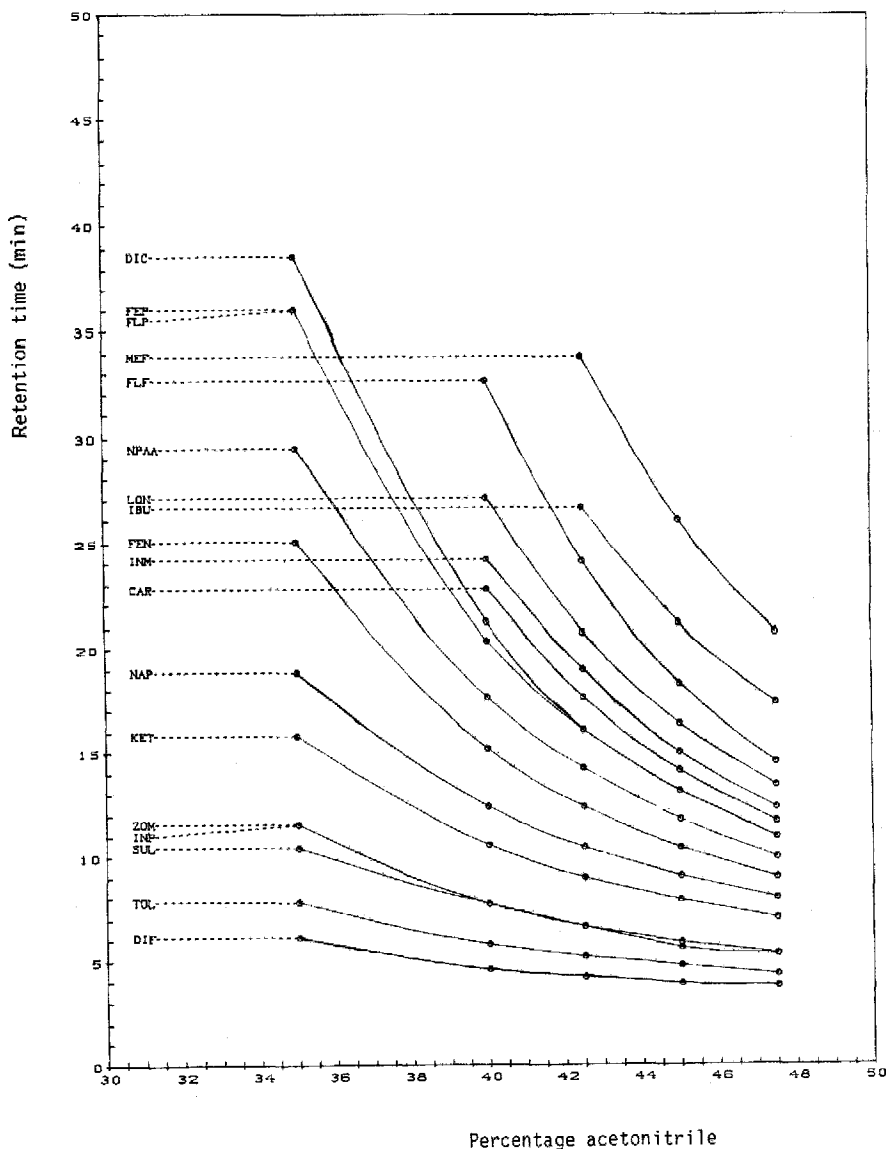

Fig. 3. Retention times as a function of the volume ratio of acetonitrile—0.05 M acetate buffer (pH 4.5).

TABLE III

TABLE FOR THE IDENTIFICATION OF NSAIDs (SOLVENT-PROGRAMMED ELUTION)

No.	Substance	k	Concentration (μg/ml)
1	Diflunisal	3.77	10
2	Tolmetin	3.77	10
3	Tiaprofenic acid	3.77	10
4	Sulindac	5.02	10
5	Indoprofen	5.08	10
6	Zomepirac	5.39	10
7	Ketoprofen	5.98	10
8	Nifluminic acid	6.24	10
9	Naproxen	6.59	10
10	Fenbufen	7.46	10
11	NPAA	7.69	10
12	Pirprofen	7.98	10
13	Flurbiprofen	8.54	10
14	Fenoprofen	8.54	10
15	Diclofenac	8.54	10
16	Carprofen	8.75	10
17	Indomethacin	8.86	10
18	Lonazolac	9.06	10
19	Flufenamic acid	9.39	10
20	Ibuprofen	10.01	50
21	Mefenamic acid	10.55	10

Separation of the whole group (cf. Fig. 5) of substances investigated was achieved by solvent programming (Table III) using the following instrumental conditions: acetonitrile—0.05 M acetate buffer, pH 4.5 (from 25 to 55% acetonitrile in 30 min); column temperature, 35°C; flow-rate, 0.8 ml/min; detection wavelength, 254 nm; sensitivity, 0.16 a.u.f.s.

Using mixtures of acetontirile—water, reasonable separation could not be achieved. By employing a mixture of acetonitrile—0.05 M phosphate buffer, pH 2.5 (50:50), separation could be achieved within 30 min. Equal results could be achieved by using a 45:55 mixture of acetonitrile—0.05 M acetate buffer, pH 4.5. This mixture was subsequently used because of the mild conditions and the decreased danger of growing micro-organisms in the buffer solution. Alternate use of both buffers affords an additional means of differentiation because the NSAIDs show different retention behaviour in solvent mixtures using the two different buffers.

Diflunisal and nifluminic acid both show a marked tailing when using acetate buffer. The former substance even shows marked tailing when phosphate buffer was used for chromatography.

Column oven temperature

Investigation of the influence of oven temperature on the retention behaviour of the substances investigated showed an almost proportional relation, i.e. shortening of the retention times when the temperature was increased. Only flufenamic acid was eluted earlier than expected (Fig. 4). An
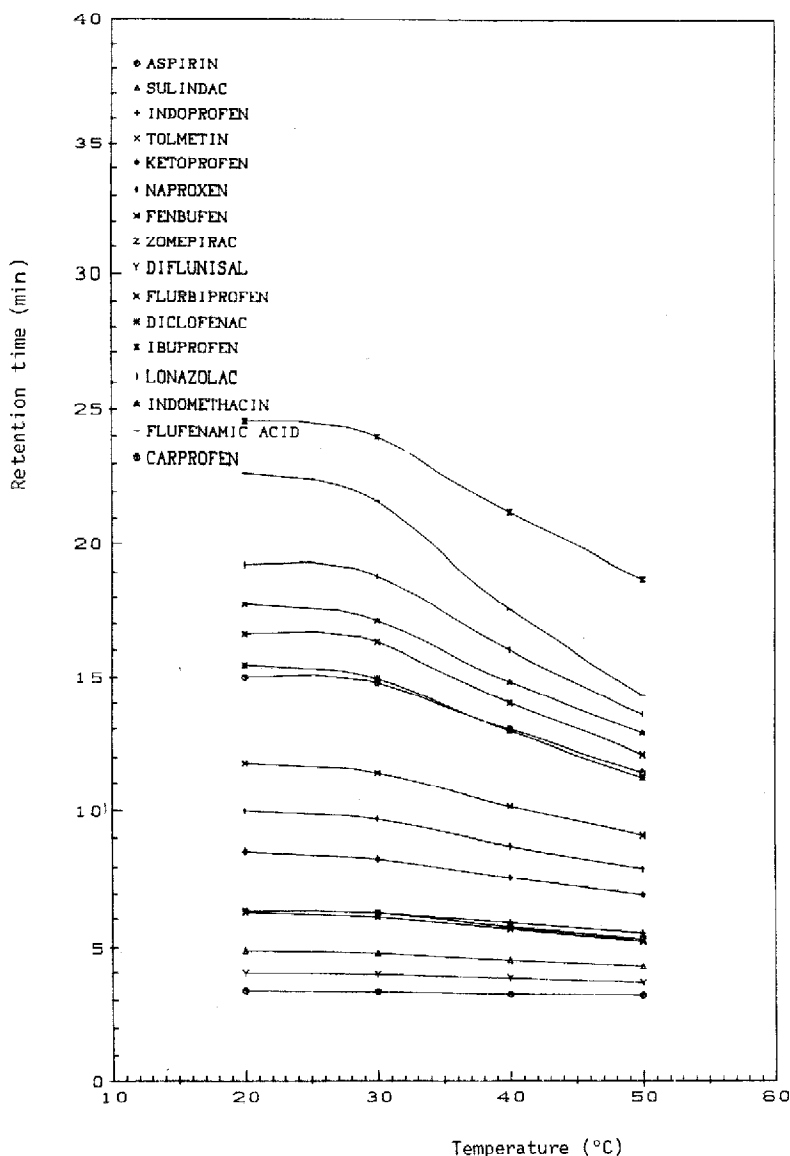
Fig. 4. Retention times as a function of the column temperature using acetonitrile—0.05 M acetate buffer, pH 4.5 (50:50).

oven temperature of 35°C was therefore chosen for all subsequent investigations because it turned out to be the best compromise between a low enough viscosity of the solvent mixture, minimized degassing problems and optimum separation power of the systems.

Interference by other drugs

Drugs such as paracetamol, pyrithyldione, salicylic acid amide, bromisoval, phenacetin, mephenytoin, phenytoin, glutethimide, methaqualone and salicylic acid methyl ester are extracted into the acid fraction as well and are eluted in the same range (Fig. 6 and Table IV), but they can easily be differentiated by other methods such as gas—liquid chromatography, TLC and spot tests.
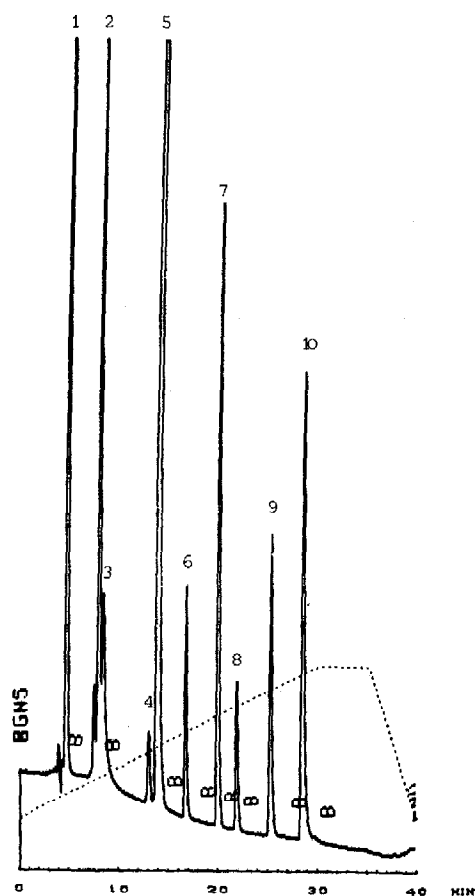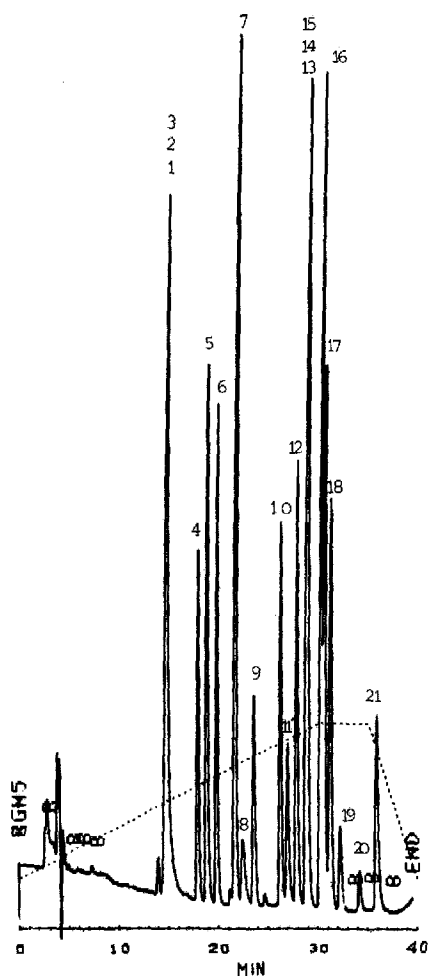
Fig. 5. Chromatogram showing the separation of NSAIDs containing a free carboxyl group. Column: Lichrosorb RP-18 (5 μm; 250 × 4 mm I.D.); pre-column: Perisorb RP-8 (30 × 4 mm I.D.). Mobile phase: acetonitrile—0.05 M acetate buffer (pH 4.5); gradient elution 25—55% acetonitrile in 30 min. Column temperature: 35° C. Flow-rate: 0.8 ml/min. Detection: UV 254 nm; sensitivity 0.16 a.u.f.s. (For identification of peaks see Table III.)

Fig. 6. Chromatogram showing possibly interfering substances coextractable at acidic pH. (For conditions of separation see Fig. 5; for identification of peaks see Table IV.)

The barbiturates are eluted in this range as well, but apart from the aryl-substituted members of the group they show a much lower sensitivity and can be differentiated by other methods.

Elution of the members of the group of pyrazolidines is shown in Fig. 7 and Table V. This group poses the greatest difficulties in differentiating from the other NSAIDs.

Chromatography of the urine extracts

The naturally occurring substances in urine are eluted at k values of 0—1.5 using an isocratic mixture and at k values of 0—2 using solvent-programmed

TABLE IV

IDENTIFICATION OF POSSIBLY INTERFERING PEAKS (SOLVENT-PROGRAMMED ELUTION)

No.	Substance*	k
1	Paracetamol	0.48
2	Pyrithyldione	1.53
3	Salicylamide	1.68
4	Bromisoval	3.17
5	Phenacetin	3.44
6	Mephenytoin	4.35
7	Phenytoin	5.39
8	Glutethimide	6.00
9	Methaqualone	7.11
10	Salicylic acid methyl ester	8.4

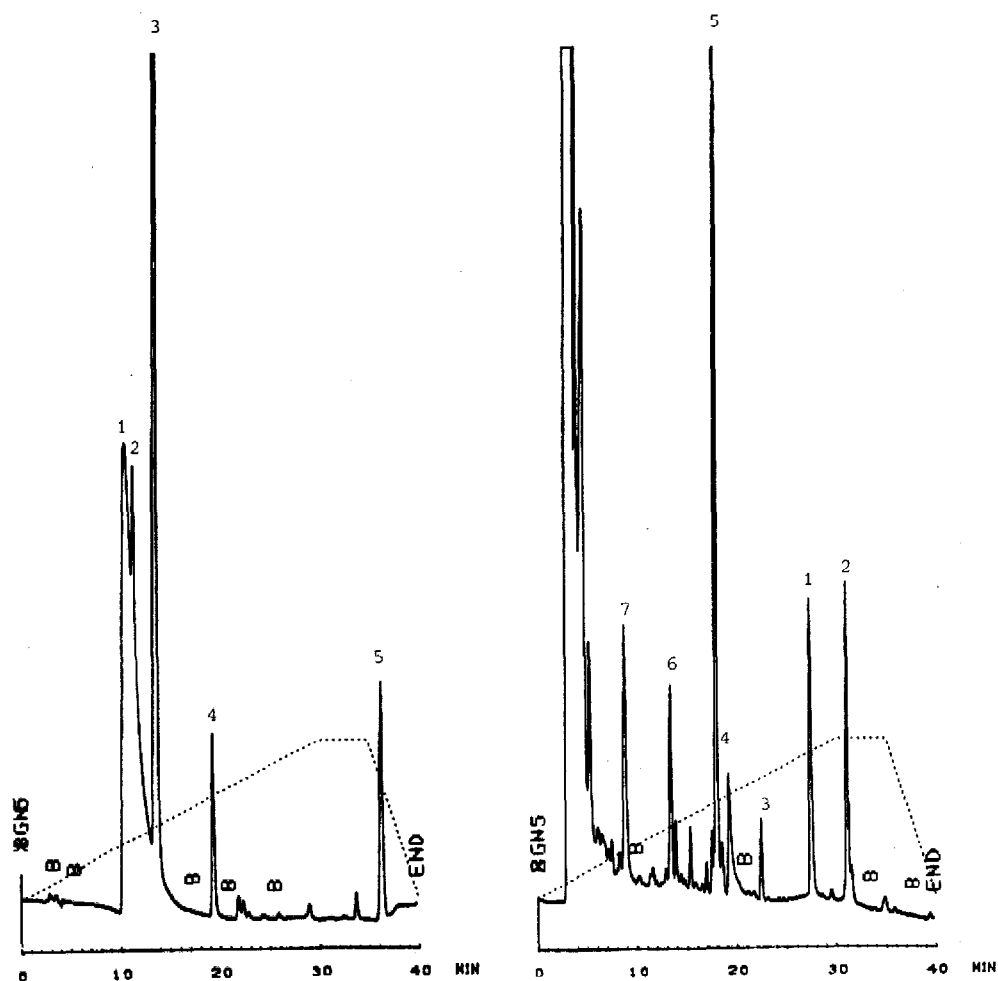*Registered or proposed International Nonproprietary Name.

Fig. 7. Chromatogram showing NSAIDs pertaining to the group of pyrazolidines. (For conditions of separation see Fig. 5; for identification of peaks see Table V.)

TABLE V

IDENTIFICATION OF PEAKS OF THE PYRAZOLIDINES (SOLVENT-PROGRAMMED ELUTION)

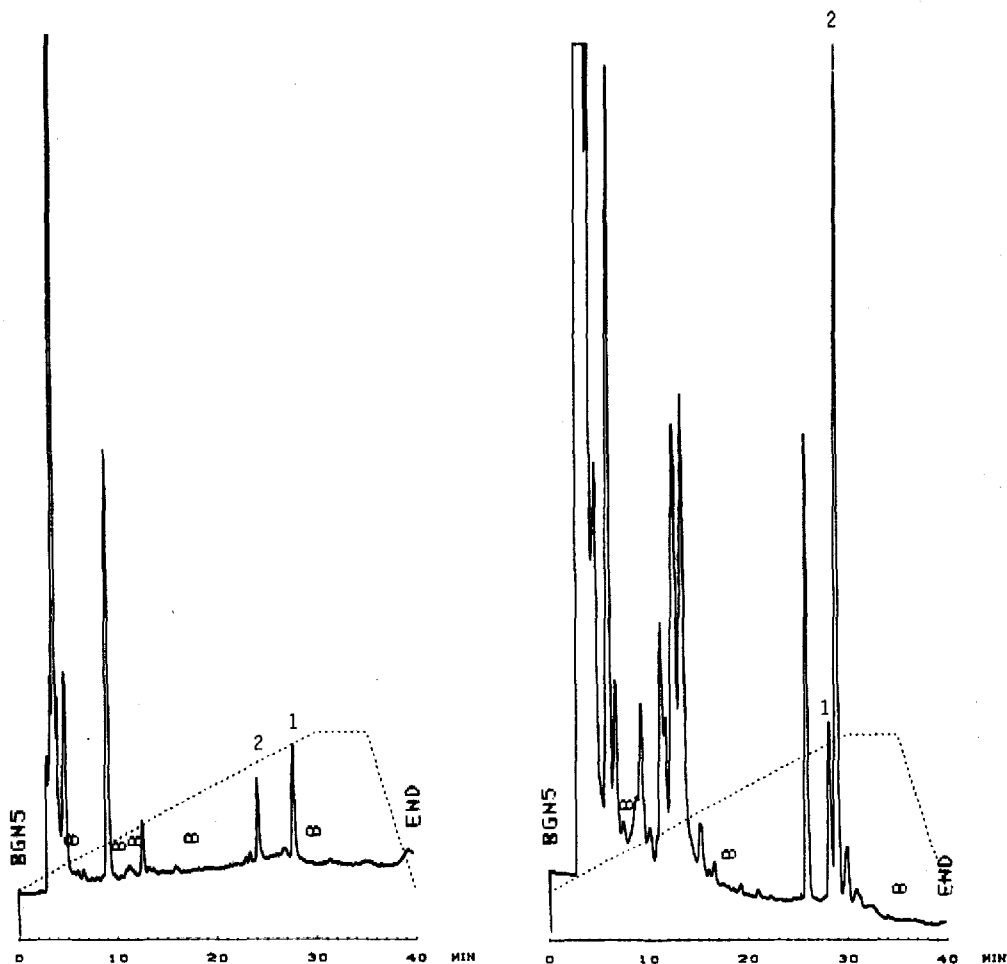| No.	Substance	k
1	Kebuzone	2.43
2	Mofebutazone	2.70
3	Phenazone	3.7
4	Phenylbutazone	5.20
5	Feprazone	10.63

Fig. 9. Chromatogram of a urine extract containing naproxene. (For conditions of separation see Fig. 5.) Peaks: 1 = NPAA; 2 = naproxen.

Fig. 10. Chromatogram of a urine extract containing pirprofen. (For conditions of separation see Fig. 5.) Peaks: 1 = NPAA; 2 = pirprofen.

elution. As the members of the group of NSAIDs elute with k values of more than 4, there is no interference with other urine contents (Figs. 8, 9 and 10).

CONCLUSION

The methods described above can be used as part of a toxicological screening programme to detect and tentatively identify NSAIDs containing free carboxyl functions in urine extracts. Although the time needed for a solvent-programmed run (45 min) is a disadvantage, analysis times can be drastically shortened by using isocratic mixtures for subsequent quantitative analysis. A further disadvantage is that acetylsalicylic acid cannot be detected by this procedure.

As is shown in Figs. 6 and 7, there are quite a few interfering acidic and neutral substances but, contrary to the group of NSAIDs, they can readily be differentiated by other methods. The procedure seems to be very useful as part of a general systematic screening programme in general-unknown cases. Investigations as to the usefulness of a photo diode array detector to increase the sensitivity and to enhance the discrimination power of the system by allowing multi-wavelength detection are underway and will be reported in a forthcoming paper.

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