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Liquid chromatography with amperometric detection for the determination of non-steroidal anti-inflammatory drugs in plasma

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ABSTRACT

A simple and sensitive liquid chromatographic method with electrochemical detection is described for the quantitative determination of the non-steroidal anti-inflammatory drugs diflunisal, indomethacin, naproxen, piroxicam and sulindac in human plasma Isolation of the drug from the biological fluid is achieved using a Sep-pak RP18 cartridge Separation of plasma components occurs on a reversed-phase C_{18} column with a mobile phase consisting of methanol–water–phosphate buffer For the amperometric detection the potential of + 0 9 V was set on the working electrode The detection limit of the assay is 10–20 ng/ml The method showed good concordance for plasma samples containing the drugs (r = 0 999) and can be readily utilized for clinical pharmacokinetic studies.

INTRODUCTION

Since the introduction of acetylsalicylic acid, more than thirty non-steroidal anti-inflammatory drugs (NSAIDs) have become commercially available for use in the treatment of osteoarthritis and other rheumatic diseases.

Several liquid chromatographic procedures have been described for the quantitation of NSAIDs in human plasma. A large number of papers deal with the assay of one or several compounds of this group using UV [1–11] or fluorescence [12–14] detection. A selection of the commonly used NSAIDs is shown in Fig. 1, and each structure is seen to contain an electroactive group, capable of undergoing easy oxidation. Except for two reports concerning naproxen and diclofenac [15,16], the NSAID group has not been investigated by high-performance liquid chromatography (HPLC) using electrochemical detection.

The present paper reports a liquid chromatographic (LC) method, which is capable of measuring many of the commonly available NSAIDs in plasma. This method includes a clean-up procedure by a Sep-Pak cartridge and electrochemical detection after separation on a reversed-phase column. Amperometric detec-

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tion of NSAIDs was the preferred procedure because of the increased sensitivity of detection compared to UV detection. The assay detects diflunisal, indomethacin, naproxen, piroxicam and sulindac.

EXPERIMENTAL

Reagents and materials

Diflunisal, indomethacin and sulindac were kindly supplied by Merck, Sharp and Dohme (Sydney, Australia), naproxen by Syntex (Sydney, Australia) and piroxicam by Pfizer (Sydney, Australia). These substances were used without further purification. All other chemicals were of analytical-reagent grade. Water was double-distilled from an all-glass apparatus.

Apparatus

The chromatographic analysis was performed on a modular system consisting of an LKB Model 2150 pump (Bromma, Sweden) equipped with an Rheodyne Model 7125 injector with 20-μl loop, an ESA Coulochem Model 5100 A detector (Bradford, MA, U.S.A.) coupled in series with a Shimadzu Model SPD-6A variable-wavelength UV detector (operated at 254 nm). The analytical column used was a Brownlee RP18 (5-μm packing, 100 mm × 4.6 mm I.D.) with a mobile phase composed of 0.025 m*M* phosphate buffer (pH 3.0) in 75% methanol. The mobile phase was prepared by dilution of a 0.25 m*M* stock ammonium phosphate

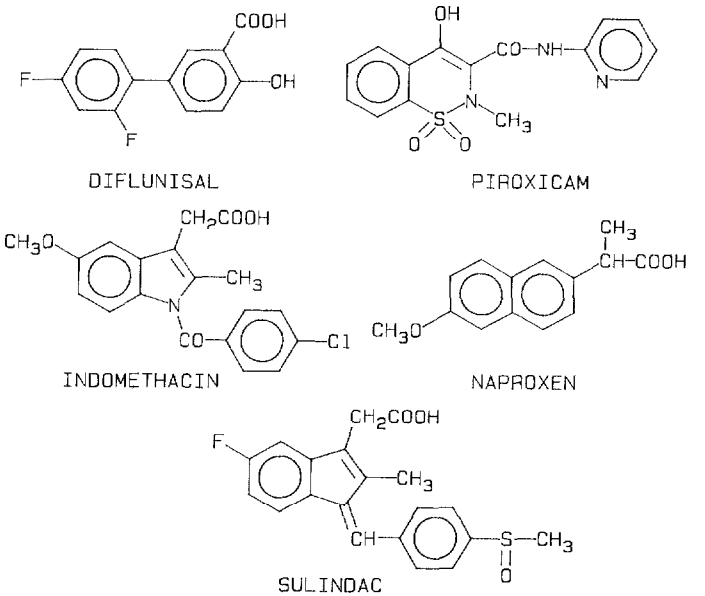

Fig 1 Structures of some electroactive non-steroidal anti-inflammatory drugs

buffer solution (adjusted to pH 3.0 with orthophosphoric acid) and was filtered through a 0.2-μm Millipore filter immediately before use. For piroxicam, it was found necessary to change the pH and methanol concentration of the mobile phase to prevent endogenous plasma constituents interfering with the peak of the drug. Thus piroxicam was chromatographed with a pH 7.0 mobile phase (containing 60% methanol).

Solutions

Stock NSAID solutions containing 50 μg/ml of each compound in methanol were prepared monthly and stored at -20°C. Working standards were prepared daily by dilution of freezer stocks with the appropriate mobile phase and processed identically.

Sample preparation

To an aliquot of plasma (20–200 μl) in a 2.0-ml Eppendorf tube were added 0.1 ml of methanol, 1 μg of internal standard (20 μl of a methanolic solution), 0.1 ml of stock buffer solution (pH 3.0) and 0.1 ml of water. The capped tube was shaken for 2 min on a vortex mixer and centrifuged at 1800 *g* for 10 min at room temperature. The supernatant was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) which had been previously washed with 2 ml of methanol and 5 ml of water and acidified with 1 ml of stock buffer solution. The cartridge was washed with 5 ml of water to eliminate most of the water-soluble plasma constituents, which are not adsorbed on the solid support. Each drug was then eluted with 2 × 5 ml of methanol. The combined methanol washing was evaporated to dryness *in vacuo* and the extracted residue was redissolved in 1 ml of mobile phase. Due to the low recovery of piroxicam using solid-phase (Sep-Pak) extraction with methanol (recovery $>70\%$), we preferred the liquid extraction procedure of Migulla *et al.* [17], which gave a mean recovery greater than 90% (using acetonitrile after adjusting the pH to 4.8).

Recovery studies

Four replicate samples containing, respectively, 0.1, 0.5, 1 0 and 5.0 μg of each NSAID and 1.0 μg of internal standard were prepared in blank plasma and taken through the sample preparation procedure described above. The absolute peak-height ratios obtained for the extracted samples were compared with those of fresh standards of the NSAID and internal standard prepared directly in mobile phase (extracted *versus* non-extracted).

Quantitation

Calibration curves were constructed for each drug separately prepared in (i) mobile phase and (ii) plasma extract, by spiking with a known amount of the drug in question. The standard curves were constructed by plotting the peak-height ratios (drug to internal standard) *versus* the concentration of the drug 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. Plasma samples from volunteers were analysed at the same time as calibration standards.

RESULTS AND DISCUSSION

Under the described conditions, the peaks corresponding to the NSAID studied here were well resolved, sharp and symmetrical. No endogenous compound extracted at the same time interfered with these peaks. Curves showing the variation of current intensity with the applied potential (the so-called hydrodynamic voltammogram) were determined for the studied compounds (Fig. 2). The oxidation potential of piroxicam (0.6 V) is the lowest, and that of naproxen (0.86 V) is the highest, in this series of compounds. However, no precise electrochemical relationship can exist between these compounds since the most electroactive group is not necessarily the same for each of the studied NSAIDs.

The half-wave potentials were read from the hydrodynamic voltammograms and are listed with the retention times in Table I. An applied oxidation potential of +0.9 V is satisfactory for all these compounds and was used for this assay procedure to maximize the responses.

Diflunisal, indomethacin and naproxen each contain an electrochemically oxidizable phenolic or methoxy group. The similarity in electrochemical and chemical properties of indomethacin and naproxen allows the use of one as internal standard for the assay of the other. Thus, indomethacin was used as internal standard for the assay of diflunisal, piroxicam and sulindac, while naproxen was the internal standard for indomethacin.

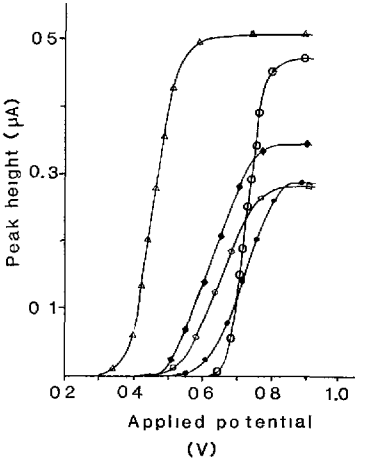

Fig 2 Hydrodynamic voltammograms of 1 mM piroxicam (△), indomethacin (◆), diflunisal (○), sulindac (◇) and naproxen (●) carried out in mobile phase

TABLE I

RETENTION TIMES, HALF-WAVE POTENTIALS AND DETECTION LIMITS OF NSAIDs IN PLASMA DETERMINED BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

Drug	Internal standard	Retention time (min)	Half-wave potential (V)	Detection limit (ng/ml)
Diflunisal		8 0	+0.73	10
	Indomethacin	14 6	+0 63	
Indomethacin		14 6	+0 63	20
	Naproxen	10 0	+0 72	
Naproxen		10 0	+0 72	15
	Indomethacin	14 6	+ 0 63	
Piroxicam		6 6	+0 46	10
	Indomethacin	19 2	+0.63	
Sulindac		7 6	+0 64	15
	Indomethacin	14 6	+0 63	

Sulindac has a sulfone group, which could be the site for the electrochemical oxidation of the compound. Preliminary studies carried out on piroxicam and some of its degradation products seem to indicate that the hydroxyl group in position 4 is the electroactive group at the potential used, and that it undergoes anodic oxidation.

Figs. 3 and 4 show typical amperometric chromatograms from drug-free plas-

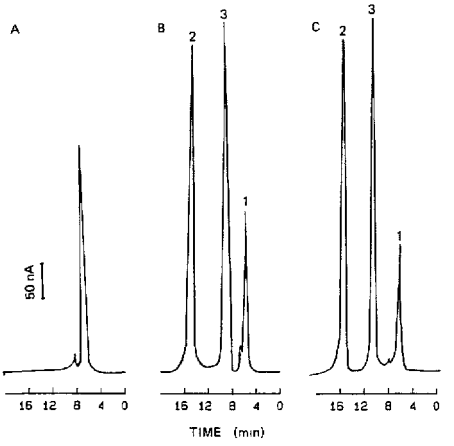

Fig 3 LC–electrochemical detection assay of NSAIDs in plasma extracts (A) Drug-free plasma, (B) patient plasma obtained 2 h after oral administration of 250 mg diflunisal (peak 3) spiked with 1 μg/ml indomethacin (peak 2), (C) patient plasma obtained 3 h after oral administration of 100 mg indomethacin (peak 2) spiked with 1 μg/ml naproxen (peak 3)

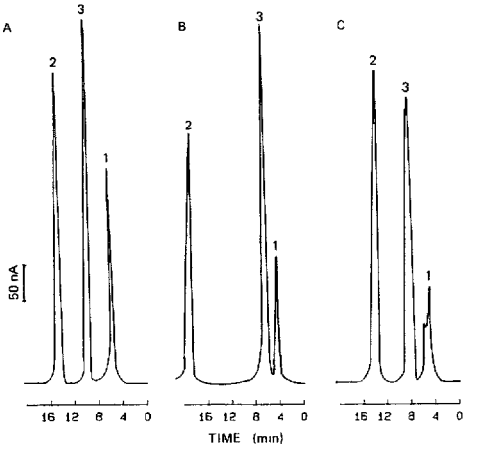

Fig 4 LC–electrochemical detection assay of NSAIDs in plasma extracts. (A) Patient plasma obtained 2 h after oral administration of 250 mg naproxen (peak 3) spiked with 1 μg/ml indomethacin (peak 2), (B) patient plasma obtained 4 h after oral administration of 20 mg piroxicam (peak 3) spiked with 1 μg/ml indomethacin (peak 2); (C) patient plasma obtained 2 h after oral administration of 200 mg sulindac (peak 3) spiked with 1 μg/ml indomethacin (peak 2)

ma and plasma extracts (with internal standard) from patients who had received the particular NSAID. Blank extracts for all analysed plasma samples do not show interfering peaks.

Recovery measurements on spiked plasma revealed a significant difference between the traditional liquid–liquid and solid-phase extraction. The separation of NSAIDs from a biological fluid using solvent extraction is in most cases relatively difficult and not precise because of the hydrophilic character of the drugs. Our preliminary experiments, and the results of some previous workers, on liquid organic extraction of NSAIDs using diethyl ether [18,6], dichloromethane [19,20], acetonitrile [21,22] and a mixture of organic solvents [23,24] show that these compounds are not extracted reproducibly with organic solvents. Inadequate to poor recovery values (range 40–100%) were reported by different workers even though the same solvent was used. Thus solid-phase extraction was the preferred procedure here (except for piroxicam). In our experiments the average recovery was sufficiently high (<90%) and in some cases quantitative. In all cases the percentage recovery was essentially unaffected by the concentration of the drug.

A linear relationship was established between the peak-height ratio and concentration for each NSAID when dissolved in the mobile phase, for the concentration range 0.02–10 μg/ml. Using the same procedure, nearly identical lines were obtained for analysis of each drug when added to plasma. For each plot, over the concentration range investigated, the regression coefficient was greater than 0 999. The slopes of the regression lines were in good agreement, intercept

TABLE II

PRECISION OF THE LC–ELECTROCHEMICAL DETECTION METHOD FOR THE MEASUREMENT OF NSAIDs IN PLASMA (n = 3–6)

Drug	NSAID concentration (μg/ml)		Coefficient of variation (%)
	Expected	Found (mean ± S D)	
Diflunisal	0 1	0 108 ± 0 016	14 81
	0 5	0 510 ± 0 018	3 53
	1 0	1 002 ± 0 008	0 80
	5 0	4 984 ± 0.015	0 30
Indomethacin	0 1	0.108 ± 0.013	12 03
	0 5	0 512 ± 0.011	2 15
	1 0	1 008 ± 0.008	0 79
	5 0	4 976 ± 0.038	0 76
Naproxen	0 1	0.128 ± 0.022	17 19
	0 5	0.510 ± 0 022	4 3
	1 0	1.010 ± 0 010	0 99
	5 0	4.994 ± 0 038	0 76
Piroxicam	0 1	0.122 ± 0 015	12 29
	0 5	0.512 ± 0 025	4 88
	1 0	1.004 ± 0 023	2 29
	5 0	5 084 ± 0 189	3 72
Sulindac	0 1	0 110 ± 0 007	6.36
	0.5	0 498 ± 0 013	2.61
	1.0	1 004 ± 0 005	0.50
	5.0	5 006 ± 0 028	0.56

values were insignificant and the correlation coefficients demonstrated good definition of the calibration curves.

The detection limit of the assay was determined as where the signal-to-noise ratio became 3:1. The limits are given in Table I. Using amperometric detection the limit in all cases was better than that with UV detection by a factor of five- to twenty-fold, depending on the molar absorptivity of the drug at the most appropriate wavelength.

To determine the precision and accuracy of the assay method, three to six replicate samples were analysed at each of four concentrations with the results summarized in Table II. In all but one instance the coefficient of variation was less than 5%. There were no detrimental effects in the plasma samples and no losses of drugs from stock solution over a period of fourteen days, when stored at

−20°C. The working standards were found to be stable over one week storage at 4°C. Nevertheless the standards were prepared daily as a precautionary step.

In summary, the LC procedure reported here has proven to be simple, concise and reproducible. The method as described can be the method of choice for analysis of electroactive NSAIDs in biological fluids, being superior to any previous one in terms of sensitivity and specificity. Amperometric detection provides high sensitivity for these compounds and allows the characterisation of important NSAIDs while utilizing a small sample volume (20–200 μl). One of the advantages of this method is the use of one NSAID as internal standard for assay of the others.

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