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Determination of non-steroidal anti-inflammatory drugs in pharmaceuticals and human serum by dual-mode gradient HPLC and fluorescence detection

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) such as piroxicam and mefenamic acid are commonly prescribed to treat inflammation, pain and fever. Similarly acetylsalicylic acid is used to prevent strokes and heart attacks. A rapid and selective method was developed for the simultaneous assay of three NSAIDs and salicylic acid via HPLC with fluorescence detection. The separation was performed using a "dual-mode" gradient (acetonitrile–0.1% aqueous orthophosphoric acid) and the analysis was completed within 7 min using an ACE® column C18, 5 μm, 150 mm × 4.6 mm. Naproxen was used as internal standard. The proposed method is simple, selective as well as with a good sensitivity reaching LOD lower than 2 pmol (0.05 μM) and was applied for quantitative analysis in pharmaceuticals and in human serum samples. The mean recovery was more than 95% and the within-day and between-days precisions were found to be satisfactory having RSD within the acceptable limits (<10%).

Keywords: NSAIDs; Acetylsalicylic acid; Mefenamic acid; Piroxicam; Salicylic acid; "Dual-mode" gradient; Fluorescence

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed agents worldwide to treat a variety of pain-related conditions, including arthritis and other rheumatic diseases. In addition, epidemiological studies have shown that long-term use of NSAIDs reduces the risk of developing Alzheimer's disease and delays its onset [1–3]. Acetylsalicylic acid (ASA) has anticoagulant properties and prevents strokes and heart attacks (reduction of cardiovascular risks) [4,5]. ASA is frequently used for the treatment of fever and minor pain and is available as a widely used over-the counter (OTC) drug. NSAIDs are included in many cold and allergy preparations. Piroxicam and mefenamic acid are used mainly to treat rheumatoid arthritis and osteoarthritis [6,7]. Mefenamic acid has also been found to produce closure of patent ductus arteriosus in premature neonates [8]. The combination of aspirin–mefenamic acid is more effective as an analgesic than both drugs alone [9].

Several methods have been reported for the assay of NSAIDs in pharmaceuticals and biological fluids; in general these works deal with a single compound or a few compounds, and employ capillary electrophoresis [10–15], spectrofluorimetry [16–20], flow injection fluorimetry [21] or gas chromatography coupled to mass spectrometry (GC/MS) [22–24]. GC methods are time-consuming and have been largely replaced by HPLC methods with ultra-violet (UV) [25-35] detection, electrochemical detection [36], or mass spectrometry [37]. To our best knowledge, very few studies describe the analysis of NSAIDs via HPLC with fluorescence detection [38].

Simultaneous assay of the more widely used NSAIDs would provide a number of advantages including:

- Several drugs could be analysed using the same separation conditions which might be very convenient for clinical and

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toxicological screening as well as in routine pharmaceutical analysis.
- Identification of the NSAID peak could be carried out accurately and expeditiously using a simultaneous analytical system.
- Drug–drug interaction studies, which might require a simultaneous measurement system to detect more than one drug in biological samples.

This work describes the analysis of three NSAIDs and salicylic acid (the hydrolysis product and major metabolite of acetylsalicylic acid) in serum via HPLC with fluorescence detection. Fluorescence detection was employed as it can provide excellent selectivity and sensitivity. A "dual-mode" gradient RP-HPLC separation was used to improve resolution and shorten the analysis time [39–41]. In this paper, we describe a rapid, simple, sensitive, cost-effective and selective method for the analysis of NSAIDs with a LOD lower than 2 pmol (0.05 μM). This method was successfully applied to the determination of each compound in human serum sample, as well as commercially available pharmaceutical formulations.

2. Experimental

2.1. Reagents and materials

Acetylsalicylic acid (ASA) Mw = 180.16 g mol^{-1}, salicylic acid (SA) Mw = 138.12 g mol^{-1}, piroxicam (PIR) Mw = 331.3 g mol^{-1}, mefenamic acid (MF) Mw = 241.3 g mol^{-1}, Na naproxen (IS), (Fig. 1) (Sigma St. Quentin, France), orthophosphoric acid 85% (Merck, Darmstadt, Germany) were used as received. Methanol and acetonitrile were of HPLC gradient grade (Fischer, Illkirch, France). All aqueous solutions were prepared using high-purity water obtained from a Milli Q® water purification system (Millipore, St Quentin, France). A glass vacuum-filtration apparatus was employed for the filtration of the aqueous solutions, using 0.20 μm membrane filters (Millipore). Dissolution of compounds was enhanced by sonication in an ultrasonic bath (Elma®, Germany). A small vortex mixer and a centrifugation system Biofuge A (Heraeus-Sepatech, Germany) were employed for sample pre-treatment.

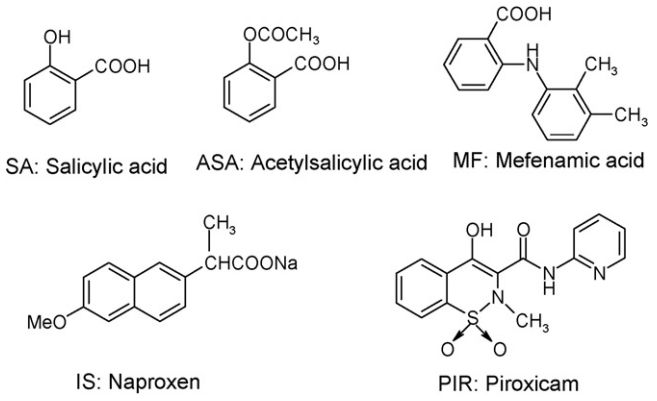

Fig. 1. Chemical structures of NSAIDs.

Serum samples were provided from "Etablissement Français du Sang" (Purpan, Toulouse, France). Commercial pharmaceuticals were purchased from a local drugstore. The pharmaceutical dosage forms tested were Piroxicam *Merck*® (10 mg per capsule), Ponstyl *Pfizer*® (MF, 250 mg per capsule), Aspro *Roche*® (ASA, 500 mg per tablet).

2.2. Preparation of standard solutions and samples

All stock standard solutions of NSAIDs, SA and IS were prepared at a concentration of 1 mg mL^{-1}. ASA was prepared in acetonitrile–orthophosphoric acid (99:1, v/v). SA and PIR were prepared in acetonitrile. IS was prepared in methanol. MF was prepared in acetonitrile–DMSO (4:1, v/v). All standard solutions were stored at 4 °C and protected from light (except ASA which was freshly prepared). Working standard solutions were prepared from these stocks by suitable dilution with acetonitrile–orthophosphoric acid (99:1, v/v). All working standards contained the IS, which was 12 μM. Human serum samples (100 μL) were spiked with known amounts (20 μL) of each compound and the IS. The samples were treated with 200 μL of 0.1% orthophosphoric acid in acetonitrile to precipitate the protein and diluted with the same solvent until the total volume was 0.4 mL. The samples were then vortexed for 30 s and centrifuged for 10 min at 12,000 rpm. The supernatant was transferred directly to the injection vial.

2.3. Procedure for the assay of dosage forms

The contents of l0 capsules or 10 finely ground tablets were weighed and mixed. The powder equivalent to one capsule (or one tablet) of each drug was accurately weighed out and dissolved via a 5 min sonication in the same solvent as the standards. The solution was diluted with the same solvent to a known volume in a calibrated flask and any remaining residue was removed by filtration through a dry filter paper. All solutions were stored at 4 °C and protected from light except Aspro *Roche*® which was freshly prepared. Appropriate dilutions were made before the analysis to obtain concentrations within the linear calibration range.

2.4. Instrumentation and chromatographic conditions

The HPLC/fluorescence system included an automatic injector (Waters 717 plus, Waters Corp., Milford MA), a degasser (Waters In-Line), a quaternary pump (Waters 600) with an oven (Waters) coupled to a fluorescence detector (Waters 474). Data acquisition was performed using Waters Millenium^{32} software, version 3.2. The chromatographic separation was performed on an analytical column ACE® C-18 with trimethylsilane (TMS) endcapping, 5 μm, 150 mm × 4.6 mm (AIT, France). The mobile phase was acetonitrile (solvent A) and 0.1% aqueous orthophosphoric acid (solvent B), and the elution was performed by programming both concentration and flow rate of mobile phase. A 10 min gradient program shown in Table 1 was employed using a temperature of 32 °C. The backpressure observed was lower than 2000 psi (138 bar), the injected volume was 20 μL.

Table 1
HPLC dual-mode gradient program

Time (min)	Flow rate (ml/min)	Acetonitrile (%)
0	1	40
3.5	1	55
5.5	2	70
7	3	70
8	3	70
9	3	40
10	3	40

Fluorescence detection was performed at 290 nm (λ_{ex}) and 445 nm (λ_{em}), with a bandwidth of 40 nm, a photomultiplier tube gain was set at 1000, and attenuation was fixed at 1, the electronic filter time constant was 3 s and the detection cell volume was 16 μL.

2.5. Standardization, sensitivity, and determination of reproducibility

Five different calibration standards of a mixture of ASA, SA, PIR, IS, and MF were prepared in acetonitrile–orthophosphoric acid (99:1, v/v). The standard curves were used to determine recovery of the chemicals from serum and pharmaceutical samples using the internal standard method. The response factors for each concentration were calculated from the ratio of peak area of analyte to that of the internal standard. Five determinations were performed for each concentration level. Validation of the procedure was determined by the constancy of the relationship between each concentration and the corresponding factor. Stability was examined by repeated injections the same day or on different days.

2.6. Recovery

The recoveries of different concentrations of each NSAID after protein precipitation were determined by comparing the response factor of precipitated drug-spiked serum samples with the response factor obtained from direct injections containing the same concentration of the standard drug. The recovery assessment was made on the pharmaceutical formulation samples instead of preparing placebos, so known amounts of each reference standard compound were spiked into their corresponding formulation in order to obtain three different levels of addition for each analyte. The recovery was calculated as follow:

$$\text{Recovery (\%)} = \left[\frac{\text{measured concentration} - \text{original concentration}}{\text{spiked concentration}}\right] \times 100 \quad (1)$$

Within-day precision was determined by five analyses of spiked samples at three different concentrations. Between-days precision was determined by analysis of spiked samples on five different days.

Table 2
Chromatographic parameters obtained in HPLC assay of NSAIDs

Compound	Capacity factor (k')	Peak asymmetry (A_s)	Resolution factor (R_s)	Selectivity factor (α)
ASA	1.16	1.13		
SA	1.82	1.14		
PIR	2.45	1.03		
MF	4.58	1.01		
ASA–SA			2.29	1.57
PIR–ASA			5.65	2.11
MF–ASA			17.12	3.95
MF–PIR			12.01	1.87

3. Results and discussion

3.1. Chromatography

Investigation of the influence of oven temperature on the retention behavior showed that an oven temperature of 32 °C

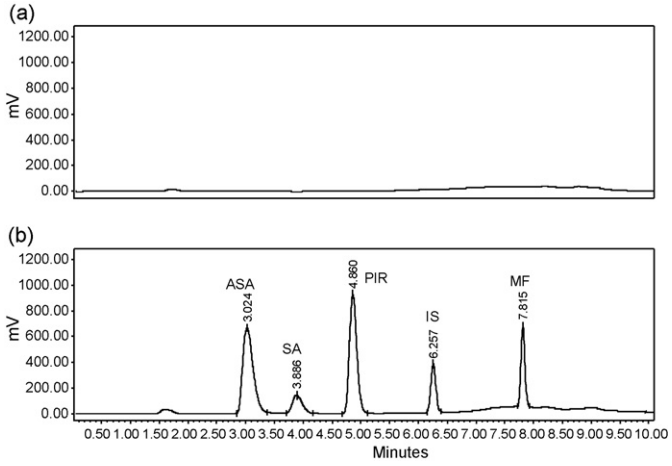

Fig. 2. Chromatograms: (a) the changes in the fluorescence of the effluent due to acetonitrile gradient; (b) NSAIDs in standard acetylsalicylic acid (ASA), salicylic acid (SA), piroxicam (PIR), naproxen (IS), mefenamic acid (MF).

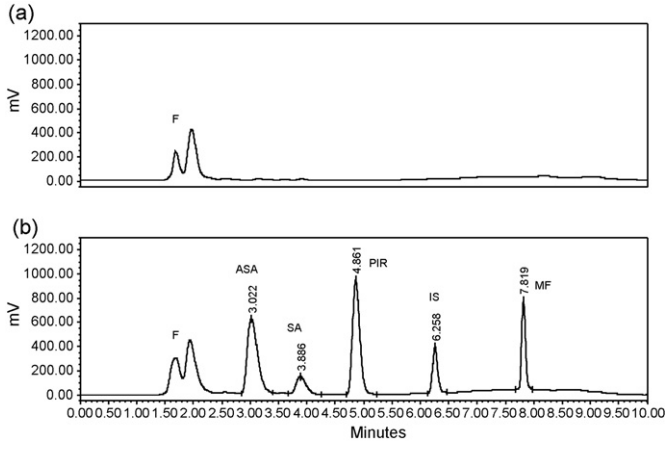

Fig. 3. Chromatograms of (a) blank serum showing (F) fluorescent endogenous compounds without interference with drugs being tested; (b) NSAID-spiked serum sample acetylsalicylic acid (ASA), salicylic acid (SA), piroxicam (PIR), naproxen (IS), mefenamic acid (MF).

Table 3
Performance parameters for the quantification of NSAIDs

	ASA	SA	PIR	MF
Regression equation	$y = 0.0017(\pm 0.0001)x + 0.0263(\pm 0.0018)$	$y = 1.202(\pm 0.05)x + 0.0063(\pm 0.0004)$	$y = 0.0361(\pm 0.0001)x - 0.0694(\pm 0.0299)$	$y = 0.017(\pm 0.0003)x + 0.0169(\pm 0.0110)$
Correlation coefficient	0.9993	0.9990	0.9990	0.9989
Linear range	27.8–2222 μM	0.11–4.4 μM	0.15–106 μM	0.2–104 μM
LOD	13.9 μM (277 pmol)[a]	0.05 μM (1.1 pmol)[a]	0.075 μM (1.5 pmol)[a]	0.10 μM (2.0 pmol)[a]
LOQ	27.80 μM	0.11 μM	0.15 μM	0.20 μM

All the concentrations mentioned above correspond to the actual concentrations in the serum samples after 4-fold dilution. y = peak area ratio [peak area for each analyte divided by the peak area for the IS on the same chromatogram] and x = analyte concentration (μM).
[a] The on-column LOD.

was the best compromise between minimizing the viscosity of the solvent mixture, minimizing degassing problems, minimizing the organic solvent employed and optimizing the separation. An acidic mobile phase consisting of acetonitrile–0.1% aqueous orthophosphoric acid was used to suppress ionization and to take advantage of the properties of the reversed-phase systems, this optimized the resolution and we observed no interference due to endogenous substances in serum or in pharmaceutical samples. The problem associated with the HPLC analysis of ASA is the hydrolysis tendency in protic solvents such as water and methanol, in water the rate of hydrolysis is dependent on pH with the best stability at pH 2–3; at pH 7 about 20% of ASA is degraded to SA within one day [42]. The orthophosphoric acid was added to the solvent and to the mobile phase to suppress the rate of degradation (hydrolysis) of ASA.

3.1.1. Dual-mode gradient elution

Flow-rate programming was introduced to reduce the time needed for separation and re-equilibration. This is a well accepted technique; programming of the organic concentration and the flow rate is commonly termed "dual-mode gradient" elution [39]. Yokoyama et al. [40] showed that dual-mode gradient chromatography was very reproducible, and Paci et al. [41] demonstrated a validation of a "dual-mode gradient" HPLC method for pharmaceutical quality control. Däppen and Molnar [43] showed that gradient elution improves separation and a segmented gradient leads to further improvement. Allan et al. [44] demonstrated that the gradient elution provides better detection compared to isocratic elution. The gradient program was optimized to obtain lower baseline fluctuations due to the small change in acetonitrile concentration required in the gradient elution. Fig. 2(a) shows the changes of the fluorescence of

Table 4
Within-day and between-days reproducibility data for NSAIDs (a) in the serum, (b) in pharmaceuticals

Theoretical concentration (μM)	Within-day ($n = 5$)		Between-days ($n = 5$)		Recovery (%)
	Measured concentration average (μM ± SD)	C.V. (%)	Measured concentration average (μM ± SD)	C.V. (%)	
ASA					
27.8[a]	26.1 ± 1.5	5.7	26.5 ± 1.9	7.2	95.3
228[a]	222 ± 14	6.3	220 ± 18	8.1	96.5
2222[a]	2173 ± 37	1.7	2191 ± 41	1.8	98.6
228[b]	225 ± 5	2.2	222 ± 4	1.8	97.4
556[b]	569 ± 12	2.1	557 ± 9	1.6	100.2
1112[b]	1085 ± 11	1.0	1072 ± 21	1.9	96.4
PIR					
0.15[a]	0.143 ± 0.006	4.2	0.141 ± 0.003	2.1	94.0
1.5[a]	1.47 ± 0.07	4.8	1.40 ± 0.05	3.6	93.3
106[a]	104.9 ± 2.4	2.3	105.8 ± 3.8	3.5	99.8
7.5[b]	7.45 ± 0.11	1.5	7.35 ± 0.12	1.6	98.0
15[b]	14.60 ± 0.16	1.1	14.30 ± 0.09	0.6	95.3
30[b]	29.8 ± 0.6	2.0	29.1 ± 0.6	2.1	97.0
MF					
0.2[a]	0.187 ± 0.007	3.7	0.192 ± 0.003	1.6	96.0
4[a]	3.96 ± 0.11	2.8	3.91 ± 0.16	4.1	97.8
104[a]	102.1 ± 1.4	1.4	103.0 ± 2.6	2.5	99.0
20[b]	21.6 ± 0.2	0.9	19.1 ± 0.4	2.1	95.5
40[b]	38.9 ± 0.5	1.3	37.9 ± 0.7	1.8	94.8
80[b]	78.2 ± 1.3	1.7	79.1 ± 1.5	1.9	98.9

Table 5
HPLC methods for the analysis of NSAIDs

Compounds	Matrix	IS	LOD (ng mL^{-1})	Chromatographic conditions						Refs.
				Anal. time (min)	Injected volume (μL)	Flow rate (mL min^{-1})	Elution mode	Detection	Column[a]	
ASA, **SA**, **PIR**, **MF**	Serum (100 μL) pharmaceuticals	NAP	15–2500	10	20	1–3	Dual-mode gradient (ACN/0.1% H_3PO_4)	Fluorescence	ACE® C 18, 5, 150 × 4.6	Present work
MF, IND	Plasma (50 μL)	IND or MF	60	10	20	0.9	Isocratic (ACN/10 mM H_3PO_4, 60:40, v/v) pH 2.6	UV (280 nm)	Vydac C18; 5, 250 × 4.6	[8]
MF	Serum (70 μL)	DIC	15	10	50	1	Isocratic (ACN/H_2O, 50:50, v/v) pH 3 (H_3PO_4)	UV (280 nm)	Techsphere C8; 3, 150 × 4.6	[25]
ASA, **SA**, PAR, CAF, PHE	Pharmaceuticals	–	90–170	13	nr[b]	2	Isocratic (ACN/H_2O, 25:75, v/v) pH 2.5 (H_3PO_4)	UV (207 nm)	Bio Sil HL C18; 5, 250 × 4.6	[27]
PIR	Plasma (250 μL)	NAP	20	12	50	1.5	Isocratic (0.1 M CH_3COONa/ACN/triethylamine, 61:39:0.05, v/v/v) pH 4	UV (330 nm)	Novapak C18; 4, 250 × 4.6	[28]
MF, **PIR**, DIC, FEB, FEL, FLB, IBU, IND, KET, LOX, NAP, SUL	Urine (1 mL)	IND or NAP	nr[b]	35	10–30	0.9	Isocratic (50 mM phosphate buffer/ACN, 58:42, v/v) pH 5	UV (230 or 320 nm)	Inertsil ODS-2; 5, 150 × 4.6	[29]
ASA, **SA**, GA, SLA	Plasma (200 μL)	MBA	75–100 pg; on-column	10	10	1	Isocratic (ACN/H_2O/H_3PO_4, 18:74:0.09, v/v/v) pH 2.5	UV (237 nm)	Novapak C18; 4, 150 × 3.9	[30]
PIR	Plasma (500 μL), Urine (1 mL)	NAP	50	5	100	2.5	Isocratic (ACN/0.1 M CH_3COONa, 33:67, v/v) pH 3.3	UV (330 nm)	Sphrisorb C18; 5, 250 × 4.5	[31]
MF, **PIR**, DIF, FEN, IBU, IND, KET, NAP	Plasma, serum	–	1000–5000	5	40–100	1.5–2.5	Isocratic (ACN/CH_3COONa) pH 4.2–4.8	UV (different for each compound)	Hypersil ODS; 5, 250 × 4	[32]
PIR, DIC, FEN, KET, NAP, PHZ, SUL	Plasma (200 μL)	PTA	50–2000	30	20	1	Isocratic (ACN/0.03% H_3PO_4, 45:55, v/v) pH 2.5 ± 0.2	UV (254 nm)	Bonapak C18; 10, 300 × 3.9	[33]
MF, CAR, DIC, DIF, FEB, FEN, FLB, FLU, IBU, IND, INP, KET, LON, NAP, NIF, PIP, SUL, ZOM	Urine (10 mL)	NPAA	nr[b]	45	10–50	0.8	Gradient (ACN/0.05 M Acetate buffer) pH 4.5	UV (254 nm)	Hibar C18; 5, 250 × 4	[34]
ASA, **SA**, GA, SLA	Plasma (100 μL), Urine (1 mL)	OTA or OAA	500	12	20	2	Isocratic (H_2O/MeOH/CH_3COOH, 64:25:1, v/v/v)	UV (238 nm)	μBondapak C18	[35]
PIR, DIF, IND, NAP, SUL	Plasma (20–200 μL)	IND	10–20	20	20	nr[b]	Isocratic (MeOH/0.025 mM phosphate buffer)	Amperometric at +0.9 V	Brownlee C18; 5, 100 × 4.6	[36]
SA	Pharmaceuticals	–	1	5	40	1.5	Isocratic (MeOH/2.5% CH_3COOH, 54:46, v/v)	Fluorescence	ODS C18; 10, 250 × 4.6	[38]
ASA, **SA**, **MF**, DIC, IBU	Pharmaceuticals	–	0.2–0.4 μM	5	20	1	Isocratic [ACN/(0.17 mM CH_3COOH + 0.25 mM CH_3COONa), 65:35, v/v]	Contactless conductivity	Nucleosil 120–5 C18; 5, 250 × 4.6	[47]
ASA, **MF**, ACM, ACT, DIC, FEN, FLB, FLU, IBU, IND, KET, LOX, NAP, OXA, PHZ, TOL	Plasma (500 μL)	–	10–900	7	5	0.4	Isocratic (ACN/10 mM $HCOONH_4$, 50:50, v/v) pH 3.5	MS (ESI)	Cosmosil C18-MS-II; 3, 100 × 2	[37]

ACM: acemetacin, ACT: acetaminophen, CAF: caffeine, CAR: carprofen, DIC: diclofenac, DIF: diflunisal, FEB: fenbufen, FEL: felbinac, FEN: fenoprofen, FLB: flurbiprofen, FLU: flufenamic acid, GA: gentisic acid, IBU: ibuprofen, IND: indomethacin, INP: indoprofen, KET: ketoprofen, LON: lonazolac, LOX: loxoprofen, MBA: 2-methyl-benzoic acid, NAP: naproxen, NIF: nifluminic acid, NPAA: *N*-phenyl-anthranilic acid, OAA: *o*-anisic acid, OTA: *o*-toluic acid, OXA: oxaprozin, PAR: paracetamol, PHE: phenobarbital, PHZ: phenylbutazone, PIP: pirprofen, PTA: *p*-toluic acid, SLA: salicyluric acid, SUL: sulindac, TOL: tolfenamic acid, ZOM: zomepirac. Bold abbreviations : molecules studied in this study.

[a] Column dimensions, in the order: particle size (μm), length × internal diameter (mm).

[b] nr: not reported.

the effluent due to the acetonitrile gradient elution. The retention time reproducibility was good with a relative standard deviation of less than 3% ($n = 10$). System suitability tests were performed and chromatographic parameters were calculated from experimental data, such as capacity factor (k'), peak asymmetry factor (A_s), selectivity factor (α) and resolution factor (R_s) are given in Table 2. The capacity factor ($1 < k' < 10$), selectivity factor ($\alpha > 1$), peak asymmetry ($1 < A_s < 1.2$) and resolution factor

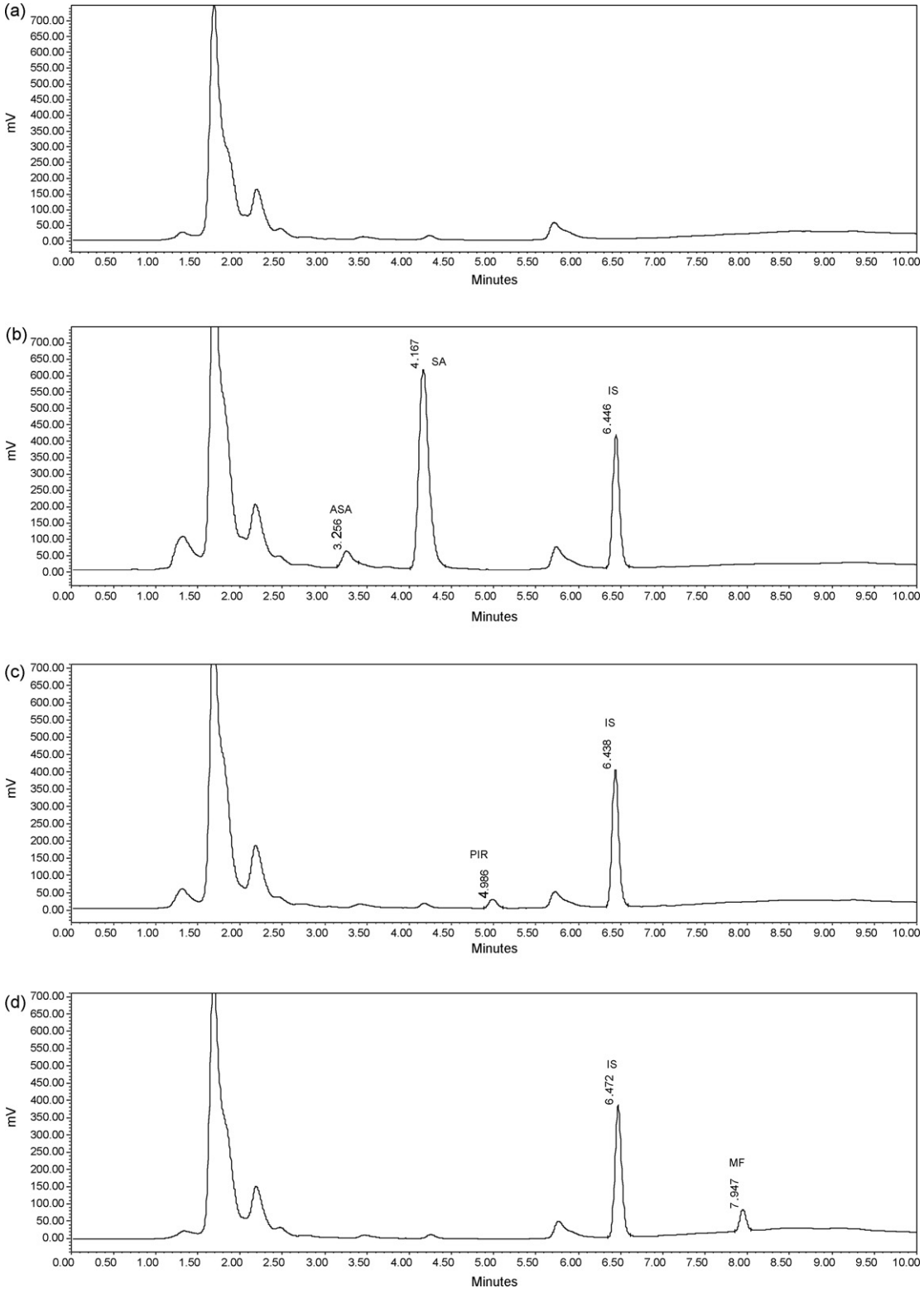

Fig. 4. Chromatograms of (a) blank serum, (b) serum sample 1 h after administration of 500 mg ASA p.o., (c) serum sample 2 h after administration of 20 mg PIR p.o. and (d) serum sample 2 h after administration of 250 mg MF p.o.

($R_s > 2$) were found to be satisfactory. Retention times were 3.02, 3.89, 4.86, 6.26, 7.82 min for ASA, SA, PIR, IS and MF, respectively. Fig. 2(b) shows representative chromatogram obtained with HPLC-fluorescence in the standards, and Fig. 3 shows that there is no interference from the endogenous compounds in the spiked serum sample.

3.2. Linearity and sensitivity [45,46]

Calibration curves with seven points were constructed by injecting a series of standard mixtures covering the tested concentration range. The equations were obtained by least-squares linear regression analysis of the peak area ratio of analyte/internal standard versus analyte concentration. The limit of detection (LOD) was defined as the compound concentration that produced a signal-to-noise ratio greater than three, while the limit of quantification (LOQ) of the assay was taken to be the concentration equal to or greater than 10 times the signal-to-noise ratio. The lowest concentration used for evaluation was chosen to indicate the lower limit of quantification for each compound. LOD and LOQ values for each compound based upon these criteria are shown in Table 3.

3.3. Precision and accuracy [45,46]

Table 4 summarizes the results of the determination of reproducibility regarding within-day and day-to-day assay precision and the recovery in the spiked samples. The intra-assay precision of the method based on within-day repeatability was performed, by replicate injections ($n = 5$) of three standard solutions covering three different concentration levels: low, medium and high, where peak areas were measured, in comparison to the peak area of the internal standard. The inter-assay precision (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of five different days. The measured concentrations had RSD values <9%. Accuracy of the methods, evaluated by means of recovery determinations, was determined by replicate analysis of three different concentrations. These data show that the statistical evaluation of this method is satisfactory. The salicylic acid content in tablets was found to be lower than 0.1% of the acetylsalicylic acid, which is in accordance with USP XXIV. All analytes were found to be stable for at least 24 h in the autosampler. This was confirmed by replicate injections of standard samples. After 3 months, stock solutions (stored at −20 °C and protected from light) did not show any degradation (peak areas ≈100% of the peak areas of freshly prepared solutions).

3.4. Application

The applicability of the method developed is illustrated by the analysis of serum samples collected from a healthy volunteer having taken separately 500 mg of ASA, 250 mg of MF and 20 mg of PIR per oral as a single dose. Fig. 4 shows the chromatograms of serum samples 2 h post-dose (1 h in case of ASA). Chromatogram of the corresponding blank serum is also displayed. These chromatograms show that no interferences originating from the metabolites of these compounds were observed.

3.5. Comparison with other methods for the compounds of interest

The obtained results compare our method over previously published ones (Table 5). Our method involves a simple work-up with better on-column LOD for MF, PIR and SA. Rouini et al. [25] developed an HPLC method using UV detection for the determination of only MF in serum. Their method provides almost the same retention time for MF as ours ($t_r = 7.4$ min), but it needs a preliminary liquid/liquid extraction of the sample with a consecutive evaporation step, which is time-consuming and tedious. Other workers [e.g. 28, 31] reported HPLC methods using UV detection for the determination of PIR in plasma. These studies involved a direct protein precipitation but they used larger plasma volumes (200–500 μL) and larger injected volumes (50–100 μL). Kubáň et al. [47] accomplished a simultaneous analysis of five NSAIDs in pharmaceuticals by HPLC using conductivity detection. Although the analysis time was shorter and the LOD for ASA was better than ours, they did not apply the method to the analysis of serum sample and the chromatographic peaks suffer from either peak tailing or poor resolution. In this work, we applied the method for the quantitative analysis in serum sample; this method required a slightly longer analysis time than Kubáňs method to eliminate the interferences of the endogenous compounds in serum samples but the sensitivity of our method is 3-4 fold better for SA and MF. Other workers [32,34,36] have reported the analysis of different NSAIDs, but different chromatographic conditions in the composition of the mobile phase and the detection wavelength were used. Suenami et al. [37] developed a LC/MS procedure for the analysis of NSAIDs. Although LC/MS provides good sensitivity and selectivity and provides information about the structure, the higher cost of a MS system and its cost of operation make it less attractive for routine analytical work.

4. Conclusion

HPLC coupled with fluorescence detection is a simple, rapid, selective and sensitive method and suitable for the routine analysis of ASA, PIR, MF in pharmaceuticals. The proposed method could also be used for the determination of salicylic acid as impurity in dosage forms containing acetylsalicylic acid. The work up procedure is simple, using deproteinization of serum samples and direct injection and without prior liquid–liquid phase extraction or solid phase extraction. The recovery from serum is more than 90%. The dual-mode gradient HPLC method developed for NSAIDs analysis has several advantages: standard instrumentation, a simple mobile phase, a simple gradient program, a highly acceptable separation given in reasonable time, and low running costs. The analytical results were reproducible and quantitative. The main advantage of the method is the ability to simultaneously analyze the four compounds under similar conditions.

The simultaneous measurement of NSAIDs concentrations in biological samples is required in clinical and toxicological pharmacokinetic studies, as well as in therapeutic monitoring.

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