ELSEVIER

Journal of Chromatography B, 692 (1997) 375–388

JOURNAL OF CHROMATOGRAPHY B

Simultaneous analysis of several non-steroidal anti-inflammatory drugs in human urine by high-performance liquid chromatography with normal solid-phase extraction

Toshio Hirai*, Shozo Matsumoto, Ikuo Kishi

Medical Research Laboratories, Lederle (Japan) Ltd., 1-6-34 Kashiwa-cho, Shiki-shi, Saitama 353, Japan

Received 2 August 1996; revised 1 November 1996; accepted 11 November 1996

Abstract

A practical and reproducible high-performance liquid chromatographic method using normal solid-phase extraction has been developed for the simultaneous analysis of twelve non-steroidal anti-inflammatory drugs (NSAIDs) in human urine. A urine specimen mixed with acetate buffer pH 5.0 was purified by solid-phase extraction on a Sep-Pak Silica cartridge. The analyte was chromatographed by a reversed-phase Inertsil ODS-2 column using a phosphate buffer–acetonitrile at pH 5.0 as the mobile phase, and the effluent from the column was monitored at 230 or 320 nm. Absolute recoveries were greater than 73% for all of the twelve NSAIDs. The present method enabled simple manipulation and isocratic HPLC with UV analysis as well as high sensitivity of 0.005 μg/ml for naproxen, and 0.05 μg/ml for sulindac, piroxicam, loxoprofen, ketoprofen, felbinac, fenbufen, flurbiprofen, diclofenac, ibuprofen and mefenamic acid as the quantitation limit in human urine using indomethacin as an internal standard.

Keywords: Naproxen; Indomethacin; Sulindac; Piroxicam; Loxoprofen; Ketoprofen; Felbinac; Fenbufen; Flurbiprofen; Diclofenac; Ibuprofen; Mefenamic acid

1. Introduction

Since acetylsalicylic acid was introduced in 1899, non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used as the first-choice agents in the treatment of patients with rheumatoid arthritis (RA).

The commercially available NSAIDs can broadly be divided into eight groups according to their structures: (a) salicylic acid derivatives, (b) phenylbutazone and related compounds, (c) pyrazolone derivatives, (d) indomethacin and related compounds, (e) arylalkanoic acid and related compounds, (f) anthranilic acid derivatives, (g) oxicam derivatives and (h) miscellaneous drugs, and the groups of (a), (d), (e) and (g) are frequently used for RA therapy.

Recently, it has been shown that cyclooxygenase (COX), a key enzyme of prostaglandine synthesis, exists in two isoforms [1,2]; COX-1 which provides the cytoprotective effect in the digestive organs and kidney [3], and COX-2 which is induced under inflammatory conditions [4,5]. It has been thought, therefore, that the currently used NSAIDs show analgesic, antiphlogistic and antipyretic effects by inhibition of COX-2 but appear with gastrointestinal,

*Corresponding author.

PII S0378-4347(96)00509-9

renal and other common side effects in NSAIDs by simultaneous inhibition of COX-1 [6,7]. In the case of long-term NSAIDs therapy, a high incidence of severe side effects, including gastrointestinal ulcer and nephrosis, has been reported, especially in RA therapy [8–10]. It is also of concern that many medications, including D-penicillamine, steroids and methotrexate used as antirheumatic agents, antihypertensives and antianxietics, as well as other drugs used, lead to complications when they are frequently co-administered to RA patients, and the results of the drug interactions involve severe side-effects [11,12]. Hence, it is considered that the monitoring of NSAIDs is of importance in order to improve the toxicological management of long-term NSAID therapy.

Although a number of high-performance liquid chromatographic (HPLC) methods have been described for the determination of commercially available NSAIDs in biological fluids, these papers deal with the assay of a single compound or a few compounds [13–36]. Battista et al. [37] accomplished a screening method for 18 NSAIDs in human urine by HPLC with UV 254 nm, but the gradient elution was employed and several compounds were overlapped. Streete [38] also reported the determination of eight NSAIDs in human plasma or serum by HPLC with UV detection, but different chromatographic conditions in the composition of the mobile phase and the detection wavelength were used for each compound. These methods also lacked sensitivity. Singh et al. [39] developed a simultaneous analysis of seven NSAIDs in horse plasma and urine by using either photodiode-array detection or a gas chromatography–mass spectrometry system, but the sensitivity depended on the gas chromatographic–mass spectrometric analysis. Kazemifard and Moore [40] accomplished a high sensitivity of 10–20 ng/ml as the detection limit for five NSAIDs in plasma by using electrochemical detection and reversed solid-phase extraction (Sep-Pak C_{18} cartridge), but only electroactive compounds are applicable.

A method combining simple manipulation and a wide use of instruments with no special assembly would seem to be very useful in routine clinical use. For this purpose the authors [41] previously established a favourable sample clean-up procedure using normal solid-phase extraction for drug analysis in urine using HPLC with UV detection, and successfully applied it to the determination of folic acid antagonist methotrexate and its metabolite 7-hydroxymethotrexate. Applying this sample clean-up procedure in this study, a simultaneous analytical method for twelve NSAIDs used for RA therapy in human urine by isocratic HPLC has been developed.

2. Experimental

2.1. Materials and reagents

All substances and their abbreviations used in this study are shown in Fig. 1.

FEN and FEL were kindly supplied by Lederle Laboratories (Pearl River, NY, USA). DIC, FLB, IBP, IND, KEP and MFA were obtained from Wako (Osaka, Japan), SUL and PIR were from Sigma-Aldrich Japan (Tokyo, Japan) and NAP was from Seikagaku Kogyo (Tokyo, Japan). LOX was extracted from commercial preparations. The extraction was made with ethanol, and the crude extract was recrystallized from ether-hexane. Sep-Pak Silica cartridge used for sample treatment and was purchased from Waters (Milford, MA, USA). Acetonitrile was HPLC grade and other chemicals used in this study were of analytical grade, and they were all from Wako (Osaka, Japan) or Kanto (Tokyo, Japan).

2.2. Instrumentation and chromatography

The chromatographic system consisted of a Waters 600E solvent delivery pump (Milford, MA, USA) equipped with a JASCO 851-AS intelligent autosampler (Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength detector (Kyoto, Japan). A Millennium 2010 Chromatography Manager (Waters Japan, Tokyo, Japan) was used for chromatographic peak integration.

Chromatography was performed on a reversed-phase Inertsil ODS-2 column (5 μm particle size, 150×4.6 mm I.D. from GL Science, Tokyo, Japan) using 50 m*M* phosphate buffer–acetonitrile (58:42, v/v) at pH 5.0 as the mobile phase at a flow-rate of 0.9 ml/min under an ambient temperature. The effluent from the column was monitored at 230 nm without any description. The capacity factor (k') was

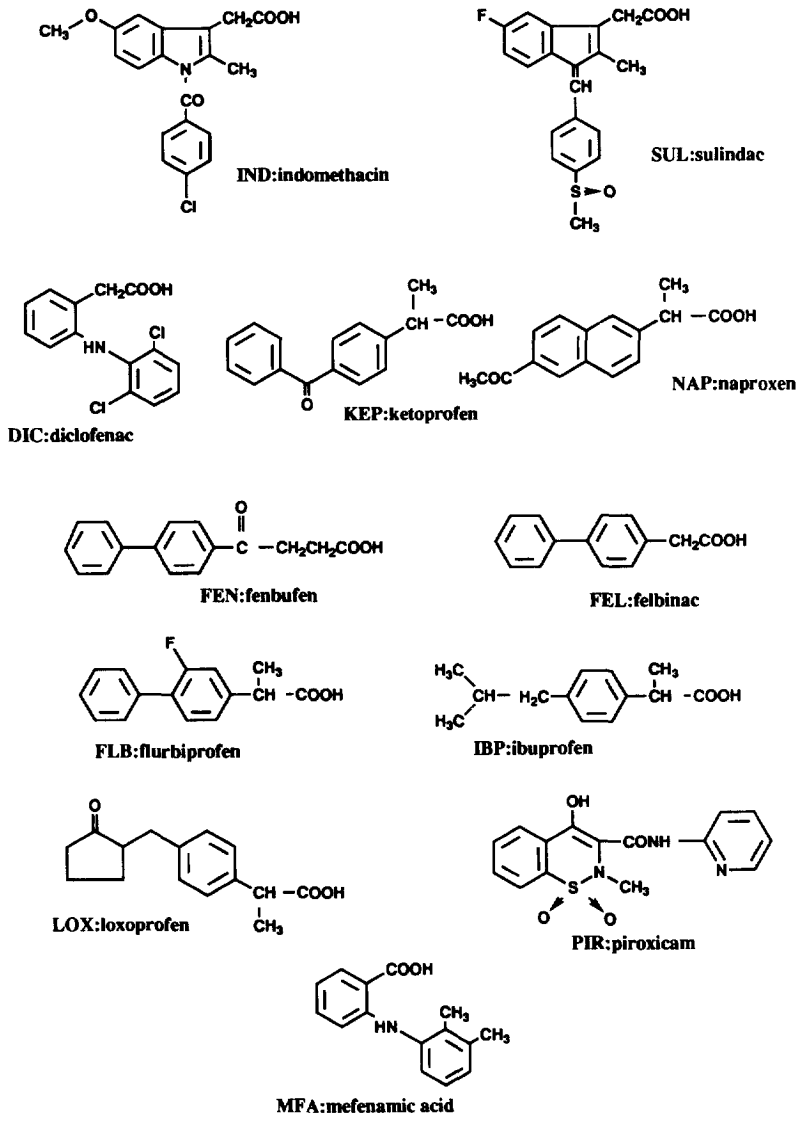

Fig. 1. Structures and abbreviations of NSAIDs used in this study.

calculated as $(t_r - t_o)/t_o$, where t_r is the retention time of the analyte and t_o is the retention time of the void elution determined by the injection of methanol.

2.3. Solutions

The 1 mg/ml stock solution of each NSAID without PIR was prepared in methanol. In PIR, 0.5 or 0.6 mg/ml stock solution was prepared because of its poor solubility in methanol. All stock solutions were stable for at least 1 month when stored at −20°C in a freezer.

Working solution I used for the recovery study was made by mixing with proper amounts of each of twelve NSAIDs stock solutions followed by dilution with methanol, containing 5 μg/ml for NAP, 60 μg/ml for PIR and 50 μg/ml for SUL, LOX, KEP, FEL, FEN, FLB, DIC, IND, IBP and MFA. Working solution II used for the preparation of standard calibration curves and other quality-control samples

was prepared in the same manner as above, without IND which was used as an internal standard, containing 5 μg/ml for NAP and 50 μg/ml for SUL, PIR, LOX, KEP, FEL, FEN, FLB, DIC, IBP and MFA. The internal standard solution (20 μg/ml) was made by a dilution of the IND stock solution with methanol. Further dilutions of working solutions I and II were made with methanol, and these working and internal standard solutions were freshly prepared each week and stored at 4°C in a refrigerator.

2.4. Sample preparation

The Sep-Pak Silica cartridge used for sample treatment was previously washed with 10 ml of ethyl acetate, and dried by aspiration of air.

An aliquot of 100 μl of internal standard solution was evaporated to dryness in a water bath at 37°C, 1.0 ml of urine sample was added, and it was vortex mixed. A 250 μl volume of 1 *M* acetate buffer pH 5.0 was added to the sample, vortex mixed, and an aliquot of 250 μl of the resulting mixture was directly loaded onto a Sep-Pak Silica cartridge, and dried by aspiration of air. The cartridge was then eluted with 3 ml of ethyl acetate, and the eluate was evaporated to dryness under a stream of nitrogen in a water bath at 37°C. The dried residue was reconstituted with 200 μl of mobile phase, and 10–30 μl of the reconstituent was injected onto HPLC.

'Spiked samples' were prepared as follows: proper amounts of working solutions I and II or their dilutions were evaporated to dryness under a stream of nitrogen in a water bath at 37°C, 1.0 ml of drug-free human urine was added and vortex mixed. The standard calibration samples were freshly prepared each day, and others were frozen at −20°C until analytical use.

The recovery and hydrolysed samples were assayed without internal standard.

2.5. Biological samples

Drug-free urine used in this study was obtained from healthy investigators.

For method application: (A) urine of 0–4 h post dose was collected from a healthy volunteer who received once orally 150 mg of IBP, and (B) urine of 0–10 h post dose was collected from a healthy volunteer who received once orally 200 mg of NAP. Urine pH and volume were measured, and the sample aliquots of ca. 100 ml each were frozen at −20°C until analytical use.

2.6. Method validation

2.6.1. Recovery and reproducibility

Absolute recovery was determined for urine with 0.02, 0.1 and 0.5 μg/ml as the final concentration for NAP, 0.24, 1.2 and 6.0 μg/ml for PIR, and 0.2, 1.0 and 5.0 μg/ml for SUL, LOX, KEP, FEL, FEN, FLB, DIC, IND, IBP and MFA, by assaying 6–8 replicate samples at each concentration. The absolute peak heights of NSAIDs obtained for the extracted samples were compared with those of known amounts of fresh standards prepared in mobile phase.

Within day reproducibility was also evaluated during this experiment.

2.6.2. Linearity

The linearity was assessed over the concentration range 0.005–1.0 μg/ml urine for NAP, and 0.05–10.0 μg/ml urine for SUL, PIR, LOX, KEP, FEL, FEN, FLB, DIC, IBP and MFA by assaying duplicates at each of 7 concentrations. In the low concentration range (0.05–1.0 μg/ml urine) of SUL and PIR, a wavelength of 320 nm was also used for the chromatographic peak detection.

2.6.3. Inter-assay accuracy and precision

To determine the inter-assay accuracy and precision, drug-free human urine was spiked with each analyte at three different concentrations: 0.005, 0.05 and 0.5 μg/ml urine for NAP, and 0.05, 0.5 and 5.0 μg/ml urine for SUL, PIR, LOX, KEP, FEL, FEN, FLB, DIC, IBP and MFA. In the lowest concentration (0.05 μg/ml) of SUL and PIR, a wavelength of 320 nm was used for the peak detection in the place of 230 nm. The reproducibility was assessed over 5 consecutive days.

2.7. Quantitation

The concentrations of NSAIDs in the sample urine were calculated by comparison of their respective NSAIDs/internal standard peak height ratios with

those of calibration samples prepared by spiking drug-free human urine with NSAIDs.

2.8. Deconjugation

The urine samples of drug-free and spiked human urine containing 0.5 μg/ml for NAP, 6.0 μg/ml for PIR, and 5.0 μg/ml for SUL, LOX, KEP, FEL, FEN, FLB, DIC, IND, IBP and MFA as the final concentration were used in this experiment.

Three kinds of hydrolyses were examined, as follows: (1) Enzymatic hydrolysis: 0.5 ml of sulfatase type H1 from *Helix pomatia* (Sigma, Product No. S 9626)–1 *M* acetate buffer of pH 5.0 solution (containing 900 U of β-glucuronidase and 50 U of sulfatase) was added to 1.0 ml of urine, and incubated at 37°C for 24 h; (2) Acidic hydrolysis: 1.0 ml of 6 *M* hydrochloric acid was added to 1.0 ml of urine, and incubated at 60°C for 1 h; and (3) Alkaline hydrolysis: 1.0 ml of 1 *M* sodium hydroxide was added to 1.0 ml of urine, and allowed to stand for 1 h.

After hydrolysis, each of the resulting mixtures was assayed according to the method described in Sections 2.2–2.4.

3. Results

3.1. Selection of detector wavelength

As shown in Fig. 2, the UV absorption spectra of the compounds used in this study exhibited differences in maximum absorbance; LOX and IBP showed no measurable absorption above 240 nm. In this study, therefore, we set the wavelength to 230 nm. NAP had a maximum absorption at this wavelength, and showed a sensitivity relatively higher than the other compounds. In SUL and PIR, there

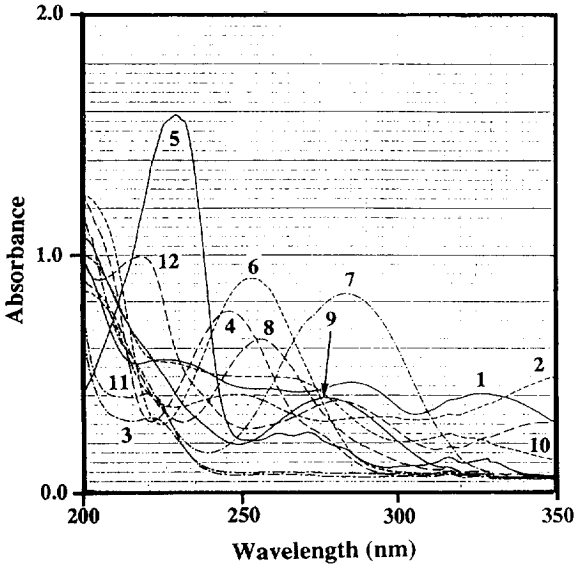

Fig. 2. UV absorption curves for SUL (1), PIR (2), LOX (3), KEP (4), NAP (5), FEL (6), FEN (7), FLB (8), DIC (9), IND (10), IBP (11) and MFA (12). Solvent: 50 m*M* phosphate buffer–acetonitrile (58:42, v/v) at pH 5.0; concentration: 10 μg/ml each.

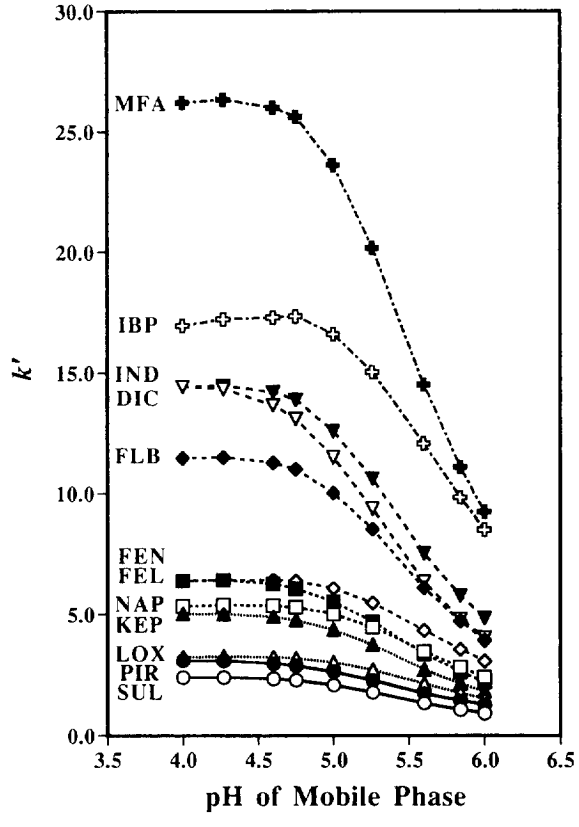

Fig. 3. Relation between *k'* values of NSAIDs and pH of mobile phase. Column: Inertsil ODS-2, 5 μm, 150×4.6 mm I.D. Mobile phase: 50 m*M* phosphate buffer–acetonitrile (58:42, v/v), 0.9 ml/min, room temperature. Monitor: UV 230 nm.

were small differences in the intensity of absorption curves between 230 and 350 nm.

3.2. Chromatographic separation

In our preliminary study, a phosphate buffer–acetonitrile mobile phase was the most suitable for the separation of acidic NSAIDs by reversed-phase HPLC. As shown in Fig. 3, the k' values in all substances used in this study rapidly decreased in response with increases in the pH of the mobile phase above 5.0, whereas there were little changes up to pH 4.5 under the chromatographic conditions described in Section 2. Although relatively stable k' values were obtained in all compounds at pH 4.0, compounds of LOX and PIR, FEL and FEN, and DIC and IND were overlapped. At pH 6.0, on the other hand, slight changes in the pH of the mobile phase affected the separation. A complete separation with constant k' values in all twelve compounds could be achieved at pH 5.0.

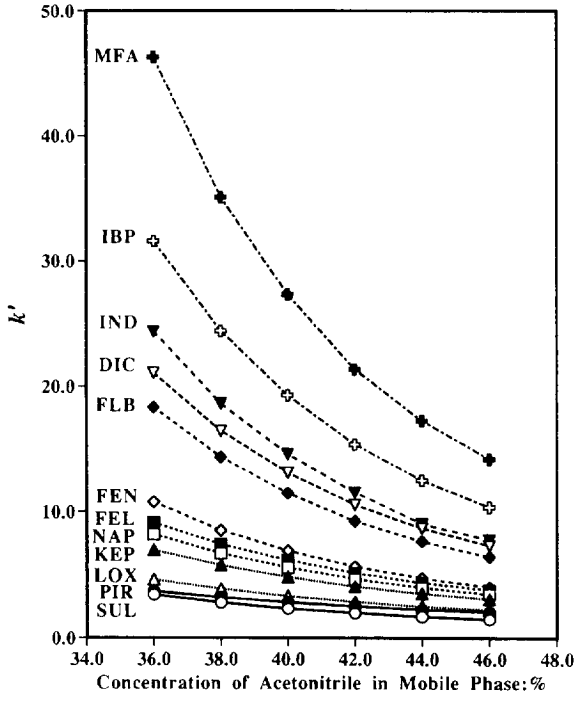

Fig. 4. Relation between k' values of NSAIDs and concentration of acetonitrile in mobile phase. Mobile phase: 50 m*M* phosphate buffer–acetonitrile at pH 5.0. The other chromatographic conditions are the same as for Fig. 3.

Fig. 4 shows a relation between the k' values and the concentration of the acetonitrile in mobile phase at pH 5.0. Increasing the contents of the acetonitrile in the mobile phase decreased the k' values proportionally in all twelve compounds.

Fig. 5 shows typical chromatograms of standard, drug-free and spiked human urine samples obtained according to the method described in Section 2. The peaks corresponding to the compounds used in this study were well separated and were sharp and

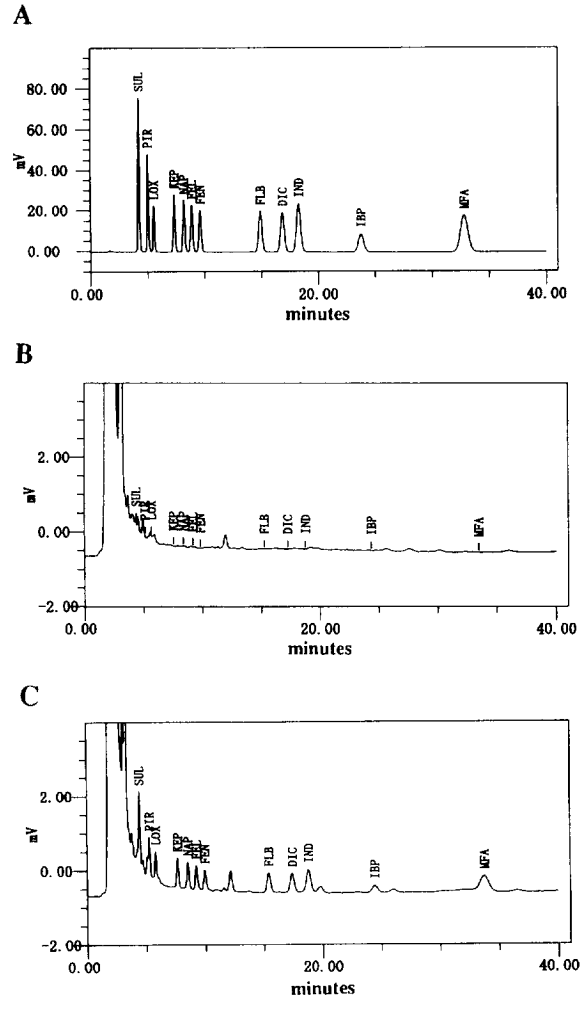

Fig. 5. Chromatograms of (A) standard, (B) drug-free human urine and (C) urine spiked with 0.02 μg/ml as the final concentration for NAP, 0.24 μg/ml for PIR, and 0.2 μg/ml for SUL, LOX, KEP, FEL, FEN, FLB, DIC, IND, IBP and MFA. The chromatographic conditions are given in Section 2.

symmetric, and chromatography was favourably done within 40 min by using the mobile phase containing 42% of acetonitrile. No endogenous peak interfered at the same time positions corresponding to the compounds, without SUL and PIR, even at a detection wavelength as low as 230 nm. Slight interfering peaks were observed in SUL and PIR, but were solved by shifting the detector wavelength to 320 nm (Fig. 6).

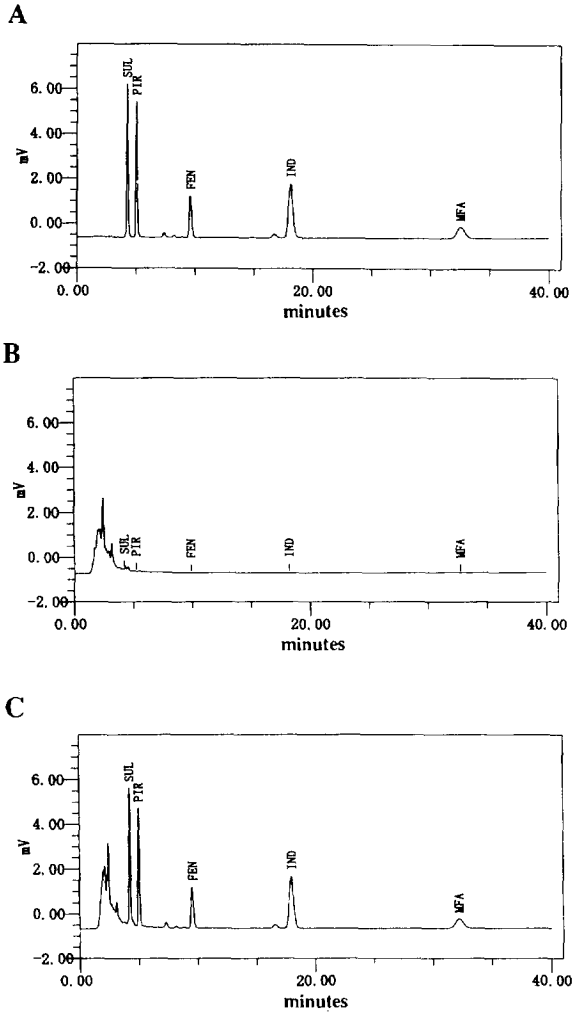

Fig. 6. Chromatograms of (A) standard, (B) drug-free human urine and (C) urine spiked with 1.2 μg/ml as the final concentration for PIR, 2.0 μg/ml for IND, and 1.0 μg/ml for SUL, FEN and MFA. The chromatographic conditions are the same as for Fig. 5, with the UV detector set at 320 nm.

3.3. Recovery and precision

Table 1 shows the absolute recovery and precision for each of twelve NSAIDs used in this study, by normal solid-phase extraction. Recoveries were greater than 73% from human urine over the concentration range 0.02–0.5 μg/ml for NAP, 0.24–6.0 μg/ml for PIR, and 0.2–5.0 μg/ml for SUL, LOX, KEP, FEL, FEN, FLB, DIC, IND, IBP and MFA. In all cases within day coefficients of variation were better than 6.4%.

Table 1
Within-day reproducibility and absolute recovery for the determination of NSAIDs in human urine

Compound	Amount added (μg/ml)	*n*	Recovery (mean±S.D.) (%)	C.V. (%)
SUL	0.2	6	74.9±3.30	4.41
	1.0	8	76.6±3.34	4.37
	5.0	7	79.0±2.00	2.54
PIR	0.24	6	76.7±0.90	1.17
	1.2	8	73.1±1.24	1.69
	6.0	7	80.0±0.95	1.19
LOX	0.2	6	94.6±6.04	6.39
	1.0	8	95.1±2.34	2.46
	5.0	7	94.3±2.03	2.15
KEP	0.2	6	93.8±1.62	1.71
	1.0	8	92.6±2.40	2.59
	5.0	7	93.5±1.76	1.88
NAP	0.02	6	92.9+1.48	1.59
	0.1	8	91.8±2.32	2.53
	0.5	7	92.6±2.10	2.27
FEL	0.2	6	95.0±2.05	2.16
	1.0	8	91.2±2.43	2.66
	5.0	7	91.9±1.91	2.08
FEN	0.2	6	87.3±1.43	1.64
	1.0	8	88.6±2.09	2.36
	5.0	7	90.4±2.24	2.47
FLB	0.2	6	89.6±1.71	1.91
	1.0	8	89.8±2.54	2.83
	5.0	7	91.0±2.07	2.28
DIC	0.2	6	88.4±0.87	0.99
	1.0	8	88.6±2.32	2.62
	5.0	7	90.5±2.09	2.31
IND	0.2	6	87.0±1.30	1.50
	1.0	8	86.2±2.65	3.08
	5.0	7	87.9±2.28	2.60
IBP	0.2	6	73.4±4.51	6.15
	1.0	8	80.9±2.99	3.69
	5.0	7	78.0±2.32	2.98
MFA	0.2	6	81.1±2.49	3.07
	1.0	8	78.2±2.25	2.88
	5.0	7	82.1±2.54	3.09

3.4. Linearity

A linear relationship was established between the peak height ratio and concentration for each NSAID in spiked urine samples. Table 2 lists the correlation coefficients, γ, slope, x- and y-axis intercept for each NSAID when IND was used as an internal standard. The calibration lines were linear over the concentration range 0.005–1.0 μg/ml for NAP, 0.1–10.0 μg/ml for SUL and PIR, and 0.05–10.0 μg/ml for LOX, KEP, FEL, FEN, FLB, DIC, IBP and MFA. In the low concentration range (0.05–1.0 μg/ml) of SUL and PIR, linear regressions were also obtained by shifting the detector wavelength at 320 nm. The correlation coefficients, γ, were better than 0.999 in all cases.

3.5. Inter-assay variability

The inter-assay accuracy and precision for each NSAID in urine are summarized in Table 3. The coefficients of variation at the lowest concentration (0.05 μg/ml) were less than 5% for FEN and MFA, 5–10% for PIR (detected at 320 nm), LOX, KEP, NAP (0.005 μg/ml), FEL and DIC, and 10–15% for SUL (detected at 320 nm), FLB and IBP when IND was used as an internal standard.

3.6. Study on hydrolysis

When enzymatic hydrolysis was carried out with 1.0 ml of human urine containing twelve NSAIDs used in this study and 0.5 ml of enzyme solution (Sulfatase–1 *M* acetate buffer pH 5.0) at 37°C for 24 h, recoveries of IND, DIC and MFA were decreased. In the case of acidic hydrolysis by strong acid (3 *M* HCl as the final concentrations at 60°C for 1 h), SUL, PIR, IND and MFA were decreased and DIC disappeared. When alkaline hydrolysis (0.5 *M* NaOH as the final concentrations at ambient temperature for 1 h) was conducted, IND was lost and SUL was decreased. LOX, KEP, NAP, FEL, FEN, FLB and IBP were constantly recovered with or without these three hydrolysing processes. There were hardly any interfering peaks on the chromatogram during all three hydrolysing procedures when drug-free human urine was subjected to the assay. These results are shown in Fig. 7.

3.7. Method application

Chromatograms obtained in the analysis of sample A (a 0–4 h urine sample collected from a healthy volunteer who received once orally 150 mg of IBP) and sample B (a 0–10 h urine sample collected from a healthy volunteer who received once orally 200 mg

Table 2
Linear estimation for the determination of NSAIDs in human urine

Compound	Range (μg/ml)	Monitor UV (nm)	Linear estimation		
			x	y	γ
SUL	0.1–10.0	230	0.9554	−0.0302	0.99990
	0.05–1.0	320	0.5108	0.0058	0.9995
PIR	0.1–10.0	230	1.5819	−0.0355	0.9998
	0.05–1.0	320	0.6260	−0.0142	0.9998
LOX	0.05–10.0	230	2.2403	−0.0308	0.99994
KEP	0.05–10.0	230	1.8275	−0.0103	0.99995
NAP	0.005–1.0	230	0.19433	−0.00023	0.99995
FEL	0.05–10.0	230	2.3403	−0.0127	0.99990
FEN	0.05–10.0	230	2.6588	−0.0248	0.99994
FLB	0.05–10.0	230	2.5377	0.0355	0.9997
DIC	0.05–10.0	230	2.8057	0.0206	0.99990
IBP	0.05–10.0	230	6.1130	0.0601	0.9991
MFA	0.05–10.0	230	3.9479	0.0417	0.99990

IND was used as an internal standard.

Table 3
Inter-assay accuracy and precision for the determination of NSAIDs in human urine ($n=5$)

Compound	Amount added (μg/ml)	Amount found (mean±S.D.) (μg/ml)	C.V. (%)	Bias (%)
SUL	0.05	0.050±0.0070	13.9	0.0
	0.5	0.529±0.0327	6.2	5.9
	5.0	5.038±0.1972	3.9	0.8
PIR	0.05	0.050±0.0044	8.8	0.0
	0.5	0.480±0.0191	4.0	−3.9
	5.0	0.496±0.0662	1.3	−0.8
LOX	0.05	0.055±0.0054	9.8	10.8
	0.5	0.509±0.0487	9.6	1.8
	5.0	5.036±0.1495	3.0	0.7
KEP	0.05	0.049±0.0036	7.2	−1.2
	0.5	0.496±0.0202	4.1	−0.8
	5.0	0.500±0.1126	2.3	−0.0
NAP	0.005	0.0048±0.00040	8.3	−4.0
	0.05	0.049±0.0026	5.3	−1.8
	0.5	0.499±0.0139	2.8	−0.1
FEL	0.05	0.048±0.0046	9.5	−3.2
	0.5	0.488±0.0238	4.9	−2.4
	5.0	4.984±0.1174	2.4	−0.3
FEN	0.05	0.058±0.0026	4.5	15.2
	0.5	0.488±0.0254	5.2	−2.4
	5.0	4.957±0.1177	2.4	−0.9
FLB	0.05	0.051±0.0056	11.1	1.6
	0.5	0.494±0.0250	5.1	−1.2
	5.0	4.936±0.1275	2.6	−1.3
DIC	0.05	0.056±0.0047	8.3	12.8
	0.5	0.502±0.0393	7.8	0.4
	5.0	4.944±0.1475	3.0	−1.1
IBP	0.05	0.049±0.0072	14.7	−1.6
	0.5	0.483±0.0344	7.1	−3.4
	5.0	4.853±0.1960	4.0	−2.9
MFA	0.05	0.049±0.0024	4.9	−2.4
	0.5	0.496±0.0241	4.9	−0.8
	5.0	4.929±0.1292	2.6	−1.4

IND was used as an internal standard.

of NAP) are shown in Figs. 8 and 9, respectively. Enzymatic and acidic hydrolysis were also carried out according to the methods described in the Section 2. The urinary concentration and excretion rate of each intact were 2.31 μg/ml and 0.46% of dose (0–4 h) for IBP, and 1.79 μg/ml and 1.25% of dose (0–10 h) for NAP, respectively. On the other hand, the total values (free+conjugate) obtained by deconjugation were ca. 50% higher in the acidic than they were in the enzymatic hydrolysis for IBP, whereas they were consistent for both hydrolyses for NAP (Table 4).

4. Discussion

A direct injection of diluted urine [15–20,39] or liquid–liquid extraction as the sample clean-up procedure [21–30,37] are frequently used for determining NSAIDs in urine by HPLC. In the direct injection, however, they lack in sensitivity and the removal of the interferences is insufficient. For the liquid–liquid extraction procedure, on the other hand, a complex manipulation, e.g., back extraction and/or some derivatization techniques included in order to increase the sensitivity, is often carried out

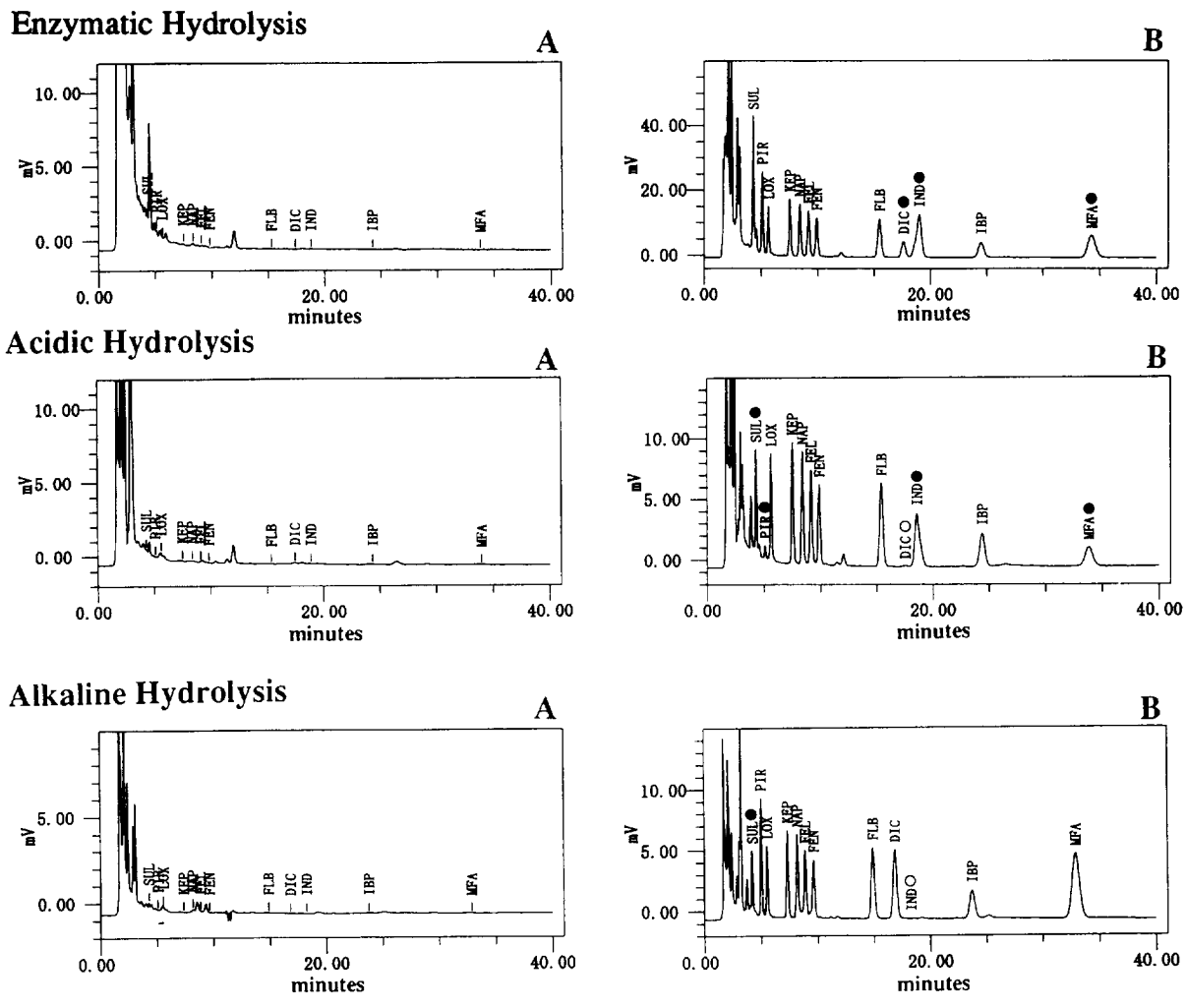

Fig. 7. Chromatograms of the hydrolysed human urine extracts. (A) Drug-free human urine; (B) urine spiked with 0.5 μg/ml as the final concentration for NAP, 6.0 μg/ml for PIR, and 5.0 μg/ml for SUL, LOX, KEP, FEL, FEN, FLB, DIC, IND, IBP and MFA. (○) Peak disappeared; (●) recovery decreased. Each hydrolysing condition is shown in Section 2, and the chromatographic conditions are the same as for Fig. 5.

to avoid the endogenous interferences, and almost all of them are not applicable in the simultaneous analysis of the plural number of NSAIDs. Kazemifard and Moore [40] reported the isolation of diflunisal, IND, NAP, PIR and SUL in plasma by reversed solid-phase extraction (Sep-Pak C_{18} cartridge), but the recovery of PIR was low and did not apply to urine analysis. Under these circumstances a favourable sample purification technique corresponding to the simultaneous analysis of a lot of NSAIDs (more than ten compounds) in urine by HPLC has not yet been established.

The authors [41] previously established, using normal solid-phase extraction (Sep-Pak Silica cartridge), a simple and highly sensitive analytical procedure for folic acid antagonist methotrexate and its main metabolite 7-hydroxymethotrexate in human urine by HPLC with UV detection. Then we found over 70% of several acidic NSAIDs are recovered in the fraction of ethyl acetate which is the first

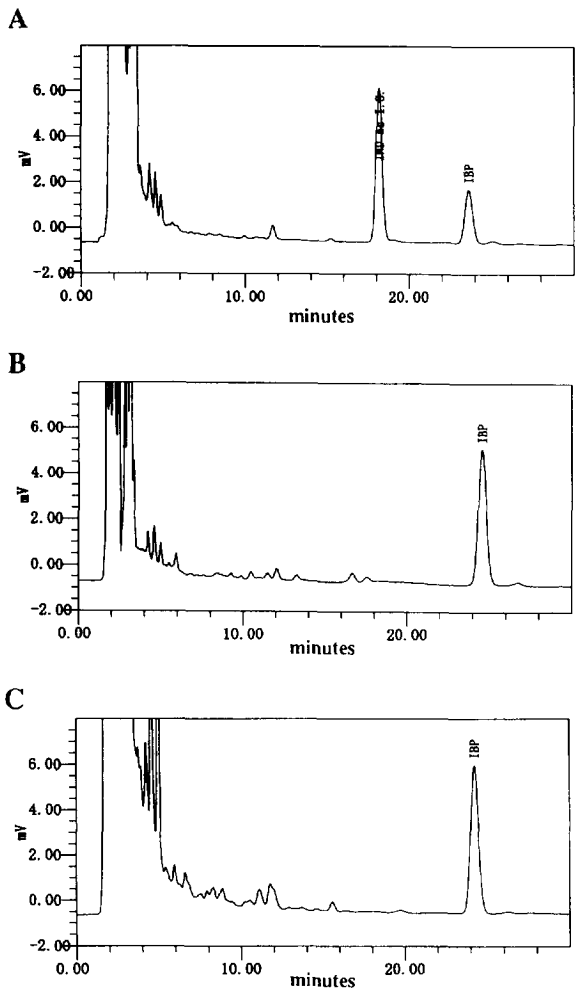

Fig. 8. Chromatograms obtained in the analysis of 0–4 h urine after single oral administration of 150 mg of IBP to a healthy volunteer. (A) Drug-free analysis; (B) acidic hydrolysis; (C) enzymatic hydrolysis. UV detection at 230 nm.

washing solvent of the Sep-Pak Silica cartridge loaded urine sample. In this experiment, we accordingly applied this sample clean-up procedure to the determination of twelve NSAIDs used for RA therapy in human urine by isocratic HPLC with UV detection. In the results, urinary interferences were able to be favourably removed without washing the cartridge loaded urine sample, using a sample clean-up procedure described in Section 2, thereby leading to a high sensitivity of 0.005 μg/ml for NAP, and 0.05 μg/ml for LOX, KEP, FEL, FEN, FLB, DIC, IBP and MFA as the quantitation limit in human urine using IND as an internal standard, using HPLC with UV detection at 230 nm. Slight interfering peaks were observed in SUL and PIR, but were solved by shifting the detector wavelength to 320 nm, and this also led to 0.05 μg/ml as the quantitation limit.

In humans, it is well known that urinary excretion of the parent is very low, as small as 5% of the dose in many acidic NSAIDs. Almost all of the parent and its phase I metabolites are eliminated mainly via phase II metabolism by conjugation to their corresponding glucuronides or sulfates [13,17,21,25, 27,29,42–47]. Several deconjugating procedures, i.e., enzymatic, acidic or alkaline hydrolysis, are accordingly performed for urine analysis of each individual NSAID used in this study, as listed in Table 5. We searched for the possibility of common hydrolysing conditions in the twelve NSAIDs used in this study, by examining the conditions in each of three hydrolyses with reference to Table 5. Ten compounds except SUL and IND were constantly recovered from urine with or without the alkali treatment, whereas IND was decreased or disappeared during all three hydrolysing processes. The data suggested that IND is not suitable as an internal standard for total parent analysis (hydrolysing procedure). However, LOX, KEP, NAP, FEL, FEN, FLB and IBP might be suitable for use for not only free but also total parent analysis because these were constantly recovered with or without three hydrolysing processes. SUL was constantly recovered only when the conditions of enzymatic hydrolysis applied. Urinary interferences could also favourably be removed from urine treated with each of the three kinds of hydrolyses, by using normal solid-phase extraction developed in this study. These results and Table 5 suggest: (1) normal solid-phase extraction with Sep-Pak Silica cartridge is available as a sample clean-up procedure for not only free but also total parent (free+conjugate) analysis of NSAIDs in urine by HPLC; (2) alkaline hydrolysis is applicable for the common hydrolysing conditions of ten compounds except SUL and IND used in this study if the conjugates of NAP, FEL, FEN, FLB and DIC are completely hydrolysed (although we can not yet confirm this); (3) in NAP, FEL, FEN and FLB, however, the total parent can be analyzed by using acidic hydrolysis; and (4) en-

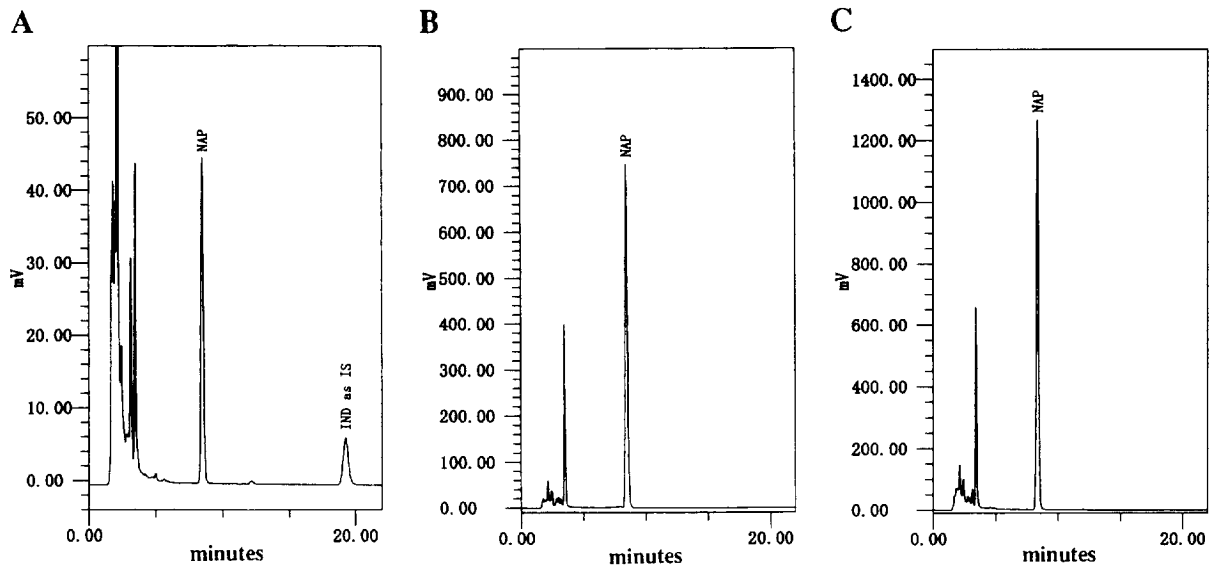

Fig. 9. Chromatograms obtained in the analysis of 0–10 h urine after single oral administration of 200 mg of NAP to a healthy volunteer. (A) Drug-free analysis; (B) acidic hydrolysis; (C) enzymatic hydrolysis. UV detection at 230 nm.

Table 4
Urinary excretion of parent compound following single oral administration of IBP or NAP to healthy volunteer

Compound	Parameter	Free	Deconjugation with 6 *M* HCl	Deconjugation with sulfatase
IBP	Obtained (μg/ml)	2.31	10.78	7.12
(Dose: 150 mg)	$Excretion_{0-4\ h}$ (mg)	0.69	3.23	2.14
	$\%Excretion_{0-4\ h}$	0.46	2.15	1.43
NAP	Obtained (μg/ml)	1.79	40.33	39.58
(Dose: 200 mg)	$Excretion_{0-10\ h}$ (mg)	2.49	56.06	55.02
	$\%Excretion_{0-10\ h}$	1.25	28.03	27.51

Table 5
Published methods for deconjugation of twelve NSAIDs in urine

Compound	Method	Reference
SUL	Glusulase (DuPont), pH 5.2, 1 h at 37°C	[13]
PIR	β-Glucuronidase Bovin Type B1 (Sigma), pH 5.0, 24 h at 27°C	[30]
	1 *M* NaOH, ambient temperature for 2 h (0.4 *M*)	[20]
LOX	0.5 or 1 *M* NaOH, ambient temperature for 30–60 min (0.25–0.5 *M*)	[22,29]
KEP	1 *M* NaOH (0.25 *M*)	[23]
NAP	β-Glucuronidase and sulfatase (Sigma), pH 5.0, 3 h at 37°C	[34]
	2.5 *M* HCl, 20 h at 50°C (1.25 *M*)	[17]
FEL, FEN	Concentrated HCl, ambient temperature for 5 min (6 *M*)	[21]
FLB	6 *M* HCl, 30 min at 90°C (3 *M*)	[27]
DIC	4 *M* HCl (0.36 *M*)	[18]
IND	4 *M* HCl, ambient temperature for 10 min (0.36 *M*)	[16,19]
IBP	6 *M* HCl, 30 min at 60–90°C (3 *M*)	[24,28]
	1 *M* NaOH, ambient temperature for 20–30 min (0.33 *M*)	[15,25,26]
MFA	NaOH	[39]

Final concentration is given in parentheses.

zymatic hydrolysis can be employed for SUL. Furthermore, mild acidic hydrolysis is needed for IND because of the lability (*N*-acylation) of the IND molecules in alkaline conditions [18,48] and the isomerization of IND acyl glucuronide to IND iso acyl glucuronide, registant to β-glucuronidase cleavage under physical conditions [19].

When enzymatic and acidic hydrolysis were carried out on the urine sample A (dosed IBP), total values (free+conjugate) of parent obtained by deconjugation were ca. 50% higher in the acidic than they were in the enzymatic hydrolysis. This discrepancy may be explained by labile IBP acyl glucuronide formed by intramolecular acyl migration to IBP iso acyl glucuronide, registant to β-glucuronidase cleavage, under physical conditions [49,50]. When the same experiment was conducted on the urine sample B (dosed NAP), on the other hand, the obtained values were consistent with both hydrolyses. NAP acyl glucuronide is also isomerized to its corresponding β-glucuronidase-registant iso acyl glucuronide of NAP [17,51]. But this iso acyl glucuronide is unstable at pH 7 and 8 [17]. It is thought, therefore, that NAP iso acyl glucuronide was cleaved in urine because the pH of urine sample B was 6.9.

5. Conclusions

The present method could be used to remove urinary interferences by using a normal solid-phase extraction as the sample clean-up procedure, thereby enabling simple manipulation and isocratic HPLC with UV analysis as well as achieving a high sensitivity of 0.005 μg/ml for NAP, and 0.05 μg/ml for SUL, PIR, LOX, KEP, FEL, FEN, FLB, DIC, IBP and MFA as the quantitation limit in human urine using IND as an internal standard. As a part of clinical support, it may be suitable for simultaneous screening and quantitation of several NSAIDs in urine.

References

[1] D.A. Kujubu, B.S. Fletcher, B.C. Varnum, R.W. Lim and H.R. Herschman, J. Biol. Chem., 266 (1991) 12866.
[2] B.S. Fletcher, D.A. Kujubu, D.M. Perrin and H.R. Herschman, J. Biol. Chem., 267 (1992) 4338.
[3] J.A. Mitchell, P. Akarasereenont, C. Thiemermann, R.J. Flower and J.R. Vane, Proc. Natl. Acad. Sci. USA, 90 (1993) 11693.
[4] W. Xie, J.G. Chipman, D.L. Robertson, R.L. Frikson and D.L. Simmons, Proc. Natl. Acad. Sci. USA, 88 (1991) 2692.
[5] T. Hla and K. Neilson, Proc. Natl. Acad. Sci. USA, 89 (1992) 7384.
[6] J.E. Dinchuk, B.D. Car, R.J. Focht, J.J. Johnston, B.D. Jaffee, M.B. Covington, N.R. Contel, V.M. Eng, R.J. Collins, P.M. Czerniak, S.A. Gorry and J.M. Trzaskos, Nature, 378 (1995) 406.
[7] J.J. Li, M.B. Norton, E.J. Reinhard, G.D. Anderson, S.A. Gregory, P.C. Isakson, C.M. Koboldt, J.L. Mosferrer, W.E. Perkins, K. Seibert, Y. Zhang, B.S. Zweifei and D.B. Reitz, J. Med. Chem., 39 (1996) 1846.
[8] C.A. Berntsen and R.H. Freyberg, Ann. Intern. Med., 54 (1961) 938.
[9] I. Caruso and P.G. Bianchi, Br. Med. J., 1 (1980) 75.
[10] L.F. Prescott, Drugs, 23 (1982) 75.
[11] A. Gabriellie, P. Leone and G. Daniele, Br. Med. J., 294 (1987) 176.
[12] D.E. Furst, R.A. Herman, R. Koehnke, N. Erickson, L. Hash, C.E. Riggs, A. Porras and P. Veng-Pedersen, J. Pharm. Sci., 79 (1990) 782.
[13] R.J. Stubb, L.L. Ng, L.A. Entwistle and W.F. Bayne, J. Chromatogr., 413 (1987) 171.
[14] R. Bruno, A. Iliadis, I. Julien, M. Guego, H. Pinhas, S. Cunci and J.P. Cano, Br. J. Clin. Pharmacol., 26 (1988) 41.
[15] A. Shah and D. Jung, J. Chromatogr., 378 (1986) 232.
[16] A. Avegerinos and S. Malamataris, J. Chromatogr., 495 (1989) 309.
[17] T.B. Vree, M. van den Biggelaar-Martea and C.P.W.G.M. Verwey-van Wissen, J. Chromatogr., 578 (1992) 239.
[18] A. Avegrinos, Th. Karidas and S. Malamataris, J. Chromatogr., 619 (1993) 324.
[19] T.B. Vree, M. van den Biggelaar-Martea and C.P.W.G.M. Verwey-van Wissen, J. Chromatogr., 616 (1993) 271.
[20] A. Argerinos, S. Axarlis, J. Dragatsis, Th. Karidas and S. Malamataris, J. Chromatogr. B, 673 (1995) 142.
[21] G.E. van Lear, F.S. Chiccarelli, P.A. Bonenfant and A. Barr, J. Pharm. Sci., 67 (1978) 1662.
[22] H. Nagashima, Y. Tanaka and R. Hayashi, J. Chromatogr., 345 (1985) 373.
[23] R.T. Foster and F. Jamali, J. Chromatogr., 416 (1987) 388.
[24] D.A. Nicoll-Griffith, T. Inaba, B.K. Tang and W. Kalow, J. Chromatogr., 428 (1988) 103.
[25] B. Chai, P.E. Minkler and C.L. Hoppel, J. Chromatogr., 430 (1988) 93.
[26] G. Geisslinger, K. Dietzel, D. Loew, O. Schusten, G. Rau, G. Lachmann and K. Brune, J. Chromatogr., 491 (1989) 139.
[27] M.P. Knadler and S.D. Hall, J. Chromatogr., 494 (1989) 173.
[28] A.C. Rudy, K.S. Anliker and S.D. Hall, J. Chromatogr., 528 (1990) 395.
[29] H. Naganuma and Y. Kawahara, J. Chromatogr., 530 (1990) 387.

[30] P.A. Milligan, J. Chromatogr., 576 (1992) 121.
[31] R. Mehvar, F. Jamali and F.M. Pasutto, Clin. Chem., 34 (1988) 493.
[32] D. Grandjean, J.C. Beolor, M.T. Qunicon and E. Savel, J. Pharm. Sci., 78 (1989) 247.
[33] P. Hubert and J. Crommen, J. Liq. Chromatogr., 13 (1990) 3891.
[34] J.V. Andersen and S.H. Hansen, J. Chromatogr., 577 (1992) 362.
[35] M.R. Wright and F. Jamali, J. Chromatogr., 616 (1993) 59.
[36] I. Niopas and K. Mamzoridi, J. Chromatogr. B, 656 (1994) 447.
[37] H.J. Battista, G. Wehinger and R. Henn, J. Chromatogr., 345 (1985) 77.
[38] P.J. Streete, J. Chromatogr., 495 (1989) 179.
[39] A.K. Singh, Y. Jang, U. Mishra and K. Granley, J. Chromatogr., 568 (1991) 351.
[40] A.G. Kazemifard and D.E. Moore, J. Chromatogr., 533 (1990) 125.
[41] T. Hirai, S. Matsumoto and I. Kishi, J. Chromatogr. B, 690 (1997) 267.
[42] R.E. Harman, M.A.P. Meisinger, G.E. Davis and F.A. Kuehl, J. Pharmacol. Exp. Ther., 143 (1964) 215.
[43] H. Stierlin and J.W. Faigle, Xenobiotica, 9 (1979) 611.
[44] E.J.D. Lee, K. Williams, R. Day, G. Graham and D. Champion, Br. J. Clin. Pharmacol., 19 (1985) 669.
[45] R.K. Verbeek, C.J. Richardson and K.L.N. Blocka, J. Rheumatol., 13 (1986) 789.
[46] G.J. Szpunar, K.S. Albert, G.G. Bole, J.N. Dreyfus, G.F. Lockwood and J.G. Wagner, Biopharm. Drug Dispos., 8 (1987) 273.
[47] P.A. Todd and S.P. Clissold, Drugs, 40 (1990) 91.
[48] M.S. Bernstein and M.A. Evans, J. Chromatogr., 229 (1982) 179.
[49] P.C. Smith, J. Hasegawa, P.N.J. Langendijk and L.Z. Benet, Drug Metab. Dispos., 13 (1985) 110.
[50] M. Castillo and P.C. Smith, J. Chromatogr., 614 (1993) 109.
[51] M. Fead, Drug Metab. Rev., 15 (1984) 1213.