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Simultaneous analysis of naproxen, nabumetone and its major metabolite 6-methoxy-2-naphthylacetic acid in pharmaceuticals and human urine by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method for simultaneous determination of naproxen (NAP), nabumetone (NAB) and its major metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA), was developed for the application to pharmaceuticals and human urine. Isocratic reversed-phase HPLC was employed for quantitative analysis using triethylamine and 1-heptanesulfonic acid sodium salt (HSA) as ion-pair reagents. Urine samples were purified by solid-phase extraction using Bond-Elut Certify **II** cartridges containing reversed-phase and anion exchange functionalities. The HPLC assay was carried out using a Wakosil ODS 5C18 column (5 μm, 150 × 4.6 mm, i.d.). The mobile phase consisted of 0.5 g of HSA dissolved in 1000 ml of a mixture of acetonitrile, water and triethylamine (500:500:1, v/v) adjusted with phosphoric acid to pH 3. The calibration curves of NAP and NAB showed good linearity in the concentration range 32–160 μg/ml with UV detection (270 nm) for pharmaceuticals. In the low concentration ranges (8–96 ng of NAP per ml, 24–288 ng of NAB per ml and 5.6–67.2 ng of 6-MNA per ml), the calibration curves were also obtained with fluorimetric detection (excitation 280 nm, emission 350 nm) for biological fluids. The correlation coefficients were better than 0.999 in all cases. The lower limits of detection (defined as a signal-to-noise ratio of about 3) were approximately 0.3 ng for NAP, 1.5 ng for NAB and 0.2 ng for 6-MNA. The procedure described here is rapid, simple, selective, and is suitable for routine analysis of pharmaceuticals and pharmacokinetic studies in human urine samples.

Keywords: Naproxen; Nabumetone; 6-Methoxy-2-naphthylacetic acid; Pharmaceuticals; Human urine; Solid-phase extraction

1. Introduction

Naproxen (NAP) [(*S*)-2-(6-methoxy-2-naphthyl) propionic acid] and nabumetone (NAB) [4-(6-methoxy-2-naphthyl)-butan-2-one] are non-

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steroidal anti-inflammatory drugs with analgesic and antipyretic properties (Fig. 1) [1]. These compounds are used in the treatment of rheumatoid arthritis and other painful musculoskeletal disorders. 6-Methoxy-2-naphthylacetic acid (6-MNA) is the major metabolite of NAB, formed by side-chain oxidation, which shows greater anti-inflammatory activity than NAB itself [2]. These naphthalene derivatives are related to the inhibition of cyclooxygenase, an enzyme involved in the arachidonic acid conversion pathway, resulting in a decrease of prostaglandin synthesis [3,4].

Several high-performance liquid chromatographic (HPLC) methods have been published for the individual determination of NAP and NAB in pharmaceutical preparations [5–7] and biological fluids, serum and urine samples [7–14]. However, there have been no reports concerning the simultaneous determination of NAP, NAB or 6-MNA by isocratic HPLC.

Direct injection of diluted urine [8,10,11] and liquid–liquid extraction as a sample clean-up procedure [7,9,13,14] are frequently used for the determination of naphthalene derivatives in biological fluids. However, these methods lack sensitivity and removal of interfering substances is insufficient. For the liquid–liquid extraction procedure, back extraction and/or some derivatization techniques induced to increase the sensitivity are often carried out to avoid interference by endogeneous substances. For this purpose, we established a sample clean-up procedure using solid-phase extraction by Bond-Elut Certify **II** cartridges [15–17] containing reversed-phase and anion exchange functionalities for the analysis of these naphthalene derivatives in human urine samples using HPLC with fluorimetric detection.

Here, we describe simple, sensitive, accurate and precise simultaneous analytical procedures for the determination of three naphthalene derivatives using reversed-phase ion-pair isocratic HPLC. Pharmaceuticals after extraction by solvent were analyzed by HPLC–UV detection. After efficient purification by solid-phase extraction on Bond-Elut Certify **II** cartridges, urine samples were directly analyzed with HPLC-fluorimetric detection. These procedures were used to determine NAP, NAB and 6-MNA in pharmaceutical preparations and human urine.

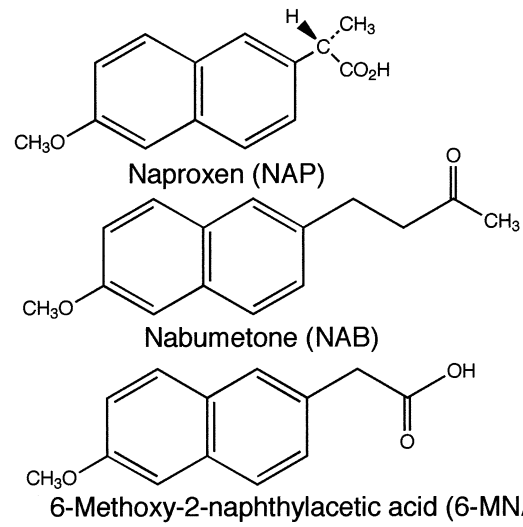

Fig. 1. Structures and abbreviations of drugs investigated in this study.

2. Experimental

2.1. Chemicals and reagents

NAP and NAB were purchased from Sigma (St. Louis, MO, USA). 6-MNA was from Biomol Research Laboratories (Plymouth Meeting, PA, USA). 1-Heptanesulfonic acid sodium salt (HSA), triethylamine and HPLC-grade acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). Methyl *p*-toluate was from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). All other reagents were of analytical grade.

2.2. Apparatus

A Shimadzu (Kyoto, Japan) HPLC system consisting of the following components was used; model LC-6A pump, a model CTO-10A column oven, a model SPD-6A UV detector, a model RF-10A fluorescence detector, a model C-R6A integrator/recorder and a model SIL-10AD auto-injector fitted with a 50 μl injection loop. Column temperature was 40°C and chromatographic separation was carried out on a Wakosil 5C18 column (5 μm, 150 × 4.6 mm i.d., Wako Pure Chemical Industries). An ultrasonic bath (model B-42H,

Branson Co., Shelton, CT, USA) was used to dissolve the samples in ethanol.

For urine sample preparation, we used Bond-Elut Certify **II** cartridges (3-ml capacity containing 200 mg sorbent; Varian, Harbor City, CA, USA) preconditioned with 4 ml of methanol and 4 ml of water. Care was taken to prevent the drying of cartridges. A vacuum manifold capable of holding 12 sample preparation cartridges (GL Sciences, Tokyo, Japan) was used for the simultaneous sample extraction.

2.3. Standard solutions

Standard stock solutions of NAP (0.8 mg/ml), NAB (0.8 mg/ml), 6-MNA (0.14 mg/ml) and an internal standard stock solution of methyl *p*-toluate (3.6 mg/ml) were prepared by dissolving appropriate amounts of respective compounds in ethanol at room temperature. All stock solutions were stored at −20°C in a freezer.

2.4. Chromatographic conditions

Chromatography was performed under isocratic conditions, at a flow-rate of 1.0 ml/min. The mobile phase consisted of 0.5 g of HSA dissolved in 1000 ml of a mixture of acetonitrile, water and triethylamine (500:500:1, v/v) adjusted with phosphoric acid to pH 3. The solution was filtered and degassed for 10 min in an ultrasonic bath. The column effluent was monitored at 270 nm using a detector range of 0.32 absorbance unit of full scale (aufs) for pharmaceuticals. Fluorimetric detection was achieved by a fluorescence detector set at an excitation wavelength of 280 nm and an emission wavelength of 350 nm for the urine samples. The chart speed was 1 mm/min. An aliquot of sample solution (10 μl) was injected onto the analytical column with an auto-HPLC injector.

2.5. Calibration assay

For pharmaceuticals, 1, 2, 3, 4 and 5 ml of NAP and NAB standard stock solution were pipetted into 25 ml volumetric flasks, followed by the addition of 2 ml of the internal standard stock solution to the corresponding flasks and then made up with ethanol to the mark. NAP and NAB mixed standard ethanol solutions of 32, 64, 96, 128, 160 μg/ml were prepared.

For human urine samples, 0.5 ml of NAP and 1.5 ml of NAB standard stock solutions were pipetted into 100-ml volumetric flasks, 1 ml of 6-MNA standard stock solution was pipetted into 50-ml volumetric flask, and made up with ethanol to the mark. Then, 0.5, 1, 2, 4, or 6 ml of these NAP, NAB and 6-MNA diluted standard solutions were pipetted into 25 ml volumetric flasks, followed by the addition of 6 ml of the internal standard stock solution to the corresponding flasks and made up with ethanol to the mark. Aliquots of 1 ml of these three naphthalenes mixed solutions were pipetted into 10-ml volumetric flasks and then made up with ethanol to the mark. The diluted three naphthalenes mixed standard solutions containing 8, 16, 32, 64, and 96 ng/ml for NAP; 24, 48, 96, 192, and 288 ng/ml for NAB; and 5.6, 11.2, 22.4, 44.8, and 67.2 ng/ml for 6-MNA were prepared.

An aliquot (10 μl) of each solution was then injected onto the analytical column. All the measurements were performed in duplicate for each concentration on 5 different days over 1 week. The peak areas were measured and the peak area ratios of analyte to internal standard (y) were then plotted against the respective concentration of NAP, NAB and 6-MNA (x). Least square linear regression analysis was used to determine the slope, y-intercept and the correlation coefficients of the standard plots.

2.6. Sample preparation for pharmaceuticals

The contents of ten tablets of NAP and NAB were finely ground and mixed well in a mortar. An accurately weighed powdered sample containing 400 mg (labeled amount) of NAP or 800 mg (labeled amount) of NAB was transferred into a 200 ml volumetric flask. The volume was adjusted with ethanol and the solution was sonicated for 30 min in an ultrasonic bath. Portions of these solutions were then filtered through 0.5 μm PTFE filters (Toyo Roshi Kaisha, Tokyo, Japan). For NAP, 4 ml of the internal standard solution was

added to 2.5 ml of the filtrate and ethanol was added to bring the vol. up to 50 ml. For NAB, 8 ml of the internal standard solution was added to 2.5 ml of the filtrate and ethanol was added to bring the vol. up to 100 ml. An aliquot (10 μl) of each solution was injected onto the column. Four different tablets (100 mg per tablet of NAP) and one tablet (400 mg per tablet of NAB) of commercially available pharmaceuticals were analyzed for the statistical evaluation of the assay.

2.7. Preparation of human urine samples

Drug-free urine samples used in this study were obtained from healthy investigators. They were frozen at −20°C until analytical use.

The recovery was assessed at three concentration levels of 6.4, 25.6 and 76.8 ng NAP per ml urine; 19.2, 76.8 and 172.8 ng NAB per ml urine; and 4.5, 17.9 and 53.8 ng 6-MNA per ml urine. To 460 μl aliquots of urine was added 40 μl of different concentrations (0.08, 0.32, 0.96 μg NAP per ml; 0.24, 0.96, 2.88 μg NAB per ml; and 0.056, 0.224, 0.672 μg 6-MNA per ml) of the three naphthalene standard solutions. The samples were then diluted with 500 μl of water and with 1 ml of methanol. Aliquots of 1 ml of diluted methanolic urine samples were applied to pre-conditioned Bond-Elut Certify **II** cartridges and slowly drawn through the cartridge. The cartridges were washed with 4 ml of water and dried for 5 min by aspiration with a moderate to strong vacuum (5–6 in. mercury). The analytes were eluted with 6 ml of n-hexane-ethyl acetate (1:1). These n-hexane-ethyl acetate (1:1) solutions were evaporated to dryness in a water bath at 40°C. The residues were redissolved in 200 μl of ethanol containing methyl *p*-toluate (0.9 mg/ml ethanol), and aliquots of 10 μl were injected into the chromatograph. Recoveries were determined from the peak-area ratios (NAP, NAB and 6-MNA/internal standard) of extracts with those of standard solutions.

For practical application of our method, urine was collected 0–4 h after a single oral administration of 200 mg of NAP or 0–4 h after a single oral administration of 800 mg of NAB. A 500 μl portion of each urine sample was directly used to determine NAP, NAB and 6-MNA. The urine samples were diluted a 100-fold for the determination of NAP and NAB glucuronides. NAP and NAB glucuronides were determined as NAP and NAB after alkaline hydrolysis [12]. Alkaline hydrolysis was achieved by adding 250 μl of 1 mol/l sodium hydroxide solution to 0.5 ml of diluted urine and standing at ambient temperature for 1 h. The mixture was neutralized with 250 μl of 1 mol/l hydrochloric acid, and diluted with 1 ml of methanol. A 1-ml portion of deconjugated urine sample was used to determine NAP or NAB as described above.

3. Results and discussion

3.1. Chromatography

In our preliminary study, a mobile phase consisting of acetonitrile and water mixture using triethylamine and HSA as ion-pair reagents was the most suitable for separation of these naphthalene derivatives by reversed-phase HPLC. The UV absorption spectra of the NAP and NAB exhibited similar wavelengths of maximum absorbance. Therefore, we set the detector wavelength to 270 nm for the determination of NAP and NAB in commercial tablets. The fluorimetric detection method showed an excellent sensitivity for quantifying NAP, NAB and 6-MNA in biological fluids, often exceeding the sensitivity of most spectrophotometric methods. In addition, fluorometry offers the advantage of having less interference in the chromatogram due to fewer fluorescent compounds present in the biological fluids than UV absorbing molecules. Fluorescence emission scanning of naphthalenes and methyl *p*-toluate (internal standard) following excitation at a wavelength of 280 nm indicated that the internal standard yielded lower fluorescence intensity at the emission wavelength of 350 nm than naphthalenes. This was the reason for the requirement of a high concentration of the internal standard in ethanol (0.9 mg/ml).

In the analyses of these compounds, we found that 6-MNA and NAP were weakly retained on the reversed-phase sorbents when the mobile

phase consisted of a mixture of acetonitrile, water and triethylamine (500:500:1, v/v) adjusted with phosphoric acid to pH 3. There was an asymmetrical and tailed NAB peak without HSA as a mobile phase additive. They showed reduced resolution, and increased interference from unretained matrix compounds hampered accurate determination. The addition of HSA to the eluent is particularly useful for the separation and detection of several drugs, such as mepenzolate [18] and metoprolol [19], because it provides in appropriate capacity factors for complexes formed with the counter ion. Therefore, to enhance the retention of three naphthalenes on reversed-phase HPLC, the hydrophobic ion-pair reagent HSA was used as a mobile phase additive.

Consequently, 0.5 g of HSA in the 1000 ml of mobile phase was selected, because the peaks corresponding to the compounds were well separated and were sharp and symmetrical, and chromatography could be completed within 13 min. No significant changes in standard retention or column efficiency were observed over long periods (several months). Under these chromatographic conditions, the capacity factors of NAP, NAB, 6-MNA and internal standard were 2.8, 6.3, 1.9 and 4.6, respectively.

3.2. Solid-phase extraction of biological samples

Urine samples are often used to obtain pharmacokinetic information. To enable the direct determination of three naphthalene derivatives by HPLC without derivatization, a purification step prior to their determination was based on solid-phase extraction. Solid-phase extraction techniques for drug analysis in biological samples would be very useful in routine clinical use. For comparison, we selected a solid-phase extraction method using Bond-Elut Certify **II** cartridge prepacked with a special, nonpolar C8 sorbent and a strong anion exchanger for this investigation. To test the suitability of the solid-phase extraction of three naphthalenes in aqueous samples and of the eluting solvent, the extraction was first tested without urine matrix, so that three naphthalenes could be efficiently retained on the cartridges. This was found to be suitable.

After washing the extraction cartridges with water to remove polar and non-ionic endogenous components, quantitative desorption of three naphthalenes was obtained by elution of the cartridges with 6 ml of n-hexane-ethyl acetate (1:1). Urinary interference was removed by eluting the cartridges loaded with urine samples with n-hexane-ethyl acetate (1:1), thereby yielding a high sensitivity and recovery. In addition, the clean-up method with Bond-Elut Certify **II** cartridges and subsequent evaporation has some excellent features with regard to simplicity and reproducibility compared with many conventional extraction procedures. Therefore, the present method seemed to be useful for monitoring three naphthalenes in clinical practice.

3.3. Analytical performance characteristics

A linear relationship was established between the peak area ratio and concentration for each standard solution of three naphthalenes when methyl *p*-toluate was used as an internal standard. For pharmaceuticals, the calibration curves of NAP and NAB in the concentration range 32–160 μg/ml showed good linearity with a detection wavelength of 270 nm at a sensitivity of 0.32 aufs. In the same manner, for urine samples, the calibration curves in the low concentration ranges 8–96 ng of NAP per ml, 24–288 ng of NAB per ml and 5.6–67.2 ng of 6-MNA per ml showed good linearity with a fluorescence detector (excitation 280 nm, emission 350 nm). The correlation coefficients were better than 0.999 in all the cases. The intercepts with the *y*-axis were not significantly different from the origin. The instrumental detection limits based on a signal-to-noise ratio of about 3 for standard solutions of NAP, NAB, and 6-MNA were 30, 150 and 20 ng/ml, respectively. The lower limits of quantification in human urine were 24, 120 and 16 ng/ml, respectively, using the procedure described above.

The accuracy of the method for the analysis of urine samples was determined by recovery experiments. Control urine samples were spiked with three different concentrations of NAP, NAB and 6-MNA and subjected to the assay. The recoveries and relative standard deviations (R.S.D.) after six

Table 1
Accuracy and precision of the method for HPLC analysis of naphthalene derivatives in human urine ($n = 6$)

Sample	NAP			NAB			6-MNA		
	Amount added (ng/ml)	Recovery (%, mean ± S.D.)	R.S.D. (%)	Amount added (ng/ml)	Recovery (%, mean ± S.D.)	R.S.D. (%)	Amount added (ng/ml)	Recovery (%, mean ± S.D.)	R.S.D. (%)
Urine	6.4	92.6 ± 3.8	4.1	19.2	93.6 ± 4.0	4.3	4.5	92.5 ± 4.2	4.5
	25.6	98.1 ± 3.7	3.8	76.8	97.1 ± 3.5	3.6	17.9	93.5 ± 3.2	3.5
	76.8	95.8 ± 3.6	3.8	172.8	96.2 ± 4.1	4.3	53.8	95.0 ± 3.8	4.0

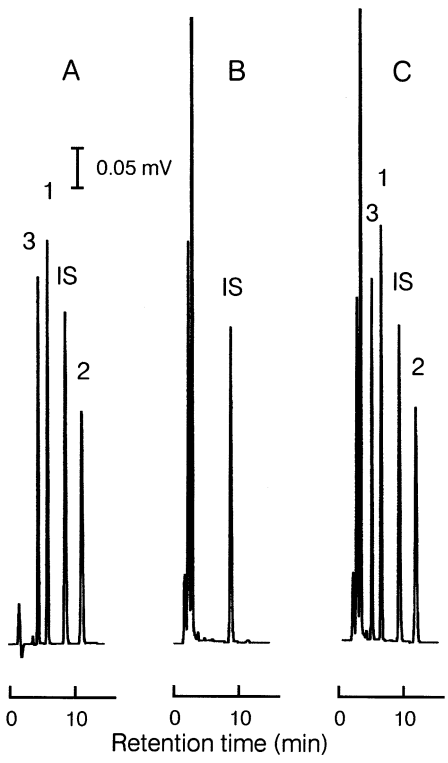

Fig. 2. Chromatograms of (A) standard solution containing 32 ng NAP per ml, 96 ng NAB per ml and 22.4 ng 6-MNA per ml, (B) drug-free human urine and (C) urine spiked with 25.6 ng NAP per ml, 76.8 ng NAB per ml and 17.9 ng 6-MNA per ml as the final concentrations. Chromatographic conditions are described in the text. Peaks, 1, NAP; 2, NAB; 3, 6-MNA; IS, methyl *p*-toluate.

replicate repeated analyses are given in Table 1. The recoveries of three naphthalenes from the urine samples were greater than 90% at each concentration examined. Assay precision expressed, as R.S.D., was better than 4.5%. Using this pretreatment technique, it is possible to analyze naphthalene derivatives in urine samples within 13 min (Fig. 2). Alkaline hydrolysis is applicable for common hydrolysis conditions of the glucuronide conjugates of drugs in human urine. There were hardly any interfering peaks on the chromatograms during alkaline hydrolysis when drug-free human urine was subjected to the assay. Three naphthalenes were consistently recovered from urine with or without alkaline treatment. The possibility of interfering peaks was checked by the analysis of more than ten different urine blanks.

3.4. Method application

The specificity of simultaneous analysis of NAP and NAB with isocratic elution was confirmed by the results of content uniformity tests of NAP and NAB performed on commercial NAP-1 and NAB-1 tablets. The average percentages according to the manufacturer's specification in ten tablets were 101.1 ± 1.6 and $99.8 \pm 1.5\%$, respectively, in the range 103.5–98.2%. The results of content uniformity tests indicated compliance to

Table 2
Assay results for the analysis of NAP and NAB in pharmaceutical preparations by HPLC in the presence of methyl *p*-toluate as an internal standard ($n = 5$)

Sample	Pharmaceutical forms	Manufacturer's specification (mg per tablet)	Manufacturer's specification (%, mean ± S.D.)	R.S.D. (%)
NAP 1	Uncoated tablets	100	101.04 ± 2.32	2.30
NAP 2	Uncoated tablets	100	100.84 ± 2.23	2.21
NAP 3	Uncoated tablets	100	101.12 ± 1.58	1.56
NAP 4	Uncoated tablets	100	100.32 ± 1.54	1.54
NAB 1	Film coated tablets	400	99.48 ± 1.59	1.60

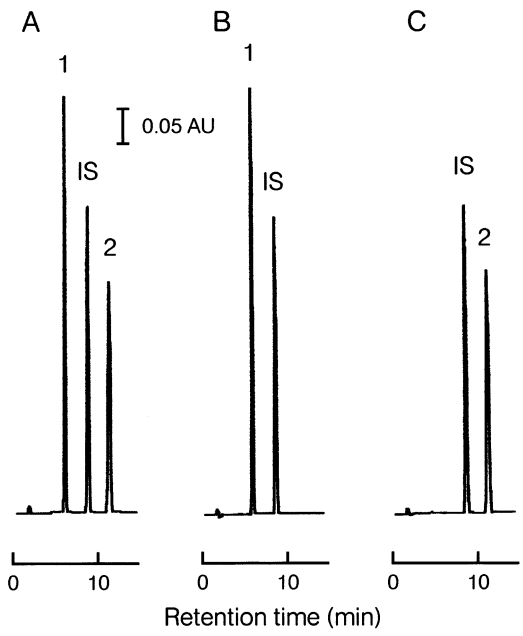

Fig. 3. Chromatograms of the pharmaceuticals, (A) standard solution containing 96 μg/ml for NAP and NAB, respectively; (B) tablets containing NAP; and (C) tablets containing NAB. Chromatographic conditions are described in the text. Peaks, 1, NAP; 2, NAB; IS, methyl *p*-toluate.

specifications of pharmaceuticals and supported the specificity of this method. The applicability of the method was tested by analyzing NAP and NAB in commercial pharmaceutical preparations. The results obtained were in good agreement with the manufacturer's specification (Table 2), and no interference was observed in the chromatograms from the excipients (Fig. 3). The assay results indicated that the proposed analytical method could be used for the determination of NAP and NAB in commercial pharmaceuticals.

As an example of the application of the method, the urinary excretion of NAP, NAB and 6-MNA were further investigated in the urine from healthy volunteers after receiving 200 mg of NAP and 800 mg of NAB orally, respectively. Fig. 4 shows the chromatograms obtained in the analysis of 0–4 h urine samples collected after administration. The urinary concentration and intact excretion rates were 0.33 μg/ml and 0.10% of dose for NAP and 0.11 μg/ml for 6-MNA, respectively. On the other hand, the total value

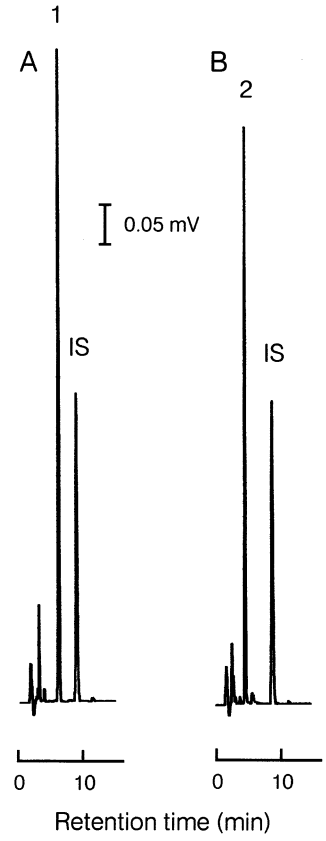

Fig. 4. Chromatograms obtained in the analysis of 0–4 h urine after a single oral administration of (A) 200 mg of NAP and (B) 800 mg of NAB to healthy volunteers. Chromatographic conditions are described in the text. Peaks, 1, NAP; 2, 6-MNA; IS, methyl *p*-toluate.

(free + conjugate) obtained by deconjugation with alkaline hydrolysis was 4.52 μg/ml and 1.40% of dose for NAP and 3.10 μg/ml for 6-MNA. NAB concentration of 0–4 h deconjugated urine sample was below the limits of detection, in agreement with previous studies [1,20]. The present method using Bond-Elut Certify **II** cartridges could be used for routine pharmacokinetic analysis after the administration of NAP and NAB to urine samples.

4. Conclusions

An isocratic HPLC method using HSA and triethylamine as ion-pair reagents was established

for the simultaneous analysis of NAP, NAB and 6-MNA in pharmaceuticals and human urine. For pharmaceuticals, the method was applied to commercial tablets, and was shown to be free of interference from excipients normally used in pharmaceutical formulations. For urine samples, the method could be used to remove endogeneous interference by solid-phase extraction on Bond-Elut Certify **II** cartridges as a sample clean-up procedure, thereby enabling simple manipulation and HPLC with fluorimetric detection as well as achieving a high degree of sensitivity. Due to the minimal sample preparation, and its good accuracy and precision, this method appears to be very useful for the quality control of pharmaceuticals and clinical monitoring.

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