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Simultaneous quantitative determination of naproxen, its metabolite 6-O-desmethylnaproxen and their five conjugates in plasma and urine samples by high-performance liquid chromatography on dynamically modified silica

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ABSTRACT

The glucuronides of the anti-inflammatory drug naproxen and its metabolite 6-O-desmethylnaproxen have been produced on a preparative scale by enzymatic synthesis. 6-O-Desmethylnaproxen, the glycine conjugate of naproxen and the O-sulphate of 6-O-desmethylnaproxen were prepared by chemical synthesis. Naproxen and the purified metabolite and conjugates were used as standards for the analytical investigation of the metabolic pattern of naproxen in humans. A reversed-phase high-performance liquid chromatographic method based on bare silica dynamically modified with cetyltrimethylammonium ions has been developed. The system was optimized to give a separation of naproxen, 6-O-desmethylnaproxen and five conjugates. Using this method it is also possible to deduce the relationship between the amount of the intact ether-glucuronide and acyl-glucuronide of 6-O-desmethylnaproxen.

INTRODUCTION

Naproxen, (*S*)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid, is a non-steroidal, anti-inflammatory drug used in the treatment of rheumatic and other inflammatory diseases. Naproxen is a major drug in the group of 2-arylpropionic acid derivatives used in the treatment of these diseases. Naproxen today is one of the twenty most commonly used drugs in USA [1]. Pharmacokinetic studies have shown that after oral administration of [^{3}H]naproxen more than 90% of the radioactivity is found in urine and only 1–2% is excreted with the faeces [2]. Naproxen is metabolized by 6-O-desmethylation in animals and humans. The drug is excreted as this compound and also as a glucuronic acid conjugate, whereas the glycine conjugate of naproxen has only been reported in animal studies [3,4]. The 6-O-desmethylated metabolite (DM-naproxen) is excreted unchanged as well as conjugated with sulphate and glucuronic acid [4,5] (Fig. 1).

A number of chromatographic methods have been reported for the quantitative determination of naproxen and DM-naproxen in biological fluids [6–8]. Some of these papers also describe methods for the determination of the conjugates, but this is performed indirectly as a measurement of the difference for the parent drug or metabolite

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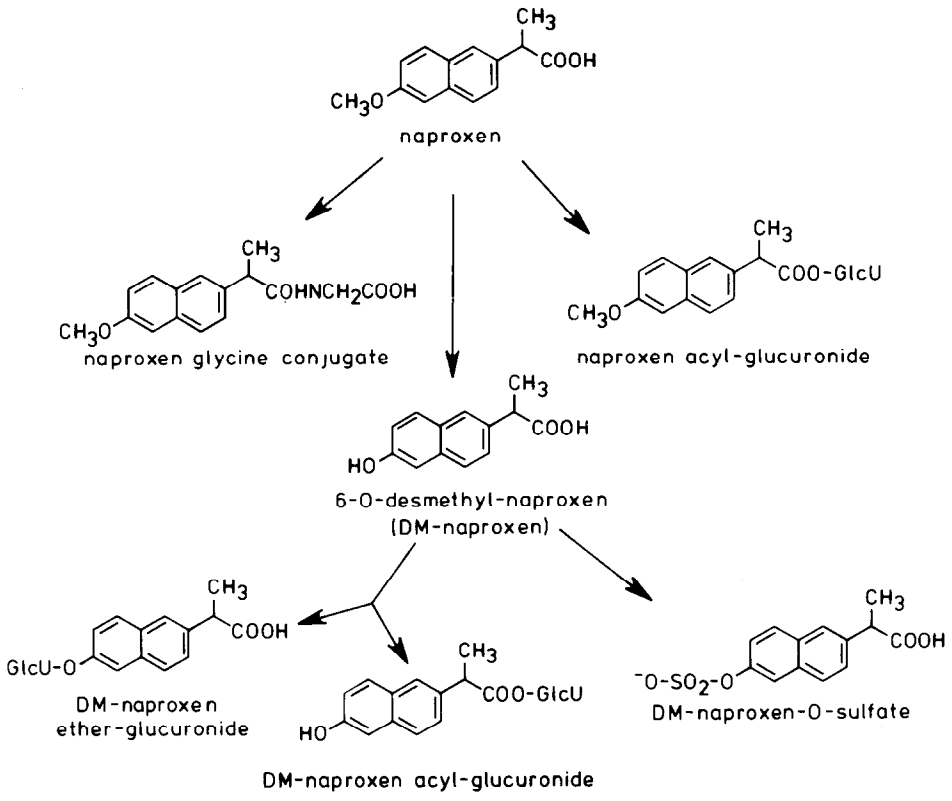

Fig. 1. Metabolic pathways of naproxen.

before and after chemical or enzymatic hydrolysis of the conjugates.

The determination of conjugates has in most instances been performed in an indirect way because of a lack of reference materials. However, different glucuronides or other types of conjugates may have the same aglycon, and furthermore some glucuronides are resistant to cleavage by β-glucuronidase [9]. In such instances less, or even false, information about the pharmacokinetics are obtained. For naproxen, the number of glucuronides of DM-naproxen and thus the metabolic pattern of naproxen is unknown.

This paper describes methods for the preparative enzymatic or chemical synthesis of one metabolite of naproxen and its five conjugates (Fig. 1). The synthesized metabolites and conjugates were used as reference materials in the development of a high-performance liquid chromatographic (HPLC) method, where the seven substances are separated on dynamically modified silica. The chromatographic method is used to assay the drug, the metabolite and their conjugates in urine obtained from a single-dose study of the metabolic pattern of naproxen in humans.

EXPERIMENTAL

Apparatus

A Kontron (Tegimenta, Switzerland) liquid chromatograph consisting of a Model 420 pump, a Model 460 autosampler [a Rheodyne (Cotati, CA, USA) 7125 loop injector with a 1.5-ml loop was used for preparative work], a Model 430 UV–visible detector operated at 232 nm, a Model 480 column oven and a Model 450 data system was used. A Jasco (Tokyo, Japan) 821-FP spectrofluorimeter operated at an excitation wavelength of 330 nm and an emission wavelength of 355 nm was also used.

NMR experiments were performed either on a 60-MHz Varian (Sunnyvale, CA, USA) EM360L instrument or on a 400 MHz Bruker (Rheinstetten, Germany) AMX 400 WB instrument.

Chemicals

Disodium UDP-glucuronate (UDP-GA) and β-glucuronidase (*Escherichia coli*) (200 U/ml) were from Boehringer (Mannheim, Germany). Naproxen and disodium 6-O-desmethylnaproxen-O-sulphate were a gift from Syntex (Palo Alto, CA, USA). Chlorosulphonic acid and hydriodic acid (hydrogen iodide, 47%) were of synthesis grade from Aldrich (Steinheim, Germany), glucaro-1,4-lactone was from Sigma (St. Louis, MO, USA) and all other chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

Human plasma was obtained from the University Hospital in Copenhagen.

Chemical synthesis

DM-naproxen. Naproxen (500 mg) was dissolved in 3 ml of glacial acetic acid by heating and 10 ml of a 57% hydriodic acid solution were added. The mixture was refluxed at 130°C for 20 min. Then 100 ml of ice-cold water was added and the resultant solution was placed in the refrigerator for 1 h. The crystals were isolated by filtration, washed with cold water and recrystallized in 60% methanol. The yield was about 80% and neither naproxen nor other impurities could be detected by HPLC using UV detection at 232 nm. The optical purity of naproxen was tested by chiral HPLC [10] and was found to be 99.8%. Using the same method the chiral purity of the synthesized 6-O-desmethylnaproxen was also tested and no decrease in the optical purity could be detected. ^{1}H NMR (60 MHz in d_6-DMSO): δ = 6.6–6.7 ppm (6H, m), 3.60 ppm (1H, q, J = 7 Hz) and 1.40 ppm (3H, d, J = 7 Hz).

Naproxen glycine conjugate. Naproxen (2 g) was dissolved in 10 ml of $SOCl_2$ and one drop of dimethylformamide. The mixture was refluxed at 80°C for 30 min, and the excess $SOCl_2$ was then removed by rotory evaporation. The acyl chloride of naproxen was dissolved in 25 ml of dry toluene and cooled on ice. An ice-cold solution of 1 g of glycine in 30 ml of 1.33 *M* NaOH was slowly (5 min) added to the toluene phase with stirring. The reaction mixture was removed from the ice-bath and was stirred for 24 h at room temperature. The glycine conjugate was extracted from the water phase with 60 ml of methylene chloride after adding 4 *M* HCl to adjust to pH 1–2. The glycine conjugate of naproxen was isolated using preparative HPLC. The yield was 34% and the chromatographic purity was about 97% detected by HPLC using UV detection at 232 nm.

^{1}H NMR (400 MHz in d_6-DMSO): δ = 7.93 ppm (1H, m), 7.1–7.8 ppm (6H, m), 3.85 ppm (3H, s), 3.84 ppm (1H, q, J = 7 Hz), 3.64 ppm (1H, dd, J = 4 and 18 Hz), 3.59 ppm (1H, dd, J = 4 and 18 Hz) and 1.40 ppm (3H, d, J = 7 Hz).

DM-naproxen-O-sulphate. DM-naproxen (250 mg) was dissolved in 10 ml of dimethylformamide and cooled to −30°C. Chlorosulphonic acid (500 μl) was added slowly (5 min) with stirring. The reaction mixture was left at room temperature for 90 min after which 100 ml of ice-cold water containing 1.7 g of potassium carbonate were added. The synthesis mixture was then lyophilized. The yield was 50%. The product was not purified further as a sample of DM-naproxen-O-sulphate was obtained as a gift from Syntex.

Enzymatic synthesis of glucuronides

Influence of pH. Preparation of the hepatic microsomal fraction from New Zeeland white rabbit was performed as described previously [9]. The enzymatic synthesis was performed at 37°C with the following incubation mixtures: rabbit microsomal protein, 15 mg/ml; UDP-GA, 3.0 m*M*; DM-naproxen, 0.5 m*M*; $MgCl_2$, 5.0 m*M*; glucaro-1,4-lactone, 1.0 m*M*; and potassium phosphate buffer, 50 m*M*, (pH 6.0, 6.5 or 7.0). The total volume of each incubation mixture was 2 ml.

Temperature influence. The enzymatic syntheses were performed at 5, 25 and 37°C with the following incubation mixtures: rabbit microsomal protein, 8.4 mg/ml; UDP-GA, 5.0 m*M*; DM-

naproxen, 5.0 m*M*; $MgCl_2$, 5.0 m*M*; glucaro-1,4-lactone, 1.0 m*M*; and potassium phosphate buffer, 50 m*M* (pH 6.5). The total volume of each incubation mixture was 2 ml.

DM-naproxen acyl- and ether-glucuronide. The enzymatic synthesis was performed at 25°C with the following incubation mixture (total volume, 40.0 ml): rabbit microsomal protein, 11 mg/ml; UDP-GA, 10 m*M*; DM-naproxen, 10 m*M*; $MgCl_2$, 5.0 m*M*; potassium phosphate buffer (pH 6.5), 50 m*M*; and glucaro-1,4-lactone, 1.0 m*M*. The synthesis was stopped after 120 h by adding 100 ml of acetonitrile. After centrifugation at 1500 *g* for 10 min, the supernatant was separated by rotory evaporation. The glucuronides were isolated using preparative HPLC.

Naproxen acyl-glucuronide. The synthesis conditions were the same as those described for the sythesis of DM-naproxen glucuronides except for the substrate, naproxen, and the concentration of the protein which was 10 mg/ml. After 144 h the synthesis was stopped and the glucuronide was isolated as described for the glucuronides of DM-naproxen.

Preparative chromatography

The preparative HPLC systems used for the isolation procedure of different conjugates of naproxen and DM-naproxen were as followed: the column was a Knauer (Berlin, Germany) column (250 mm × 16 mm I.D.) packed with Polygosil 60 C-18 (Macherey-Nagel, Düren, Germany), 10 μm particle size. The column was operated at room temperature with a flow-rate of 10 ml/min. The conjugates were detected using UV detection at 332 nm. The mobile phases were methanol–100 m*M* ammonium formiate buffer pH 6.5 (45:55, v/v) for naproxen glycine conjugate and naproxen acyl-glucuronide and methanol–100 m*M* ammonium formiate buffer pH 3.0 (40:60, v/v) for DM-naproxen, acyl- and ether-glucuronides.

Sample preparation

To 200 μl of plasma, 400 μl of acetonitrile and 100 μl of 0.5 *M* potassium phosphate buffer (pH 3.0) were added. After 30 min at −18°C the mixture was centrifuged at 18 000 *g* for 4 min, and 20 μl of the supernatant were injected. The urine samples were treated in the same manner, but the addition of potassium phosphate buffer was omitted if the pH of the urine sample was below 6.0.

Analytical chromatography

The analytical column was a Knauer column (120 mm × 4.6 mm I.D.) packed with Polygosil 60, 5 μm particle size. The saturation column (150 mm × 4.6 mm I.D.), placed between the pump and the injector, was dry-packed with Li-Chroprep Si60 (Merck), 15–25 μm particle size. The columns were operated at 37°C. The final mobile phase was acetonitrile–0.2 *M* potassium phosphate buffer (pH 7.5)–water (65:30:105, v/v) containing 1.5 m*M* cetyltrimethylammonium (CTMA) bromide. The flow-rate was 1.5 ml/min.

RESULTS AND DISCUSSION

Enzymatic synthesis of glucuronides

Enzymatic synthesis of glucuronides is usually performed at pH 7.4–8.0 and at 37°C to give the maximum reaction rate for the transfer of the glucuronic acid moiety from the co-enzyme to the substrate [11,12]. However, many papers report that acyl-glucuronides are unstable in neutral and basic solutions [13–15], and that the stability of acyl-glucuronides is affected by the temperature [15].

When the formation of ether- and acyl-glucuronides is studied at pH 6.0, 6.5 and 7.0 using DM-naproxen as substrate (Fig. 2a and 2b), the net yield of the acyl-glucuronide, *i.e.* the formation (conjugation) minus the hydrolysis, is greater at pH 6.0, whereas the net yield of the ether-glucuronide is greater at pH 7.0. At pH 6.5, the yield is nearly the same as at pH 6.0 for the acyl-glucuronide whereas the yield of the ether-glucuronide, although lower than at pH 7.0, is about twice the yield of acyl-glucuronide after 20 h. Therefore, pH 6.5 is the most favourable choice for the simultaneous enzymatic synthesis of the two glucuronides of DM-naproxen.

When the formation of ether- and acyl-glucu-

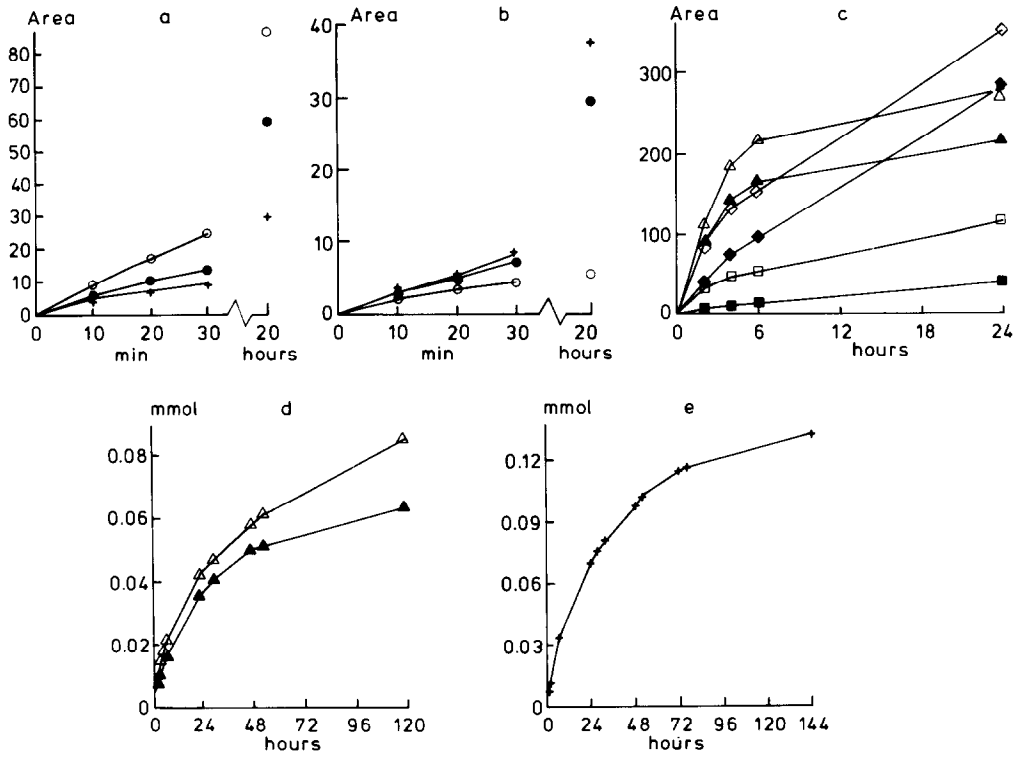

Fig. 2. (a) Formation of DM-naproxen ether-glucuronide during enzymatic synthesis at pH 6.0 (+), 6.5 (●) and 7.0 (○). Experiments performed at 37°C. (b) Formation of DM-naproxen acyl-glucuronide during enzymatic synthesis at pH 6.0 (+), 6.5 (●) and 7.0 (○). Experiments performed at 37°C. (c) Formation of DM-naproxen ether- and acyl-glucuronide during enzymatic synthesis at 5°C [(□) and (■) for ether- and acyl-glucuronide, respectively], 25°C [(◇) and (◆) for ether- and acyl-glucuronide, respectively] and 37°C [(△) and (▲) for ether and acyl-glucuronide, respectively]. (d) Formation of DM-naproxen ether- and acyl-glucuronide during the preparative enzymatic synthesis. (△) DM-naproxen ether-glucuronide; (▲) DM-naproxen acyl-glucuronide. Experiments performed at 25°C. (e) Formation of naproxen acyl-glucuronide during the preparative enzymatic synthesis. Experiments were performed at 25°C. For further details see under Experimental.

ronides is studied in a similar experiment at different temperatures (Fig. 2c), it is found that the initial formation rates of both glucuronides are faster at 37°C, but after 24 h the total yields are larger at 25°C, this probably being due to the increased stability of the enzyme or to decreased decomposition of UDP-GA or the glucuronides by micro-organisms (it was not possible to prevent the growth of micro-organisms during the trial). At 5°C the reaction rates are slow and the total yields are low.

The preparative enzymatic syntheses of the glucuronides of naproxen and DM-naproxen were performed at 25°C in six to seven days (Fig. 2d and e). In these experiments an optimum yield of glucuronides is needed using as little as possible of the UDP-GA, as this substance is rather expensive. The yields of the ether- and acyl-glucuronide of DM-naproxen were 33 mg (21%) and 25 mg (16%), respectively. No formation of diglucuronides of DM-naproxen was observed during the synthesis. The acyl-glucuronide was hydrolysed by β-glucuronidase(*E. coli*) as well as by 1 *M* NaOH, whereas the ether-glucuronide was resistent to cleavage by 1 *M* NaOH at room temperature but not to cleavage by β-glucuronidase (*E. coli*). The purity was 87 and 74% for the ether- and acyl-glucuronide, respectively, calculated as the free acids, determined after cleavage by β-glucuronidase. The yield of the acyl-glucu-

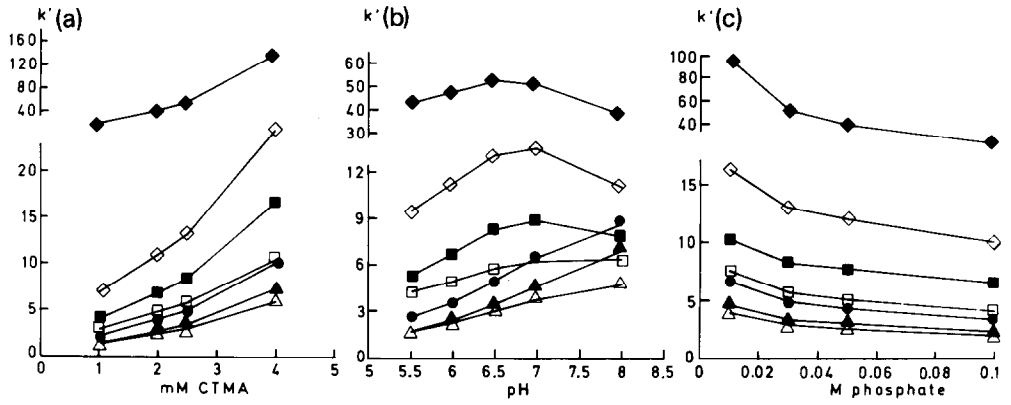

Fig. 3. (a) Relationship between the concentration of CTMA in the eluent and the capacity factor (k') for the seven analytes. Column, Polygosil 60, 5 μm, 120 mm × 4.6 mm I.D.; eluent, methanol–acetonitrile–0.2 *M* potassium phosphate (pH 6.5)–water (20:18:15:47, v/v) with CTMA added in the concentrations indicated. (◇) Naproxen; (□) naproxen glycine conjugate; (●) naproxen acyl-glucuronide; (■) DM-naproxen; (△) DM-naproxen ether-glucuronide; (▲) DM-naproxen acyl-glucuronide; and (◆) DM-naproxen-O-sulphate. (b) Relationship between pH of the buffer in the eluent and the capacity factor (k') for the seven analytes. Column as in (a); eluent, methanol–acetonitrile–0.2 *M* potassium phosphate (pH 5.5–8.0)–water (20:18:15:47, v/v) with 2.5 m*M* CTMA added. Symbols as in (a). (c) Relationship between the phosphate concentration in the buffer added and the capacity factor (k') for the seven analytes. Column as in (a); eluent, methanol–acetonitrile–0.2 *M* potassium phosphate (pH 6.5)–water [20:18:(5–50):(57–12), v/v] with 2.5 m*M* CTMA added. Symbols as in (a).

ronide of naproxen was 54 mg (33%) and the purity was 75% calculated as the free acid, determined after cleavage by β-glucuronidase.

Chromatography

The dynamically modified silica approach has already proved to be a valuable separation method in the analysis of biological samples, especially in the simultaneous analysis of the parent drug, metabolites and conjugates [16,17], which often seems to be difficult because of the large variation in polarity. In this technique, bare silica is used as a column packing material, but the addition of CTMA bromide to the aqueous eluent results in a reversed-phase HPLC system [18]. The presence of CTMA ions also implies the formation of very hydrophobic ion-pairs with anions. Thus this system is suitable for the analysis of the intact glucuronides as well as other anionic species.

To obtain a satisfactory separation of naproxen, DM-naproxen and their five conjugates, the chromatographic system was investigated with respect to the concentration of CTMA bromide in the eluent, the pH of the buffer, the concentration of the buffer and the nature of the organic modifier (methanol or acetonitrile).

CTMA. When the concentration of CTMA is increased, the amount of CTMA adsorbed onto the silica surface and the amount of CTMA in the mobile phase are increased. As expected, the capacity factor (k') of all analytes is increased when the CTMA concentration is increased (Fig. 3a), partly as a function of the increased amount adsorbed as the stationary phase and partly because of increased ion-pair formation between CTMA ions and the anionic analytes at pH values greater than 4–5. Little change in selectivity is seen between the seven analytes, but DM-naproxen-O-sulphate, containing an additional anionic sulphate group, is more affected than the other analytes which only contain the carboxylate group.

pH. The pK_a value of the silanol groups on the silica surface is 6.5–7.0. When the pH of the buffer in the eluent is increased, the ionization of the silanol groups is also increased and more CTMA groups are adsorbed on the surface. As seen in Fig. 3b, the retention of the three glucuronides and the glycine conjugates is increased in the range 5.5–8.0, whereas the three other analytes

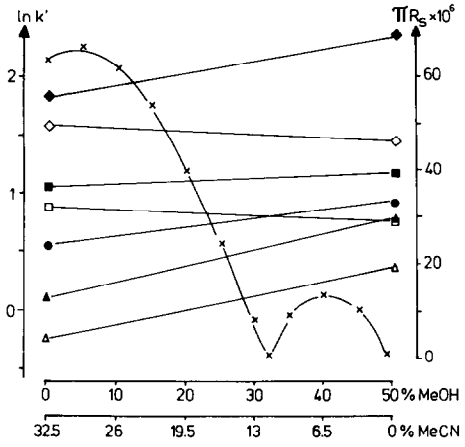

Fig. 4. Relationship between the organic modifier in the eluent and the natural logarithm to the capacity factor (lnk') for the seven analytes. Optimization diagram with respect to product resolution (ΠR_s) is shown (×). The product resolution is the product of the resolutions (R_s) calculated for each pair of neighbouring peaks at a given composition of the mobile phase. Column as in Fig. 3a. Eluent: methanol–0.2 M potassium phosphate (pH 7.5)–water (50:15:35, v/v) with 1.5 mM CTMA added or acetonitrile–0.2 M potassium phosphate (pH 7.5)–water (65:30:105, v/v) with 1.5 mM CTMA added. Symbols as in Fig. 3a.

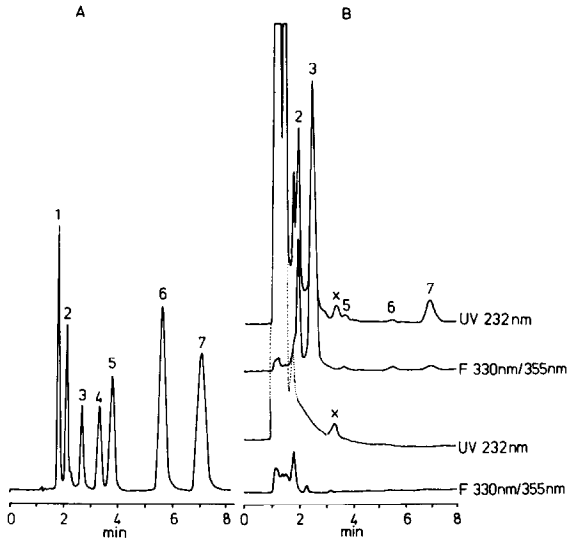

Fig. 5. (A) Chromatogram of standards of (1) DM-naproxen ether-glucuronide, (2) DM-naproxen acyl-glucuronide, (3) naproxen acyl-glucuronide, (4) naproxen glycine conjugate, (5) DM-naproxen, (6) naproxen and (7) DM-naproxen-O-sulphate. Chromatographic conditions as in Fig. 4, mobile phase b. UV detection at 232 nm. (B) Chromatogram of urine from volunteers before and after receiving a dose of 250 mg naproxen orally. Chromatographic conditions as in Fig. 4, mobile phase b. UV detection at 232 nm and fluorescence (F) excitation at 330 nm and emission at 355 nm. Peaks as in (A).

have a maximal retention at pH 6.5–7.0. Fig. 3b also shows the great change in selectivity when the buffer pH is changed.

Buffer concentration. The influence of the buffer concentration is investigated by changing the concentration of the buffer in the mobile phase in the range 0.01–0.1 M. The retention of all seven analytes decreases when the concentration increases, but no change in selectivity is seen (Fig. 3c).

Organic modifier. It is well known that different modifiers or mixtures of modifiers may have a large effect on the selectivity of the chromatographic system. The pH (7.5) and the concentration of CTMA (1.5 mM) were changed relative to the initial system to obtain less retention of DM-naproxen-O-sulphate. Finally the chromatographic system was optimized according to the method of Schoenmakers *et al.* [19] to obtain an optimum resolution of the seven analytes. Two modifiers were tested, acetonitrile (32.5%, v/v) and methanol (50%, v/v), with nearly identical eluotropic effect on naproxen. The optimization diagram is given in Fig. 4. The results indicate that a mixture of 5% methanol and 29.25% acetonitrile will provide an optimum resolution of the analytes, but the use of acetonitrile alone as a modifier should give nearly the same resolution. In Fig. 5A this is seen to be true.

Assay validation

All of the seven compounds investigated absorb in the UV range and also fluoresce (Table I and Fig. 5). For six of the seven substances the limit of detection is about 0.1 μg/ml regardless of the method of detection. The detection limit for DM-naproxen-O-sulphate using fluorescence at 330 or 355 nm is 0.5 μg/ml, whereas the limit of detection using UV absorption is 0.1 μg/ml. A signal-to-noise ratio of 5 has been used as the definition of the detection limit. The limit of quantification for all the substances, except the

TABLE I

ANALYSIS OF DRUG-FREE HUMAN PLASMA SAMPLES SPIKED WITH THE SEVEN ANALYTES

Sample	Added (μg/ml)	Found (n = 6) (μg/ml)	Recovery (%)	Intra-assay coefficient of variation (%)	Method of detection[a]
DM-naproxen ether-glucuronide	1.27	1.23	97	3.8	F
	6.37	6.11	96	1.4	
	63.7	54.9	86	4.0	
DM-naproxen acyl-glucuronide	1.05	0.99	95	3.9	F
	5.27	5.22	99	2.1	
	52.7	52.9	100	1.7	
Naproxen acyl-glucuronide	1.06	1.12	106	3.7	F
	5.30	4.92	93	1.7	
	53.0	52.0	98	1.9	
Naproxen glycine conjugate	1.08	1.12	104	4.1	F
	5.40	5.90	109	3.2	
	54.0	55.0	102	1.6	
DM-naproxen	1.12	1.15	103	5.0	F
	5.58	5.67	102	2.7	
	55.8	55.9	100	2.7	
Naproxen	1.04	0.97	93	4.5	F
	5.19	4.17	80	1.0	
	51.9	46.2	89	2.5	
DM-naproxen-O-sulphate	0.86	0.84	97	6.8	UV
	4.32	4.24	98	3.0	
	43.2	41.7	97	2.5	

[a] UV = UV detection af 232 nm; F = fluorescence detection, excitation at 330 nm and emission at 355 nm.

two glucuronides of DM-naproxen, is of the same order of magnitude if the method of detection given in Table I is used. For the two glucuronides of DM-naproxen the limit of quantification in urine is about 1 μg/ml. Validation studies were conducted by adding known amounts of naproxen and the six metabolites and conjugates of naproxen to drug-free human plasma at three different concentrations. The recoveries were found to be 80–109% for all seven analytes, and the coefficients of variation were low: 1.0–4.0% at the two highest concentrations and 3.7–6.8% at the lowest concentration (Table I).

The stability of the acyl-glucuronides and the O-sulphate has been tested in 0.05 *M* phosphate buffer at different pH values. Half-lives of 18 and 26 h for naproxen acyl-glucuronide and DM-naproxen acyl-glucuronide, respectively, at pH 7.0 were found. DM-naproxen-O-sulphate is stable at pH 4–8 for 24 h with less than 5% degradation.

TABLE II

CUMULATED EXCRETION OF NAPROXEN AND THE FOUR METABOLITES AND CONJUGATES FOUND IN HUMAN URINE, 0–96 h AFTER THE ADMINISTRATION OF 250 mg NAPROXEN TO VOLUNTEERS A AND B ORALLY

Compound	Excretion (% of dose)	
	A	B
Naproxen acyl-glucuronide	44.6	43.7
DM-naproxen acyl-glucuronide	18.0	21.3
DM-naproxen-O-sulphate	10.0	9.3
DM-naproxen	1.5	0.7
Naproxen	1.1	0.7
Total	75.2	75.7

Application

The developed method has been used for the determination of the metabolic pattern of naproxen in humans. Two healthy male volunteers aged 29 and 33 years with body weights of 72 and 68 kg respectively, were given 250 mg of naproxen (Daprox) orally (volunteers A and B). Urine was collected between 0 and 96 h. Fig. 5b shows chromatograms of the urine samples using UV and fluorescence detection. Only four of the six metabolites and conjugates of naproxen could be detected in human urine. Naproxen glycine conjugate and DM-naproxen ether-glucuronide could not be detected even after cleavage of the two acyl-glucuronides by 1 *M* NaOH at room temperature. This indicates that less than 0.2% of the dose was excreted as one of these two metabolites. The total amounts of naproxen and the four metabolites and conjugates of naproxen excreted by the two volunteers in urine over 96 h are given in Table II. These results are in good agreement with the cumulative excretion of naproxen, DM-naproxen glucuronide and naproxen glucuronide found by Guelen *et al.* [8] after a rectal dose of 500 mg naproxen.

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