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Journal of Chromatography B, 721 (1999) 109–125

JOURNAL OF CHROMATOGRAPHY B

Direct assay of nonopioid analgesics and their metabolites in human urine by capillary electrophoresis and capillary electrophoresis–mass spectrometry[1]

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Received 19 June 1998; received in revised form 5 October 1998; accepted 19 October 1998

Abstract

Capillary electrophoresis (CE) was used for the analysis of nonopioid analgesics and their metabolites directly in urine samples. A simple, reliable screening method was developed that allows identification of the drug and/or its metabolites in urine after oral application of paracetamol, acetylsalicylic acid, antipyrine, ibuprofen, naproxen, ketoprofen and propyphenazone by their migration in CE and by their UV spectra recorded with a diode-array detector in a common CE–UV system with 50 m*M* borax pH 9.4 as separation buffer. For the CE–electrospray (ESI)-MS coupling a volatile 50 m*M* ammonium acetate buffer at pH 9.8 was used. In order to analyze the metabolic pattern in more detail different methods were developed for each drug. The separation of the metabolites of acetylsalicylic acid could be improved by injection of the urine sample at the cathodic side of the capillary. In order to identify antipyrine as neutral compound as well as its neutral metabolites a micellar electrokinetic chromatography (MEKC) method was developed.

Keywords: Micellar electrokinetic chromatography; Paracetamol; Acetylsalicylic acid; Antipyrine; Ibuprofen; Naproxen; Ketoprofen; Propyphenazone

1. Introduction

Nonopioid analgesics such as paracetamol (acetaminophen), acetylsalicylic acid, antipyrine (phenazone) and ibuprofen are well-known drugs which are frequently used for the treatment of fever and minor pain and are available without prescription. Besides their usual therapeutic use, chronic abuse, accidental intoxications and the intake of high doses especially of paracetamol and acetylsalicylic acid for suicide purposes have been described [1,2]. Additionally, due to their safety at normal dosage they have often been used as model compounds for the investigation of phase II (paracetamol) [3,4] or phase I biotransformations (antipyrine) [5,6] or are referred to as typical compounds for the examination of plasma protein binding (acetylsalicylic acid) or stereoselective biotransformation processes (ibuprofen). In all these cases simple and fast assays of the drugs and their metabolites in body fluids are needed for rapid and certain diagnoses and interpretations of studies.

[1]Dedicated to Professor B. Unterhalt on the occasion of his 65th birthday.

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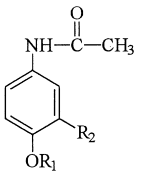
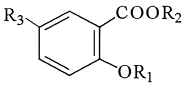
PII: S0378-4347(98)00483-6

A)



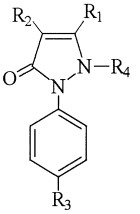
compound		R_1	R_2
paracetamol	P	H	H
paracetamol glucuronide	PG	-GlucU	H
paracetamol sulfate	PS	SO_3	H
paracetamol cysteinate	PC	H	$-S-CH_2-CH(COOH)-NH_2$
paracetamol mercapturate	PM	H	$-S-CH_2-CH(COOH)-NH-C(=O)-CH_3$
methoxyparacetamol glucuronide	MPG	-GlucU	OCH_3

B)

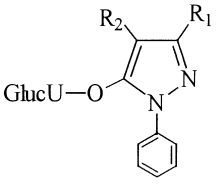


compound		R_1	R_2	R_3
acetylsalicylic acid	ASA	$-C(=O)-CH_3$	H	H
salicylic acid	SA	H	H	H
salicyluric acid	SU	H	$-NH-CH_2COOH$	H
salicylic acid acyl glucuronide	SAAG	H	-GlucU	H
salicylic acid phenolic glucuronide	SAPG	-GlucU	H	H
salicyluric acid phenolic glucuronide	SUPG	-GlucU	$-NH-CH_2COOH$	H
gentisic acid	GA	H	H	OH

C)



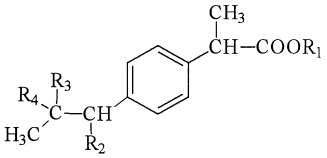
compound		R_1	R_2	R_3	R_4
antipyrine (phenazone)	Ph	CH_3	H	H	CH_3
3-hydroxymethyl antipyrine	3OHPh	CH_2OH	H	H	CH_3
antipyrine-3-carboxylic acid	PhC	COOH	H	H	CH_3
4-hydroxyantipyrine	4OHPh	CH_3	OH	H	CH_3
4,4'-dihydroxyantipyrine	44DiOHPh	CH_3	OH	OH	CH_3
norantipyrine	NPh	CH_3	H	H	H
4-hydroxyantipyrine glucuronide	4OHPhG	CH_3	-O-GlucU	H	CH_3



norantipyrine glucuronide (NPhG)

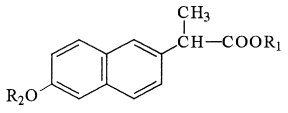
Fig. 1. Structures of the nonopioid analgesics and their metabolites (-O-GlucU=glucuronic acid).

D)



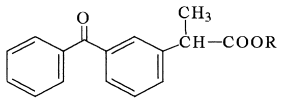
compound		R_1	R_2	R_3	R_4
ibuprofen	Ibu	H	H	H	CH_3
2'-hydroxyibuprofen	Ibu-2-OH	H	H	OH	CH_3
2'-carboxyibuprofen	IbuCOOH	H	H	H	COOH
1'-hydroxyibuprofen	Ibu-1-OH	H	OH	H	CH_3
3'-hydroxyibuprofen	Ibu-3-OH	H	H	H	CH_2OH
ibuprofen glucuronide	IbuG	-GlucU	H	H	CH_3
2'-hydroxyibuprofen glucuronide	Ibu-2-OG	-GlucU	H	OH	CH_3
2'-carboxyibuprofen glucuronide	IbuCOOG	-GlucU or H	H	H	COOH or COOGlucU

E)



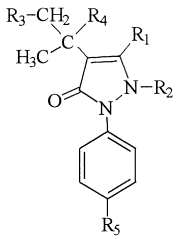
compound		R_1	R_2
naproxen	N	H	CH_3
O-demethylnaproxen	ODN	H	H
naproxen glucuronide	NG	-GlucU	CH_3
O-demethylnaproxen glucuronide	ODNG	-GlucU	H

F)



compound		R
ketoprofen	K	H
ketoprofen glucuronide	KG	-GlucU

G)



compound	R_1	R_2	R_3	R_4	R_5
propyphenazone	CH_3	CH_3	H	CH_3	H
N-demethylpropyphenazone	CH_3	H	H	CH_3	H
hydroxylated propyphenazone derivatives	CH_2OH	CH_3	H	CH_3	H
	CH_3	CH_3	OH	CH_3	H
	CH_3	CH_3	H	CH_2OH	H
	CH_3	CH_3	H	CH_3	OH
N-demethylhydroxyphenazone	CH_3	H	H	CH_3	OH

Fig. 1. (*continued*)

As illustrated by the compilation of the drug compounds and their main metabolites in Fig. 1 the analgesics undergo extensive biotransformations in man yielding phase I and to a large extent phase II metabolites of the parent drugs and their phase I products.

The metabolism of paracetamol is dominated by direct glucuronidation and sulfation (Fig. 1A). Cytochrome P-450 reactions take place to a minor extent and lead mainly to paracetamol cysteinate and paracetamol mercapturate or in small amounts to hydroxylated and methoxylated products detectable as glucuronides in urine [7].

After oral application of acetylsalicylic acid (Fig. 1B) to a healthy volunteer the drug is rapidly hydrolyzed to salicylic acid. Further biotransformation results in the main metabolite salicyluric acid and the glucuronide conjugates of salicylic acid (acyl and phenolic glucuronide) and salicyluric acid (phenolic glucuronide). Hydroxylation products like gentisic acid occur only in traces [8].

Antipyrine (Fig. 1C) undergoes a complex phase I metabolism catalyzed by different cytochrome P-450 isoenzymes yielding several hydroxylated products and demethyl and carboxy derivatives [9]. Most of these compounds are subsequently conjugated to glucuronides and sulfates. Thus, in urine the main metabolites are the glucuronides of 4-hydroxyantipyrine and norantipyrine, whereas antipyrine and other phase I and phase II products appear in lower concentrations. The metabolism of propyphenazone is very similar to antipyrine, beside additional products which are formed by hydroxylation in the side chain (Fig. 1G). Main metabolites in urine are the glucuronides of *N*-demethylated, hydroxylated and demethylhydroxyl derivatives of propyphenazone [10,11].

Ibuprofen is also metabolized by hydroxylation and optional further oxidation in the side chain and by glucuronidation of the metabolites and the parent drug to acyl glucuronides (Fig. 1D). Main metabolites are 2-carboxyibuprofen and 2-hydroxyibuprofen and their glucuronides [12,13]. Naproxen is metabolized by demethylation and glucuronidation of the parent drug and the demethyl product (Fig. 1E) [14,15]. Ketoprofen is mainly transformed into its acyl glucuronide. Phenolic compounds mentioned in the literature exist in very low concentrations (Fig. 1F) [16].

Chromatographic techniques like HPLC or more rarely GC are the common choice for the investigation of the metabolic pattern of drug substances. Thus, for all the above mentioned drugs HPLC assays are described in literature [17–22]. However, the separation of polar phase II metabolites in polar media and the complexity of the matrix might raise problems which can only be solved by time-consuming sample preparations, using gradient eluent systems with a high consumption of organic solvents or increasing analysis times.

Capillary electrophoresis is an analytical technique that has developed tremendously during the last years. It has been established in biomedical analysis as a powerful alternative or supplement to HPLC or GC [23–28]. Particularly advantageous for biomedical applications is the excellent peak resolution which enables performance of analyses directly in body fluids and the small sample volumes needed for the assays.

To date, CE has been used to analyze the drug compounds in biofluids [29–34].

The determination of paracetamol and its metabolites in urine and serum is described [35]. Stereochemical investigations of the metabolism of ibuprofen have been published recently [36]. Experiments with a hyphenated tandem mass spectrometer were carried out after solid-phase extraction of urine samples in order to detect the metabolites of ibuprofen, and in part of paracetamol and acetylsalicylic acid [37,38].

The studies described below were carried out in order to evaluate the potential of CE for the analysis of all important members of the heterogeneous group of nonopioid analgesic compounds and their metabolites directly in urine samples. Thus, a simple, rapid and robust screening method could be developed that allows detection of ingestion of these drugs by the analysis of their metabolic pattern in urine, though they differ widely in their chemical structure and characteristics.

Various CE modes were used for the development of individual methods for each of the drugs to have a closer look at the metabolic pattern. While the capillary zone electrophoresis mode itself offers optimal separation conditions for the charged phase II metabolites it was tried to separate antipyrine and its in part neutral metabolites by micellar electrokinetic chromatgraphy (MEKC). Aspects concerning

the stereoselective biotransformation of the chiral compounds ibuprofen and etodolac and their stereoselective separation as well as that of their metabolites directly in urine samples will be published elsewhere [39].

Diode-array detection (DAD) and CE–MS coupling were used for on-line identification of the analytes in urine samples.

2. Experimental

2.1. Chemicals and reagents

Paracetamol and its metabolites were obtained as described previously [35]. Acetylsalicylic acid, salicylic acid, salicyluric acid, gentisic acid, antipyrine, 4-hydroxyantipyrine, ibuprofen, ketoprofen and naproxen were purchased from Sigma–Aldrich (Deisenhofen, Germany). Norantipyrine, 3-hydroxyantipyrine, 4,4′-dihydroxyantipyrine and antipyrine-3-carboxylic acid were donated by Dr. M. Neugebauer (University of Bonn, Germany), and 2-hydroxyibuprofen and carboxyibuprofen by Dr. G. Geisslinger (University of Erlangen-Nuernberg, Germany).

The glucuronides of salicylic acid and salicyluric acid were collected from urine samples of a healthy volunteer after the intake of 1 g acetylsalicylic acid. Twenty ml of urine were purified and concentrated by solid-phase extraction. The eluent was dried under a stream of nitrogen and reconstituted in water. This solution was fractionated by HPLC with a Lichrospher RP-8 select B column and a gradient mobile phase system (1% acetonitrile in 0.5% acetic acid in water at 0 min to 35% acetonitrile at 30 min) with slight deviations following a method published by Vree et al. [40]. The glucuronides of ibuprofen and its metabolites as well as of norantipyrine, 4-hydroxyantipyrine, naproxen and O-demethylnaproxen were collected via similar procedures. *O*-Demethylnaproxen was prepared as described previously [21].

Sodium tetraborate (borax), 0.1 *M* sodium hydroxide, sodium dihydrogenphosphate, disodium monohydrogenphosphate, sodium hydrogencarbonate, acetic acid, ammonium acetate, ammonium hydroxide and methanol were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS) was supplied by Fluka (Buchs, Switzerland).

2.2. Apparatus and methods

2.2.1. Screening methods

The CE instrument used for the UV detection experiments was a Beckman P/ACE 5510 (Beckman, Munich, Germany) equipped with a diode-array detector and GoldSoftware 8.10. Electrophoretic runs were performed in an uncoated fused-silica capillary (50 μm I.D.×40/4 7 cm effective/total length) filled with a buffer consisting of 50 m*M* borax pH 9.4. The field strength was 500 V/cm. Between each run the capillary was rinsed with 0.1 *M* sodium hydroxide for 1 min followed by a rinse with the run buffer for 2 min. The temperature of the cooling system of the capillary was kept at 20°C. The detection was carried out at 235 nm. Urine samples were introduced into the capillary by hydrodynamic injection with 0.5 p.s.i. (1 p.s.i.=6894.76 Pa) for 2 s followed by the injection of water for 1 s.

The CE–ESI-MS experiments were performed with a Grom capillary electrophoresis system 100 high voltage power supply (Herrenberg, Germany) in an uncoated fused-silica capillary (50 μm I.D.×44 cm length) filled with a buffer containing 50 m*M* ammonium acetate adjusted to pH 9.8 with 50 m*M* ammonium hydroxide. Hydrostatic injection (10 cm, 10 s) was used to introduce the samples into the anodic side of the capillary. A field strength of 225 V/cm was applied. A LCQ® ion trap mass spectrometer (Finnigan, Branford, CT, USA) equipped with an electrospray interface was used in the negative ion mode for the detection of the analytes. The sheath liquid consisting of 50% methanol–49% water–1% ammonia was delivered at a flow-rate of 6 μl/min using a syringe pump.

2.2.2. Individual assays of the metabolites of some of the drugs

2.2.2.1. *Acetylsalicylic acid*

2.2.2.1.1. *Injection at the anodic side of the capillary*. The pH value of the screening method was varied between pH 8.8 and 9.4 by adjustment with 200 m*M* boric acid. A satisfactory separation was

achieved at pH 9.0. All other conditions were the same as described in Section 2.2.1).

2.2.2.1.2. *Injection at the cathodic side of the capillary*. When the sample was injected at the cathodic side a 100 m*M* phosphate buffer pH 5.1 was used which contained 1 m*M* spermine tetrahydrochloride. The applied field strength was −500 V/cm. All other conditions were as mentioned before. Prior to each run the capillary was rinsed for 1 min with 0.1 *M* phosphoric acid and for 2 min with the separation buffer. The urine samples were injected directly for 2 s followed by an injection of 0.01 *M* sodium hydroxide solution for 1s.

For the CE–MS coupling with the injection at the cathodic side a 50 m*M* ammonium acetate pH 5.0 was used at a field strength of −450 V/cm in a 44 cm capillary coated with polyacrylamide [41]. The sheath liquid contained 2% ammonia in water–methanol (50:50). Other conditions were as mentioned in Section 2.2.1).

2.2.2.2. *Antipyrine*. In order to separate the neutral analytes 100 m*M* SDS were added to the separation buffer. Due to the potential instability of some phase I metabolites at alkaline pH values and the comigration of antipyrine-3-carboxylic acid and 3-hydroxymethylantipyrine at this pH a 50 m*M* phosphate buffer at pH 7.5 was used with 100 m*M* SDS.

2.2.2.3. Ibuprofen

Beside the introduction of the sample at the anodic side in the screening method an assay was developed with injection at the cathodic side of the capillary in a buffer consisting of 100 m*M* phosphate pH 5.3 and 3 m*M* spermine tetrahydrochloride. All other parameters were the same as for the analysis of urine samples collected after the administration of acetylsalicylic acid by injecting at the cathodic side.

2.3. Collection of urine samples

Urine samples were collected from healthy volunteers after oral application of the analgesics. The collection intervals for each drug are given in the legends to the figures. Usually the presented intervals cover 0–4 h. The corresponding blank urine samples were taken over the same time range immediately before the application of the drugs.

2.4. Identification of the glucuronides by cleavage of the conjugates

2.4.1. Cleavage of phenolic or alcoholic glucuronides in β-glucuronidase assays

A 0.2 ml volume of the urine sample was added to 1.8 ml sodium acetate buffer pH 4.6 containing ca. 1000 I.U. β-glucuronidase type H-1 from *Helix pomatia* and incubated for 24 h at 37°C. The reaction was stopped by adding 2 ml of acetonitrile. The precipitated proteins were separated by centrifugation (10 min at 1200 *g*). A 0.3 ml volume of the supernatant was dried under a stream of nitrogen and reconstituted in 50 μl water. The solution was used for CE analysis.

2.4.2. Alkaline cleavage of acyl glucuronides

A 0.5 ml volume of the urine sample adjusted to pH 9 by the addition of 0.5 *M* NaOH was kept at 37°C for 1 h. Afterwards the solution was adjusted to pH 5 with 0.5 *M* HCl and analyzed by CE.

3. Results and discussion

3.1. Development of the screening method

In Figs. 2 and 3 electropherograms of urine samples collected after receiving therapeutic doses of the analgesics investigated (i.e. 500 mg paracetamol, 500 mg acetylsalicylic acid, 500 mg antipyrine, 500 mg ibuprofen, 500 mg propyphenazone, 250 mg naproxen, and 50 mg ketoprofen) are compared to electropherograms of blank urine samples.

The samples were injected at the anodic side of the capillary. Thus, the anionic analytes are moved to the detection window by the electroosmotic flow (EOF) against their own migration direction. The runs were carried out in an alkaline separation media in order to obtain both a strong EOF and the dissociation of the weakly acidic analytes. In order to avoid dispersive effects caused by the high salt content in urine samples separation buffers with high

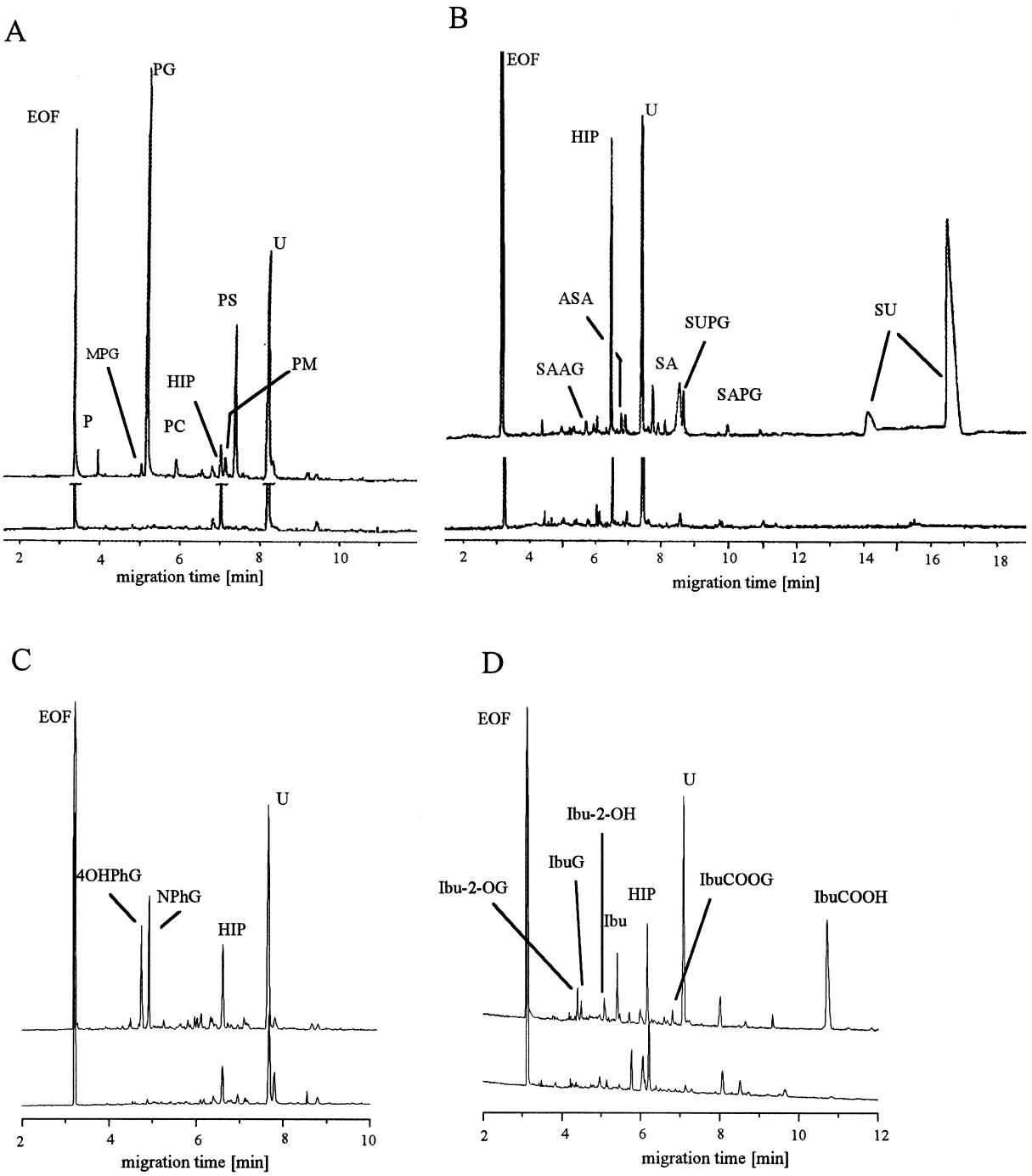


Fig. 2. Detection of nonopioid analgesics and their metabolites in urine samples, collected 0–4 h after oral application of 500 mg paracetamol (A), 500 mg acetylsalicylic acid (B), 500 mg antipyrine (C) and 500 mg ibuprofen (D) by using the screening method (abbreviations of the analytes as described in Fig. 1, HIP=hippuric acid, U=uric acid).

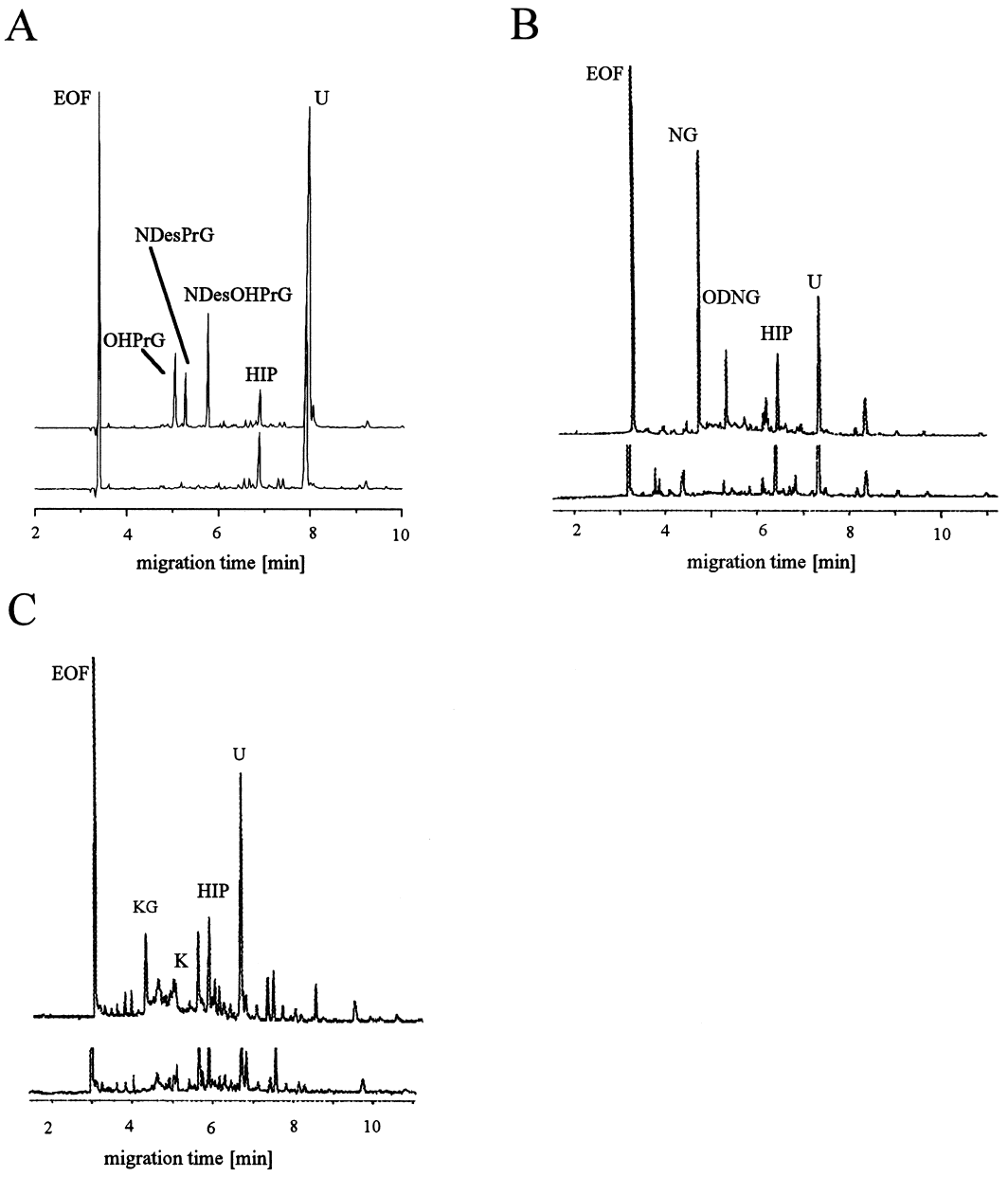


Fig. 3. Detection of nonopioid analgesics and their metabolites in urine samples, collected 0–4 h after oral application of 500 mg propyphenazone (A), 0–10 h after oral application of 250 mg naproxen (B), 0–5 h after oral application of 50 mg ketoprofen (C) by using the screening method (abbreviations of the analytes as described in Fig. 1).

ionic strengths were chosen. Simple preparation, good stability and reliable results could be obtained by using a 50 m*M* borax buffer pH 9.4. Further peak sharpening was obtained by the injection of a water zone behind the sample. This caused focusing effects of the analyte zones and thus reduced peak tailing

[35]. Satisfactory separations were achieved using untreated 50 μm capillaries (40/47 cm effective/total length) at a field strength of 500 V/cm.

The high efficiency achieved enabled avoidance of peak interferences between the analytes and matrix compounds from urine and the separation of the metabolites with similar structures. In particular, for the analysis of the polar phase II metabolites in polar media like urine samples CE appears to be favorable due to its separation principle based on the different electromigrations of these charged compounds. This is remarkable because the metabolism of the nonopioid analgesics is dominated by glucuronidation and sulfation reactions.

The identification of the analytes was carried out by spiking urine samples with authentic substances. Sulfatase and β-glucuronidase incubations were used to confirm the identification of the glucuronides and sulfates by their cleavage yielding the corresponding drug compound or phase I metabolite.

Most interest was focused on on-line identification methods that provide additional structural data on the detected compounds after direct injection of the samples. With a diode-array detector UV spectra (200–400 nm) were recorded and compared to the spectra obtained from authentic compounds under the CE conditions. In order to demonstrate that the peaks are free from interferences with matrix compounds all electropherograms obtained after the application of the drugs were compared to corresponding blank urine samples.

As can be seen in all electropherograms, apart from neutral endogenous compounds like creatinine, which migrate with the velocity of the EOF, hippuric acid and uric acid were detected as acidic endogenous compounds. These three peaks could be used as migration time markers due to their ubiquitous occurrence in urine. In some cases, especially when biological matrices are assayed, the migration time repeatabilities are known to be troublesome. When 20 different urine samples were analyzed with the herein described screening method the migration time repeatabilities of the neutral compounds, hippuric acid and uric acid were below 1%.

As illustrated in the figures the intake of a normal dose of each of the drugs could be confirmed easily by detecting different patterns of analytes related to the different drugs and their main metabolites directly in urine samples without any sample preparation. Especially the excellent separations of the phase II metabolites are remarkable. Individual features of the electropherograms for each drug compound are discussed below. Further development of the assays for the determination of paracetamol (Fig. 2A), its main metabolites paracetamol glucuronide and paracetamol sulfate in urine and serum as well as the minor metabolites paracetamol mercapturate, paracetamol cysteinate and 3-methoxyparacetamol glucuronide in urine are described [35].

For CE–ESI-MS experiments the borax buffer was substituted by a volatile 50 m*M* ammonium acetate buffer at pH 9.8 [35]. The results of the analysis of urine samples collected after the administration of antipyrine and propyphenazone are illustrated as reconstructed ionic electropherograms (RIEs) of the analyte masses and as full scan mass spectra recorded at the apex of the peaks in these RIEs in Fig. 4. The urine samples collected after the administration of acetylsalicylic acid and ibuprofen were analyzed by injecting at the anodic side (Fig. 5A and C, respectively) as well as the cathodic side with negative voltage and an acidic separation buffer (Fig. 5B and D, respectively). The RIEs of naproxen and ketoprofen and their main metabolites are shown in Fig. 6. Experiments with urine samples containing paracetamol and its metabolites under the same conditions were published in Ref. [35]. The observed results reveal the advantages of CE–ESI-MS in terms of separation selectivity. It appeared to be extremely useful in cases like the investigation of urine samples after application of propyphenazone when no metabolites were at our disposal and the identification of the peaks in the urine samples could only be supposed by cleaving experiments and by their migration behavior. The CE–ESI-MS coupling allowed us to verify these assumptions easily by the direct analysis of urine samples.

DAD and MS detection can be combined within one run. This can be useful in cases when the recorded UV spectra of the metabolites are very similar (as in the case of ibuprofen). Only by MS could the parent compound and its hydroxylated or carboxylated derivatives including their glucuronides be easily identified. In contrast, we were not able to differentiate between the glucuronides of salicylic acid by MS because they have the same mass.

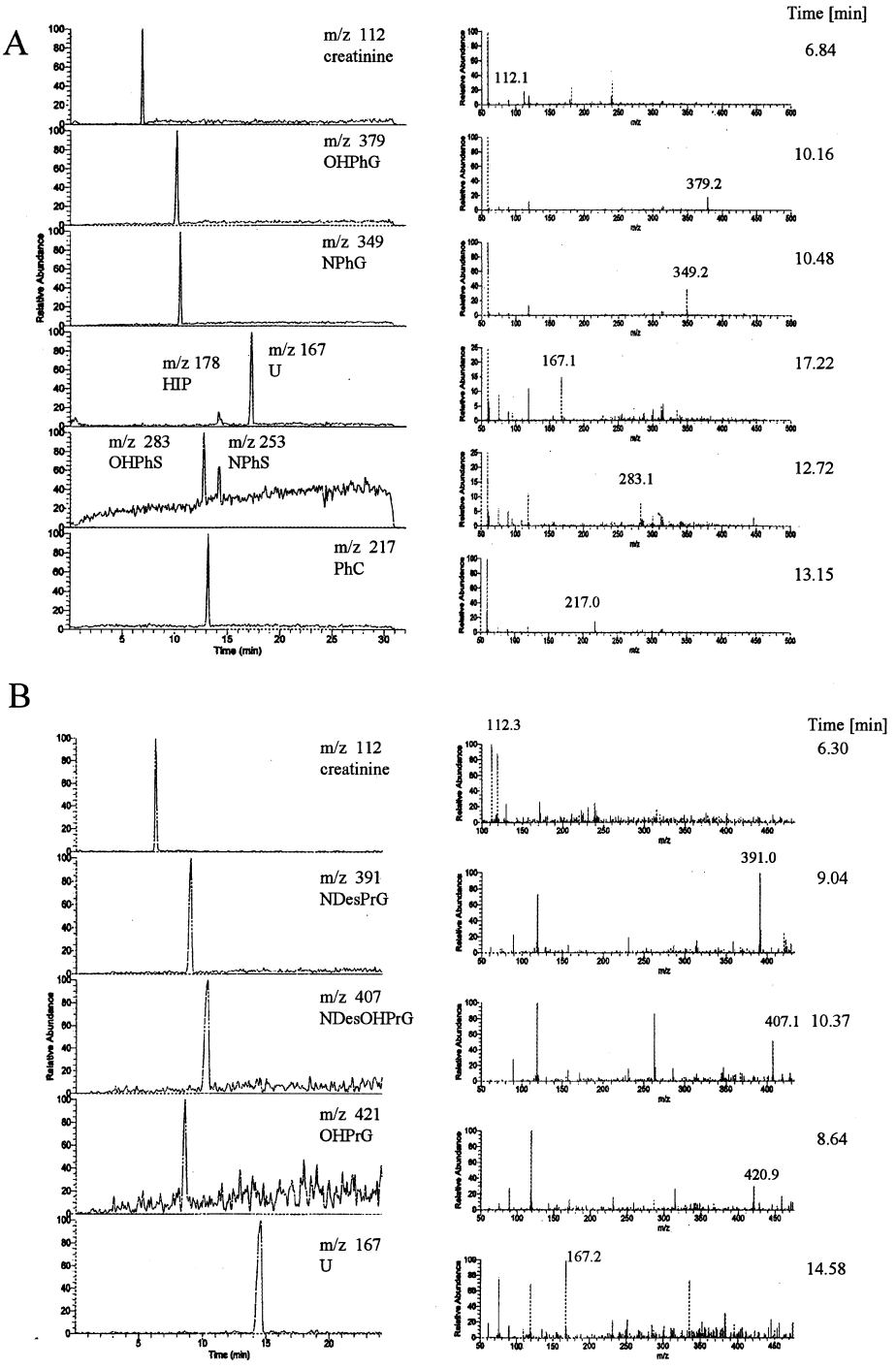


Fig. 4. Detection of nonopioid analgesics directly in urine samples by the screening method with MS detection. (A) RIEs and corresponding full scan mass spectra of the metabolites of antipyrine (abbreviations of the analytes as in Fig. 1, OHPhS and NPhS are the RIEs of the hydroxy and norantipyrine sulfate, creatinine serves as EOF marker). (B) RIEs and the corresponding full scan mass spectra of the metabolites of propyphenazone. CE conditions: 50 m*M* ammonium acetate pH 9.8, 225 V/cm, as described in Section 2.

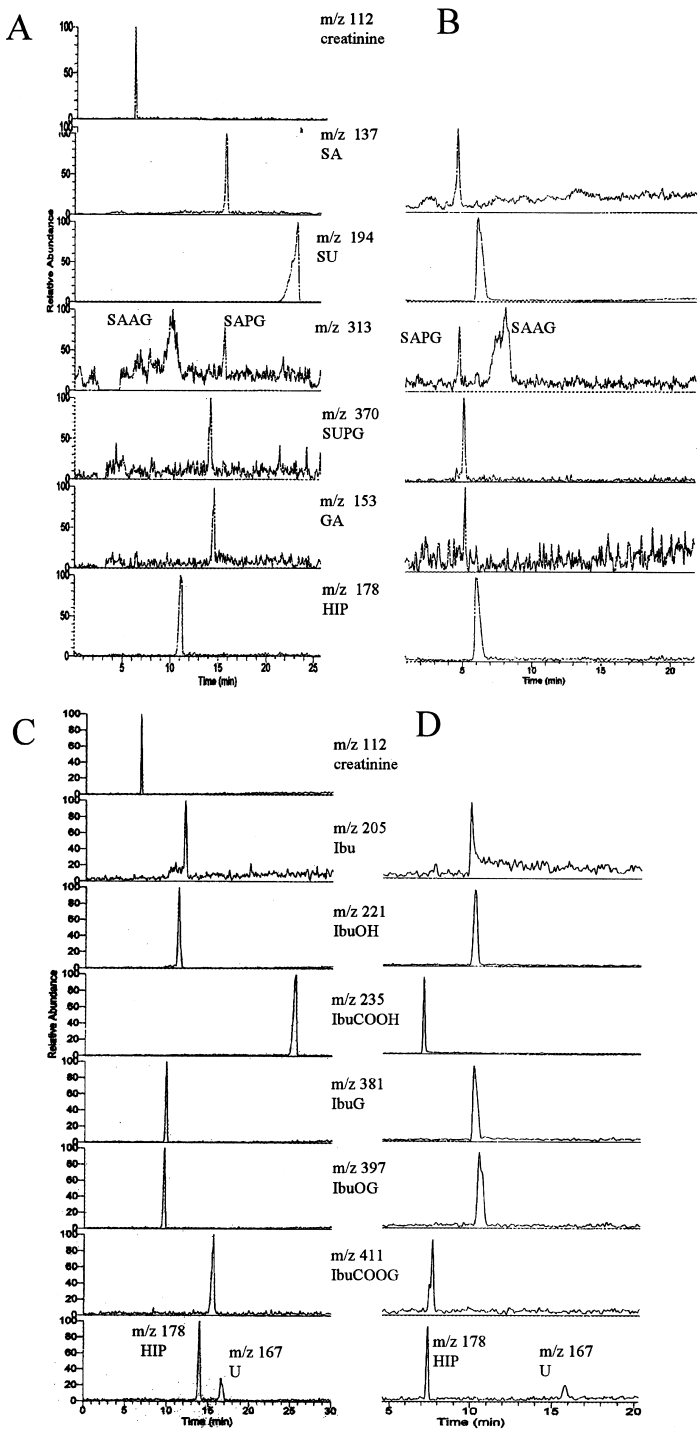


Fig. 5. (A) and (C) analysis of urine samples after the administration of acetylsalicylic acid and ibuprofen, respectively, after injection at the anodic side of the capillary (conditions: as described in Fig. 4). (B) and (D) Electropherograms obtained after injection at the cathodic side (conditions: 50 m*M* ammonium acetate pH 5.0, −450 V/cm, as described in Section 2).

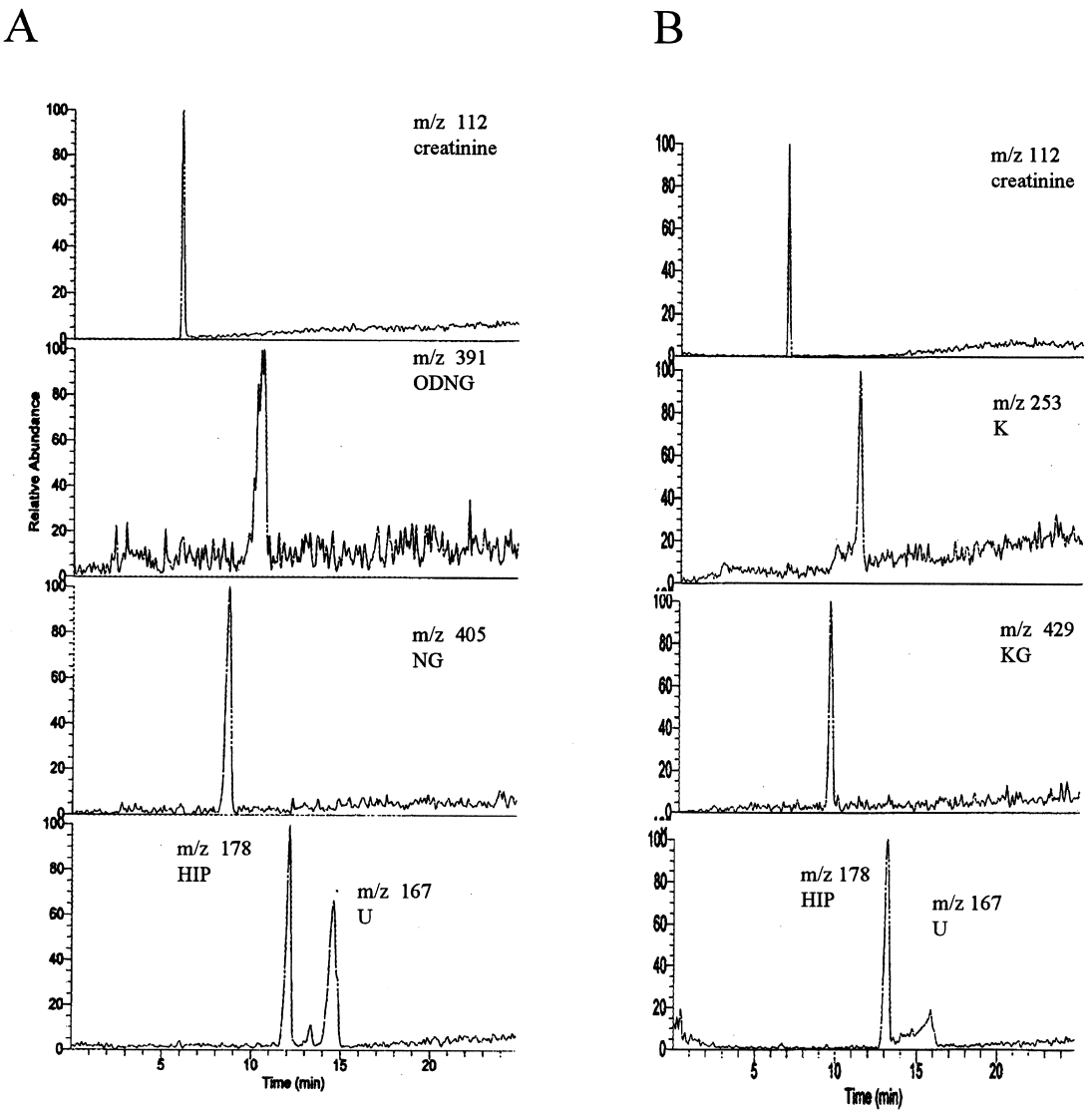


Fig. 6. Detection of nonopioid analgesics directly in urine samples by CE–ESI-MS. RIEs of the endogenous compounds in urine (creatinine, hippuric acid, uric acid) and the metabolites of naproxen (A) and ketoprofen (B) in urine (conditions: as in Fig. 4).

However, these glucuronides could be clearly identified by their UV spectra (Fig. 8, below).

3.2. Development of assays of the individual drugs and their metabolites

3.2.1. Acetylsalicylic acid

When the screening method was applied to urine samples collected after the administration of acetylsalicylic acid the following compounds could be identified: salicylic acid, salicyluric acid and their glucuronides. At pH 9.4 of the separation buffer salicyluric acid was split into a large and a small peak (Fig. 2B), which may be related to the weak acidic phenolic function in this molecule [35,42]. Because of this typical but disturbing phenomenon and the poor separation of salicylic acid and salicyluric acid glucuronide the run conditions, especially the pH of the buffer (Fig. 7B), were varied to

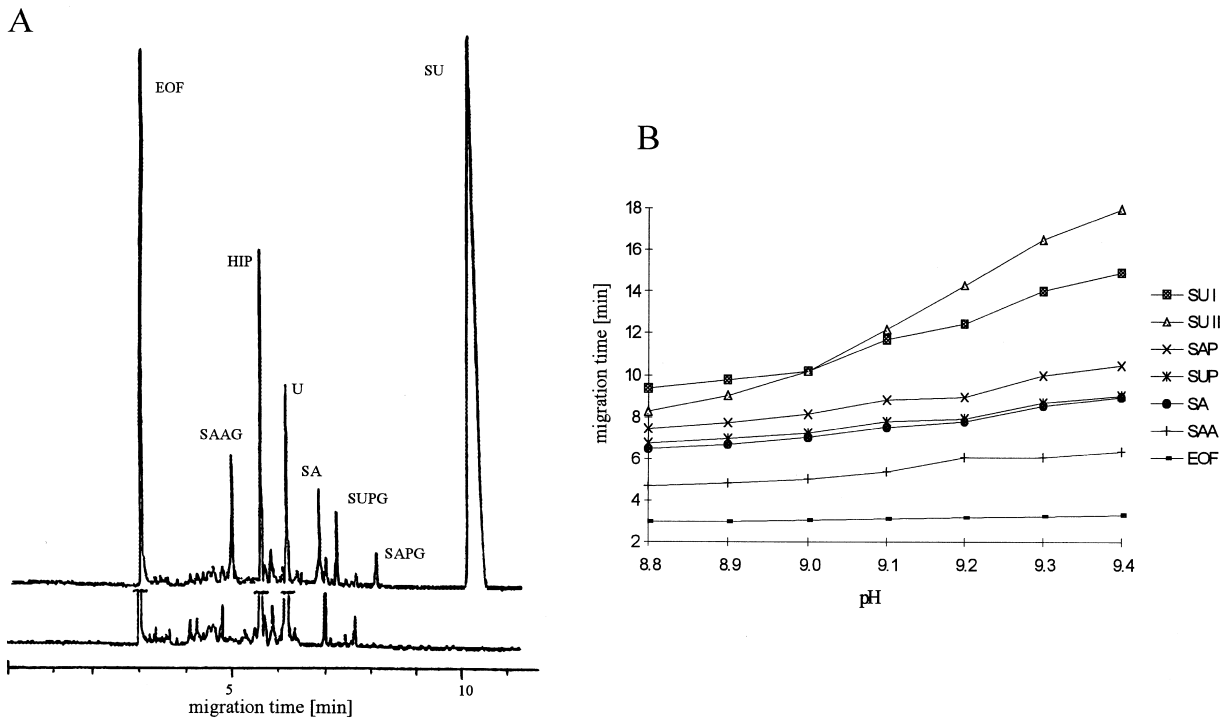


Fig. 7. Detection of the metabolites of acetylsalicylic acid directly in urine by injection at the anodic side. (A) Electropherogram achieved with the same conditions as for the screening method, except: buffer pH 9.0. (B) Diagram: dependence of the migration time of the analytes on the separation buffer pH.

overcome these problems. As can be seen in the diagram (Fig. 7B), the migration order of the larger (i.e. SU I) and the smaller peak (i.e. SU II) became reversed when the pH was changed from 9.4 to 8.8. At pH 9.0 salicyluric acid migrated as a single zone. Additionally, at this pH the separation of the other metabolites was improved (Fig. 7A). However, the method was extremely sensitive to the variation of the pH requiring a very careful buffer pH adjustment. Thus, as another option in particular for the detection of acidic compounds with a high net migration velocity like salicyluric acid assays with the injection at the cathodic side were developed. In these cases the anionic metabolites migrate to the detection window by their own electrophoretic mobility while the EOF moves in the opposite direction. In order to achieve short migration times, the pH of the separation buffer was decreased and spermine tetrahydrochloride was added to diminish or even reverse the EOF by a dynamic coating of the capillary wall. The small zone of sodium hydroxide injected behind the sample reduced occasionally occurring peak tailings. Using a 100 m*M* phosphate buffer at pH 5.1 with 1 m*M* spermine tetrahydrochloride satisfactory separations without peak splitting of salicyluric acid could be obtained. At this pH problems with the stability of acyl glucuronides in alkaline media are avoided. As can be seen in Fig. 8 the low pH value led to an improved discrimination especially of the glucuronides probably due to their pK_a values being closer to the buffer pH. Additionally, acetylsalicylic acid was detectable in the first collected urine fraction and gentisic acid could be determined in minor amounts by this method. The UV spectra recorded with the DAD reveal the usefulness of this detection for the identification of the different metabolites.

In particular, the use of dynamic coatings of the capillary wall requires the confirmation that the system is reproducible in terms of migration times and areas of the peaks. Therefore, the analysis was carried out ten times with the same buffer and the

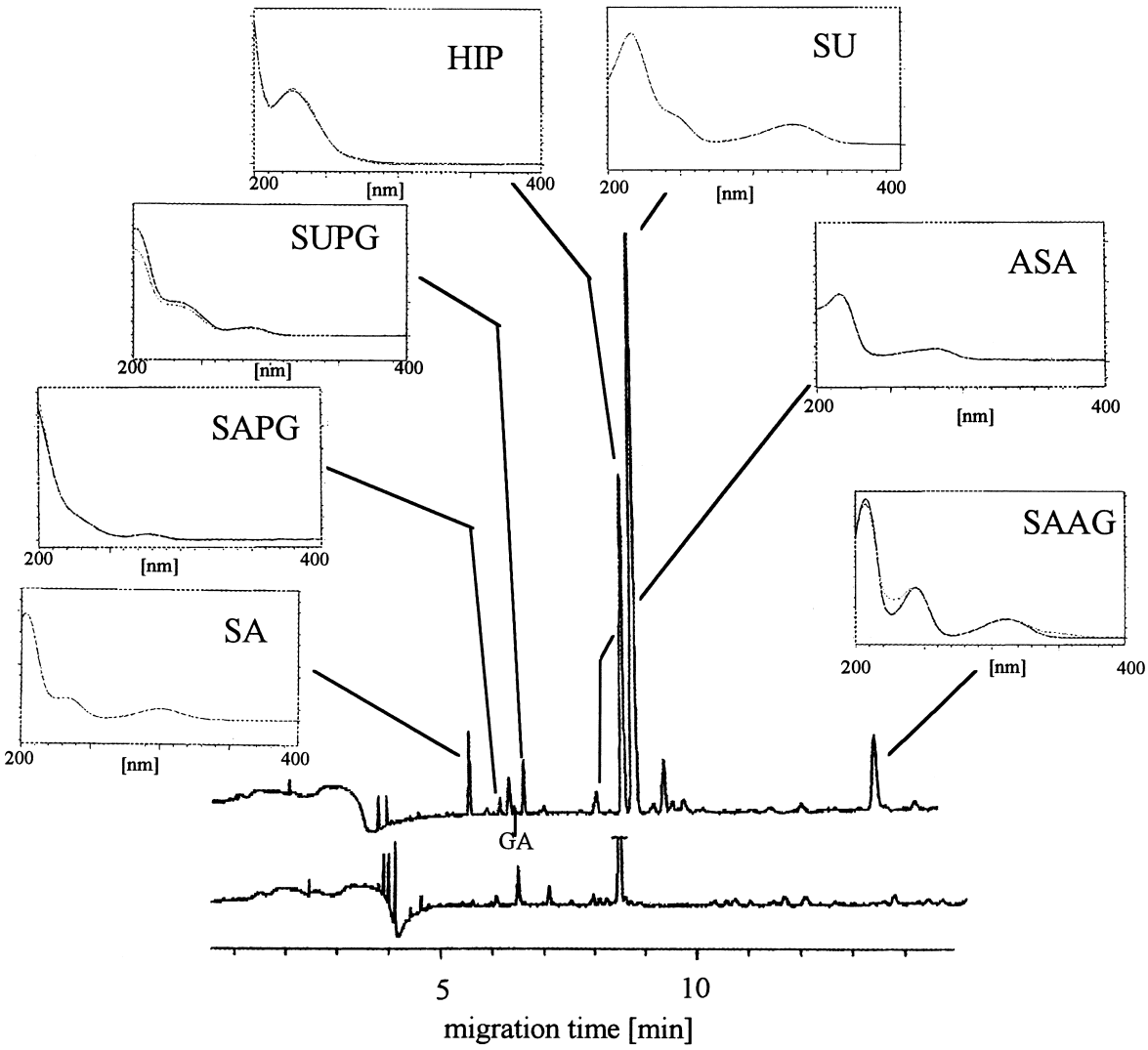


Fig. 8. Detection of acetylsalicylic acid and its metabolites by injection at the cathodic side of the capillary (conditions: 100 m*M* phosphate, pH 5.1, 1 m*M* spermine tetrahydrochloride, −500 V/cm, as described in Section 2).

same urine sample. Only slight deviations in migration times of salicylic acid, salicyluric acid, salicylic acid acyl glucuronide and hippuric acid could be observed (R.S.D. 0.1–1%), revealing the remarkable reproducibility of the system when appropriate rinse procedures were used.

3.2.2. Antipyrine

By using the screening method for the analysis of urine samples after the administration of antipyrine the significant peaks of 4-hydroxyantipyrine glucuronide and norantipyrine glucuronide could be observed in the electropherograms (Fig. 2C). The analysis by CE–MS revealed peaks in the RIEs of the masses belonging to the sulfate conjugates of hydroxy and norantipyrine (Fig. 4A).

Since antipyrine was uncharged under these conditions it migrated with the velocity of the EOF and thus could not be separated from other neutral compounds in urine. The addition of 100 m*M* SDS

to the run buffer allowed detection of not only antipyrine but also some of its neutral phase I metabolites (Fig. 9A). Higher amounts of these metabolites occurred after the cleavage of the phase II products in an β-glucuronidase assay (Fig. 9B). However, due to the comigration of antipyrine-3-carboxylic acid and 3-hydroxymethylantipyrine and the reported instability of some of the phase I metabolites under alkaline conditions other buffer systems were investigated. Especially a phosphate buffer at pH 7.5 enabled the separation of the phase I metabolites under mild conditions as presented in a standard solution in Fig. 9D. Recently, determination of the phase II metabolites instead of the actually interesting phase I product has been suggested because of the instability of the latter [43]. The method described here allows separation of both phase I and phase II products within one run under mild conditions and makes the analytical interpretation of the antipyrine test much more flexible and rapid.

3.2.3. Ibuprofen

Analyzing urine samples collected after the application of ibuprofen by using the screening method revealed that ibuprofen, its 2-hydroxy and 2-carboxy derivatives and their glucuronides could be detected (Fig. 2D). However, the lack of stability of the acyl glucuronides in alkaline media led to the development of a method with injection at the cathodic side at pH 5.3 similar to the method described for acetylsalicylic acid (Fig. 10A). In Fig. 10B an electropherogram is presented that was obtained after the alkaline cleavage of the glucuronides. The peaks appearing close to the 2-hydroxyibuprofen peak may belong to other hydroxylated metabolites according to CE–MS experiments. Aspects of the stereoselec-

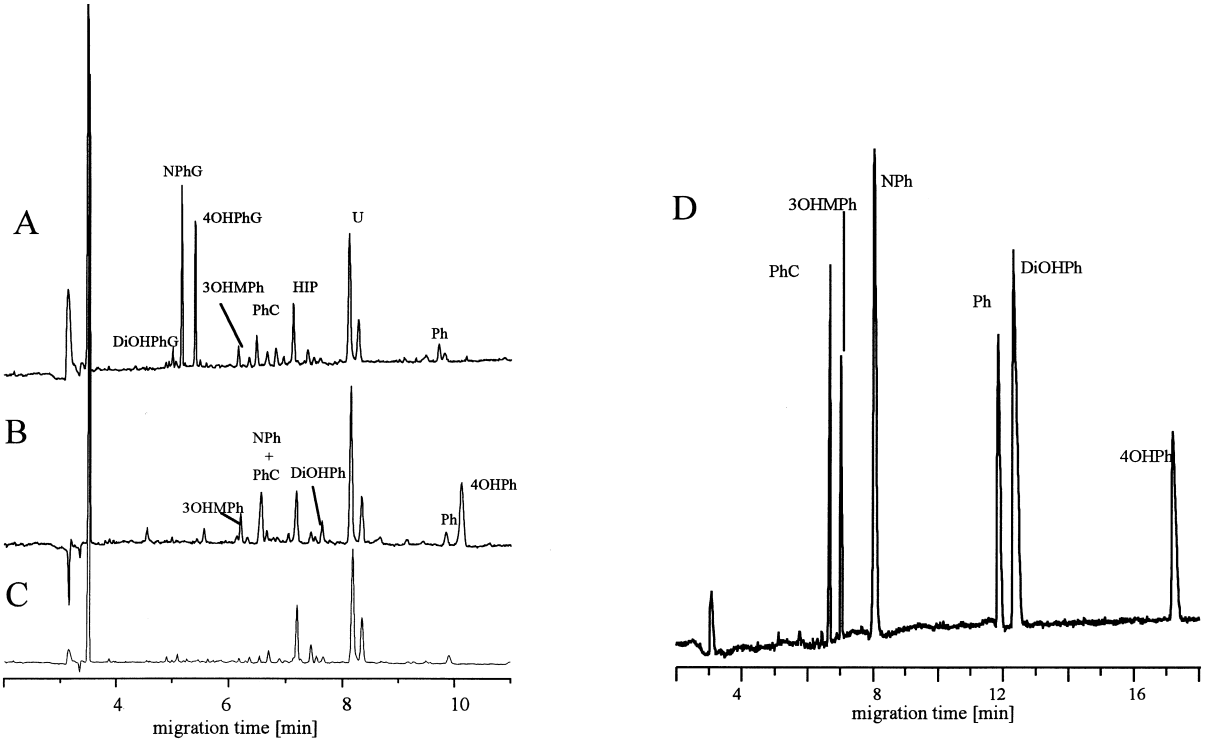


Fig. 9. Electropherograms of a urine sample after oral application of antipyrine: (A) by direct analysis (conditions: 50 m*M* borax pH 9.4, 100 m*M* SDS) and (B) after cleavage of the conjugates with β-glucuronidase; (C) blank urine; (D) separation of the phase I metabolites in a standard solution (conditions: as described above, except: buffer: 50 m*M* phosphate, pH 7.5, 100 m*M* SDS).

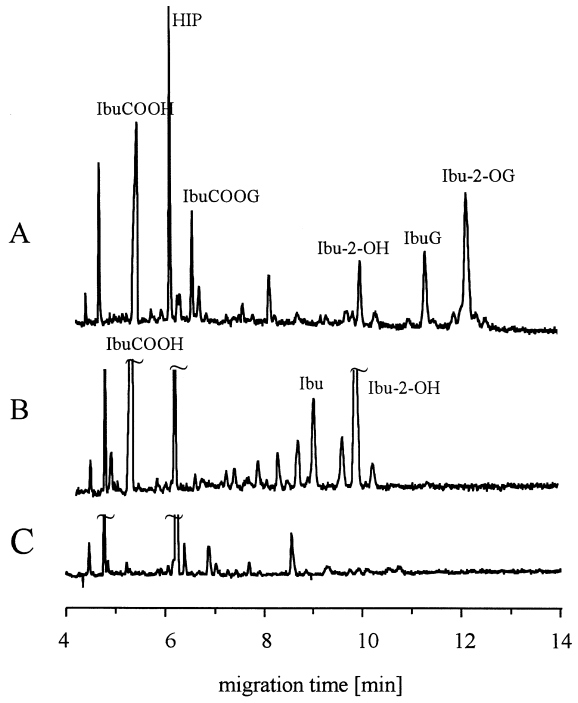


Fig. 10. Electropherograms of a urine sample after oral application of ibuprofen: (A) by direct analysis after injection at the cathodic side (conditions: 100 m*M* phosphate pH 5.3, 3 m*M* spermine tetrahydrochloride, as described in Section 2); (B) after alkaline cleavage of the conjugates in urine; (C) blank urine.

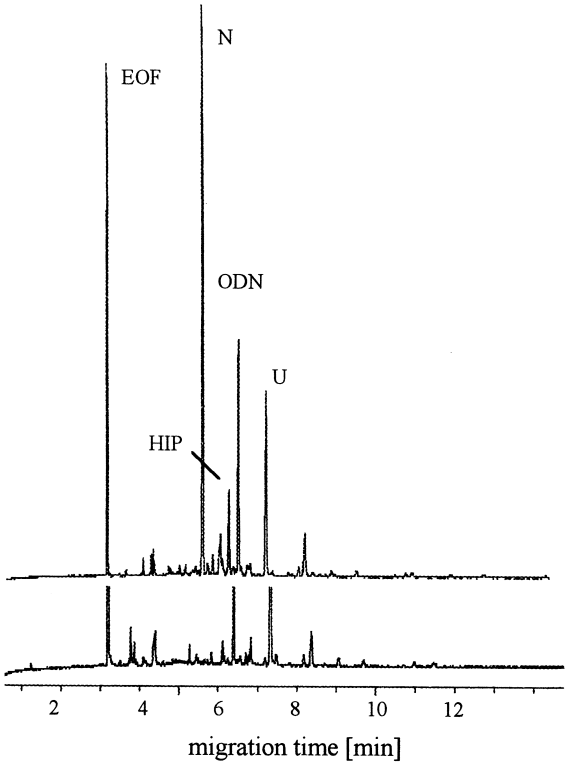


Fig. 11. : Electropherogram of a urine sample after oral application of naproxen. Prior to analysis the glucuronides were cleaved in alkaline media as described in Section 2. CE conditions: as described in Fig. 3.

tive separations of ibuprofen and its metabolites will be discussed elsewhere [39].

3.2.4. Propyphenazone

When using the screening buffer at least three peaks were detected in urine samples that could have been metabolites of propyphenazone (Fig. 3A). Because of the lack of authentic substances only the use of CE–MS gave evidence that these peaks were glucuronides of the *N*-demethyl, *N*-demethylhydroxy and hydroxy derivatives of propyphenazone (Fig. 4B).

3.2.5. Naproxen and ketoprofen

The analgesic and nonsteriodal antirheumatic drug naproxen, its demethyl derivative and their glucuronides could be separated by the screening method (Fig. 3B) and CE–MS (Fig. 6A). The parent drug and the *O*-demethyl derivative could only be detected after the cleavage of the glucuronides (Fig. 11).

Due to the low dose of ketoprofen only its glucuronide could be identified by direct analysis with CE–UV detection (Fig. 3C). It becomes apparent that there is a limit of this direct screening method when drugs which are administered at lower doses are investigated by CE–UV. In these cases on-line preconcentration procedures are necessary. In other cases the use of more sensitive detection systems could be helpful. By using CE–MS for the analysis of urine samples collected after the intake of ketoprofen small amounts of the parent drug compound could be detected (Fig. 6B).

4. Conclusions

It was demonstrated that CE is a useful analytical technique for the detection of nonopioid analgesics

such as paracetamol, acetylsalicylic acid, antipyrine, ibuprofen, propyphenazone, naproxen and ketoprofen as well as their metabolites directly in urine samples. In particular, the separation of charged drugs and metabolites in polar, complex media like urine could be performed due to the high efficiency of CE.

Screening methods based on CE–UV (DAD) and CE–MS coupling could be developed which allow a simple and rapid detection of charged drug compounds and their metabolites directly in urine samples after the application of analgesics to healthy volunteers. Neutral drugs compounds like antipyrine and its in part neutral metabolites could be separated by MEKC.

Acknowledgements

The authors wish to thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support, Dr. M. Neugebauer (University of Bonn, Germany) and Dr. G. Geisslinger (University of Erlangen-Nuernberg, Germany) for the supply of the metabolites.

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