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Determination of salicylate, gentisic acid and salicyluric acid in human urine by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Acetylsalicylic acid (Aspirin) is rapidly metabolized to salicylic acid (salicylate) and other compounds, including gentisic acid and salicyluric acid. Monitoring of salicylate and its metabolites is of toxicological, pharmacological and biomedical interest. Three capillary electrophoresis (CE) methods featuring alkaline aqueous buffers, laser-induced fluorescence (LIF) detection and no solute extraction or derivatization have been explored. A competitive binding, electrokinetic capillary-based immunoassay is developed that recognizes the presence of salicylate and gentisic acid in urine. Differentiation of the two compounds, however, is problematic. With appropriate ultraviolet excitation, many salicylate-related compounds are fluorescent so that CE with direct urine injection and LIF detection permits the determination of salicylate, gentisic acid and salicyluric acid. Using a HeCd laser with 325 nm produces interference-free monitoring of all three compounds. Using 257 nm excitation from a frequency doubled Ar ion laser, native fluorescence of an endogenous urinary compound that co-migrates with gentisic acid is observed. With wavelength-resolved fluorescence detection, however, the two substances are distinguished. Furthermore, this technique, with comparison to literature data, permits the putative assignment of several peaks to other salicylate metabolites, namely glucuronide conjugates of salicylate and salicyluric acid. All three CE-LIF techniques have been applied to toxicological patient urines and urines collected after ingestion of 500 mg acetylsalicylic acid. CE results compare favorably with those obtained by a commercial fluorescence polarization immunoassay and by a conventional photometric assay. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Salicylate; Gentisic acid; Salicyluric acid

1. Introduction

Acetylsalicylic acid (ASA, Fig. 1) is one of the most commonly used therapeutic chemicals. It has analgetic, antipyretic, antiinflammatory and anticoagulant properties and is used as free acid, calcium or magnesium salt, or lysine conjugate. Daily doses are 1.2–4 g for analgesic and antipyretic applications, up to 8 g for antiinflammatory treatment of rheumatoid arthritis and 100–300 mg for reduction of cardiovascular risks. After oral administration, ASA is readily hydrolyzed to salicylic acid (SA) by liver and blood esterases. SA is the active agent with

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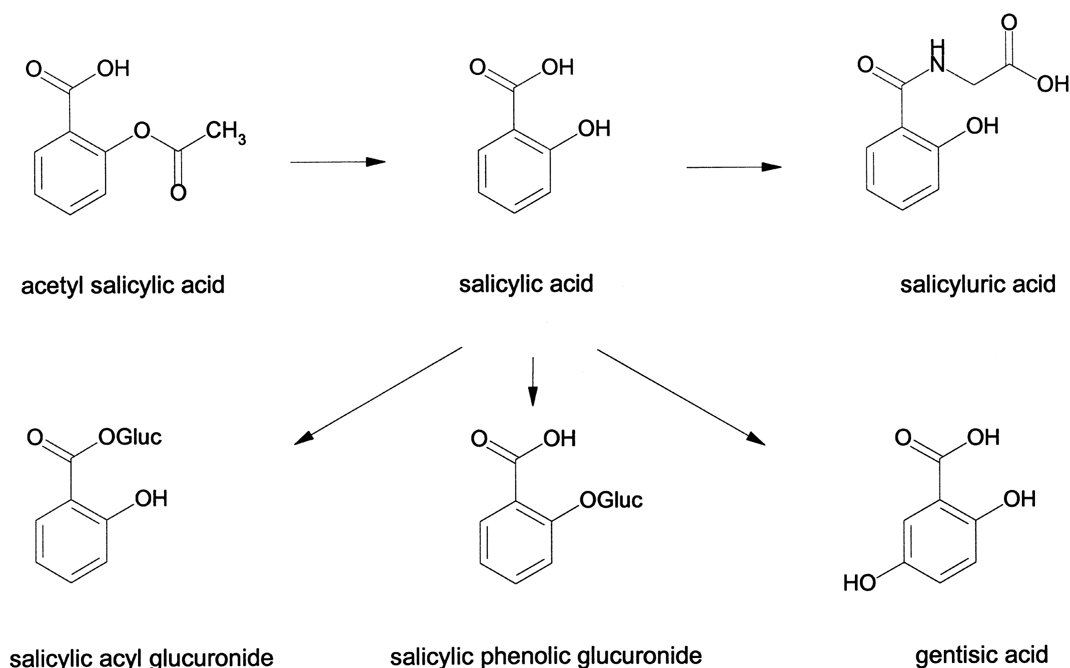

Fig. 1. Chemical structures of acetyl salicylic acid (ASA) and its metabolites salicylic acid (SA), salicyluric acid (SU), gentisic acid (GA) and two SA glucuronides.

a dose-dependent plasma half-life of 3–20 h. SA is further metabolized (see Fig. 1) to salicyluric acid (SU, conjugation with glycine), gentisic acid (GA, hydroxylation) and various glucuronide conjugates (e.g., acyl or phenolic glucuronides) [1,2]. Monitoring of SA is important in cases of drug overdose after suicidal or accidental intake of ASA (mostly observed with children and elderly people) [3,4]. As the therapeutic range in healthy people is quite large, monitoring of SA for reasons of therapy control and compliance is not common. SA is an allowed substance in sports [5] and thus is not subject of doping control in human. However, based on its antiinflammatory properties, SA is a controlled substance in racehorses. SA concentrations in equine urine (doping control of racehorse) must be lower than 750 μg/ml [6]. Furthermore, SA and derivatives are used as markers for the assessment of free radical damage in vivo caused by hydroxyl radicals [7].

SA levels in urine and plasma can be determined with the colorimetric technique of Trinder [8]. This method, however, is not specific for analysis of SA. It also reacts with SU (main urinary metabolite of SA in humans) and salicylamide, but has been found to be insensitive to GA [9]. The widely applied fluorescence polarization immunoassay (FPIA) commercialized by Abbott was designed for the determination of SA in serum. It crossreacts with GA, which is not a problem as there is almost no GA (about 1% of total salicylate [10]) in serum, and is insensitive to salicylamide, SU, and conjugates of SA and of its metabolites (Fig. 1) [9–11]. Using an assay based upon high-performance liquid chromatography (HPLC) [12,13], SA and metabolites can be analyzed individually. However, due to the complex biological matrix, there is always a requirement of time-consuming sample preparations and often gradient elution, thus increasing the analysis time. Capillary electrophoresis (CE) has been established as a powerful analytical tool in biological application because of its rapid separation, high separation efficiencies, and small sample volume. Furthermore, body fluids can be analyzed directly with little or no sample pretreatment. Three CE methods have been employed for analysis of SA and metabolites [6,14–

23], namely capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC) and capillary isotachophoresis (CITP). Caslavska et al. [14] demonstrated the use of all three methods for analysis of SA in toxicological urines and sera using fast scanning polychrome absorption detection for solute identification. For the case of MECC, Kunkel and co-workers examined reproducibility, precision, limit of quantitation and limit of detection for SA via direct injection of plasma spiked with SA [18–20] and Taylor et al. [6] analyzed SA and metabolites in human urine, equine urine and spiked equine plasma. Heitmeier and Blaschke [23] reported the use of CZE with UV and MS detection for the screening of urinary ASA, SA, SU and different glucuronides. A non-aqueous CE method examining plasma and urine samples containing ASA, SA, SU and GA was established by Hansen et al. [21]. Goto et al. [22] compared serum SA levels determined by CZE with those obtained by FPIA on the TDx. For the latter two assays, samples were extracted prior to analysis. Furthermore, CZE and MECC were proposed for the determination of free radical reaction products and metabolites of SA [7]. In these efforts, with the exception of the MS detection [23], SA and metabolites were detected via on-column UV absorption detection.

With UV excitation, SA and its major metabolites fluoresce naturally and thus can be determined by fluorescence detection [24–28]. As relatively few compounds in urine naturally fluoresce, this provides additional specificity. Using MECC with direct sample injection, this was first demonstrated in our laboratories employing two approaches, namely simultaneous UV and fluorescence detection with a homemade setup [15] and laser-induced fluorescence (LIF) detection [16]. CITP with simultaneous UV and fluorescence detection was also shown to be suitable for recognition of urinary GA, SA and SU [17]. Furthermore, LIF detection was proposed for the monitoring of the free SA-fluorescein tracer in an MECC-based immunoassay format for salicylate in serum [29]. SA, GA and SU in urine and serum have also been assayed via CZE with laser-induced resonance energy transfer (LIRET) detection [30] which is typically used for LIF detection of non-fluorescing solutes that accept the energy transferred from a donor molecule. Thus far, no comprehensive study dealing with the determination of SA and metabolites in body fluids by CZE and fluorescence detection has been undertaken.

In this paper, the focus is on CZE with LIF detection for analysis of urinary SA, GA and SU. Data obtained with (i) an electrokinetic CZE-based immunoassay (CZE-IA), (ii) CZE with on-column filter-based LIF detection using a HeCd laser with excitation wavelength at 325 nm, and (iii) CZE with sheath flow, wavelength-resolved LIF detection after excitation with a 257-nm Ar ion laser line [31–34], are presented and compared. Samples analyzed comprise a toxicological urine obtained from a patient with suspected salicylate poisoning and urines collected after self-administration of 500 mg ASA.

2. Experimental

2.1. Chemicals, reagents and origin of samples

All chemicals used were of analytical or research grade. Sodium fluorescein was purchased from Fluka (Buchs, Switzerland), GA (2,5-dihydroxybenzoic acid) was purchased from both Fluka and IGN (Aurora, OH, USA). SA (2-hydroxybenzoic acid), SU (*o*-hydroxyhippuric acid) and β-glucuronidase Type VII-A from *E. coli* were from Sigma (both Buchs, Switzerland and St. Louis, MO, USA). Sodium salicylate and naproxen were from the University Hospital pharmacy (Bern, Switzerland). Stock solutions were made with either bidistilled water (UB) or Milli-Q water (UI). Naproxen was dissolved in methanol (10 mg/ml). The TDxFLx FPIA reagent kit for SA (No. 9533-60) was purchased from Abbott Laboratories (Baar, Switzerland). The reagent pack comprises separate vials for antibody containing solution (solution S; comprising <25% sheep and rabbit salicylate antisera) and fluorescein tracer solution (solution T; comprising <0.01% salicylate fluorescein tracer in buffer) whose concentrations are not exactly disclosed [10]. The toxicological urine was collected in the departmental routine drug assay laboratory. Urine was collected 2 and 6 h after self-administration of 500 mg ASA. Our own, drug-free urine was used as blank matrix. Calibration and test urines were prepared via spiking of the appropriate amounts of SA, GA and/or SU in

blank urine. The samples were stored at −18°C until analysis.

2.2. Apparatus and methods

2.2.1. Photometric and FPIA assays

Photometric determinations of SA in serum (or plasma) and urine were made according to the colorimetric method of Trinder [8] using four calibrators in the range of 50–400 μg SA/ml (for serum/plasma) and one calibrator of 250 μg SA/ml (for urine). The controls contained 160 μg/ml SA in serum/plasma and 193 μg/ml in urine. Mean (*n*, RSD) values were calculated to be 162.1 μg/ml (7, 1.3%) and 189.2 μg/ml (26, 2.7%), respectively. For rapid, qualitative analysis of urine samples, a modified method comprising no mercuric salt was employed. The threshold value for this assay was 138.1 μg/ml. FPIA analyses on the TDxFLx (Abbott) were executed with the reagent kit provided by Abbott (see above) and following the manufacturer's instructions. This assay uses six serum calibrators containing 0–800 μg/ml (0–5792 μ*M*) SA. Reproducibility was assessed using the three controls provided by Abbott containing 75, 300 and 600 μg/ml SA, respectively. RSD values calculated from data produced during a 1-year period were 20% (n=34), 5.5% (n=37) and 3.2% (n=31), respectively.

2.2.2. CZE-IA

CZE-IA measurements were made on a P/ACE 5510 CE system (Beckman Instruments, Fullerton, CA, USA). A 50-μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 20/27 cm effective/total length was employed. The detection was performed with the LIF detector assembly (Beckman) powered by a 488-nm air-cooled Argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) and equipped with a 488-nm notch filter and a 520-nm band pass filter. Data were evaluated using the P/ACE Station Software (Beckman). A constant voltage of 12 kV (current ~68 μA) was applied and the capillary temperature was kept at 20°C. The sample carousel was at room temperature. The running buffer was composed of 50 m*M* disodium tetraborate (pH 9.4). In the morning the capillary was rinsed with 0.1 *M* NaOH, water and running buffer, 10 min each. Before each run the capillary was rinsed with the same solutions for 2, 2 and 4 min, respectively. Sample injection was made by positive pressure using 0.5 p.s.i. for 2 s.

Sodium fluorescein, the internal standard (I.S.) solution, was made fresh daily at a concentration of 200 ng/ml. For sample preparation, 25 μl free tracer (FT) solution, 25 μl antiserum solution, 5 μl I.S. solution and 5 μl of urine (blank, spiked, volunteer or toxicological urine) were mixed and vortexed for 10 s followed by incubation at room temperature for 10 min. Data evaluation was based upon ratios of peak heights (FT to I.S.). Calibration graphs were evaluated on a personal computer (PC) using SigmaPlot Scientific graphing software version 2.01 (Jandel, San Rafael, CA, USA). The calibration graphs were constructed by non-linear regression analysis based upon a four-parameter log/logit-model according to [35]

$$y = a + b/\{1 + \exp[-(c + d^{*}\ln x)]\} \quad (1)$$

where x is the solute concentration, y the peak height ratio and a, b, c and d the parameters to be determined by regression analysis.

2.2.3. CZE-LIF using the HeCd laser at 325 nm for excitation

CE-LIF measurements were made on the same P/ACE 5510 instrument as described in Section 2.2.2. A fused-silica capillary (Polymicro Technologies) of 75 μm I.D. 40/47 cm effective/total length was employed. A 325-nm HeCd laser (Model 4230 NB, LiCONiX, Santa Clara, CA, USA) and, if not stated otherwise, a 450-nm band pass filter was used. A constant voltage of 21 kV (current, ~55 μA) was applied and the capillary temperature was kept at 20°C. The sample carousel was at room temperature. The CZE running buffer was composed of 6 m*M* disodium tetraborate and 10 m*M* disodium hydrogen phosphate (pH 9.1). In the morning the capillary was rinsed with 0.1 *M* NaOH, water and running buffer, 10 min each. Between runs the capillary was rinsed for 5 min with running buffer. Sample injection was made by positive pressure using 0.5 p.s.i. for 4 s. Urine samples were applied directly, after dilution with water or after hydrolysis.

For hydrolysis, 100 μl urine were mixed with 80 μl 0.1 *M* phosphate buffer of pH 6.8 and 20 μl (100 U) β-glucuronidase Type VII-A solution from *E. coli*, recommended by Vree et al. [12]. The mixture was vortexed for 10 s and then incubated at 37°C (water bath) for 17 h.

2.2.4. Wavelength-resolved CZE-LIF using a 257-nm Ar ion laser for excitation

A laboratory-assembled CE setup with an untreated fused-silica capillary of either 73 or 88 cm length and 50 μm I.D. (Polymicro Technologies) was employed. The detection end of the capillary was directed into a sheath flow assembly, where the core stream was excited by a frequency-doubled, liquid-cooled argon-ion laser (Innova 300 FrED; Coherent, Palo Alto, CA) operating at 257 nm. The collection optics were orthogonal to the excitation beam focusing the fluorescence emission to a f/2.2 CP 140 imaging spectrograph (Instruments SA, Edison, NJ, USA) and then onto a 1024× 256 detector-array, liquid nitrogen-cooled scientific CCD (EEV 15-11; Essex, UK). For details of the assembly refer to previous publications [31–34]. Sample injection was performed electrokinetically at 2.1 kV for 10 s and the separation voltage was maintained at 21 kV (current, ~25 μA). All experiments were at room temperature. The same buffer as described in Section 2.2.3 was employed for both sheath and running buffer. Each day, the capillary was rinsed with 0.1 *M* NaOH, water and running buffer, 5 min each. Between runs the capillary was rinsed for 5 min with the running buffer. With the exception of dilutions with buffer, no sample preparation was required. Fluorescence emission from 260 to 710 nm was processed and viewed in MATLAB (the Mathworks, Natick, MA, USA) on a PC. Detection limits were calculated by choosing an optimum analyte-dependent wavelength range, integrating intensity over this range and generating a filter-based electropherogram, as described in Ref. [31]. For the data analysis using the 88-cm capillary length (the toxicological urine samples), the time axis was reduced ~21% to allow comparison of the electropherograms from the 73-cm capillary length used for all other urine experiments.

3. Results and discussion

3.1. CZE-IA

In most electrokinetic capillary-based immunoassays for drugs, labeled antigens are separated from the antibody–antigen complexes and the peak of the free tracer is typically employed for data evaluation [29,35–37]. The FPIA immunoassay kit reagents are manufactured for analysis of SA in serum and plasma [10,11] and these reagents have previously been investigated for serum analysis of SA by MECC-IA [29]. In that work, the impact of capillary length and the effect of the relative amount of the antibody solution in the incubated sample and reagent mixture was studied. Here, analysis of urinary SA in the CZE-IA format using Abbotts's SA FPIA reagents and an alkaline tetraborate buffer that was previously applied to the CZE-IA determination of urinary drugs of abuse [35,36] is reported. Typical electropherograms are presented in Fig. 2. The antibody–tracer complex is assumed to be between the I.S. and FT peaks. Its fluorescence, however, appears to be quenched. The peak height of the FT is a measure of reactivity. As expected, the peak height of FT was found to increase as the SA concentration in the sample was increased, whereas the magnitude of the tracer–antibody complex decreased (not well seen in Fig. 2). As the instrument did not inject reproducibly when operated with a 2-s injection time, the peak heights varied strongly (best seen with the I.S., RSD of peak height up to 17%, $n=6$). The peak height ratio, however, was more reproducible (RSD, <8%). A typical calibration graph is presented in Fig. 3. The CZE-IA calibration was found to permit an estimation of the SA content in urines (Table 1; for electropherograms see Fig. 4). For example, CZE-IA data obtained for the 2- and 6-h volunteer urines compare favorably with those measured by FPIA (diamonds in Fig. 3) although the FPIA was calibrated with sera and not urine.

Furthermore, the SA-based CZE-IA was employed to assess the recognition of GA and SU by the antibody against SA. The data presented in Fig. 4a–d are those obtained for the analysis of blank urine and blank urine spiked with SU, GA and SA (3 m*M* each), respectively. SU was found not to react with

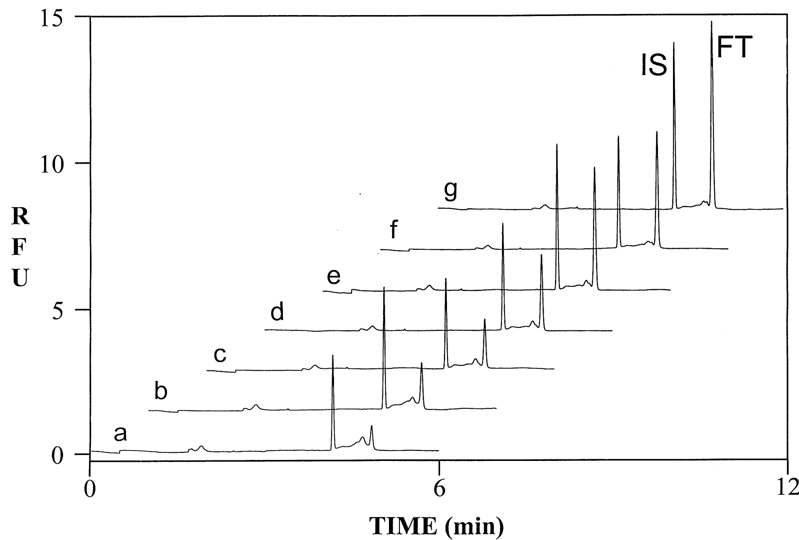

Fig. 2. CZE-IA electropherograms obtained with (a) urine blank and urine blank spiked with (b) 50, (c) 100, (d) 200, (e) 400, (f) 800 and (g) 1000 μg/ml SA. FT, free SA tracer; I.S., internal standard (fluorescein, 16.7 ng/ml). Electrophoretic conditions as discussed in Section 2.2.2.

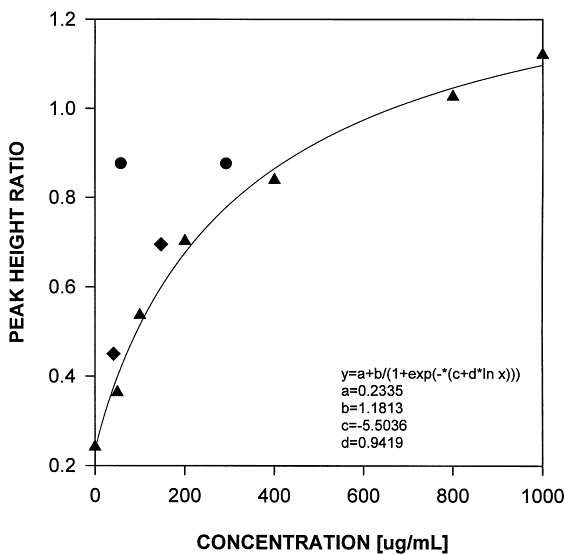

Fig. 3. Calibration graph constructed with the CZE-IA data of Fig. 2 (triangles) and nonlinear regression analysis according to Eq. (1) (solid line). Diamonds represent CZE-IA peak height ratios correlated with FPIA data for the 2-h (lower diamond) and 6-h urines (upper diamond). Dots represent data of the 5-fold diluted toxicology urine, namely the CZE-IA ratio compared with the SA concentration determined by CZE-LIF (left dot) and the sum of the SA and GA concentrations determined by CZE-LIF (right dot).

the antibody (no higher tracer signal than with blank urine). This does not come as a surprise as conjugation of SA with glycine (Fig. 1) prevents a close fit of the antibody for the steric and electronic characteristics of the hapten [38]. However, at the concentration investigated, GA was determined to produce about the same signal as SA. This means that the crossreactivity is about 100%, which compares well with the FPIA data (108% cross-reactivity) reported by Scholer et al. [9], who adapted the serum SA assay on the TDx for the analysis of urines. Since the amount of GA is significant in toxicological urines (see results obtained with CZE-LIF below), the assay of SA with the SA-based CZE-IA for the toxicological urine does not fall on the calibration curve (left dot in Fig. 3). However, by considering the sum of the GA and SA concentrations (right dot), the data point was found to fit reasonably well to the calibration curve. For CZE-IA as described here, the sensitivity (peak ratio increase compared to urine blank 0.02) limits for SA and GA were found to be about 10 and 5 μg/ml, respectively. This compares well to the SA sensitivity of 5 μg/ml of the FPIA serum assay [10]. Furthermore,

Table 1
Selected data obtained with different assays

Sample	Trinder[a] (μg/ml)	FPIA[b] (μg/ml)	CZE-IA[a] (μg/ml)	CZE-LIF (325)[c]		
				GA (μg/ml)	SA (μg/ml)	SU (μg/ml)
Tox. urine	pos (>>138)[d]	–	2085	1170	290	4910
2-h urine	105	41.3	71	ND	28.7	61.3
6-h urine	1084	147	215	5	71	2231

[a] Calibration using blank urines spiked with SA.
[b] Calibration using blank sera spiked with SA as supplied by the manufacturer of the assay kit.
[c] Calibration using blank urines spiked with GA, SA and SU.
[d] Analysis with qualitative assay only.

RSD values were determined to be typically $<10\%$ ($n=6$).

Both urinary SA data obtained by CZE-IA and FPIA were found to be significantly lower than data monitored photometrically using the method of Trinder (Table 1). This is not surprising as the Trinder method also recognizes SU and possibly conjugates of SA and SU. Thus, the Trinder method should be used on a qualitative basis when applied to urine samples, which is not the case of monitoring SA in serum. Data determined by the Trinder method revealed values that were found to be comparable to those obtained by FPIA. The correlation of the data pairs was linear ($[SA]_{Trinder}=0.983\times[SA]_{FPIA}+100.4$; $r=0.999$; $n=28$; range of data, 150–3100; units, μ*M*). However, values determined photometrically were slightly higher. Similarly, monitoring of SA by potentiometry using salicylate-selective elec-

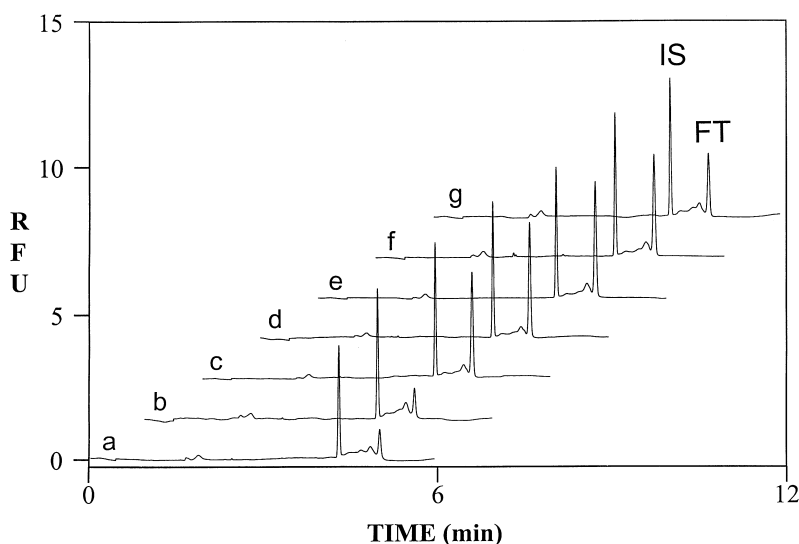

Fig. 4. CZE-IA electropherograms obtained with (a) urine blank, (b–d) blank urine fortified with (b) 3 m*M* (585.6 μg/ml) SU, (c) 3 m*M* (462.3 μg/ml) GA, (d) 3 m*M* (414.3 μg/ml) SA, (e) 5-fold diluted toxicology urine, (f) undiluted 6-h urine and (g) undiluted 2-h urine. FT, free SA tracer; I.S., internal standard (fluorescein, 16.7 ng/ml). Electrophoretic conditions as discussed in Section 2.2.2.

trodes, including those based upon Al(III) and Sn(IV) salophens [39], is expected to be sufficiently selective for the determination of SA in serum but most likely not suitable for the measuring of accurate urinary SA concentrations in presence of large amounts of GA.

3.2. CZE-LIF using the HeCd laser at 325 nm for excitation

The assay of SA and its two metabolites in urine and serum by MECC was discussed previously using simultaneous absorbance and fluorescence [15] and LIF [16] detection. In the previous manuscript [15], one peak was wrongly assigned to conjugated SU instead of GA [16]. Here the assay using CZE with the same pH 9.1 buffer (see description in Section 2.2.3) but without dodecyl sulfate micelles is presented. For the determination of the optimized fluorescence detection wavelength, standard solutions (Fig. 5A), spiked urines and the toxicological urine (Fig. 5B) were measured using four different band filters. At 366 nm, GA is not detected and at 479 nm, the responses for SA and SU are rather poor. The best LODs (see Table 2) for GA were observed at 450 nm (80 ng/ml), for SA at 405 nm (1 μg/ml) and for SU at 405 nm (150 ng/ml). For GA and SA, the LODs in spiked urine were found to be in the same ranges. For SU in urine, sample self-focusing was observed at low concentration (see below) and LODs were thus determined to be about three times better compared to the values given in Table 2. Furthermore, as the decrease in sensitivity for GA between 450 and 405 nm is large and the response loss for SA and SU is less significant (Fig. 5A), the 450-nm filter was chosen for most experiments.

Quantification of SA, GA and SU in the two volunteer urines (2- and 6-h urine) and the toxicological urine was performed with a 5-level internal calibration. While the evaluation via peak height for GA and SA was good (with $r^2=0.9998$ for both graphs), the evaluation of SU had to be done by area ($r^2=0.9951$). For GA, SA and SU at urinary concentrations of 9, 26 and 130 μg/ml, respectively, RSD values ($n=4$) were determined to be 2.8, 3.1 and 6.6%, respectively. Electropherograms of the three urine samples are depicted in Fig. 6A and

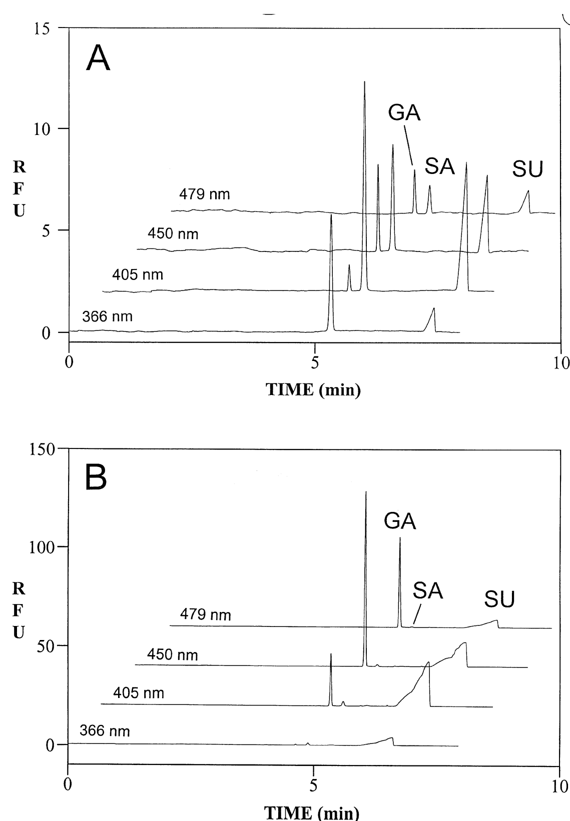

Fig. 5. CZE-LIF electropherograms measured with four different filters of (A) a standard solution containing 2 μg/ml GA, 80 μg/ml SA and 10 μg/ml SU and (B) the 30-fold diluted toxicological urine. Electrophoretic conditions as discussed in Section 2.2.3.

Table 2
Estimated LODs for analysis of an aqueous standard solution by CZE-LIF using the 325-nm HeCd laser with different band filters and by a sheath-flow, cuvette-based CZE-LIF system using a 257-nm Ar^+ laser

Band filter in nm for HeCd laser assembly	LOD (ng/ml)		
	GA	SA	SU
479	200	8000	1000
450	80	3200	400
405	160	1000	150
366	–	1600	500
257 nm Ar^+ laser (wavelength range (nm) for integration)	160 (400–500)	140 (375–455)	120 (380–470)

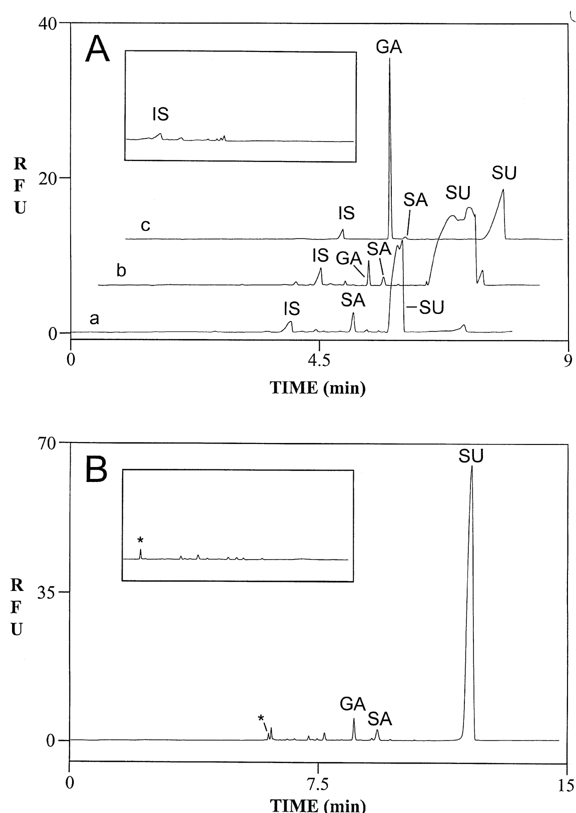

Fig. 6. (A) CZE-LIF electropherograms of (a) undiluted 2-h urine, (b) 10-fold diluted 6-h urine and (c) 100-fold diluted toxicology urine. I.S., internal standard (naproxen, 476 μg/ml). Electrophoretic conditions as discussed in Section 2.2.3. The inset depicts part of an electropherogram of an undiluted urine blank that was spiked with the I.S. only and was drawn on the same scale. (B) MECC-LIF electropherogram of 10-fold diluted 6-h urine that was obtained with a running buffer composed of 75 m*M* SDS, 6 m*M* $Na_2B_4O_7$, 10 m*M* Na_2HPO_4, a 1-s injection at 0.5 p.s.i. and a constant voltage (current) of 15 kV (70 μA). The inset depicts part of an electropherogram obtained with an undiluted blank urine and drawn on the same scale. The peak marked with an asterisk corresponds to that marked on the urine electropherogram. The detection wavelength was 450 nm in all cases.

calculated urine concentrations are given in Table 1. It is obvious that the toxicological urine contains higher concentrations of the investigated substances than the others. GA was not detectable in the 2 h urine and was found to be present at a low concentration in the 6 h urine. This compares well with the observations made by Heitmeier and Blaschke [23]. In the toxicological urine, however, the concentration of GA was found to be high. In all three urines, SU is present in a significant higher concentration compared to SA.

The cleavage of glucuronides are visualized indirectly via an increase of the SA response after enzymatic hydrolysis. In Fig. 7 the SA peaks of the hydrolyzed urines are higher than those measured with plain urines, which indicates that several compounds are converted to SA after hydrolysis. Only one peak (marked with asterisk) seen in the electropherograms of the unhydrolyzed urines was not detected after hydrolysis. Due to the lack of standards, identification of this peak was not possible. However, compared to literature [23], it may be that the vanishing peak represents one of the glucuro-

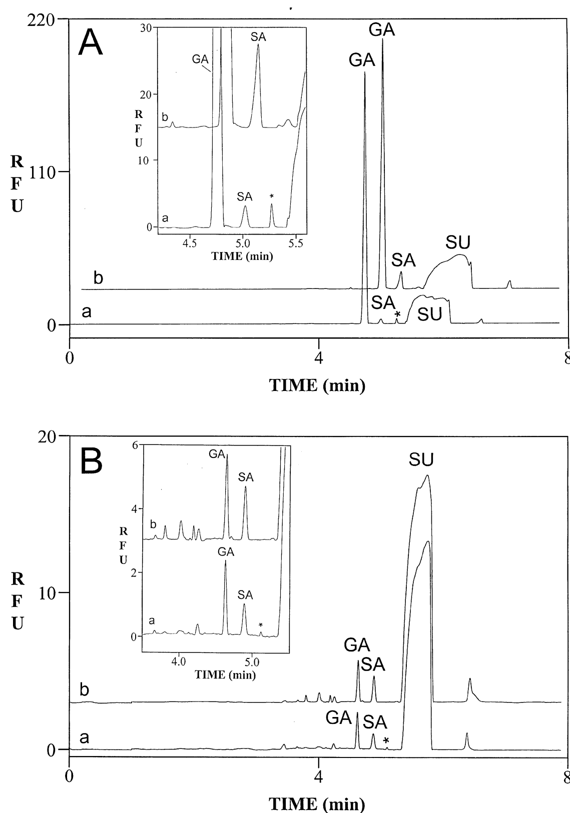

Fig. 7. CZE-LIF electropherograms obtained with enzymatically hydrolyzed urines (upper graphs) and unhydrolyzed (lower graphs) urines. (A) Ten-fold diluted toxicological urine and (B) 10-fold diluted 6-h urine. Insets depict enlargements of the part showing the SA peak. Electrophoretic conditions as discussed in Section 2.2.3. The asterisks mark peaks that are not detected after hydrolysis. The detection wavelength was 450 nm in all cases.

nides, likely salicyluric phenolic glucuronide (SUPG) based on the migration properties in a similar buffer system.

The data presented in Fig. 6B show an electropherogram of the 10-fold diluted 6-h urine that was monitored by MECC rather than by CZE under otherwise almost identical conditions. The SU peak in this system is much sharper than that observed in CZE (graph b in Fig. 6A). Although the injection time in MECC was 1 s (not 4 s as in CZE) and a different capillary cartridge was used (difference in detection sensitivity), these data demonstrate that SU is being defocused under CZE conditions. Defocusing was found to take place at SU concentrations only above 40 μg/ml. This is illustrated with the electropherograms presented in Fig. 8. Comparison of electropherograms obtained with standard solutions (Fig. 8A) and spiked blank urines (Fig. 8B) revealed that GA and SA produced very similar peaks in both cases, whereas the presence of the urine matrix has an impact on the electrophoretic behavior of SU. At SU concentrations ≤10 μg/ml (Fig. 8B), SU is detected earlier and has a sharper peak profile compared to the case without urinary matrix (Fig. 8A). After exceeding a threshold concentration of about 40 μg/ml, focusing does not take place any more (Fig. 6A). These data suggest that SU is being focused by one or several ions of like charge [40,41]. Further work is required to elucidate the exact mechanism involved.

3.3. Wavelength-resolved CZE-LIF using 257 nm excitation

With the advent of wavelength-resolved fluorescence detection in CE, spectral differentiation of analytes became a reality [31–34]. The data presented in Figs. 9–11 are 'contour' plot gray-scale wavelength-resolved electropherograms, where white marks low and black high fluorescence intensity, together with selected normalized fluorescence spectra extracted from the multiwavelength emission data. With excitation at 257 nm as employed in this work, many fluorescent compounds are detected, with SA and its metabolites (GA and SU) giving the highest response (Fig. 9 shows an example of the toxicological urine). Identification was determined by mobility or migration time and confirmed by the

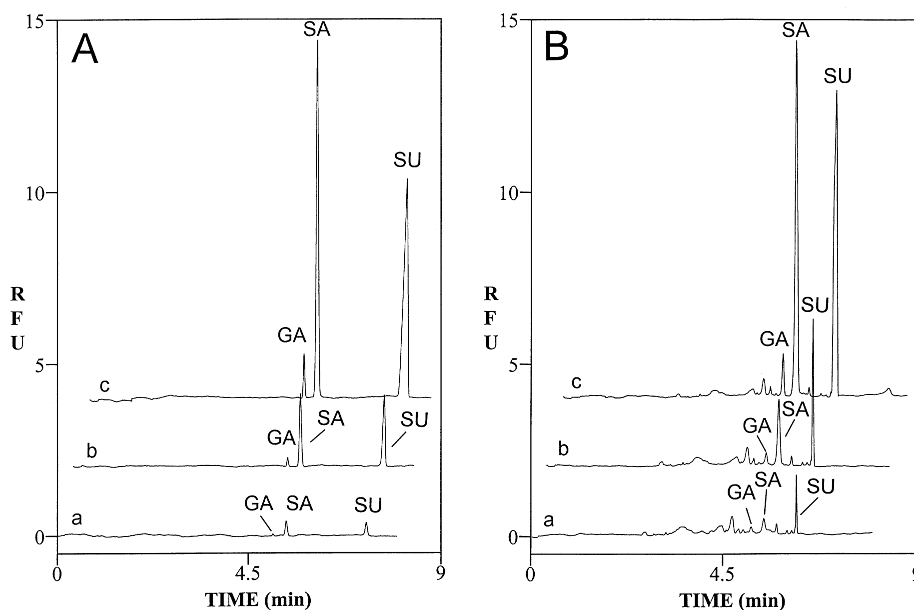

Fig. 8. CZE-LIF electropherograms measured at 405 nm with (A) standard solutions and (B) urines spiked with 0.08, 0.40 and 2.0 μg/ml GA (from bottom to top, respectively), 3.2, 16 and 80 μg/ml SA (from bottom to top, respectively) and 0.40, 2.0 and 10 μg/ml SU (from bottom to top, respectively). Electrophoretic conditions as discussed in Section 2.2.3.

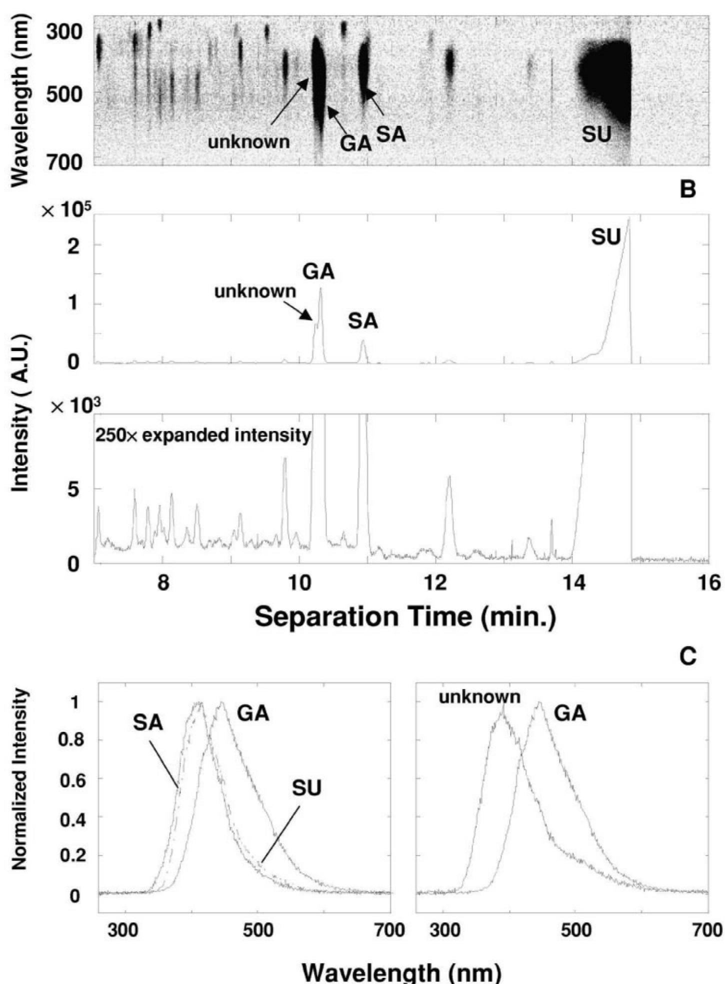

Fig. 9. (A) Wavelength-resolved and (B) extracted fluorescence-intensity electropherograms of the toxicology urine at 20-fold dilution. (C) Normalized fluorescence emission spectra of selected compounds from the electropherogram presented in (A). The *y*-axis scale units in panel B are arbitrary units (A.U.). Electrophoretic conditions as discussed in Section 2.2.4.

characteristic emission profiles (Fig. 9C). Note the richness of information produced by wavelength-resolved fluorescence detection; more than 10 unknown compounds, many with distinct emission profiles, elute between 7 and 10 min in Fig. 9. Monitoring of blank urine (Fig. 10B) revealed an unknown, endogenous substance (labeled as unknown in Figs. 9 and 10) that was detected at almost the same time as GA in the standard solution (Fig. 10A). This unknown substance was also found to be present in the toxicological (Figs. 9 and 10C) and the 2- and 6-h urines (Fig. 10D and E, respectively), and was characterized by a different emission spectrum than the GA emission profile (Fig. 9C, right panel). Using an excitation wavelength of 325 nm, however, this unknown compound was not detectable (inset of Fig. 6A). Exact comparison of migration time between the two CZE systems is problematic due to the different capillary length, operating temperature and sheath flow detection. No GA was detected in the 2- and 6-h urine samples.

An advantage of this approach is that different optimal emission wavelengths can be used for each analyte as opposed to a single compromised set.

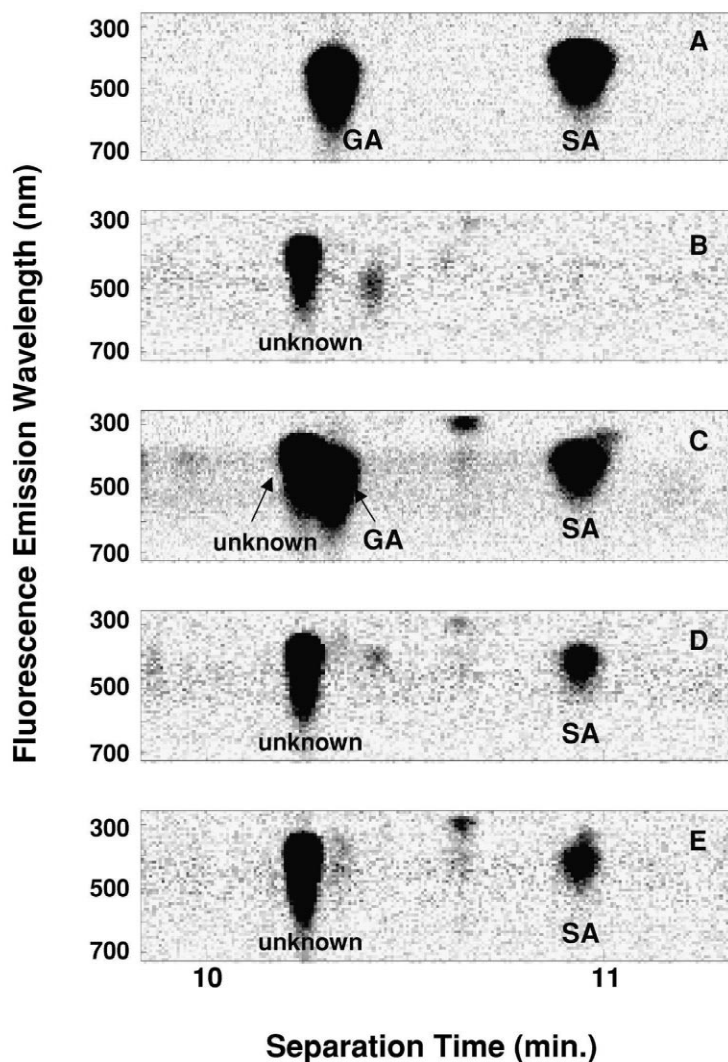

Fig. 10. Wavelength-resolved electropherograms for the time range of GA and SA detection (9.5–11.5 min). (A) Standard mixture (concentration of 0.5 m*M* each), (B) 10-fold diluted urine blank, (C) 20-fold diluted toxicology urine, (D) 10-fold diluted 2-h urine and (E) 30-fold diluted 6-h urine sample. Electrophoretic conditions as discussed in Section 2.2.4.

While 'noise' can be observed in the wavelength-resolved electropherograms (see Figs. 9 and 10), the wavelength information can be collapsed to a fluorescence-intensity electropherogram (Fig. 9B) to improve LODs. Compared to the 325-nm HeCd laser with the commercial instrument, the sensitivity of the laboratory-made system featuring a sheath flow cell and wavelength-resolved fluorescence detection is comparable for GA and about 5- to 10-fold better for SA and SU (Table 2).

Wavelength-resolved fluorescence detection was also employed in an attempt to identify the presence of other SA metabolites (Fig. 11). The peaks marked as peaks 1–6 in Fig. 11A have normalized emission spectra that are close to those observed for SA (Fig. 11B). Good agreement was observed for peaks 1, 4, 5 and 6, so these peaks are identified as putative conjugated metabolites of SA and SU, such as salicylic acyl glucuronide (SAAG), salicylic phenolic glucuronide (SAPG) and SUPG. As the emis-

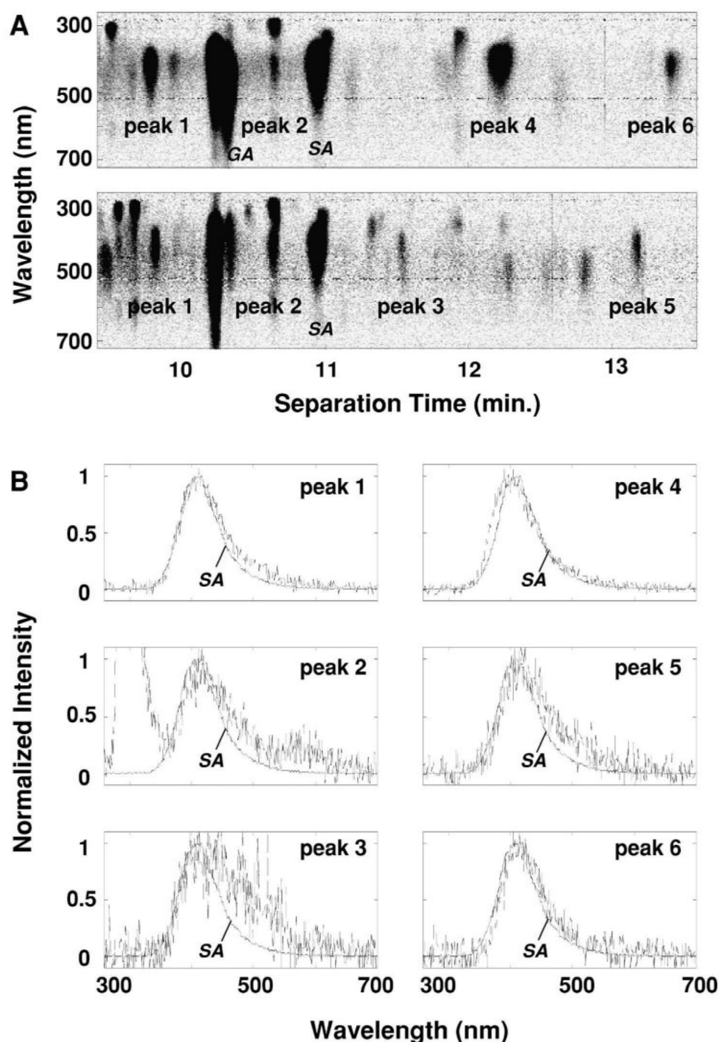

Fig. 11. (A) Wavelength-resolved electropherogram of the 10-fold diluted toxicology urine (upper) and 10-fold diluted 6-h urine sample (lower). (B) Normalized emission spectra for selected peaks (labeled as 1–6 in (A)) compared to SA. Electrophoretic conditions as discussed in Section 2.2.4.

sion spectra of SA and SU are very similar (Fig. 9C, left panel), unambiguous identification with fluorescence detection is not possible without appropriate standards. Investigations of hydrolyzed urine samples have not been undertaken.

4. Conclusion

The data presented in this paper show that urinary SA, GA and SU can be determined by CE with fluorescence detection and without sample extraction. Sample preparation is minimal as the urines only have to be filtered and diluted. Different stages of selectivity are attained with the different methods used in this work. The antibody of the electrokinetic capillary-based IA interacts with SA and GA with about the same intensity, but not with SU. However, no differentiation between SA and GA is attained with this method. Data obtained by CZE-IA and FPIA were found to compare well when no significant GA is present. However for urines of persons

with a SA intoxication, where GA level is significantly high, a combined response is thus monitored.

With CZE-LIF having a HeCd laser at 325 nm as excitation source, interference-free determination of SA, GA and SU in urine is possible. Application of 257-nm laser light produces native fluorescence of an endogenous urinary compound that comigrates with GA. With the observation of wavelength-resolved fluorescence, however, the two substances can be distinguished. Furthermore, this technique and comparison to literature data permitted the assignment of several putative SA metabolites. While such assignments are tentative, they can direct future investigations. In comparison to the use of the 325-nm laser line with on-column LIF detection, the 257-nm laser with sheath flow detection was found to provide higher sensitivity for SA and SU, this permitting the recognition of the minor, conjugated metabolites of SA.

Using an alkaline buffer, direct urine injection and CZE with LIF detection, SA and GA were found to produce nice peaks. SU injected at low concentrations (≤ 10 μg/ml) produces a sharp, focused peak. At higher concentrations, as was the case in the urines analyzed, the peak shape of SU deteriorates and thus becomes difficult to quantitate. Using micelles in the buffer, the effect was not observed. Thus, we conclude that SU is being defocused when present at concentrations $\gg 10$ μg/ml. Further work is required to identify the underlying mechanism. Although LIF with 325 nm excitation [16] and fluorescence with 220 nm excitation [15] have been reported to be well suited for analysis of GA, SA and SU by MECC, excitation at 257 nm combined with the sheath-flow detection produced an unstable and extremely high background fluorescence so that MECC was not evaluated using the wavelength-resolved technique. The benefits of using LIF for monitoring natively fluorescent compounds have been amply demonstrated in this work.

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