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Modification of a tunable UV–visible capillary electrophoresis detector for simultaneous absorbance and fluorescence detection: profiling of body fluids for drugs and endogenous compounds

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Abstract

Using fused-silica optical fibres for fluorescence light collection and bandpass filters for selection of emission wavelengths, a capillary electrophoresis detection cell of a conventional, tunable UV–Vis absorbance detector was adapted for simultaneous fluorescence (at selected emission wavelength) and absorbance (at selected excitation wavelength) detection. Detector performance is demonstrated with the monitoring of underivatized fluorescent compounds in body fluids by micellar electrokinetic capillary chromatography with direct sample injection. Compared with UV absorption detection, fluorescence detection is shown to provide increased selectivity and for selected compounds also up to tenfold higher sensitivity. Examples studied include screening for urinary indole derivatives (tryptophan, 5-hydroxytryptophan, tyrosine, 3-indoxyl sulfate and 5-hydroxyindole-3-acetic acid) and catecholamine metabolites (homovanillic acid and vanillylmandelic acid) and the monitoring of naproxen in serum, quinidine in serum and urine and of salicylate and its metabolites in serum and urine.

1. Introduction

Laser-based fluorescence has produced spectacular results for solute detection in capillary electrophoresis (CE) [1,2], with detection limits of model compounds in the yoctomole (10^{-24} mol) range [2]. Lasers suffer from a number of inherent limitations, including the availability of excitation wavelengths and high cost. The non-laser-based fluorescence detectors constructed for CE, such as those (in order of decreasing performance) using xenon arc [3] (detection limit 10^{-20} mol), mercury–xenon arc [4] (10^{-16} mol), pulsed xenon [5], deuterium [6] and tungsten [6] lamps, provide much poorer sensitivity but allow for wavelength selection over a wide range. Using such incoherent excitation sources, solute monitoring by simultaneous absorption and fluorescence detection has been described [5], but no detector of this kind is currently available.

Fluorescence detection in CE of low-molecular-mass compounds in body fluids has been shown to provide improved selectivity and often also sensitivity when compared with absorption detection. Examples discussed in the literature include the determination of urinary porphyrins [7] and thiols in blood [8], the assessment of

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metabolic disorders via the determination of urinary cysteine and homocysteine, taurine in biopsy samples of the myocardium and gluthathione in red blood cell lysates [9] and the monitoring of serum levels of methotrexate [10], anthracyclines [11], naproxen [12] and retinol [13]. It is interesting that in work dealing with endogenous compounds, mostly non-laser-based fluorescence detection was applied via adaptation of HPLC fluorescence detectors to CE, whereas drug monitoring was effected via the use of laser-induced fluorescence detection.

The laboratory in Berne has specialized in applying CE and micellar electrokinetic capillary chromatography (MECC) to the determination of drugs and metabolites in body fluids [14–20]. In view of the high potential of using fluorescence detection for drug monitoring and clinical analysis, the construction of an inexpensive, versatile, non-laser-based fluorescence detector for CE was undertaken. The cell of a conventional, tunable UV–Vis absorbance detector was adapted for simultaneous fluorescence and absorbance detection. The modification implemented is similar to that described by Albin et al. [6], using optical fibres for fluorescence light collection, optical bandpass filters for selection of emission wavelengths and a photomultiplier module for emission light detection. In this set-up, the detector can be employed (i) for simultaneous fluorescence and absorption detection where the wavelengths for excitation of fluorescence and absorption detection are identical, or (ii) for multi- or single wavelength absorption detection, for which the detector was originally designed. In both operational modes, computerized data acquisition, handling and storage are employed. Fluorescence detector performance is demonstrated with the monitoring of endogenous low-molecular-mass compounds, such as urinary indole derivatives and catecholamine metabolites. Further, combined fluorescence and absorption detection is shown to be an interesting approach for the monitoring of drugs in body fluids, including naproxen in serum, quinidine in serum and urine and salicylate and its metabolites in serum and urine.

2. Experimental

2.1. Chemicals, origin of samples and routine methods of analysis

All chemicals were of analytical-reagent or research grade. The drugs naproxen, salicylate and quinidine were of European Pharmacopoeia quality. Salicyluric acid, tyrosine (Tyr), tryptophan (Trp), 5-hydroxytryptophan (5HTrp), 5-hydroxyindole-3-acetic acid (5HIAA), homovanillic acid (HVA), vanillylmandelic acid (VMA) and 3-indoxyl sulfate (3IXS) were obtained from Sigma (St. Louis, MO, USA). Our own serum and urine were employed as blank matrices. Sera from patients were collected in our routine drug assay laboratory where they were received for drug monitoring. Salicylate and quinidine in serum were determined by automated fluorescence polarization immunoassays (FPIA) on a TDx analyser (Abbott Laboratories, Irving, TX, USA). The immunoassays were performed according to the manufacturer's instructions using their reagent kits. Patients' 0–24-h urine samples with increased concentrations of 5-HIAA, HVA or VMA were obtained from the routine clinical laboratory of the University Hospital (Berne, Switzerland), where they were analysed by HPLC. All samples were stored at −20°C until further analysis.

2.2. Electrophoretic instrumentation and running conditions

The instrument with multi-wavelength detection employed in this work has been described previously [14–17]. If not stated otherwise, it featured a 75 μm I.D. fused-silica capillary of about 50 (70) cm effective (total) length (Product TSP/075/375, Polymicro Technologies, Phoenix, AZ, USA), together with a UVIS 206 PHD fast-scanning multi-wavelength detector and a No. 9550-0155 on-column capillary detector cell (both from Linear Instruments, Reno, NV, USA). The detector allows the selection of wavelengths between 190 and 800 nm (in 1-nm increments; with a deuterium and a tungsten

lamp covering the wavelength ranges 190–365 and 366–800 nm, respectively). For all experiments, the 206 PHD detector was controlled by a Mandax AT 286 computer system (Panatronic, Zürich, Switzerland) and running the 206 detector software package version 2.0 (Linear Instruments) with windows 286 version 2.1 (Microsoft, Redmond, WA, USA). Multi-wavelength data were read, evaluated and stored as described previously [14–17].

Modifications for simultaneous absorbance and fluorescence detection were made in-house. A schematic representation of the assembly is presented in Fig. 1. The body of the detector cell was machined so that two 370 μm O.D. fused-silica optical fibres (Polymicro Technologies) could be mounted at right-angles (as shown in Fig. 1) or at 30° to the capillary axis (right-angles to the optical axis of the excitation light beam in both instances). For positioning of the two fibres in close proximity to the capillary and ball lens, the same sleeve and ferrule mounting (inset in Fig. 1) as for the fixation of the capillary was employed. The ends of the fibres were polished with successively finer grades of wet silicon carbide paper followed by slurries of alumina on microcloth. The optical assembly was cleansed using methanol. The two fibres were united and interfaced to an interchangeable filter holder which was mounted as closely as possible to an HC120-05 photosensor module (Hamamatsu, Bridgewater, NJ, USA). Bandpass (interference) filters for 340, 366, 405, 450 and 520 nm (half-bandwidth 10 ± 2 nm; transmission >30%) were purchased from Balzers (Balzers, Liechtenstein). The output signal of the photomultiplier module was interfaced directly to the data acquisition system. Fluorescence and absorption (at the excitation wavelength) data were registered simultaneously with a two-channel PC Integration Pack (version 3.0; Kontron, Zürich, Switzerland), which features automatic range switching and a dynamic sampling rate allowing sampling every 10 ms for rapidly changing signals. Data

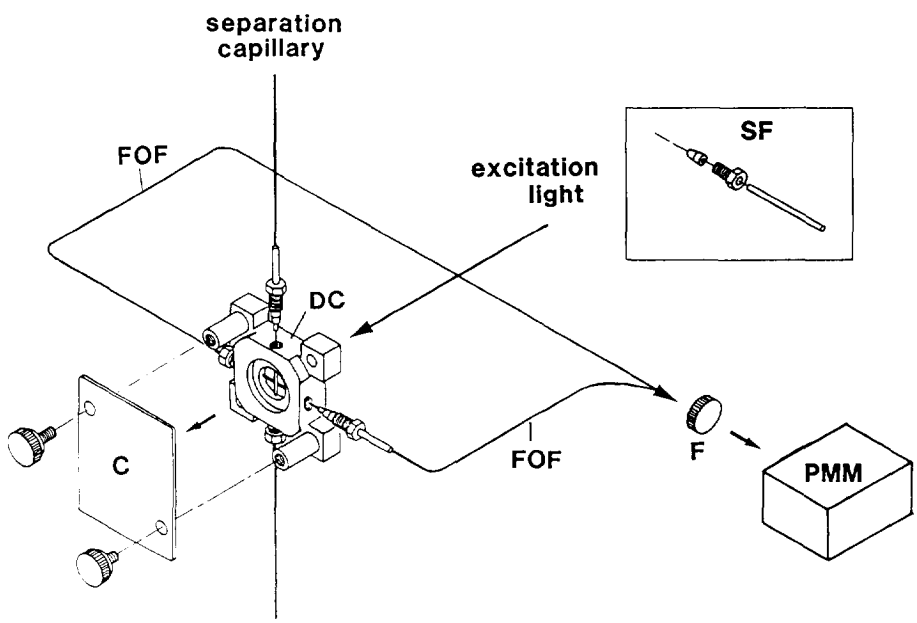

Fig. 1. Schematic representation of the detection cell for simultaneous absorption and fluorescence detection. The inset depicts an exploded view of the sleeve and ferrule mounting for the optical fibres and the separation capillary (adapted from the capillary detector cell manual of Linear Instruments). DC = detector cell body; C = cover plate with photodiode assembly for absorption light detection; FOF = fused-silica optical fibre; F = bandpass or edge filter; PMM = photomultiplier module for fluorescence light detection; SF = sleeve and ferrule mounting.

storage and computations were executed on a Mandax AT 286 computer. For simultaneous fluorescence and absorption detection, the 206 PHD detector was used in the high-sensitivity monochrome mode.

If not stated otherwise, a constant voltage of 20 kV (90 μA) was applied and the cathode was on the detector side. Sample application was effected manually through dipping the anodic capillary end into the sample vial and lifting it by ca. 34 cm for a specified time interval. Conditioning between runs was achieved by rinsing the capillary with buffer for 10 min. A buffer composed of 75 m*M* sodium dodecyl sulfate (SDS), 6 m*M* $Na_2B_4O_7$ and 10 m*M* Na_2HPO_4 (pH $\approx$ 9.2) was employed. All standard drug solutions were prepared in buffer or methanol at concentrations of 5–100 μg/ml. Blank and patients' samples were spiked by addition of known aliquots of these standard solutions to the plain body fluids.

3. Results and discussion

As described above, the detector cell constructed uses two optical fibres for fluorescence light collection (Fig. 1). First, employing Trp as a model compound, the performance of the mountings of these fibres at 30° angles was compared with that provided with fixation at right-angles. With excitation at 220 nm and emission monitored at 340 nm, fluorescence monitoring at 30° was determined to provide lower sensitivity compared with emission light collection at right-angles, the difference being attributed to difficulties in the alignment of the collection fibres. With the 90° mounting, the sensitivity was found to be about equal to that obtained by UV absorption detection at the excitation wavelength. Thus, for all the data discussed below, the mounting of the optical fibres at right-angles was employed. The relative magnitudes of the absorption and fluorescence signals are not only dependent on how the emission light is collected, but are mainly dependent on the solute, its chemical environment, and the optical specifications, including the wavelengths selected for excitation and emission. For example, with fluorescein as the sample, the fluorescence response at 520 nm was determined to be much larger than the absorption signal at 220 nm. Therefore, with fluorescence detection as described here, the detection limit can be improved compared with that obtained by UV absorption detection. Under the experimental conditions investigated, the absorbance and fluorescence detection limits ($S/N = 3$) for fluorescein were determined to be about 6.0 and 0.4 μg/ml, respectively.

As reported previously, CE techniques are attractive methods which permit monitoring of endogenous and exogenous compounds by direct injection of body fluids [15,17–20]. Resolution, however, can be hampered owing to the presence of a large number of endogenous low-molecular-mass components and peak assignment can be difficult when solutes are monitored by UV absorption. This is particularly the case with urine samples. The data presented in Fig. 2A represent an electropherogram obtained after direct injection of a tenfold diluted urine of a healthy individual and absorption detection at 220 nm. Corresponding fluorescence data with an emission band filter of 340 nm are depicted in Fig. 2B and those with the same urine spiked with 5HIAA, HVA and VMA are presented in Fig. 2C. All peaks, including those of Tyr, Trp, 5HTrp and 3IXS, were not only assigned based on their fluorescence, but also on matching multi-wavelength absorption spectra obtained by using the same detector in the fast scanning mode (data not shown).

Indoles, including Trp, 5HTrp, 5HIAA and 3IXS, which are heterocyclic amine compounds, and catecholamine metabolites, such as HVA and VMA, are present in cells and extracellular fluids of living organisms. Determination of such compounds in urine is clinically important and is typically performed by HPLC or GC [21,22]. For example, monitoring of 5HIAA in 0–24-h urine is employed for screening for carcinoid tumours, the normal excretion being <70 μmol per 24 h. The MECC data presented in Fig. 3A were obtained by injection of a tenfold diluted patient's urine, the 5HIAA concentration of which

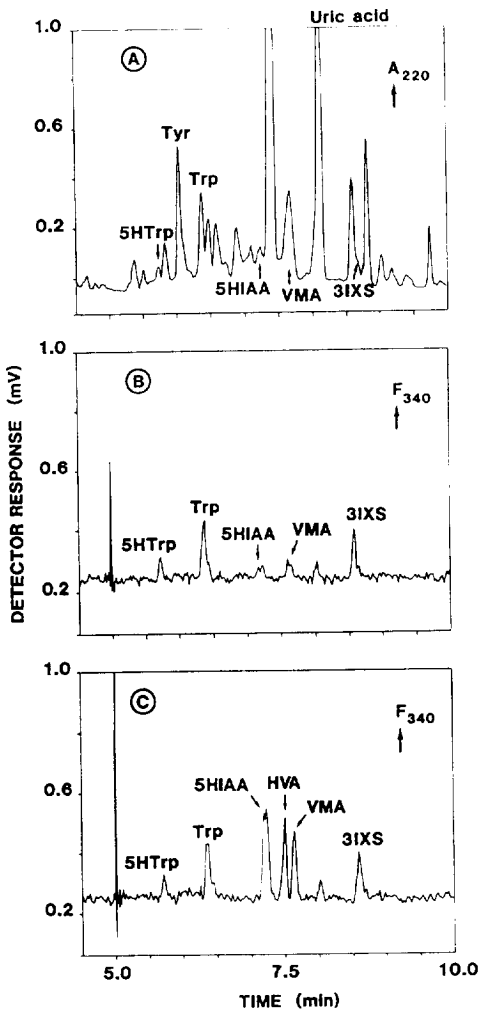

Fig. 2. MECC electropherograms of (A) tenfold diluted urine from a healthy individual obtained via absorption measurement at 220 nm, (B) the simultaneously collected fluorescence data with an emission filter of 340 nm and (C) the fluorescence data obtained with the same tenfold diluted urine but spiked with 5HIAA (5.4 μg/ml), HVA (74 μg/ml) and VMA (31 μg/ml). Sample injection time and applied voltage, 5 s and 20 kV, respectively.

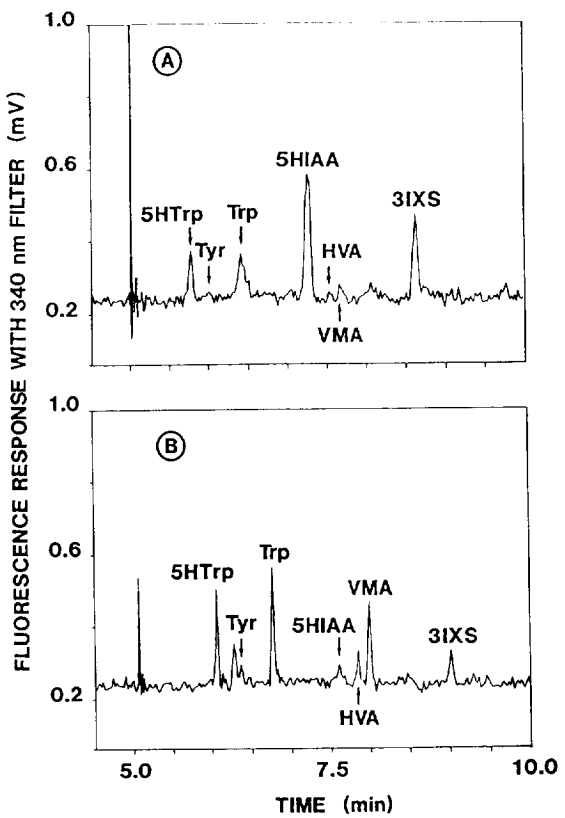

Fig. 3. MECC fluorescence (excitation at 220 nm and emission at 340 nm) data for (A) a tenfold diluted patient's urine with a pathological 5HIAA level and (B) an undiluted patient's urine with elevated concentrations of HVA and VMA. Sample injection times, 5 and 2 s, respectively. Other conditions as in Fig. 2.

was determined to be 332 μM using HPLC (data from the routine clinical laboratory). The urine volume collected was 760 ml and the excretion was calculated to be 252 μmol per 24 h. Comparison of the data in Fig. 3A with those in Fig. 2 reveals the presence of a much enlarged 5HIAA peak in the former case, indicating that elevated, pathological concentrations of 5HIAA can easily be monitored by MECC with direct injection of plain or diluted urine and on-column fluorescence detection.

HVA and VMA are catabolic products of catecholamines that are excreted in elevated amounts (>34 and >46 μmol per 24 h, respectively) in the urine of patients with various diseases, including neuroplastoma and hepatic encephalopathy [21–23]. The MECC data depicted in Fig. 3B were obtained by injection of an undiluted patient's urine with elevated excretion of HVA and VMA. The presence of both HVA and VMA could be determined unambiguously by MECC and fluorescence detection (Fig. 3B). Compared with the data reported by Issaq

et al. [23], the fluorescence detector described here appears to have higher sensitivity than the UV detector employed by them. These few data suggest that MECC with fluorescence detection could be used for the simultaneous monitoring of pathological urinary levels of Trp, 5HTrp, 5HIAA, HVA and VMA, and thus for the diagnosis of diseases. Further, with extraction prior to application to the capillary, many more basic neurotransmitters and their acidic metabolites could be detected [21,22].

To complement our previous efforts in monitoring drugs by MECC with UV absorption detection and direct sample injection [18–20], the potential of employing simultaneous fluorescence and absorption detection for drug analysis was investigated. Using naproxen, salicylate and quinidine as model drugs, electropherograms of a serum blank and the serum blank spiked with the three drugs are presented in Fig. 4A and B, respectively. The fluorescence data for the serum blank reveal a sharp peak produced by Trp. Hence this compound can be used as an endogenous marker substance. Further, the response originating from the proteins could easily be allocated. However, the compounds producing the two sharp peaks marked ? could not be identified. As can be seen from the data in Fig. 4B, all three drugs were found to produce satisfactory peaks when injected within plain serum. For naproxen, the fluorescence response with a 366-nm emission filter was found to be much stronger than that obtained with absorption detection at 220 nm. The opposite was true for salicylate, whereas for quinidine the peak height of the fluorescence signal was slightly larger than that of the UV absorption response observed at the excitation wavelength. With

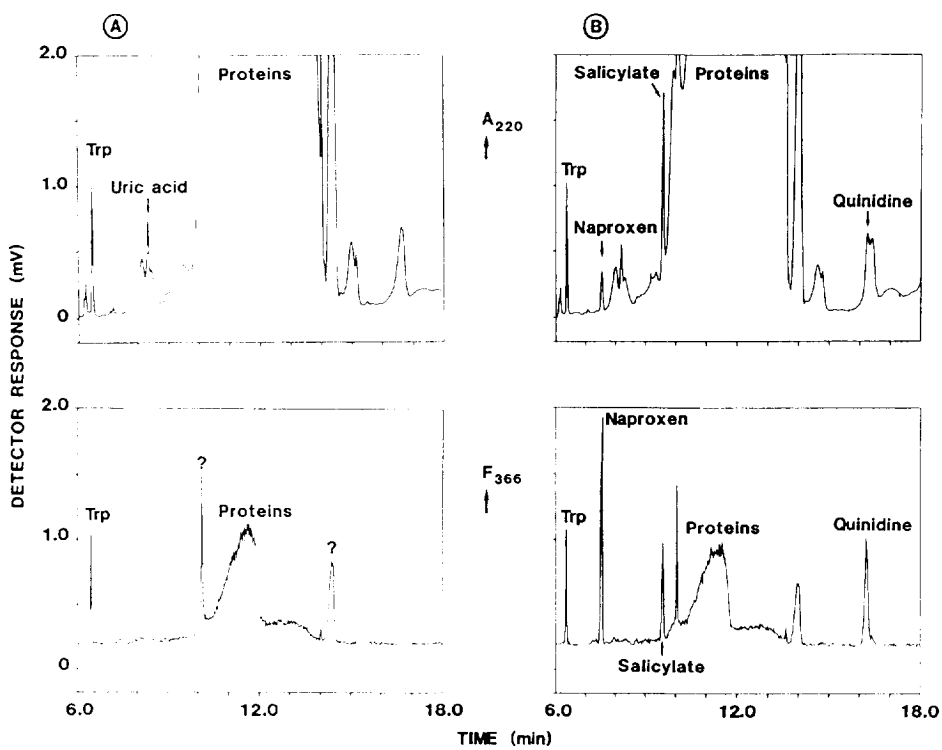

Fig. 4. MECC data for (A) serum blank and (B) serum blank spiked with naproxen (5 μg/ml), salicylate (75 μg/ml) and quinidine (13 μg/ml). The upper graphs represent UV absorption data measured at the excitation wavelength of 220 nm and the lower graphs are the fluorescence data obtained with a 366-nm bandpass filter. Injection time and applied voltage, 2 s and 20 kV, respectively.

direct serum injection, the latter drug was found almost to co-elute with another compound, this being clearly seen in the absorbance data (upper graph of Fig. 4B). However, this unknown endogenous substance appears not to fluoresce and quinidine can easily be determined via evaluation of the fluorescence data. Hence simultaneous gathering of UV absorption and fluorescence data appears to be advantageous for drug monitoring and will be further emphasized with the analysis of patients' samples discussed below.

The MECC data shown in Fig. 5 were obtained via direct injection of serum from a patient undergoing naproxen pharmacotherapy. Using MECC with on-column absorption detection, the naproxen serum level was determined to be 9.4 μg/ml [19]. As expected, fluorescence detection provides a higher response than that obtained with absorption measurement at the excitation wavelength of 220 nm. Thus, with the simple fluorescence detector sub-μg/ml serum levels of naproxen (detection limit ca. 0.2 μg/ml) can be recognized, the concentration limit being about one order of magnitude lower than that observed with UV absorption detection [19]. However, using laser-induced fluorescence detection and drug extraction, an even lower detection limit was reported [12].

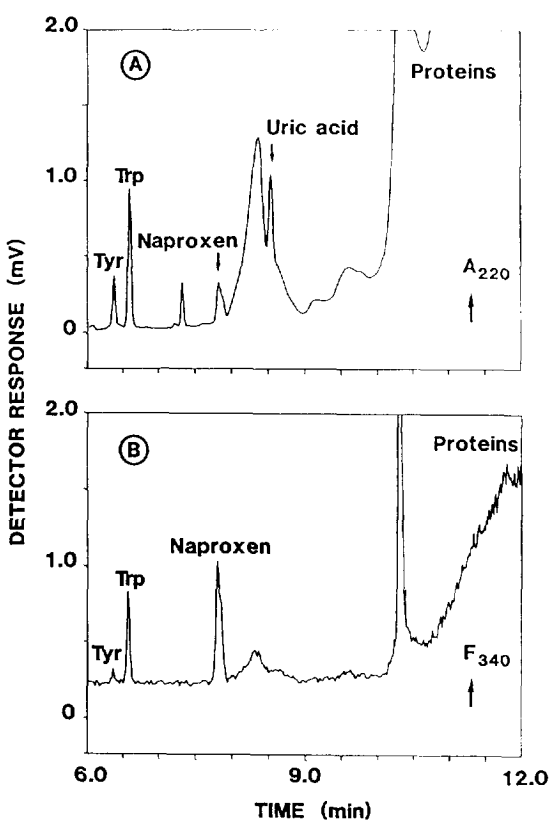

Fig. 5. MECC data for serum from a patient under naproxen pharmacotherapy. The upper graphs represent UV absorption data measured at the excitation wavelength of 220 nm and the lower graphs are the fluorescence data obtained with a 340-nm bandpass filter. Other conditions as in Fig. 4.

The data presented in Fig. 6A were obtained with a directly injected serum sample from a patient under quinidine pharmacotherapy. Using FPIA, the quinidine serum level in that sample was determined to be 10.7 μM (3.5 μg/ml), this being within the therapeutic range (6–15 μM) of this drug. As suggested according to the absorption data obtained with serum blank spiked with quinidine (Fig. 4B), this drug is co-eluting with matrix compounds (upper graph of Fig. 6A). However, using fluorescence detection with excitation and emission at 220 and 366 nm, respectively (lower graph of Fig. 6A), quinidine could readily be detected (for blank data refer to Fig. 4A). The MECC detection limit ($S/N = 3$) was determined to be 4.6 μM (1.5 μg/ml), hence MECC with direct serum injection and on-column fluorescence detection permits the determination of quinidine serum levels of pharmacological interest.

The electropherograms depicted in Fig. 6B were registered when analysing a twofold diluted urine which was collected 8–16 h after administration of 200 mg of quinidine sulfate (one tablet of Kinidin-Duriles; Astra Pharmaceutica, Dietikon, Switzerland). With urine, hardly any endogenous compounds elute after about 10 min {see Fig. 2A (complete blank data not shown) and to data reported in Refs. [17] and [24]}. Hence urinary quinidine can be monitored by both UV absorption and fluorescence detection. Further, owing to the fluorescence and an apparent similarity of the UV absorption spectrum with that of quinidine (data not shown), the peak eluting after about 14.3 min (peak marked with

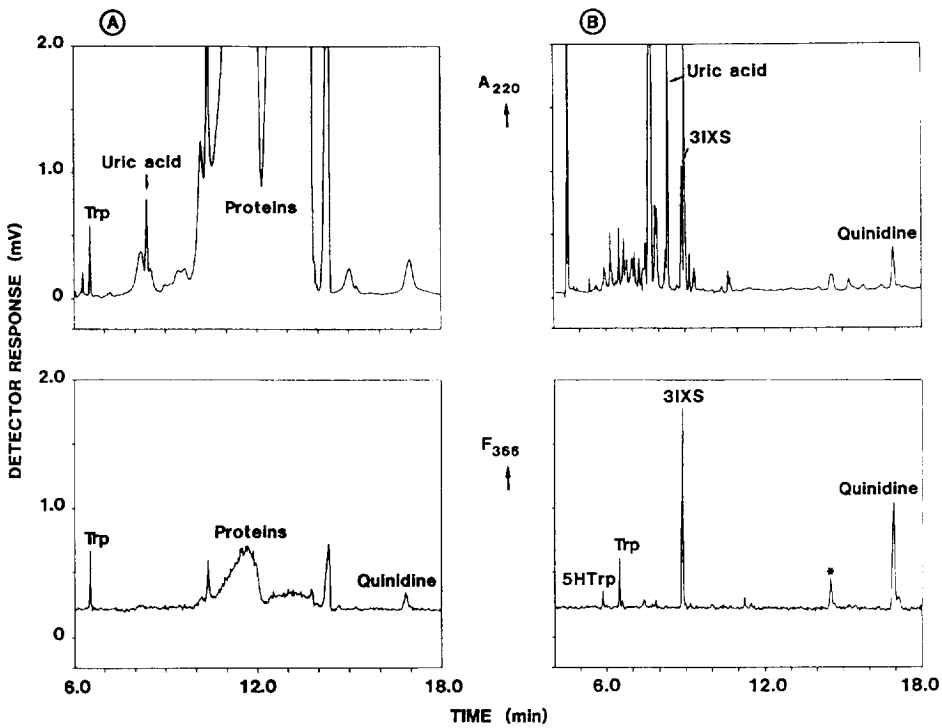

Fig. 6. MECC data obtained for (A) a patient's serum sample and (B) a twofold diluted urine from a test subject who had ingested quinidine. The upper graphs represent UV absorption data measured at the excitation wavelength of 220 nm and the lower graphs are the fluorescence data obtained with a 366-nm bandpass filter. Other conditions as in Fig. 4.

an asterisk in Fig. 6B) could be the response of 2-hydroxyquinidine, a metabolite of quinidine found in urine. Owing to the lack of a standard of this compound, however, this assumption could not be verified.

Although the fluorescence signal of salicylate is smaller than that obtained with absorption detection effected at the excitation wavelength of 220 nm (see Fig. 4B with fluorescence measured at 366 nm), it can be employed effectively for monitoring of this compound. MECC data obtained with a urine and a serum sample from a patient under suspected salicylate intoxication are presented in Fig. 7A and B, respectively. MECC data with multi-wavelength absorption data of these samples have been discussed previously (see Fig. 5 in Ref. [17]), the salicylate concentrations in urine and serum being of the order of 3 m*M* each. MECC data for tenfold diluted urine (Fig. 7A) and directly injected serum (Fig. 7B) are depicted. Both UV absorption data (at the excitation wavelength of 220 nm) and the fluorescence data with an emission wavelength of 450 nm [the use of this filter provided a slightly higher response than those employing 366-nm (Fig. 4B) and 405-nm (data not shown) filters] reveal the presence of salicylate in both samples. As discussed before [17], comparison of the urine and serum data (particularly for fluorescence data, lower graphs) reveals the presence of similar salicylate concentrations in these two body fluids. Moreover, fluorescence monitoring shows the presence of molecules of similar structure, including metabolites of drugs. For example, with the aid of the urinary fluorescence data, the presence of the

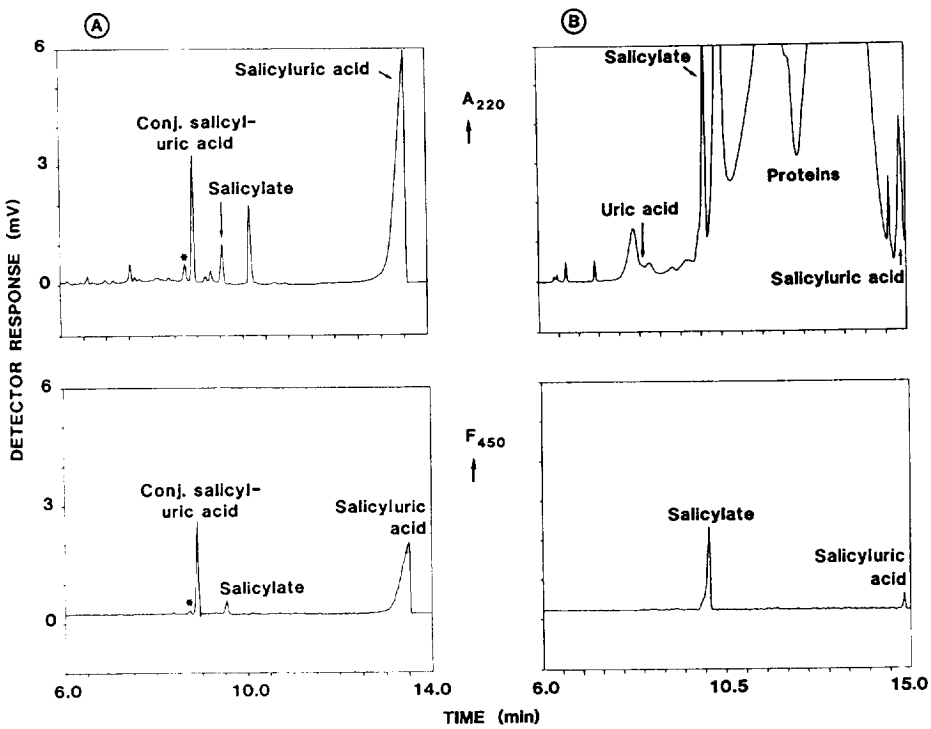

Fig. 7. MECC data obtained for (A) tenfold diluted urine and (B) serum from a patient with salicylate intoxication. The upper graphs represent UV absorption data measured at the excitation wavelength of 220 nm and the lower graphs are the fluorescence data obtained with a 450-nm bandpass filter. Other conditions as in Fig. 4.

major metabolite of salicylate, salicyluric acid and other salicylate metabolites could be suspected. Not surprisingly, using spectral analysis of the UV absorption data and rerunning the urine spiked with salicyluric acid allowed the positive identification of its peak which elutes at 13.6 min. Further, according to the UV spectrum and the fluorescence signal of the peaks eluting at 8.7 and 8.9 min, these peaks were assigned to the glucuronic acid conjugates of salicylate (marked with an asterisk in the lower graph of Fig. 7A) and salicyluric acid, respectively. In the case of the serum data (Fig. 7B), the small peak eluting at about 14.8 min could be assigned to salicyluric acid. Hence simultaneous monitoring of UV absorption and fluorescence provides valuable information both for drug monitoring and for the determination of metabolites.

4. Conclusions

Using direct injection of body fluids and looking at solutes with native fluorescence, monitoring of fluorescence with the simple detector assembly described is shown to provide increased selectivity and for selected compounds also up to tenfold higher sensitivity compared with UV absorption detection. Further, simultaneous UV absorbance (at the excitation wavelength) and fluorescence detection in MECC is shown to be attractive for screening and profiling of body fluids for disease diagnosis and/or confirmation, for therapeutic and diagnostic drug monitoring (including evaluations of intoxications) and for the assessment of drug metabolism. The detector employed allows the selection of excitation wavelengths between 195 and 800 nm (in 1-nm

increments), permits emission wavelength selection with a bandpass or a long-pass (edge) filter, and provides fluorescence and absorption data with the same time axis.

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