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Capillary electrophoresis with laser-induced fluorescence in clinical drug development
Routine application and future aspects

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Abstract

The clinical bioanalytical setting is characterized by sample volumes of <1 ml biological fluid (e.g. plasma, urine), a range of 3–4 decades of concentrations to be quantified and a limit of quantitation in the μg/l–ng/l range for sets of 100–5000 individual samples. Setup of capillary electrophoresis (CE) for routine application in this analytical field was successful for analytes accessible to fluorescence detection by using laser-induced fluorescence (LIF) detection. Empowerment of CE–LIF for routine serial analysis of thousands of samples includes improvement in autosampler techniques, thorough procedures for capillary treatment and particularly more advanced detection technology. Introduction of multi-capillary systems with charge-coupled device cameras and frequency doubled Ar-ion laser (λ=257 nm) offers this technique the chance of superiority over classical analytical assays — especially in the field of (new) low volume samples e.g. capillary blood or microdialysate encouraging clinicians to search for meaningful non-invasive samples.

Keywords: Pharmaceutical analysis; Detection, electrophoresis; Charge coupled device detection; Tramadol

1. Introduction

The feasibility of capillary electrophoresis (CE) has been shown for many drugs in pharmaceutical quality control [1–3]. Concentration measurement in clinical studies challenges this method by low limits of quantitation, large dynamic ranges (factor of 1000 within one sequence) and high sample throughput (100–5000 samples per study). CE has to compete with all other bioanalytical methods (especially LC–MS, HPLC and GC) regarding quality of results, time between sample arrival and report generation and — of course — costs. The advantages of short analysis times, small injection volumes (a few nl) and low amounts of solvent offer a good starting point for competition. Vaporization of small sample volumes and long sequences set cornerstones of method development in CE since all assays have to be validated according to international guidelines

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[4–6] with a typical accuracy of ±10% and a precision of <10% [close to limit of quantitation (LOQ) <20%].

As a key issue in the past, high LOQs restricted the wide use of CE in clinical studies. Laser-induced fluorescence (LIF) detection offers the chance to develop methods competitive to classical methods by offering different laser systems like 488 nm Ar-ion, 442 nm He–Cd and 325 nm He–Cd lasers for the CE systems.

Previous results demonstrated the potency of CE–LIF methods using a 325 nm He–Cd laser [7,8] with moxifloxacin, a new quinolone antibiotic. The comparison with other bioanalytical methods as well as advantages and challenges for its routine use in clinical studies need further discussion.

LIF in the visible and high UV range is already established as a detection system for CE. Since most drugs are excited to fluorescence in deep to medium UV (200–320 nm) the use of a frequency doubled Ar-ion lasers (λ=257 nm) may expand the potential of CE–LIF. Lasers in the deep UV range are already available but have not been routinely accessible for detection in CE due to insufficient power and stability as well as long living light fibers. First results and a validation of methods [9] have already been published.

Furthermore, the routine use of charge-coupled device (CCD) cameras offered the potential to increase sensitivity [10] and to identify peaks by emission spectra (and retention time). The simultaneous identification and quantification of the fluorescent analytes would not be possible using filter-based single-channel fluorescence detectors.

2. Material and methods

2.1. Chemicals

Moxifloxacin and ciprofloxacin were used as certified reference compounds (Bayer, Wuppertal, Germany).

Tramadol was a gift from Grünenthal (Stolberg, Germany); its phase I metabolites were synthesized according to the literature [11]. Verapamil and zolpidem were donated by Wolff (Bielefeld, Germany) and Synthelabo (Bagneux, France), respectively. Morphine was a present from Merck (Darmstadt, Germany). 3,4-Methylendioxymethylamphetamine (MDMA) and etoposid were purchased from Sigma–Aldrich (Steinheim, Germany) and Bristol Myers Squibb (Munich, Germany), respectively.

All chemicals were of analytical grade purity. Sodium tetraborate, sodium hydroxide and sodium phosphate were purchased from Merck. The water was deionized and bidestilled.

2.2. Apparatus

2.2.1. Routine application setup

CE analysis was performed on a Beckman P/ACE 5010 and Beckman P/ACE MDQ (Beckman Instruments, Munich, Germany). Both instruments were run in an air-conditioned room and were equipped with sample and capillary liquid cooling. Fluorescence was provided by an Omnichrome He–Cd laser (3074-20M, Laser 2000, Wessling, Germany) with 20 mW and 325 nm excitation wavelength. Laser and detector were connected by an optical fiber (Omnichrome POS FDS – A $^{1}/_{2}$, Laser 2000 Wessling, Germany). Details of the assays are presented in [7].

2.2.2. Experimental setup

Fig. 1 illustrates a schematic description of the experimental setup.

The CE apparatus was based on a SpectraPhoresis 100 CE system (Thermo Seperation Products, ThermoQuest Analytische Systeme, Egelsbach, Germany). Fused-silica capillaries with 375 μm outer (O.D.), 50 μm inner diameter (I.D.), an effective length of 55 cm and total length of 75 cm were used.

The UV–LIF laser source was a frequency doubled argon-ion laser (Lexel 95 SHG, Lexel Laser, Polytec, Waldbronn, Germany) operating with a power of 200 mW to provide the excitation wavelength of 257 nm. An on-column detection window was formed by removing a 4 mm section of the polyimide coating on the fused-silica tubing. This is different to most CE–LIF systems where the laser beam is focused on a spot of less than 100 μm, thus illuminating the analyte for several milliseconds only [12–14]. In the system described here, the capillary is illuminated by laser light on a length of 1.5 mm along the detection window and a height according to the I.D. of the capillary (50 μm) using a 40 mm

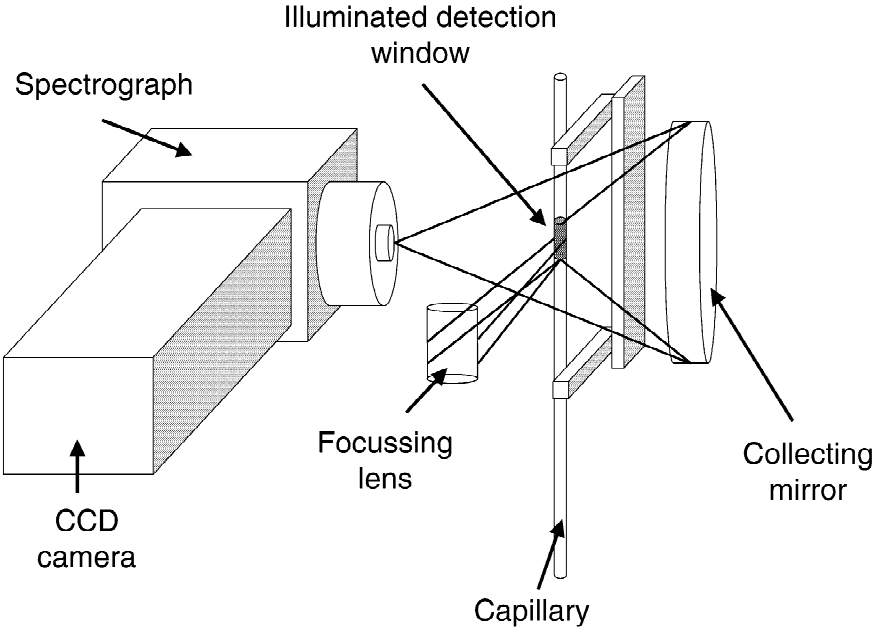

Fig. 1. Schematic description of the experimental setup.

focal length cylindrical quartz lens for focusing. The resulting fluorescence from this section was imaged onto a spectrograph with an attached intensified CCD camera (Flamestar 3, Lavision 2D Messtechnik, Göttingen, Germany). With the present CCD camera the covered spectral range is 105 nm wide, adjustable between 180 nm and 400 nm [15]. This setup was used to achieve wavelength resolution of the emitted light, allowing the additional registration of the emission spectra which were read out by the CCD camera [14,16–18]. Fluorescence was collected during the entire residence time of the analyte band in the 1.5 mm capillary section, thus enhancing the sensitivity of the LIF system significantly.

The LIF signal was collected in an angle of 90° to the excitation light with a spherical aluminum mirror (diameter 5 cm, F/number 1.1) to suppress chromatic aberration.

An imaging spectrograph (Multispec 77417, Oriel, L.O.T.-Oriel, Darmstadt, Germany) with a 1200 lines/mm grating, holographically blazed at 250 nm was employed. The CCD camera was used with a read out rate of 4.5 Hz and a binning factor 48×40 [16]. Characteristic spectral fingerprints of the analytes allowed a more guarded identification compared to the migration time alone.

The wavelength resolved CE–LIF data was processed with Mathcad 7 (Mathsoft, Cambridge, MA, USA) using customized algorithms.

2.3. Electrophoretic conditions

The limit of detection (LOD) was determined for verapamil, zolpidem, morphine and MDMA in 50 m*M* sodium phosphate buffer pH 4.5. 150 m*M* sodium tetraborate buffer adjusted to pH 10.6 with 150 m*M* sodium hydroxide was used for the assay of tramadol. The applied voltage was +25 kV and the temperature was maintained at 19.5°C. The injection was performed hydrodynamically for 3 s.

2.4. Stock solutions

Stock solutions of 1 mg/10 ml were prepared in deionized and bidestilled water and stored at +4°C. Standard solutions from 10 mg/l to 1 μg/l were prepared daily by dilution with deionized and bidestilled water.

3. Results and discussion

3.1. Routine use of CE in clinical studies

CE assays for moxifloxacin in different matrices employing a 325 nm He–Cd laser detection system were set up. Formal validation experiments according to international guidelines proved that the assays were suitable for use in clinical studies [7].

Analysis of moxifloxacin in plasma, capillary blood and blister fluid as well as in microdialysate (to determine concentrations in subcutaneous tissue and muscle) was performed. These well controlled methods generated high quality data with acceptable levels of precision, accuracy, working range and LOQ.

Besides a formal 3-day validation and its use in clinical settings, the assay for quantification of moxifloxacin in plasma was compared to results acquired by HPLC–fluorescence (FL), LC–MS–MS and bioassay (Fig. 2) [8]. Although relative deviation of single points is more expressive than r^2 to describe data for 3 to 4 orders of magnitude, correlation of CE–LIF, HPLC–FL and LC–MS–MS data was excellent ($r^2>0.990$). Results of the bioassay are systemically biased by up to 6% compared to those of CE–LIF at low to medium concentrations.

The analytical parameters of all four methods were compared (Table 1). CE–LIF results were based on the Beckman P/ACE MDQ (with microtiterplate autosampler). The primary purpose of the bioassay is to screen for active metabolites but not to achieve high sample throughput in clinical studies.

LOQ and working range were about equal for the instrumental analysis systems. Though, the excitation with the He–Cd laser at 325 nm, which is far away from moxifloxacin's five-fold higher maximum ($\lambda=$ 296 nm), and the long linearity of the LIF detector have to be highlighted. Run-time in the CE–LIF assays was shorter than in HPLC–FL and LC–MS–MS assays. Since the CE–LIF autosampler worked with only one microtiterplate (two using both sides of the capillary, though), round-the-clock analysis was limited for CE–LIF, in contrast to HPLC and LC–MS–MS.

Minimal injection volumes resulted in small volumes for work-up, presenting the key advantage of CE methodology. The work-up volume was limited

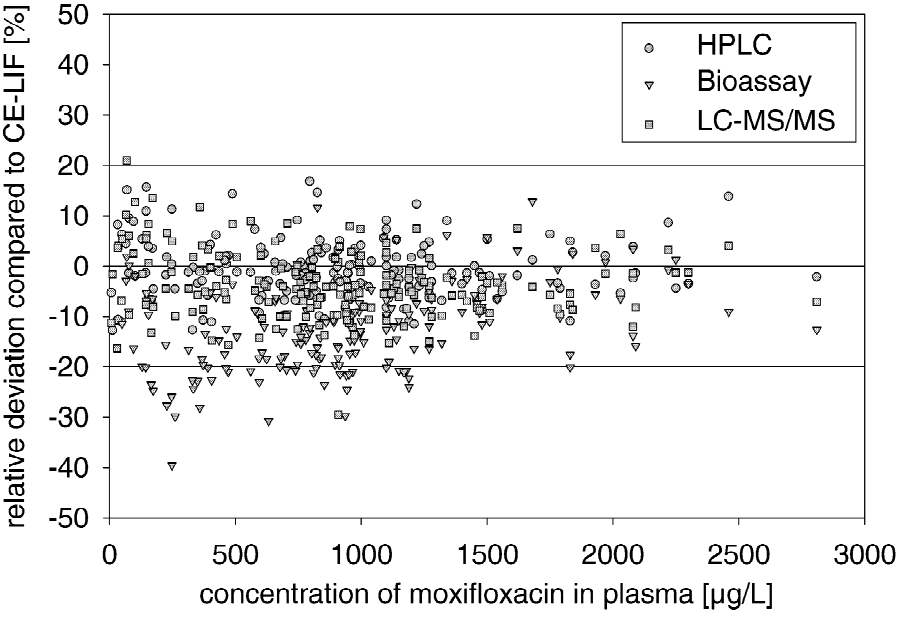

Fig. 2. Relative deviation of HPLC–FL, LC–MS–MS and bioassay results compared to CE–LIF. Determination was performed using clinical study samples.

Table 1
Analytical parameters of CE–LIF, HPLC–FL, LC–MS and bioassay for the determination of moxifloxacin in plasma

	CE–LIF	HPLC	LC–MS	Bioassay
LOQ (μg/l)	2.5	2.5	5	62.5
Working range (μg/l)	2.5–5000	10–5000	5–5000	62.5–2000
Run time per sample (min)	4.0	5.5	6.5	1 day
Time for 1000 samples (days)	6	4	4	20
Volume for work-up (μl)	10	50	50	100
Injection volume (μl)	0.02	50	30	
Costs of instrumentation	+	+	–	++
Automatisation	++	++	++	–

by evaporation, adhesion of sample to surfaces and (manual) quantitative pipetting (of hundreds of samples). Investigations with liquid handlers (Multiprobe II, Packard, Meriden, CT, USA) which can handle very small volumes are promising [19].

The assay for quantification of moxifloxacin in microdialysate was applied to one study with about 1200 samples. Analysis was performed on a Beckman P/ACE 5010 instrumentation (limited autosampler, 18 samples only). Robustness of assays was achieved by overcoming the CE specific limitations. Capillary life time was not critical. It was employed for these 4 weeks and more than 2000 injections including time thereafter by careful application of the washing steps.

Due to the influence of temperature on CE, the instrumentation was used in air conditioned laboratories and liquid cooling of the capillary had a positive influence on robustness of the system, stability of migration times (RSD<1.4%), and the absence of circuit interrupts. The challenge of sample evaporation due to the (small) sample volume was achieved by cooling the samples for at least 24 h improving also the stability of analytes.

Precision and accuracy were estimated by analyzing quality control samples (Fig. 3). Results were

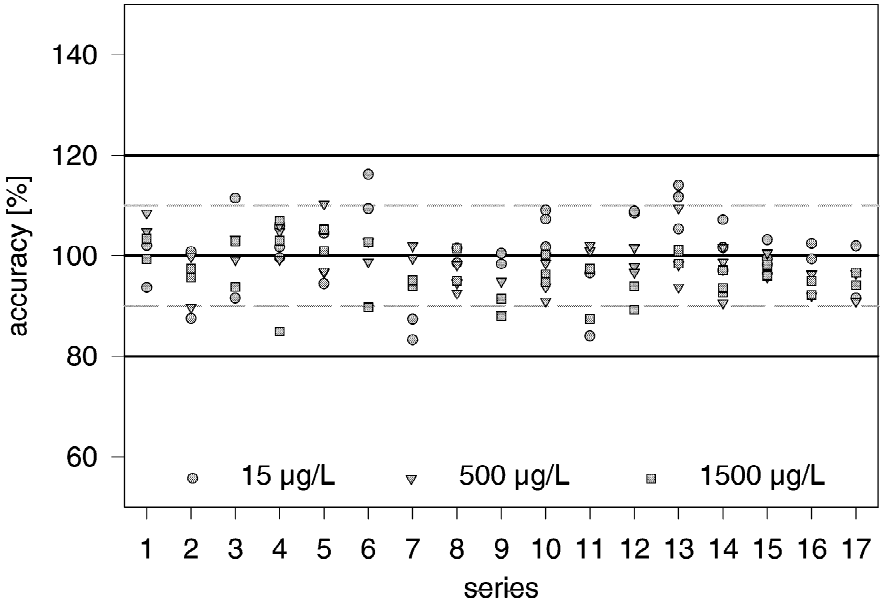

Fig. 3. QC-Chart for quantification of moxifloxacin in microdialysate (4 weeks).

Table 2
Results of ''3-day'' validation and quality controls during measurement of study

''3-day'' Validation according guidelines ($n=18$)						
Concentration (μg/l)	5	10	20	50	500	4000
Accuracy (%)	−6.64	−0.25	−0.76	−0.65	3.73	2.97
Precision (%)	13.20	7.51	4.12	4.85	4.23	4.30
QC-samples determined together with study samples						
Concentration (μg/l)	15.00	500.0	1500			
n	39	43	39			
Means (μg/l)	15.15	492.5	1447			
SD	1.183	25.94	77.40			
Accuracy (%)	0.97	−1.51	−3.51			
Precision (%)	7.81	5.27	5.35			

well within in the defined limits and comparable to those of the formal ''3-day'' validation performed according to the guidelines (Table 2).

3.2. Investigations using a 257 nm Ar-ion laser and CCD camera detection system

3.2.1. Sensitivity

To evaluate the increase in sensitivity using LIF at $\lambda_{ex}=257$ nm instead of UV detection for CE separations, the LOD of several compounds was investigated. The LOD was defined as the analyte concentration which leads to a signal-to-noise ratio (S/N) ratio) of 3:1.

The LODs for zolpidem, verapamil, MDMA, morphine (from standard solutions), tramadol (from urine samples without extraction) and, etoposid (from plasma samples after precipitation) were determined using LIF detection and were compared to those determined using instruments equipped with an UV detector (Table 3).

Table 3
Limit of detection (LOD) of CE: UV-absorption detection compared to LIF detection

Substance	UV-absorbance detection (mg/l)	LIF detection (μg/l)
Morphine	5	50
Zolpidem	1	1
Verapamil	1.2	8
MDMA	1	10
Tramadol	1	1
Etoposid	1	30

For tramadol and zolpidem the LOD was about 1000-fold higher compared to CE methods using UV detection systems. LIF detection enhanced the LOD about 150-fold for verapamil and about 100-fold for MDMA and morphine. These results demonstrate the great potential of CE–LIF for sensitive determination of fluorescent drugs.

3.2.2. Wavelength resolved detection

Very often several analytes (e.g. metabolites) with different emission wavelengths have to be evaluated in one run. Therefore it was investigated if different wavelength intervals could be used with the experimental setup to optimize sensitivity and selectivity.

Tramadol was separated from its phase I metabolites *N*-demethyltramadol and *N,O*-didemethyltramadol and the internal standard (IS) naphazoline nitrate. The emission spectra of tramadol, *N*-demethyltramadol and *N,O*-didemethyltramadol were different (Fig. 4). Therefore it was necessary to choose different wavelength intervals from the entirely collected spectral range for data processing to achieve the best S/N ratio for a certain analyte in the sample mixture (Fig. 5). The emission signal was collected in intervals from 266.5 nm to 285.5 nm, 285.5 nm to 304.5 nm, 304.5 nm to 342.5 nm, and 342.5 nm to 361.5 nm. The S/N ratios of these analytes in the different collection intervals were compared.

When excited at 257 nm a maximum of emission was found at 295 nm for tramadol and *N*-demethyltramadol. The signal was collected from 285.5

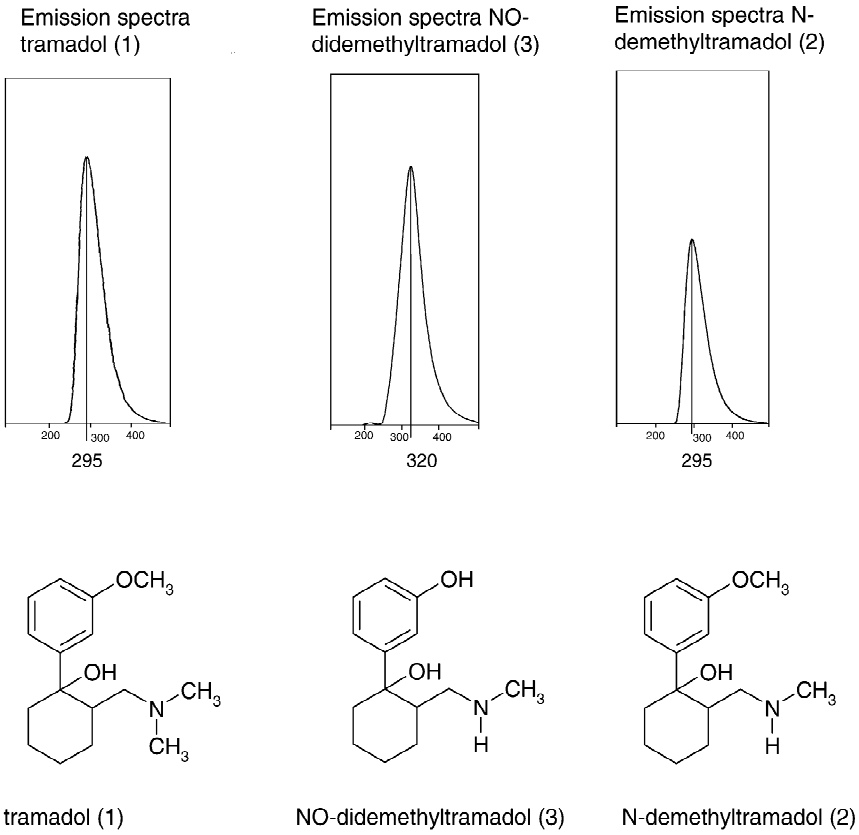

Fig. 4. Emission spectra of tramadol, *N*-demethyltramadol and *N,O*-didemethyltramadol.

nm to 304.5 nm to achieve the optimal S/N ratio for these analytes. For *N,O*-didemethyltramadol the maximum of emission was found at 320 nm. To obtain a wavelength optimized result for this metabolite the signal was collected from 304.5 to 342.5 nm. The selectivity between the internal standard and *N*-demethyltramadol was lost, but there was a 3-fold improvement of sensitivity for *N,O*-didemethyltramadol, while the sensitivity for tramadol was decreased by a factor of 4.

The separation of tramadol and its phase I metabolites showed the advantages of wavelength resolved detection. Wavelength resolved detection allowed the determination of different analytes in one mixture at their optimal detection wavelength in one run. Endogenous substrates could be separated from the interesting analytes in the same way if they show fluorescence at a different wavelength range. Analytes which are not baseline separated but show different maxima of emission could also be separated in one run.

4. Conclusion

Routine CE–LIF assays using the He–Cd laser ($\lambda = 325$ nm) and the frequency-doubled Ar-ion laser ($\lambda = 257$ nm) to measure samples in clinical studies were established successfully. Based on these assays the use of CE in a clinical setting could be demonstrated. The great improvement of the sensitivity due to the LIF in combination with the wavelength resolved detection offers the possibility of the direct quantification of fluorescent drugs and their metabolites in biological fluids. Time-consuming sample pre-treatment as extraction or pre-concentration of the analytes could be avoided.

CE–LIF can be used in bioanalytics to quantify

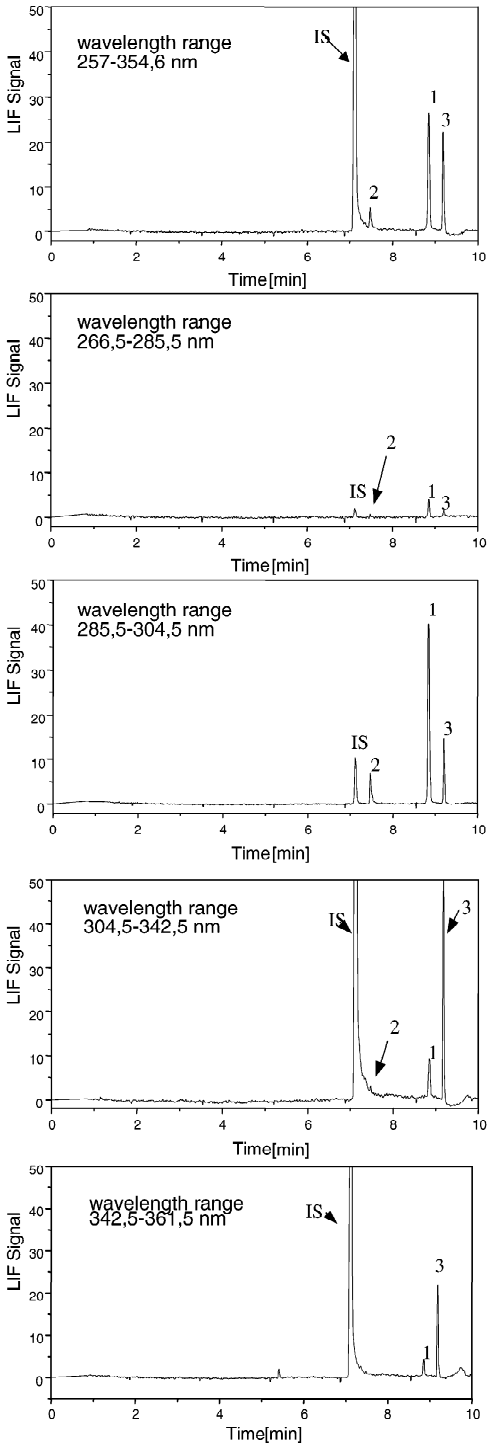

Fig. 5. Wavelength resolved detection of *N*-demethyltramadol (2), internal standard (IS), tramadol (1) and *N,O*-didemethyltramadol (3).

compounds detectable with laser-induced fluorescence. CE–LIF can be competitive to classical assays like HPLC–FL and LC–MS–MS in a clinical setting characterized by 3–4 decades of calibration range, limit of quantitation in the lower μg/l range and sample volumes of <0.1 ml. Together with microtiterplate autosamplers CE–LIF can be automated to become cost competitive with other methods.

CE–LIF will be superior in specific areas by analyzing samples of small volume to provide information in new fields of interest (e.g. microdialysate, tears, blister fluids, capillary blood). Samples from children or even newborns as well as patients at risk might be analyzed due to the small sample volume.

5. Prospects

5.1. Routine use of the Ar-ion laser

A prototype of a detection system using fiber optics is available which will be combined with a Beckmann P/ACE MDQ system. This allows the use of deep UV lasers for routine analysis in a laser class I system with no need for safety glasses and skin protection.

5.2. Multi-capillary system

Besides the wavelength resolved detection, CCD cameras offer the possibility of local resolved detection. Due to the promising results regarding the wavelength resolved detection, a multi-capillary system is going to be developed to use the local resolved detection feature of the CCD cameras. At present, multi-capillary systems are used for qualitative DNA analysis [20,21], e.g. Perkin-Elmer PE 3700 device. The purpose of our setup is to run multiple quantitative assays with one detector. Combining the different techniques CE offers a new dimension in increasing productivity by using parallel capillaries, compared to classical bioanalytical methods.

The use of 12 and 16 capillaries, respectively, offers the employment of standard microtiterplates with 96 (long side) and 384 wells (short side). First

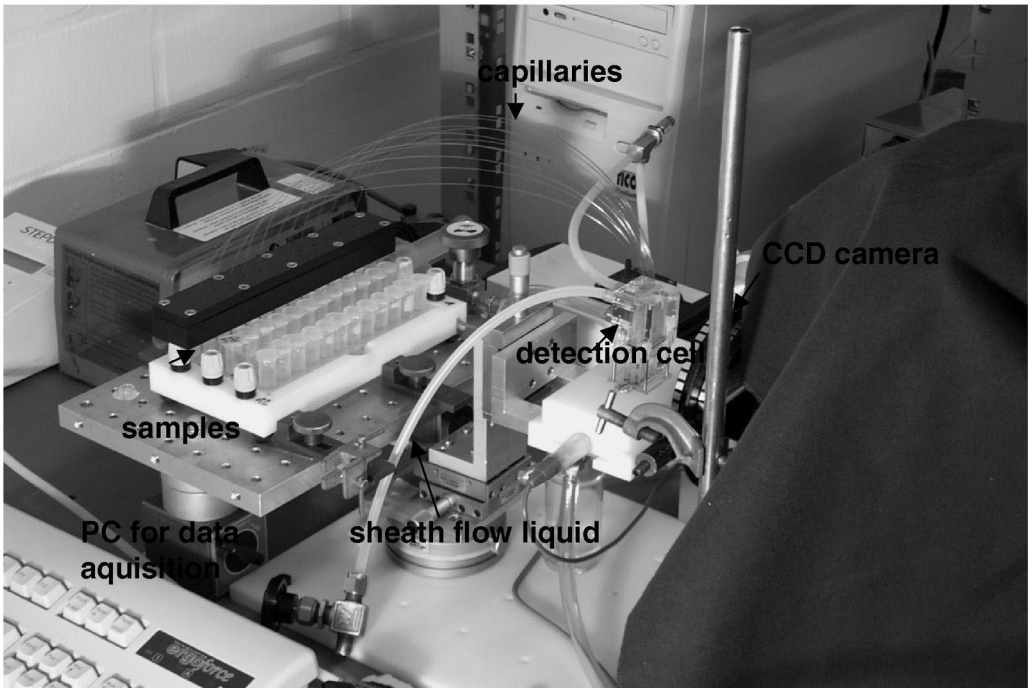

Fig. 6. Experimental setup for the multicapillary system. PC = Personal computer.

results using a system with 12 capillaries (Fig. 6) look quite promising while further experiments are ongoing.

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