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ORIGINAL ARTICLE

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HPLC with laser-induced native fluorescence detection for morphine and morphine glucuronides from blood after immunoaffinity extraction

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Abstract A new immunoaffinity solid phase extraction of morphine and its phase II metabolites, morphine-3-ß-D-glucuronide and morphine-6-ß-D-glucuronide is described. An immunoadsorber was applied which was created for the first time by the immobilisation of specific antibodies (polyclonal, host: rabbit) by the sol-gel method. The extraction method in combination with high performance liquid chromatography-fluorescence determination has been validated and shown to be applicable to blood samples of heroin victims in a low concentration range. Blood extracts were essentially free of interfering matrix components when compared to C_8-extracts. Additionally, a novel, sensitive and selective detection system for wavelength-resolved analysis of laser-induced fluorescence coupled to HPLC was developed. The analytes were excited with a frequency tripled Ti:Sa laser (λ=244 nm quasi cw). The total emission spectrum was recorded with a detection system consisting of an imaging spectrograph and a back-illuminated CCD camera. This technique of detection, combined with an extended optical path (at least 6 mm could be illuminated by the laser), resulted in an optimal fluorescence intensity of the analytes. The method permitted the analysis of morphine, morphine-3-ß-D-glucuronide and morphine-6-ß-D-glucuronide in a low concentration range and could be applied to a complex matrix such as postmortem blood samples because analyte peaks could be discriminated from matrix peaks by their characteristic emission spectra.

Keywords Morphine · Morphine-3-ß-D-glucuronide · Morphine-6-ß-D-glucuronide · Postmortem blood samples · Immunoaffinity solid phase extraction · Sol-gel · High performance liquid chromatography · Laser-induced fluorescence

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Introduction

Conjugation of morphine (M) is activated by uridine 5-diphosphoglucuronic acid (UDPGA) in the liver and intestines to the phase II metabolites morphine-3-ß-D-glucuronide (M3G) and morphine-6-ß-glucuronide (M6G). Glucuronidation via the phenolic hydroxyl group or via the secondary alcoholic group results in the main metabolite M3G and the minor metabolite M6G, respectively, with a M3G:M6G ratio of about 4–5:1 [1]. The major metabolite M3G has no analgesic activity [2, 3]. Previous studies have shown the analgesic potency of M6G to be 10–20 times greater than M [2, 4]. M6G was assumed to have an affinity to opiate receptors where an increased rate of interaction can occur [5, 6]. Recent studies suggest the existence of a specific M6G receptor [7, 8]. However, the ability of M6G to pass the blood-brain barrier has been questioned recently since a greater analgesic activity compared to M could not be observed when administered intravenously in humans [9–11].

In forensic practise the simultaneous quantification of M, M3G and M6G is important to elucidate the cause and manner of death. Methods using solid-phase extraction and HPLC with different detectors [12–14] have been described. Various capillary electrophoresis (CE) separation modes [15–19] have been performed for the analysis of opiates.

Particularly in the field of forensic science, the application of specific antibodies plays an important role for the identification of biological traces or for immunohistochemical detection [20, 21]. For purification and concentration of biological samples, immunoaffinity chromatography using immobilised antibodies is a simple and efficient technique. This technique has already been successfully applied for on-line or separate purification and extraction in several cases [22–24], e.g. for the extraction of estradiol [25], clenbuterol [26], amanitin [27], tetrahydrocannabinol [28, 29], benzodiazepines and their metabolites [30, 31], LSD [32, 33] and morphine and its metabolites [34, 35]. We developed a new immunoadsorber for the extraction of morphine and its glucuronides by the immobilisation of specific antibodies against morphine and morphine glucuronides [36] applying the sol-gel method. This new technique enables the free access of analytes to the antibody binding sites without covalent linkage of the antibodies to a matrix [37].

For the detection of M, M3G and M6G, we have developed a rapid HPLC separation which has been combined with a fluorescence detector for validation of the extraction method, and with a novel detection method using wavelength-resolved laser-induced fluorescence (LIF). In comparison with the capillary electrophoresis coupled to LIF detection, the new HPLC-LIF technique allows the injection of enhanced sample volumes, which results in enhanced fluorescence intensity. A frequency tripled Ti:Sa laser (λ=244 nm cw) was used for excitation and the total fluorescence spectra were recorded by a CCD camera. Processing the wavelength-resolved LIF data with custom-made algorithms adapted to the specific fluorescence spectra of M, M3G and M6G, has improved the analysis and resulted in optimal S/N ratios.

Materials and methods

Chemicals and experimental set-up

Morphine hydrochloride trihydrate was purchased from Merck (Darmstadt, Germany), morphine-6-ß-D-glucuronide dihydrate, morphine-3-ß-D-glucuronide, apomorphine hydrochloride from Sigma (Deisenhofen, Germany) and aqueous stock solutions of 1 mg/ml were made. Polyclonal antisera against M, M3G and M6G were made as described previously [36]. Acetonitrile was of HPLC grade and all other chemicals were of analytical grade. Phosphate-buffered saline pH 8.0 (PBS) contained 0.137 mol NaCl, 0.008 mol Na_2HPO_4 $2H_2O$, 0.001 mol KH_2PO_4, 0.003 mol KCl and 0.003 mol NaN_3/l H_2O. A mixture of methanol/H_2O (95:5 v/v) was applied for the elution of analytes from the affinity phase. For the solid phase extraction, Reserp cartridges, 100 mg/1 ml, 100 pk, purchased from Restek (Sulzbach, Germany) were used. The extraction buffer (pH 9.3) contained 0.01 mol/l $(NH_4)_2HCO_3$ and was adjusted to pH 9.3 with diluted ammonia. Elution was performed with ethanol.

For separation a 250×4 mm column, 5 µm particle size (RP select B, Merck, Darmstadt, Germany) with a 4×4 mm guard column (same type) was used.

Apparatus 1

A Hewlett-Packard Series II 1090 liquid chromatograph and a Hitachi Merck F-1050 fluorescence spectrometer with a mercury-xenon lamp and a 12 µl flow-cell were used (in the following termed "conventional HPLC-fluorescence detection"). The excitation wavelength was 235 nm and emission was recorded at 345 nm.

Apparatus 2

The arrangement of apparatus 2 is presented in Fig. 1. A HPLC pump (LKB HPLC-Pump 2248, Pharmacia) was interfaced to a UV-LIF detector which consisted of a frequency tripled Ti:Sa laser (Tsunami, Spectra Physics, Darmstadt, Germany) running at 720 nm, 80 MHz of 100 fs pulses creating a quasi-continuous wave (cw) light of 244 nm with 50 mW. The flow-cell (Semi-Micro B0631133, 100 µl sample volume, Perkin Elmer, Überlingen, Germany) was illuminated with a 3×1 mm laser beam along its 3 mm light path using a spherical lens (f=200 mm). Behind the flow-cell a mirror reflected the beam back on the same path, hence doubling the effective laser power. The fluorescence signal was collected perpendicular to the excitation light with two UV lenses (UV Nikkor, 105 mm, f# 4.5, Nikon, USA). In order to block scattered excitation light from the spectrograph, a UV long pass filter (>290 nm) was incorporated. The lenses formed a 1:1 image on a spectrograph (MS260i, LOT Oriel, Darmstadt, Germany) using a 400 l/mm grating blazed for 350 nm, resulting in a spectral resolution of 5 nm. The spectral range covered was 270–390 nm. The attached intensified CCD camera (Dyna Vision, LaVision Biotec, Bielefeld, Germany) was peltier-cooled and UV-enhanced (quantum efficiency >65%, 16 bit dynamic range) and was used with a read-out rate of 1 Hz and hardware binning factor of 1×64. The wavelength-resolved data were processed with custom algorithms using commercial software (Davis 6.2, LaVision, Goettingen, Germany). The custom algorithm gave the option to choose the spectral range of interest even after the measurement had been done.

The mobile phase consisted of 90% 10 mmol KH_2PO_4, 2 mmol 1-heptanesulfonic acid, adjusted to pH 2.5 with H_3PO_4 and 10% acetonitrile. The flow rate was 1.5 ml/min (isocratic mode), temperature was maintained at 20°C and the injection volume was 20 µl.

Preparation of the immunoadsorber

The antisera [36] were diluted with PBS pH 7.5, and purified with a 0.45 µm filter. The antibody fraction was isolated by adsorption to protein G and the anti-BSA

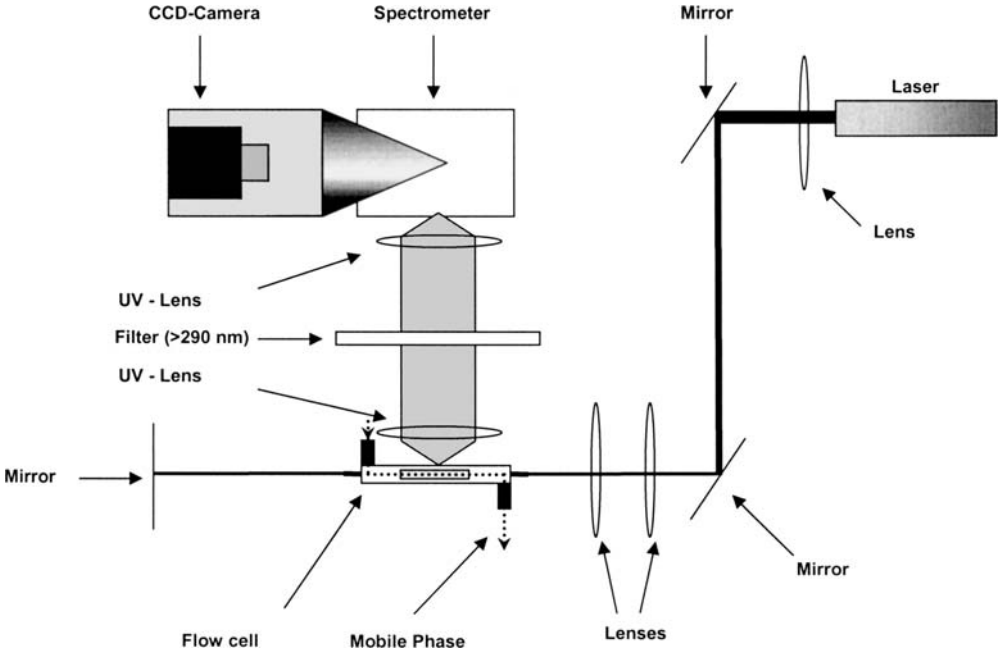

Fig. 1 Schematic description of the experimental set-up, apparatus 2

fraction was extracted by precipitation with BSA. Dialysis against PBS, pH 7 turned the cleaned-up antibody solutions into a definite ionic solution.

After the clean-up, the protein content was determined by the bicinchoninic acid method [38]. The concentration of IgG of the cleaned-up antisera was 5 mg/ml for anti-M, 4 mg/ml for anti-M3G and 3 mg/ml for anti-M6G.

Three different sol-gels, each loaded with specific antibodies, were prepared. Each silica sol was prepared by mixing 0.4 ml of 0.04 M aqueous hydrochloric acid, 1.5 ml of bi-distilled water and 6.8 ml tetramethoxysilane under stirring. The mixture was sonicated for 30 min. under ice-cooling. For each immunoadsorber, an aliquot of cleaned-up antiserum containing 8 mg IgG was suspended in ice-cooled PBS (pH 7). Equal aliquots of antisera and silica sol were mixed and gelation occurred within 2 min. The crystallising process was continued at 4°C until a weight loss of 50% was achieved. The silicate glass was

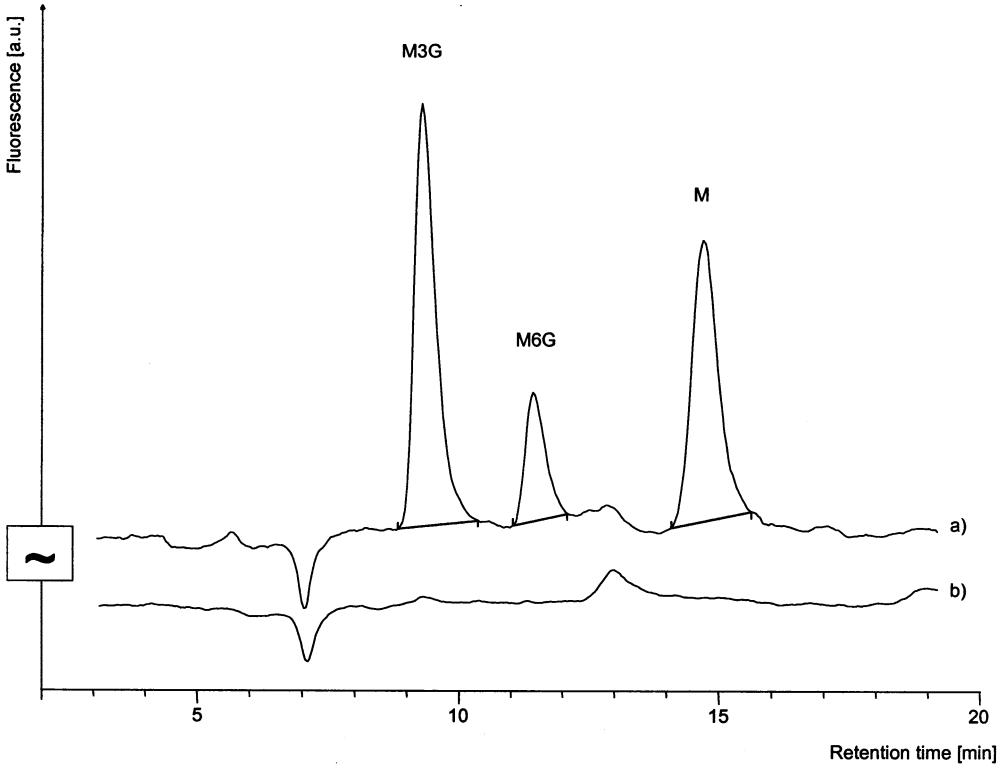

Fig. 2 Chromatogram of a sol-gel extracted spiked blood sample *a* M, M3G, M6G 250 ng/ml blood each and *b* chromatogram of a sol-gel extracted blank blood sample

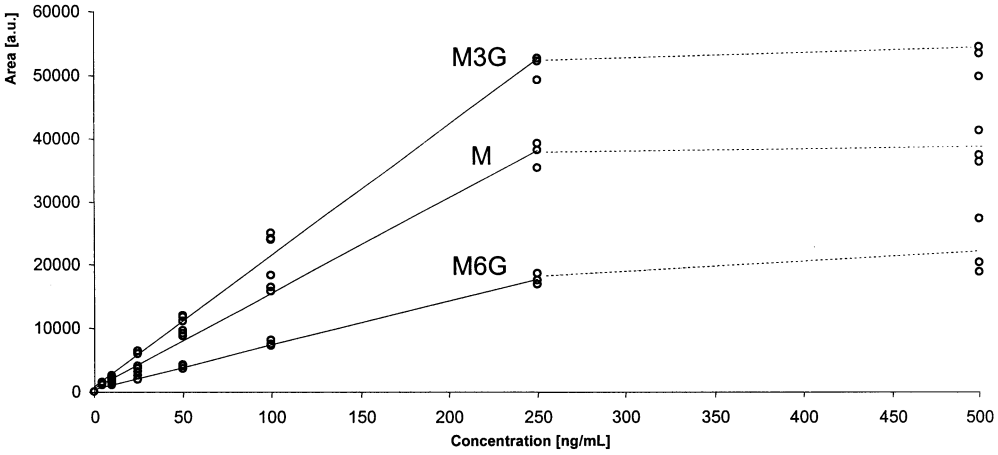

Fig. 3 Calibration curves: M, M3G und M6G, Sol-gel extracts from spiked blood samples. The *dashed line* marks the non-linear range

ground in a mortar, 120 mg of each sol-gel was packed into a 3 ml polypropylene column, washed with PBS (pH 7) and the columns were stored in PBS at 4°C.

Spiked blood calibrators

Pooled blank blood collected from healthy volunteers, was spiked with M, M3G, and M6G in a concentration range 1–500 ng/ml in order to evaluate specificity, recovery, precision and accuracy.

Extraction procedure

Sol-gel extraction Aliquots of 150 µl blood (spiked blood samples for the validation or blood sample from forensic cases) were added to 100 µl water and subsequently 100 µl methanol was added. After 10 min sonication, addition of 1 ml PBS, pH 8.0 and centrifugation (10 min, 8000 *g*), the supernatant was transferred to a 360 mg aliquot of PBS pre-conditioned sol-gel and incubated for 30 min under gently shaking at room temperature. The charged sol-gel was washed with 4×1ml PBS pH 8.0, 1×0.5 ml H_2O and 1×0.5 ml acetone/H_2O (95:5 v/v).

Elution was performed with 6×1 ml of methanol/H_2O 95:5 (v/v) under gently shaking for 20 min at room temperature. After evaporation to dryness (50°C/N_2) the extract was reconstituted with 100 µl HPLC mobile phase.

For the determination of the capacity of the immunoadsorber the pre-conditioned columns were overloaded separately each with 500 ng M, M3G and M6G in PBS. After washing the columns, elution was performed and the capacity was determined by HPLC (apparatus 1).

Solid phase extraction Aliquots of 500µl blood (spiked blood, concentration of morphine 250 ng/ml) were added to 2 ml of buffer solution (pH 9.3). After 10 min. of gentle shaking and centrifugation (10 min, 8000 *g*) the supernatant was transferred to the methanol-activated and buffer-conditioned C_8-cartridges. After passing of the sample and washing with buffer, the solid phases were dried, elution was performed and the capacity was determined by HPLC (apparatus 2).

Results and discussion

Validation of sol-gel extraction combined with conventional HPLC fluorescence detection

The specificity was determined by a comparison of the chromatograms of blank blood with a chromatogram of a blood sample spiked with M, M3G and M6G (Fig. 2). No interference of M, M3G and M6G peaks at the corresponding retention times could be observed. Due to the high specificity of the immobilised antibodies which was demonstrated in former studies [35, 36], no interference

Table 1 Interassay and intra-assay precision and accuracy for the determination of morphine, M3G and M6G from blood samples

Assay	Concentration (ng/ml blood)			Precision (CV %)		Accuracy (%)	
	Added	Range	Mean value	Intra-assay	Interassay	Intra-assay	Interassay
M	25	25.08–27.43	26.42	6.5–9.3	3.4	103.2–109.7	105.6
	100	99.00–101.03	100.05	2.7–6.6	1.0	99.0–101.0	100.0
	250	251.37–255.23	253.82	3.1–4.6	0.8	100.5–102.1	101.5
M3G	25	24.58–26.20	25.26	1.4–8.0	3.3	98.3–104.8	101.0
	100	95.13–99.83	97.56	5.1–8.5	2.4	95.1–99.8	97.6
	250	250.57–254.67	252.16	1.4–9.5	0.9	105.5–101.9	100.9
M6G	25	25.17–26.03	25.72	4.0–6.9	1.9	100.7–104.1	102.9
	100	94.97–100.70	98.43	1.2–4.9	3.1	95.0–100.7	98.4
	250	246.23–260.13	252.44	2.3–5.0	2.8	98.5–104.1	101.0

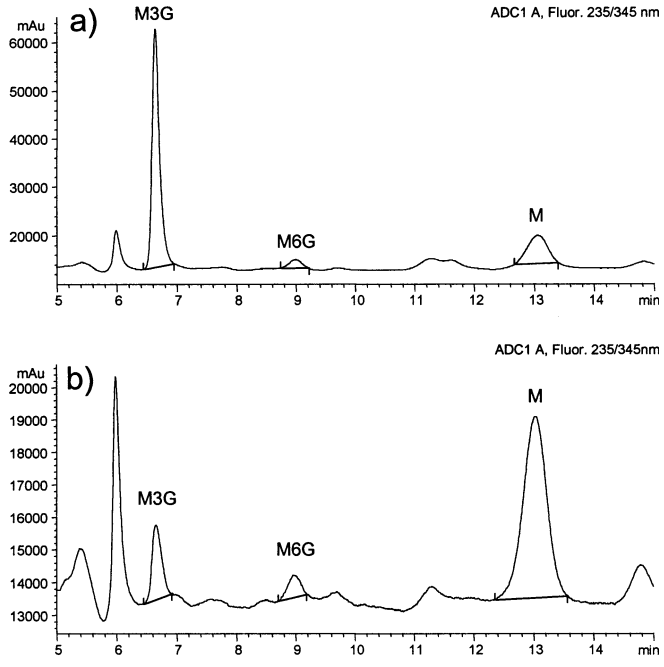

Fig. 4 Chromatograms of sol-gel extracts of blood samples from two heroin victims **a** M: 646 ng/ml, M3G: 248 ng/ml, M6G: 32 ng/ml blood, **b** M: 72 ng/ml, M3G: 304 ng/ml, M6G: 57 ng/ml blood. The differences in the peak heights of the two chromatograms are due to different dilutions of the blood samples which were necessary because the analyte concentrations exceeded the column capacity

could be observed in the case of one analyte at a very high concentration.

The capacity of the columns was determined by overloading the immunoadsorber with the analytes. In the concentration range above 300 ng of each analyte, a considerable decrease of the recovery could be observed. For this reason the column capacity was limited to 250 ng for each analyte. In cases of higher concentrations (e.g. see Table 2, victims 3, 4 and 5) the sample amount had to be reduced by diluting the sample.

For recovery experiments, blood samples were spiked in duplicate at 6 concentration levels (5–250 ng/ml blood), extracted and analysed. The recovery was calculated from the ratio of the slope of the regression line obtained with aqueous standard solutions without extraction procedure and spiked blood samples after the extraction. The recovery was 81, 84 and 79% for M, M3G and M6G, respectively.

Calibration curves were recorded with spiked and extracted blood samples at 6 concentration levels (0, 5, 10, 25, 50, 100, 250 ng/ml blood). Each point of calibration was based on data from three separate runs. A linear correlation was found with correlation coefficients higher than 0.993 for each analyte. Due to the column capacity of 300 ng of each analyte, linearity could not be obtained when overloading the columns with spiked blood samples of higher concentrations (see Fig. 3). Due to this fact, samples have been diluted if the concentration of one of the analytes was higher than 250 ng/ml.

In Table 1 the results of precision and accuracy studies are listed. The results meet the requirements for the validation of bioanalytical methods [39] although an internal standard could not be used. Various structurally related substances i.e. apomorphine, hydromorphone and N-(3-aminopropyl)-normorphine-3-β-D-glucuronide [36] were tested for their usage as internal standard. Probably due to the high specificity of the antisera used for the preparation of the immunoadsorber, these substances could not be extracted with sufficient recovery efficiency.

For the determination of the limit of detection (LOD), drug-free blood samples (n=6) were spiked in the range of 1–25 ng/ml of the analytes. The evaluation of the signal-to-noise ratios (S:N) resulted in the LOD of 5 ng/ml for M and 10 ng/ml for M3G and M6G with S:N >5 for each analyte. The limit of quantitation (LOQ) was defined as the lowest concentration included in the calibration series with both intra-assay and interassay precision within a 20% CV limit [39] and was 25 ng/ml blood for M, M3G and M6G.

Forensic application

The sol-gel extraction, combined with conventional HPLC-fluorescence detection was applied for the simultaneous determination of M, M3G and M6G in routine blood

Table 2 Determination of M and its metabolites in blood samples from heroin victims and from heroin consumers

Case No.	Concentration HPLC-fluorescence				Concentration GC/MS	
	M (ng/ml)	M3G (ng/ml)	M6G (ng/ml)	ΣBound M1 (ng/ml)	Free M (ng/ml)	Bound M2 (ng/ml)
Consumer 1	94	141	72	131	108	n.a.
Consumer 2	97	42	n.d.	26	84	n.a.
Consumer 3	153	n.d.	n.d.	-	131	n.a.
Consumer 4	27	70	n.d.	43	35	n.a.
Consumer 5	89	45	26	44	72	n.a.
Victim 1	121	248	n.d.	153	161	123
Victim 2	169	186	25	130	188	152
Victim 3	413	397	132	327	412	308
Victim 4	646	248	32	173	608	164
Victim 5	72	304	57	223	81	288

M1 Sum of morphine glucuronides calculated as M.
M2 Difference of total morphine after conjugate cleavage and free M.
n.d. Not detectable.
n.a. Not analysed.

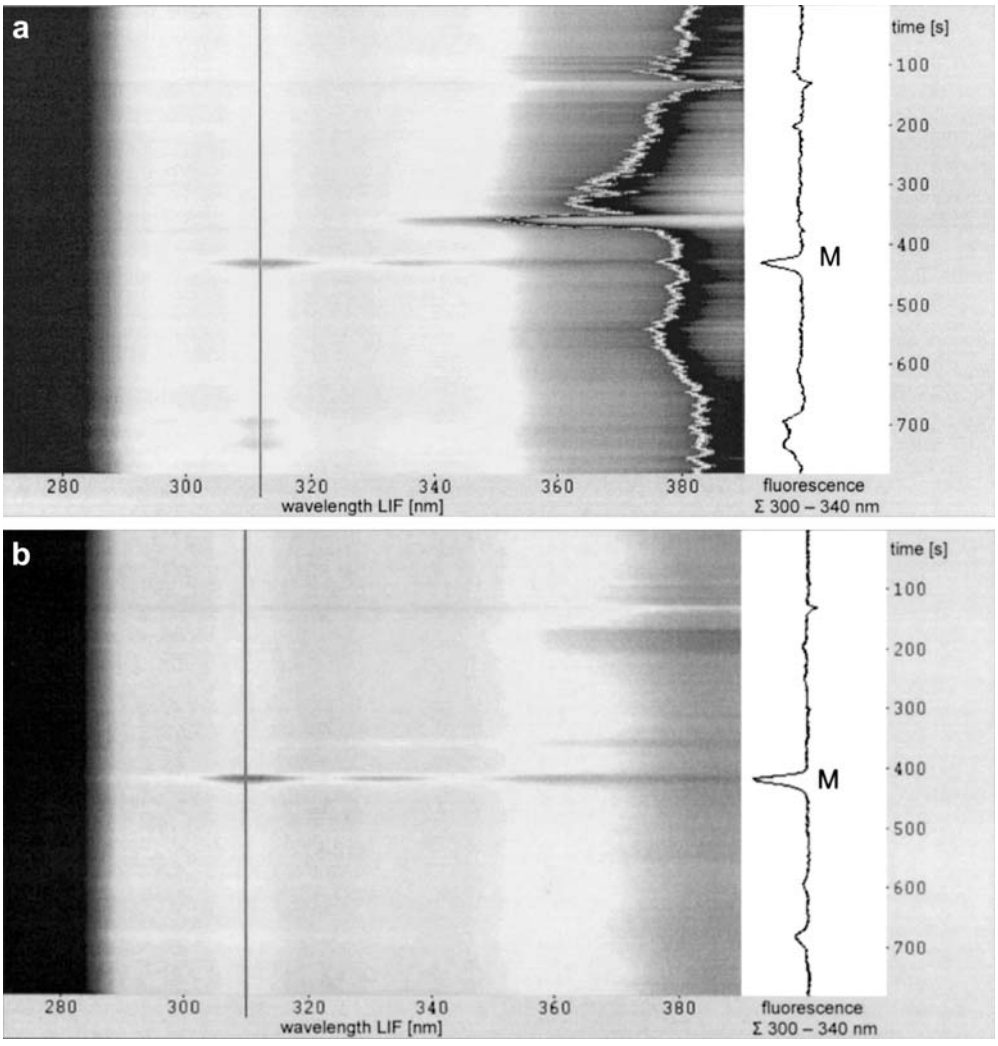

Fig. 5 Chromatogram (*right part*) and spectrum (*left part*) of a C_8-extract **a** and a sol-gel extract **b** of a spiked blood sample. Concentration: 250 ng morphine/ml blood

samples from heroin victims and heroin consumers. In forensic casework information on the ratio of morphine and morphine metabolites can give indications of the time, elapsed since the last intake of heroin. As M6G is an active metabolite, a differentiation between M3G and M6G is necessary to estimate the heroin action. Typical chromatograms of heroin victims are shown in Fig. 4. The differences in the peak heights of the two chromatograms are due to different dilutions of the blood samples which were necessary because the analyte concentrations exceeded the column capacity. The concentrations of M, M3G and M6G were compared with the findings of a confirmation GC/MS method [40] and the results summarised in Table 2 show a close correlation of both methods.

Sol-gel extraction, combined with HPLC-LIF

Sol-gel extracts from spiked blood were also analysed by the HPLC-LIF method. With this method, the whole fluorescence spectrum of a total run is detected and qualitative and quantitative detection can be performed simultaneously. The option to choose the spectral range of interest adapted to the native fluorescence of the analytes and the matrix, leads to an increased specificity and sensitivity (LOD <5 ng/ml blood for M, M3G and M6G). Chromatograms of sol-gel extracts from spiked blood samples and blood samples from heroin victims did not show any matrix fluorescence. However, the C_8-solid phase extracts of spiked blood samples showed a very intensive matrix fluorescence. An example for comparable fluorescence spectra is presented in Fig. 5.

Table 3 Determination of M, M3G and M6G in spiked blood samples, comparison of HPLC conventional fluorescence and HPLC-LIF

Concentration (ng/ml)	HPLC-fluorescence (ng/ml)	HPLC-LIF (ng/ml)
M 50	48	47
M 100	99	102
M3G 50	55	53
M3G 100	110	113
M6G 50	43	58
M6G 100	90	120

In order to compare both detection systems, sol-gel extracts from spiked blood samples in 2 concentrations (50 and 100 ng/ml blood) were analysed. The results (Table 3) show an acceptable conformance of both techniques.

Sensitivity and selectivity could be enhanced by the spectrally resolved HPLC-LIF method. This innovative detection enables isolated ranges of a full emission spectra to be analysed and the resulting optimisation of the S/N ratio for each analyte due to the current matrix. This manner of detection also represents a method for simultaneous identification and determination of the naturally fluorescent analytes. Especially for the development of new methods, discrimination between matrix and analytes is helpful.

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