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Journal of Chromatography B, 772 (2002) 11–18

JOURNAL OF
CHROMATOGRAPHY B

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Advanced fibre optical scanning in thin-layer chromatography for drug identification

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Received 7 June 2001; received in revised form 13 November 2001; accepted 13 December 2001

Abstract

A systematic toxicological analysis procedure using high-performance thin layer chromatography in combination with fibre optical scanning densitometry for identification of drugs in biological samples is presented. Two examples illustrate the practicability of the technique. First, the identification of a multiple intake of analgesics: codeine, propyphenazone, tramadol, flupirtine and lidocaine, and second, the detection of the sedative diphenhydramine. In both cases, authentic urine specimens were used. The identifications were carried out by an automatic measurement and computer-based comparison of in situ UV spectra with data from a compiled library of reference spectra using the cross-correlation function. The technique allowed a parallel recording of chromatograms and in situ UV spectra in the range of 197–612 nm. Unlike the conventional densitometry, a dependency of UV spectra by concentration of substance in a range of 250–1000 ng/spot was not observed.
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Keywords: Drug identification; Fibre optical scanning

1. Introduction

Planar chromatography is a cheap and simple separation technique which works well for the analysis of large sample numbers. High-performance thin layer chromatography (HPTLC) is the method of choice for complex and dirty samples when impurities remain absorbed at the stationary phase. HPTLC is well suited for the separation of drugs over a large polarity range. Therefore, planar chromatography is an important separation technique in the field of forensic blood and urine analysis. While the use of thin layer chromatography in combination with colour reactions to visualise chromatographic spots is wide spread [1–5], only some papers describe HPTLC drug identification using in situ UV spectra [6–8].

In analytical toxicology usually it is not known whether a poison was taken from a person showing symptoms of a specific intoxication, or when a poison was taken, it is also often not known which one was used. Therefore, screening procedures must be established which are able to identify a great number of substances as accurately and as quickly as possible [9]. Urine is a good biosample for sys-

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PII: S1570-0232(01)00617-1

tematic toxicological analysis because the concentrations of drugs or other poisons are normally high in urine. The purpose of our work is to demonstrate an application of fibre optical scanning densitometry for identification of drugs in urine in clinical and forensic toxicology.

2. Experimental

2.1. Equipment

An automatic thin layer sampler TLS 100 (Baron, Insel Reichenau, Germany) was used for application of samples onto the HPTLC plate.

For direct spectrophotometry of HPTLC plates a device developed by Fachhochschule Offenburg and Fachhochschule Giessen-Friedberg, Germany was designed. It consists of a diode array spectrophotometer (J&M, Aalen, Germany) working in a range of 198–612 nm with an average optical resolution of 0.8 nm. A home-made reflection attachment of 50 identical optical fibres with a diameter of 100 μm transports light of different wavelength from a deuterium lamp to the HPTLC plate and back to the diode array detector. In order to get high intensities of light, the light emitting and light detecting fibres were arranged in two lines of 25 fibres side by side. In this arrangement a nearly parallel light beam results. The HPTLC plate is placed horizontally on a mechanical stage which can be moved by use of two motors from Micropack Stuttgart, Germany. The whole device needs no lenses, filters or slit-width adjustment [10].

2.2. Materials and chemicals

HPTLC plates (silica K60 F254) and all chemicals (p.a. quality) were purchased from Merck, Darmstadt, Germany. Amitriptyline, carbamazepine, clozapine, codeine, diphenhydramine, morphine, and paracetamol were purchased from Sigma, Deisenhofen, Germany. Flupirtine, lidocaine and propyphenazone were obtained from Apotheke des Klinikums der Friedrich-Schiller-Universität, Jena, Germany.

Doxepine was donated by Pfizer. Trimipramine was obtained from Arzneimittelwerk Dresden, Dresden, Germany.

A methanolic extract of tablets of opipramol (Insidon®, 50 mg opipramol·2HCl per tablet; Novartis Pharma, Nürnberg, Germany), and venlafaxine (Trevilor®, 75 mg venlafaxine per tablet; Wyeth-Pharma, Münster, Germany) were additionally used.

Tramadol was prepared as solution from Tramal®-Tropfen, (100 mg/ml tramadol·HCl; Grünenthal, Stolberg, Germany) in methanol.

2.3. Sample preparation

In analytical toxicology, the substances that have to be analysed for example pharmaceuticals, drugs of abuse, pesticides, etc., are frequently unknown before analysis. The main problem is the diagnosis or the definite exclusion of an intoxication. Therefore, a two-step liquid extraction procedure of urine was performed to differentiate between acid and basic components. A liquid–liquid extraction enabled an extraction of substances with very different chemical properties especially when the substances have to be isolated from a heterogeneous matrix [9].

2.3.1. Acid urine extract

A 1-ml aliquot of urine was mixed with 200 μl sulphuric acid (0.25 mol/l) and extracted with 3 ml diethylether. After centrifugation, the organic layer was separated and evaporated to dryness by means of a stream of argon at 40 °C. The residue was reconstituted in 50 μl methanol–ethyl acetate (1:1, v/v).

2.3.2. Basic urine extract

The remaining aqueous phase of the acid extraction procedure was alkalised with 0.2 ml sodium hydroxide (0.5 mol/l) and adjusted to pH 9 by addition of sodium hydrogen carbonate; 3 ml ethyl acetate was used for extraction. After centrifugation, the organic layer was separated and evaporated to dryness under a stream of argon at 40 °C. The residue was reconstituted in 50 μl methanol–ethyl acetate (1:1, v/v).

2.4. Application and chromatography

The application of the acid and basic urine extracts was made by an automatic thin layer sampler TLS

100 (Lothar Baron Laborgeräte, Insel Reichenau, Germany) according to the following scheme:

- Start position x: 10 mm
- Start position y: 10 mm
- Spot width: 4 mm
- Gap: 6 mm
- Trace distance: 10 mm

A 5- and 15-μl aliquot of each urine extract were applied on a 10×10 cm HPTLC plate, prewashed with mobile phase. The HPTLC plate was developed in a saturated developing chamber with ethyl acetate–methanol–ammonia (85:10:5, v/v/v) as mobile phase. The separation distance was 50 mm.

For *Rf* correction on one track a set of four reference substances was additionally placed on each plate (morphine, codeine, caffeine, and trimipramine). The concentration of these reference substances were 500 ng/spot.

For the scan of the whole track 450 spectra in a range of 198–610 nm were measured over a distance of 45 mm. Each spectrum was measured within 0.5 s.

2.5. Reference spectra and identification

For identification 500 ng/spot of each drug was applied on a HPTLC plate and developed under the same chromatographic conditions as the urine specimen. The resulting spectra were stored as reference spectra.

Opipramol and venlafaxine solution was prepared by powdering one tablet of Insidon® and Trevilor®, respectively, by a glass pestle in a glass vial and digested with the corresponding volume of methanol to get a concentration of 0.1 mg/ml. The solution of tramadol was made by adding the correspondent volume of Tramal® in an adequate volume of methanol to get a concentration of 0.1 mg/ml.

For identification of unknown substances a computer based comparison of in situ UV spectra with library entries was made and the cross-correlation function between the unknown spectrum and the reference spectra of the library was calculated [11]. Additionally the corrected *Rf* values were determined.

3. Results

For application in systematic toxicological analysis, urine of 15 authentic cases was analysed.

To illustrate the efficiency of the system two chromatograms of patient urine samples are presented as an example. A HPTLC separation over a distance of 45 mm of the basic extract of an urine sample is shown in Fig. 1. Each data point consists of distance, wavelength and intensity. The simultaneous detection of in situ UV spectra during the scanning of the track allows the parallel depiction of densitograms at different wavelengths. The contour-plot in the centre of Fig. 1 shows at the abscissa the distance of a signal from the starting point of measure. The wavelengths (range from 200 to 400 nm) are plotted as ordinate while the intensity is expressed as grey-shade index in the area between abscissa and ordinate. On the left, the in situ UV spectrum at 35.4 mm is plotted which is marked by the vertical black cursor line in the contour plot. The densitogram at 204.3 nm is shown at the top of Fig. 1 which is marked by the black horizontal cursor line in the contour plot.

At 4.9 mm, we see the point of application and at 44 mm we can see the front of the mobile phase. We can distinguish several peaks.

In this chromatogram, four analgesics could be identified: codeine at 13.6 mm (1), propyphenazone (identified by its metabolite) at 17.4 mm (2), tramadol at 30.8 mm (3), and flupirtine at 33.9 mm (4). Additionally the local analgesic lidocaine at 35.4 mm (5) was detected in this specimen. The unknown spectrum of the peak 4 at 33.9 mm in Fig. 1 is compared with the library spectrum of flupirtine in Fig. 2. The best cross-correlation fit value of 95.52% was observed for flupirtine.

The second example illustrated the identification of the sedative diphenhydramine in another urine specimen (Fig. 3). The unknown spectrum of the peak 1 at 26.7 mm in Fig. 3 is compared with the library spectrum of diphenhydramine in Fig. 4. The best cross-correlation fit value [11] of 95.09% was observed for diphenhydramine. In both cases, no drugs were found in the acid urine extract.

The identifications were achieved by comparing the unknown spectra with library spectra using the cross-correlation function [11] in combination with

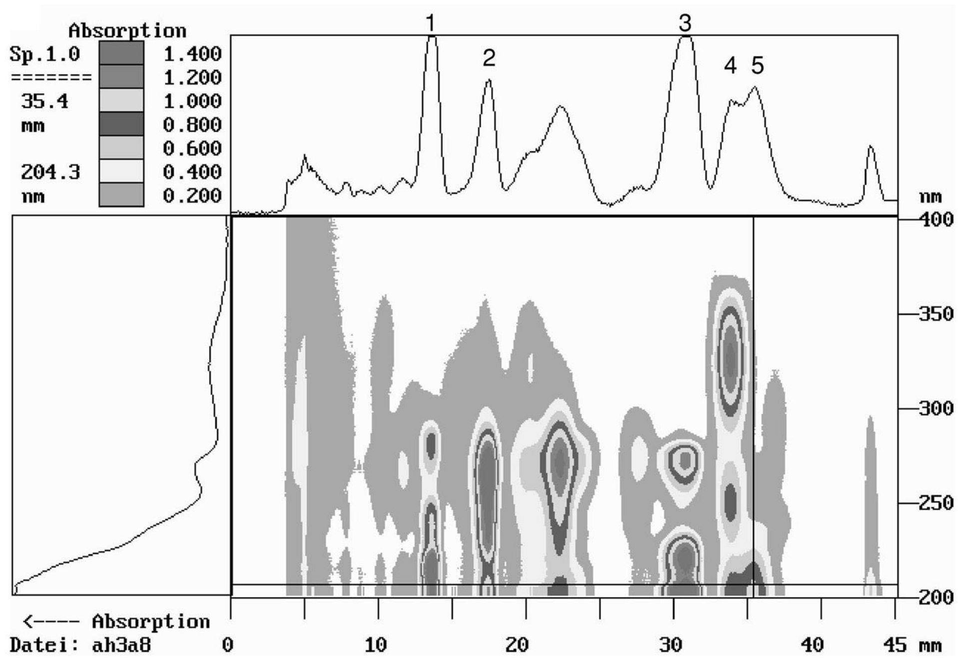

Fig. 1. Contour plot of an urine extract with codeine (1), popyphenazone metabolite (2), tramadol (3), flupirtine (4) and lidocaine (5). The chart shows the densitogram at 204.3 nm at the top. The in situ UV spectrum of lidocaine at 35.4 mm is plotted at the left.

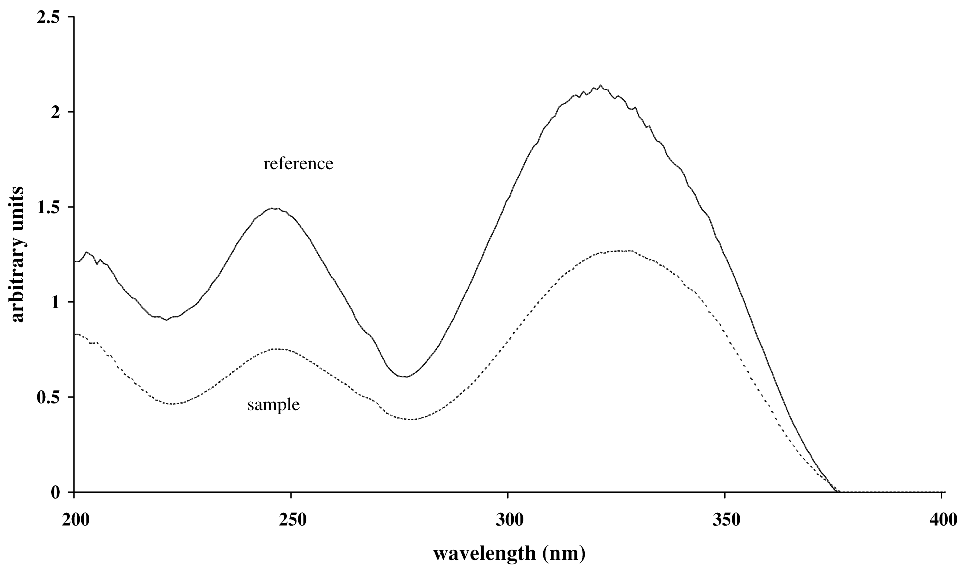

Fig. 2. Comparison of the spectrum of peak 4 at 33.9 nm taken from Fig. 1 and library spectrum of flupirtine (- - - sample; — library spectrum). Values of intensity of the ordinate are in arbitrary units.

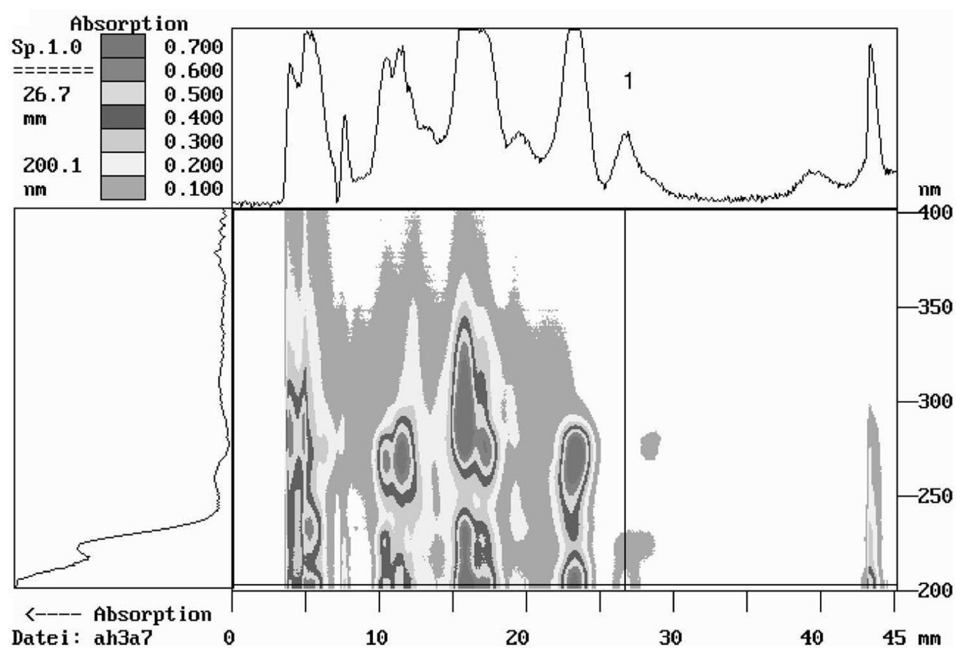

Fig. 3. Contour plot of an urine extract containing diphenhydramine (1). The chart shows the densitogram at 200.1 nm at the top. The in situ UV spectrum of diphenhydramine at 26.7 mm is plotted at the left.

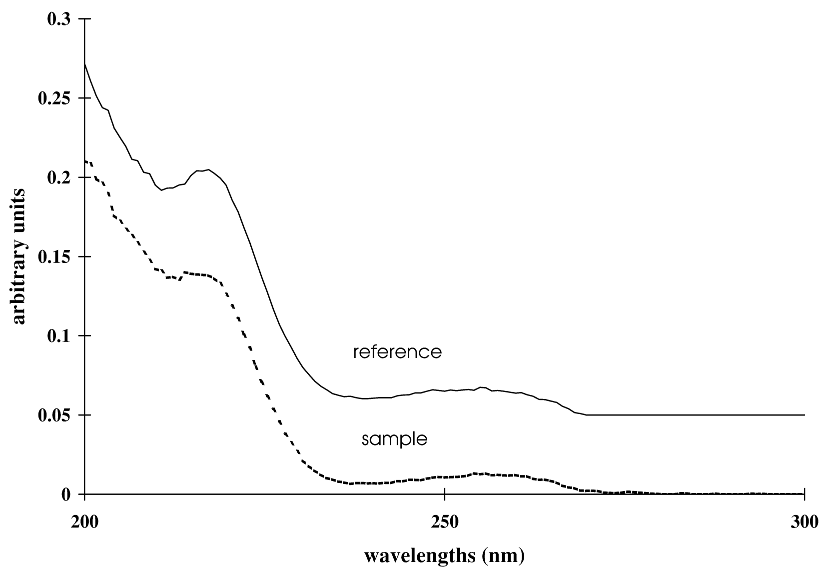

Fig. 4. Comparison of spectrum at 26.7 mm taken from Fig. 3 and library spectrum of diphenhydramine (- - - sample; — library spectrum). Values of intensity of the ordinate are in arbitrary units.

the corrected *Rf* values according to Ref. [5]. The cross-correlation function leads to fit values between zero and a 100% percent of correspondence. In Table 1, the fit values calculated by the cross-correlation function which provided identification of the detected substances in the 15 urine samples including their corrected *hRf* values are listed.

4. Discussion

The innovation of the presented HPTLC analysis is the combination of a diode array detector, a new light fibre arrangement, stable plate illumination and accurate formation of a very small scanning slit. Spatial resolutions on the TLC plate of better than 160 μm are possible. It takes less than 4 min to scan 450 spectra simultaneously in a range of 198–610 nm. The basic improvement of the technique is the use of highly transparent glass fibres which provide excellent transmission at 200 nm and the use of a special fibre arrangement [10]. All these aspects show a substantial improvement of in situ densitometric analysis in drug screening. Also it improves the search in spectra libraries for drug identification in forensic samples.

In situ UV–VIS spectra can be registered continuously across the whole developing distance and a peak purity test can be easily achieved. Therefore there is neither a need for multiple scanning at different wavelengths nor time consuming manual input from peak positions of interest to record in situ UV spectra.

Ebel and Kang [12] reported that some substances showed concentration dependent shifts of absorbance maxima. Demme et al. [8] shows that the identification of substances by in situ UV spectra depends on their concentration. In our experiments we did not observed any dependence of in situ UV spectra at concentrations of 250–1000 ng/spot of the tested substances listed in Table 1. In Fig. 5, in situ UV spectra of flupirtine at 250, 500, 750, and 1000 ng per spot are shown. If we compare the 1000 ng spectrum with the other spectra of flupirtine we observed fit values of 99.95% (750 ng), 99.72% (500 ng), and 99.23% (250 ng) using the cross-correlation function, that means nearly complete agreement.

We demonstrate an application of fibre optical scanning densitometry for identification of drugs in urine in clinical and forensic toxicology. A simple and effective procedure leads to identification of substances which are able to produce toxic symptoms in man.

An abuse of analgesics suspected by the physicians could be proven in the first example. All findings could be confirmed by GC–MS analysis.

Table 1
Drugs found in 15 urine samples with corrected *hRf* values (*hRfc*) and theoretical values according to Ref. [5] including category and fit value in percent of library match by cross-correlation

Substance name	Category	*hRfc*[a]	*hRfc* found	Fit [%] by cross-correlation
Amitriptyline	Antidepressant	69	69	90.01
Carbamazepine	Anticonvulsant	56	56	91.75
Clozapine	Neuroleptic	55	55	96.45
Codeine	Analgesic, potent antitussive	35	35	99.13
Diphenhydramine	Sedative, antihistamine	65	65	95.09
Doxepin	Antidepressant	63	63	99.19
Flupirtine	Analgesic	–	73	95.52
Lidocaine	Local anesthetic, antiarrhythmic	80	80	95.41
Morphine	Potent analgesic	20	20	99.29
Opipramol	Antidepressant	38	38	89.14
Paracetamol	Analgesic	45	45	99.15
Propyphenazone metabolite	Metabolite of analgesic	–	41	98.26
Tramadol	Potent analgesic	78	71	99.97
Trimipramine	Antidepressant	80	80	90.87
Venlafaxine	Antidepressant	–	70	95.99

[a] Corrected *hRfc* values of flupirtine, propyphenazone metabolite and venlafaxine are not reported in Ref. [5].

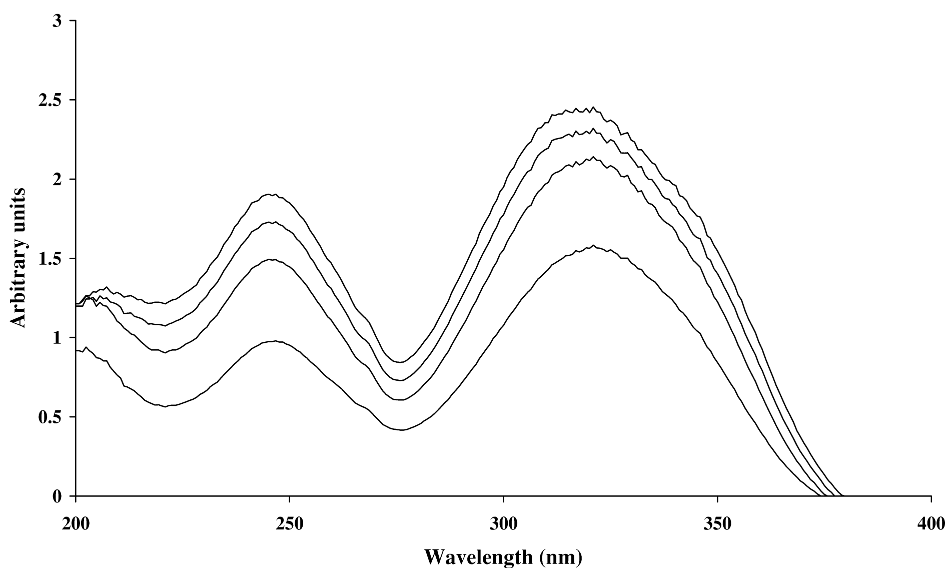

Fig. 5. In situ UV spectra of flupirtine at 250, 500, 750, and 1000 ng per spot.

The concentrations in serum of first example determined by GC–MS were: codeine 0.2 mg/l propyphenazone 10 mg/l, tramadol 0.3 mg/l, and flupirtine 9 mg/l. Lidocaine was not quantified in serum. The intake of different analgesics simultaneously and the high concentration of flupirtine in serum of 9 mg/l (therapeutic range 0.5–1.5 mg/l [14]) confirmed the medical anamnesis. Lidocaine, which was additionally identified in the urine specimen had been administered during catheterization as local anaesthetic.

In spite of partial coelution of the peaks 4 and 5 in Fig. 1, an identification could be performed. Peak 4 could be identified as the analgesic flupirtine and peak 5 could be identified as the local anesthesic lidocaine. The contour plot in Fig. 1 shows characteristic differences of both spectra at 33.9 and 35.4 nm. Especially the identification of lidocaine in conventional densitometric analysis is difficult because of its non-characteristic in situ UV spectrum and its maximum of absorption at 204.3 nm. Lidocaine could be identified by its in situ UV spectrum. Up to now the UV range below 300 nm was excluded for fibre optic densitometry [13].

The second example (Figs. 3 and 4) demonstrates the identification of diphenhydramine in another urine specimen. The case history of an intake in suicidal intention of the sedative diphenhydramine 2 days before hospitalisation could be verified. Like lidocaine, diphenhydramine showed an UV maximum at 200.1 nm. Similar to lidocaine, the spectrum is not very characteristic with the exception of the clearly visible shoulder at 222.7 nm.

A fit of 100% for identification of unknown substances via cross-correlation function means full agreement of sample and reference. Fit values of above 90% were observed when the unknown compound was identified. Confirmatory visualisation of sample and reference spectra as shown in Fig. 3 increases the certainty of identification.

The in situ UV spectra library needed only one entry per substance in contrast to conventional densitometry because a dependence on concentration in a range of 250–1000 ng/spot was not observed for the substances listed in Table 1.

New glass fibre optics enable the detection of substances with maximum of UV absorption especially at short wavelengths. Therefore, a wide range of toxicologically relevant drugs are detectable by the method.

The examples clearly underline that in situ UV spectrometry in combination with a glass fibre optical photo diode array detector is a new tool for the selective and sensitive identification of pharma-

ceuticals for systematic toxicological analysis purposes.

Acknowledgements

The authors express their appreciation to Merck (Darmstadt, Germany), Camag (Muttenz, Switzerland), J&M (Aalen, Germany), Polymicro Technologies (Phoenix, AR, USA) for technical assistance and helpful discussions.

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