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Analysis of urinary drugs of abuse by a multianalyte capillary electrophoretic immunoassay

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Abstract

This paper characterizes a novel multianalyte competitive binding, electrokinetic capillary-based immunoassay for urinary methadone, opiates, benzoylecgonine (cocaine metabolite) and amphetamines. After incubation of 25 μl urine with the reactants for several minutes in the presence of an internal standard, a small aliquot of the mixture is applied onto a fused-silica capillary and the unbound fluorescein labelled drug tracers are monitored by capillary electrophoresis with on-column laser induced fluorescence detection. The multianalyte assay is shown to be rapid, simple, quantitative, capable of recognizing urinary drug concentrations ≥30 ng/ml and suitable for screening of patient urines. Data are demonstrated to compare well with those obtained by routine screening methods based on enzyme multiplied immunoassay techniques and fluorescence polarization immunoassays. The electrokinetic capillary assay has been validated via analysis of external quality control urines and confirmation analysis of patient urines using GC–MS.

Keywords: Immunoassay; Validation; Methadone; Opiates; Benzoylecgonine; Amphetamines

1. Introduction

For the determination of drugs of abuse in body fluids, many instrumental techniques based upon immunological and chromatographic principles have been developed [1–5]. Most immunoassays are competitive binding assays in which drug and labelled drug molecules competitively interact with the binding sites of an antibody raised against a drug or a metabolite. Immunological techniques are very attractive because of their ease of performance and speed of analysis. Thus, they are typically employed for screening purposes in settings that have to handle a large number of samples, including those associated with clinical toxicology, surveillance of drug substitution programs, forensic science, drug testing in the workplace and doping control, to name but a few. On the other hand, immunoassays often lack specificity and sometimes sensitivity and are thus inappropriate for confirmation of the presence of a specific drug or metabolite. Chromatographic methods, particularly gas chromatography–mass spectrometry (GC–MS) [4] and high-performance liquid chromatography (HPLC) with multiwavelength solute detection [5], are typically employed for confirmatory testing. Alternatively, in the past decade, instrumentation for electrokinetic separations in fused-silica capillaries of very small I.D. (25–75 μm) has become available and has been found suitable for drug monitoring in body fluids, including plasma, serum, saliva and urine, hair and other tissues [6–10]. A comprehensive concept for tox-

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icological drug screening and confirmation by micellar electrokinetic capillary chromatography (MECC) [11–15] and capillary zone electrophoresis (CZE) [15–17] has been developed and successfully applied to the monitoring of urinary drugs of abuse. In that approach, confirmation testing is based upon on-column, fast scanning UV absorption detection of the solutes and comparison of extracted normalized spectra with those of standards. Furthermore, the presence of urinary methadone and its major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) has also been confirmed using CZE coupled to MS [18].

The combination of immunochemistry and capillary electrophoresis (CE) has recently attracted considerable attention. A number of competitive binding drug assays using labelled drugs as fluorescent tracers emerged. These procedures have in common that small amounts of body fluid (20–50 μl), antibody solution and tracer are incubated prior to application of a tiny aliquot of the mixture (nl to pl volume) onto the fused-silica capillary and separation of the unbound fluorescent tracer and antibody–tracer complex by CZE or MECC with on-column laser induced fluorescence (LIF) detection. Chen and co-workers reported the feasibilities for immunological determination of digoxin in serum [19], morphine and phencyclidine (PCP) in urine [20] and urinary morphine, PCP, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC) and benzoylecgonine [21] by CZE using drug-cyanine conjugates as tracers and LIF detection with a He–Ne laser (excitation: 543 or 633 nm; emission: 590 or 690 nm). Other approaches described employ fluorescein labelled tracers and LIF detection with an air cooled Ar ion laser (excitation: 488 nm; emission: 520 nm). Thormann et al. reported the monitoring of urinary methadone [18], benzoylecgonine [9] and amphetamine and analogs [17] by MECC and CZE-based immunoassays using reagents from commercial fluorescence polarization immunoassay (FPIA) kits. Choi et al. described the use of various antibodies for CZE-based immunological analysis of methamphetamine in urine [22,23]. Furthermore, Schmalzing and co-workers described CZE immunoassays for the determination of serum levels of cortisol [24–26] and thyroxine [27], Steinmann et al. discussed MECC-based immunoassays for various drugs in serum [28,29], von Heeren et al. [30] as well as Chiem and Harrison [31,32] explored chip-based monitoring of serum theophylline by MECC and CZE, respectively. Alternatively, Liu et al. developed an enzyme immunoassay for digoxin in serum which is based upon an incubation of the sample with the reagents of a commercial enzyme multiplied immunoassay technique (EMIT) prior to CZE analysis with UV detection of the enzymatic reaction product and remaining reactant [33]. Phillips and Chmielinska [34] reported an immunoaffinity CZE method for analysis of cyclosporine and some of its metabolites in tears in which part of the capillary was coated with monoclonal antibody fragments onto which the solutes of interest could be selectively adsorbed prior to their release and analysis by CZE with UV absorbance detection.

Typical automated solution-based immunoassays for drugs of abuse that are in widespread use, including those based on FPIA, EMIT, cloned enzyme donor immunoassays (CEDIA) and kinetic interaction of microparticles in solution (KIMS), represent one analyte immunoassays (e.g. for methadone or benzoylecgonine) or assays for one group of analytes, such as opiates and amphetamines. Other approaches, such as those with discrete placement of antibodies in spatially separated zones on a solid support onto which binding of different antigens can take place (e.g. the Triage 7 and 8 systems comprising immunoassays for seven and eight analytes or group of analytes, respectively [35] or the Testcup-5 system of Roche Diagnostic Systems that combines a cup for urine collection and a panel with five assays [36]), permit simultaneous screening for multiple components or multiple groups of analytes. Similarly, Parson et al. described a five-analyte system for qualitative drugs of abuse screening that is based on formation of agglutinated complexes in a multichambered disposable vessel [37]. These noninstrumental multianalyte immunoassays were developed for on-site testing of drugs of abuse. CE has also been found suitable for the performance of simultaneous immunoassays of more than one analyte (or more than one group of analytes) in a single sample. Examples of CE-based multianalyte immunoassays reported in the literature include the analysis of morphine and PCP in urine [20], urinary morphine, PCP, THC and benzoylecgonine [21],

salicylate and paracetamol in serum [29] and paracetamol, theophylline and quinidine in serum [29]. In these papers, the feasibility of performing multiple immunoassays in the liquid phase capillary format has been documented only. No reports showing analysis of real world samples and unambiguous validation of the assays could be found. Thus, a multianalyte CE-based immunoassay using four different sets of Abbott's TDxFLx FPIA reagents, namely those for methadone (M), opiates (O), the cocaine metabolite benzoylecgonine (C) and amphetamine/methamphetamine (A), was developed in our laboratory. This four-analyte immunoassay, referred to as the MOCA multianalyte immunoassay, was consequently applied to the screening of drugs of abuse in fortified blank urines, commercial quality control urines and patient samples and was validated. Data obtained with the MOCA assay are shown to be quantitative and to be in agreement with data resulting from routine urinary screening using EMIT and FPIA. Furthermore, positive screening results were confirmed by GC–MS.

2. Experimental

2.1. Chemicals, origin of urine samples and routine immunological screening for drugs of abuse

All chemicals used were of analytical or research grade. Racemic methadone was obtained from the University Hospital pharmacy (Bern, Switzerland). $Na_2B_4O_7 \cdot 10H_20$ was purchased from Merck (Darmstadt, Germany) and sodium fluorescein and Rhodamine 123 were obtained from Fluka (Buchs, Switzerland). Patient urines were collected in the departmental routine drug assay laboratory where they were received for toxicological drug screening. External quality control urines were purchased from Cardiff Bioanalytical Services (UKNEQAS for drugs of abuse, Cardiff, UK). Our own urine was used as a blank matrix. All urines were first screened for the presence of methadone, opiates, amphetamine/methamphetamine and the cocaine metabolite benzoylecgonine using four different automated enzyme multiplied immunoassay techniques (EMIT d.a.u.; Syva, San Jose, CA, USA) on a Cobas Fara II centrifugal analyzer (F. Hoffmann-La Roche, Diagnostica, Basel, Switzerland). Samples that gave an equal or higher response than the cutoff calibrators (300 ng/ml methadone, 300 ng/ml morphine, 1000 ng/ml D-methamphetamine, 300 ng/ml benzoylecgonine for the four tests, respectively) were interpreted as positive. The EMIT assays were performed according to the manufacturer's instructions. Then, the samples were stored at −18°C until analysis. Before use, all urines were centrifuged at about 1500 *g* for 5 min or filtered through disposable 0.45 μm filters (model FP 030/2, Schleicher & Schuell, Keene, NH, USA).

2.2. Immunoassay reagents and FPIA urine testing

TDxFLx FPIA reagents (two different lots of each) for methadone (No. 9676-60), opiates (No. 9673-60), amphetamine/methamphetamine II (No. 1A99-60) and cocaine metabolite (No. 9670-60) were obtained from Abbott Laboratories (Baar, Switzerland). Each reagent pack comprises separate vials for antibody containing solution (solution S; all comprising sheep antisera (Table 1)) and fluorescein tracer solution (solution T with tracer concentration <0.01%) whose concentrations are not exactly disclosed [38]. TDxFLx multiconstituent Low, Medium and High controls (No. 9687-12; Abbott) containing eight unlabelled drugs (Table 2) that were spiked into pooled human urine were employed for cali-

Table 1
FPIA immunoassay reagents employed in this work

Name of FPIA reagent kit	Antibody solution S (%)	Tracer solution T (%)	Unlabelled calibrator/control substance
Methadone	<25	<0.01	Methadone
Opiates	<1	<0.01	Morphine
Amphetamine/methamphetamine II	<25	<0.01	D-Amphetamine
Cocaine metabolite	<1	<0.01	Benzoylecgonine

Table 2
TDxFLx multiconstituent controls for abused drug assays[a]

Compound	Low level (ng/ml)	Medium level (ng/ml)	High level (ng/ml)
Methadone	300	750	2000
Morphine	250	500	800
D-Amphetamine	500	1500	4000
Benzoylecgonine	500	1500	3000
Secobarbital	300	800	1500
Phencyclidine	35	100	250
Nordiazepam	300	600	1000
THC	50	100	150

[a] These controls and dilutions thereof were employed for calibration of the multianalyte CZE-based immunoassay.

bration. Furthermore, the fluorescein tracer of the TDxFLx tricyclic antidepressant reagent kit (No. 9681-60; Abbott) was used as internal standard (I.S.). All TDxFLx products contain 0.1% sodium azide as a preservative. These reagents were employed for the electrokinetic capillary-based immunoassays. Furthermore, all urines investigated were also tested by automated FPIA on the TDxFLx Analyzer (Abbott Laboratories, Irving, TX, USA). The FPIA assays were performed according to the manufacturer's instructions. Using this technology for three concentration levels (Table 2), imprecisions ($n=6$) were determined to be between 2 and 6%.

2.3. Sample preparation

If not stated otherwise, 25 μl of each of the four T solutions, 5 μl of the I.S. solution (10 μl Hamilton syringe) and 25 μl of urine (blank, patient urine, or control urine) or 25 μl of diluted urine (dilution with urine blank or water) were pipetted into a small Eppendorf vial and vortexed for 10 s. Then 25 μl of each of the four S solutions were added, vortexed for about 10 s, and the whole mixture was incubated at room temperature for about 10 min. For analysis, the plastic sample vial was cut down and inserted into the 'thermostat vialholder' of the CE instrument.

2.4. Instrumentation, running conditions and data evaluation

CZE was performed on a P/ACE System 5510 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) featuring automated capillary rinsing, sampling, temperature control of the capillary, data collection, storage and evaluation. A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 47 cm (40 cm effective length)×75 μm I.D. was employed. Solute detection was effected with the LIF detector assembly (Beckman) that was powered by a 7 mW, 488 nm air cooled Argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) and was equipped with a 488 nm notch filter and a 520 nm band pass filter. Maximum RFU were set to 10 or 100. Data were evaluated using the P/ACE Station Software (Beckman). A constant voltage of 13 kV (current: about 87 μA; anode on sampling side) was applied and the capillary temperature was kept at 20°C. The sample carousel was at ambient temperature. Before runs the capillary was conditioned with 0.1 *M* NaOH and water by application of positive pressure (5 p.s.i. for 3 min each; 1 p.s.i.=6894.76 Pa). Sample injection occurred by positive pressure using 0.5 p.s.i. for 1 s. To prevent carry over of sample, the capillary end was then dipped into a vial containing running buffer prior to being placed into the buffer vial employed to perform the run. Between runs the capillary was rinsed with running buffer for 5 min. The buffer was composed of 50 m*M* disodium tetraborate (pH 9.3). Quantitation was based upon multi-level, internal calibration using the ratio between the peak heights of the free tracer and the I.S. Calibration graphs were constructed by non-linear regression analysis based upon a four-parameter log/logit-model according to Ref. [28]

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

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. The parameter estimation was performed on a personal computer using SigmaStat Statistical Analysis System version 1.01 (Jandel Scientific, Corte Madera, CA, USA).

2.5. Confirmation by GC–MS

A 3 ml sample of urine was hydrolyzed via addition of 26 mg β-glucuronidase (Sigma) and 2 ml of pH 6 citrate buffer (Merck No. 1.09437.1000) followed by incubation at 60°C for 2 h. Then 6 ml of a pH 7 phosphate buffer was added and the sample was centrifuged for 5 min at 3000 rpm. Extraction was effected using Bond Elut Certify cartridges and a Vac Elut setup (both from Analytichem International, Harbor City, CA, USA). The cartridges were conditioned immediately prior to use by passing sequentially 3 ml methanol and an equal volume of water through the columns. The vacuum was turned off to prevent column drying. The columns were loaded by slowly drawing (approximately 2 ml/min) of the sample. The columns were then rinsed sequentially with 3 ml of water, 3 ml of pH 4.5 acetate buffer and 3 ml of methanol followed by drying via slowly applying full vacuum for about 2 min. Elution occurred with freshly prepared 2.5 ml dichloromethane–isopropanol (80:20, v/v) containing 2% (v/v) concentrated ammonium hydroxide solution. The eluate was evaporated under a gentle stream of nitrogen in a water bath at 45°C. The residue was reconstituted in 80–100 μl of dichloromethane and put in a micro-GC vial.

Aliquots of 1 μl sample were injected into a Model HP 5890 GC (Hewlett-Packard, Widen, Switzerland) equipped with a temperature programmer and a model HP 7673A autosampler (Hewlett-Packard). A DB-XLB column of 20 m×0.18 mm I.D. and 0.18 μm coating (J&W, Folsom, CA, USA) was used together with a splitless injector which included a liner (RDL-1076-H-22ga. Chambered split/splitless, R&D, Rancho Cordova, CA, USA). The temperatures of the injector and interface were 260 and 280°C, respectively. Initial and final column temperatures were 70 and 310°C, respectively. The temperature was increased by ramping first from time 1.0 to 9.67 min (70 to 200°C) at a rate of 15°C/min and then from time 9.67 to 15.78 (200 to 310°C) at a rate 18°C/min. The total run time was 24 min. The MS detector was a model HP 5970 MSD (Hewlett-Packard) operating in the scan acquisition mode between 33 and 450 amu or in the single ion monitoring mode. An HP 59970 MS ChemStation (version 3.2, Hewlett-Packard) was employed as data station and for data evaluation. Data acquisition was initiated 4 min after sample injection. Monitored mass spectra were compared to those stored in the NBS library (HP5997373 No. 22 Revision 3.1) or the NIST library (version 4.01). Using the scan acquisition mode, detection limits were found to be in the range of 1–50 ng/μl extract.

3. Results and discussion

3.1. Basic characteristics of the multianalyte CZE-based immunoassay for drugs of abuse

Electrokinetic multianalyte immunoassays are based on the CE separability of labelled antigens from each other and from the antibody–antigen complexes. Employing equal amounts of urine, tracer solution T and antibody containing solution S for single-analyte CE-based immunoassays of methadone [18], benzoylecgonine [9] and amphetamine/methamphetamine [17] was found to produce unambiguous concentration dependent peak heights of the free tracers, signals that could be simply employed for urine screening. As example, electropherograms obtained for methadone are shown in panel A of Fig. 1. With urine blank, the free tracer (M) and the antibody–tracer complex (C_M) could be separated and detected by LIF. Under the employed conditions, M was detected after about 8.9 min of current application. The magnitude of the M peak increases as the urinary methadone concentration is increased. The opposite is true for C_M which indicates that the competitive reaction can be monitored with this configuration. The sensitivity limit of this screening assay is about 10 ng/ml and the reproducibility assessed via determination of the peak height of M was determined to be 6.8% ($n=5$, urine blank supplemented with 80 ng/ml methadone).

Extension to multianalyte immunoassays with FPIA reactants results in different dilutions and

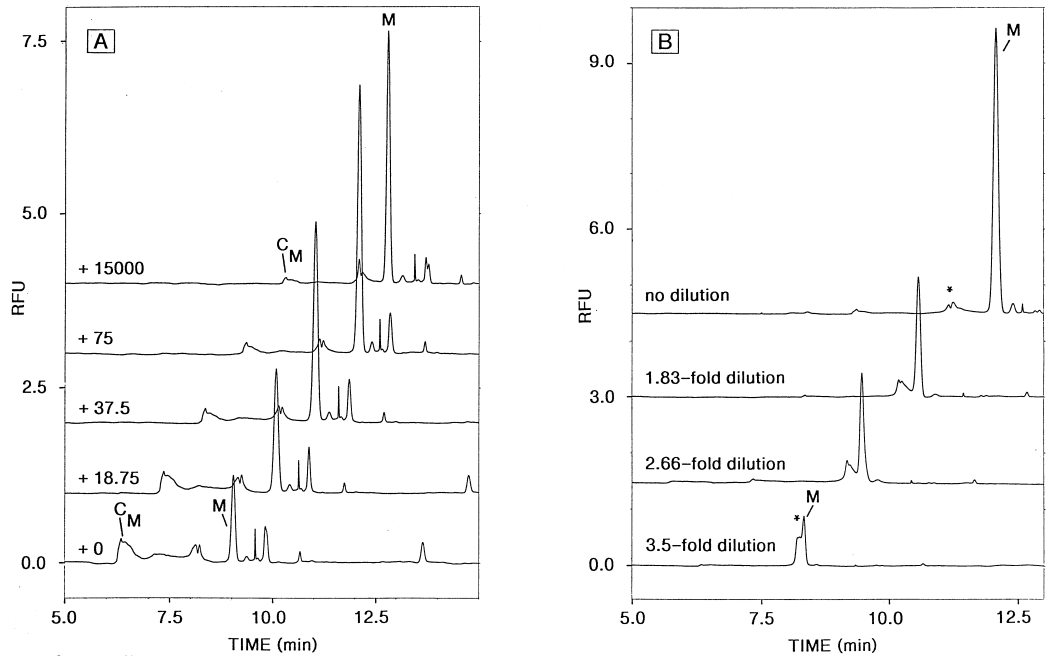

Fig. 1. CZE single-analyte immunoassay data for urinary methadone. (A) Electropherograms obtained with blank urine (bottom graph) and blank urine spiked with 18.75, 37.5, 75 and 15000 ng/ml of methadone. Each sample comprised 25 μl aliquots of urine, solution T and solution S. Electropherograms are depicted with a 1 min *x*-axis shift and a 1 RFU unit *y*-axis shift (from bottom to top). (B) Electropherograms of 3.50-, 2.66-, 1.83- and 1.00-fold diluted reagent mixture for a blank urine fortified with 200 ng/ml methadone. The *x*-axis and *y*-axis shifts are 1 min and 1.5 RFU, respectively. Key: M, free tracer of methadone; C_M, antibody–methadone tracer complex; RFU, relative fluorescence unit.

thereby other conditions. For the methadone assay, effects resulting from dilution of the reaction mixture with running buffer are presented in Fig. 1B. The data of the top graph were obtained using a fortified urine (200 ng/ml methadone) together with equal amounts of solutions S and T (10 μl each, no dilution). Under these conditions, the electropherogram monitored is comparable to those depicted in Fig. 1A. Analysis of diluted reaction mixtures (3.50-, 2.66- and 1.83-fold dilution obtained via addition of 75, 50 and 25 μl running buffer, respectively, to the mixture prior to incubation with antiserum) provided the other graphs shown in Fig. 1B. Not surprisingly, dilution resulted in smaller peaks of M and thus also somewhat diminished sensitivity. Furthermore, resolution between M and an unknown set of peaks (marked with asterisk, peaks that could be part of the antibody–tracer complex system or originate from the tracer [18]) becomes poorer. Having the same total dilution but differences in the composition of the reactants, provided differences in electropherograms as well. Data obtained with 10 μl of the high level control urine (Table 2) combined with 10 μl of appropriate S and T solutions and running buffer to provide a constant reaction mixture volume of 90 μl (i.e. addition of 60, 40, 20 and 0 μl of running buffer for single-, double-, triple- and quadruple-analyte immunoassays, respectively) are presented in Fig. 2. Electropherograms resulting from various double-analyte and triple-analyte immunoassay combinations are shown in panels A and B, respectively. Single-analyte (methadone) and quadruple-analyte immunoassay data are depicted in both panels (bottom and top graphs, respectively). With methadone alone (3-fold total dilution), an electropherogram that is similar to those shown in Fig. 1B was obtained (bottom graph of Fig. 2A and B).

Comparison of the various electropherograms presented in Fig. 2 documents that there is a great deal of pattern changes observed, e.g. addition of the

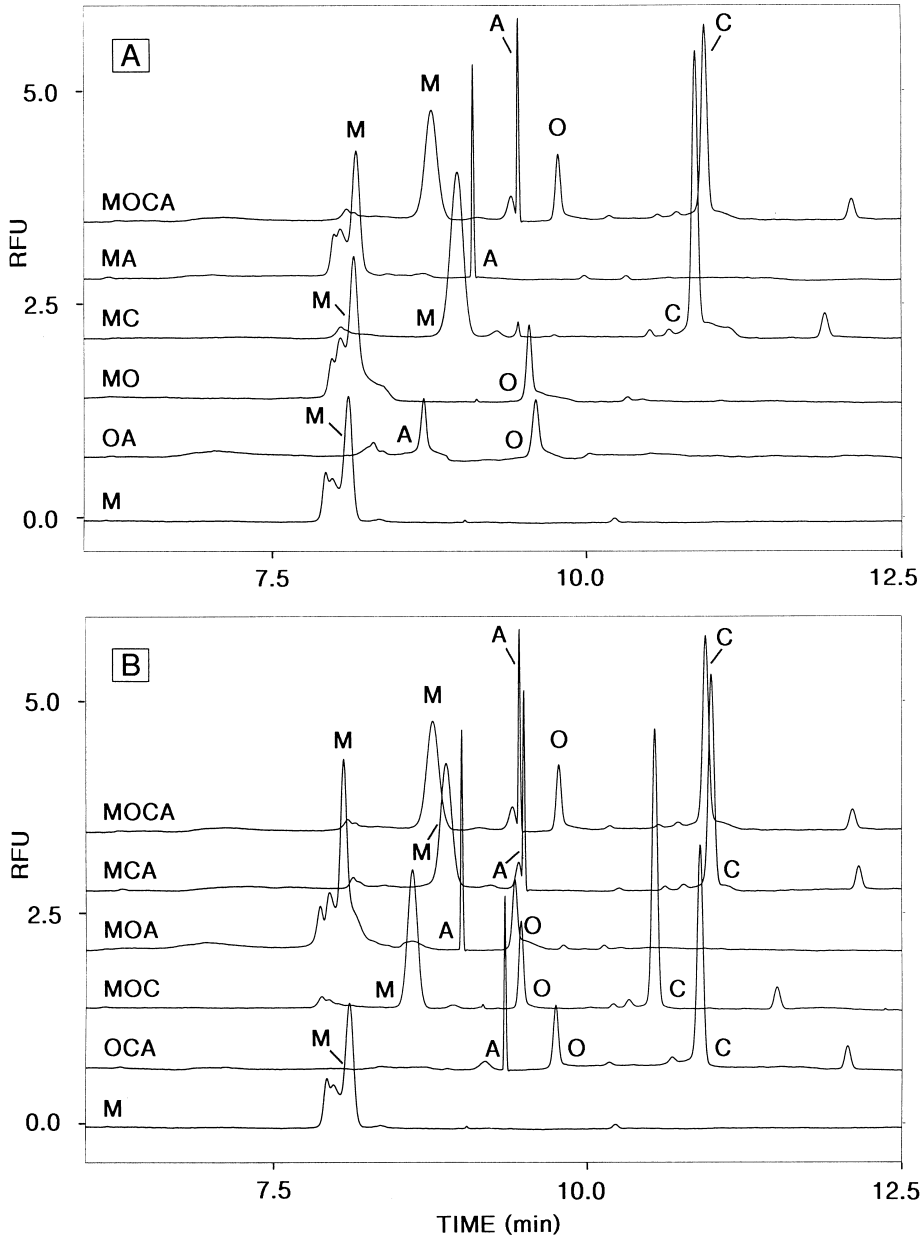

Fig. 2. Electrokinetic multianalyte immunoassay data of (A) double-analyte systems and (B) triple-analyte systems in comparison to the quadruple-analyte configuration (top electropherogram) and to methadone alone (bottom electropherogram). Labels M, O, C and A refer to the free tracers of methadone, morphine, benzoylecgonine and D-amphetamine, respectively. The *y*-scale offset is 0.7 RFU units and there is no *x*-axis shift.

reactants for benzoylecgonine results in delayed detection of the free tracers M and A and provides increased resolution for M. The origin of these changes could be based upon solute–wall interactions. As electrokinetic patterns were found to be reproducible (see below), no further work was devoted to elucidate the factors determining the differences in the detector responses. All further work was

performed with all four reagent sets combined and the obtained four-analyte immunoassay was referred to as the MOCA assay.

Before the MOCA assay was applied to testing of patient urines, inclusion of an internal standard was investigated. First, fluorescein (4.35 ng/ml sample) and rhodamine 123 (about 30 ng/ml) were added as anionic and cationic marker substances, respectively. Although fluorescein was found to produce a nice peak at about 18 min, it was omitted from the selection because it reached the detector too late (about 7 min after the free tracer of C, cf. top graph of Fig. 2). Furthermore, the cationic marker substance was monitored as a broad peak (at about 5 min, i.e. in front of the compounds of interest) and was therefore not further used. The search continued using a number of TDxFLx fluorescein tracers, including those for acetaminophen (detected after 12.4 min), quinidine (10.1 min), theophylline (15.5 min), tricyclic antidepressants (12.8 min), amikacin (8.8 min) and ethosuximide (15.2 min). Based on peak shape, peak magnitude and detection time, the fluorescein tracer for tricyclic antidepressants was used as I.S. (Fig. 3).

3.2. The MOCA four-analyte immunoassay

The data presented in Fig. 3 represent MOCA immunoassay data that were produced as described in Sections 2.3 and 2.4. The electropherograms were

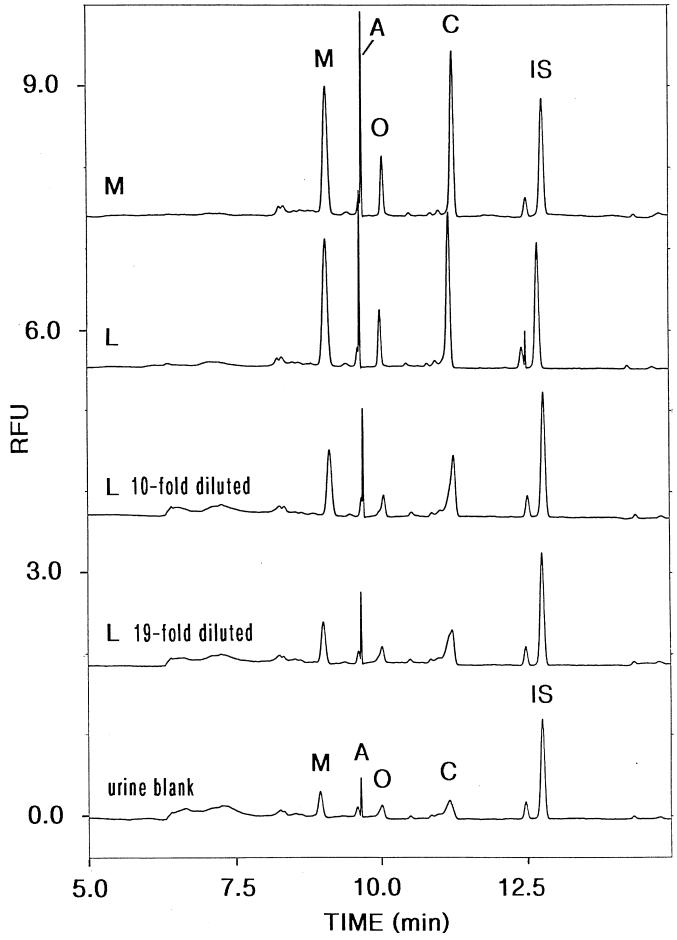

Fig. 3. MOCA multianalyte immunoassay calibration electropherograms. L and M refer to data obtained with the low level and medium level multiconstituent controls, respectively. The *y*-scale offset is 1.85 RFU units and there is no *x*-axis shift. Other conditions as for Fig. 2.

obtained with urine blank, low level control urine that was diluted 19-fold with urine blank, 10-fold diluted low level control urine, low level control urine and medium level control urine (from bottom to top, for drug concentrations refer to Table 2). These data reveal that peak heights for all free tracers are increasing as the drug concentration is increased. Thus, quantitation could simply be effected by multilevel internal calibration using ratios of tracer peak heights with the peak height of the I.S. Typical calibration graphs are depicted in Fig. 4. Overall, the four graphs were found to be quite similar, indicating that the sensitivity for the four analytes is not too different. In all cases, peak height ratios for the 19-fold diluted low control urine were determined to be significantly higher than those measured for urine blank. Thus, this assay is shown to properly recognize methadone, morphine, benzoylecgonine and D-amphetamine concentrations that are >16, >14, >27 and >27 ng/ml, respectively, values that are significantly lower compared to those typically used as cut-off values in immunological screening assays (e.g. 300 ng/ml, cf. Section 2.1). Thus, the MOCA four-analyte immunoassay is demonstrated to be more sensitive than the EMIT d.a.u. screening procedures used in our departmental drug assay laboratory. These assays operate with two calibrator urines only, the lower defining cutoff levels of the drugs and the higher representing a positive control (no multilevel calibration). The multianalyte immunoassay described here provides higher flexibility. The cutoff can be reliably set to as low as 30 ng/ml. Not surprisingly, the MOCA immunoassay is characterized with a sensitivity that is comparable to that obtained in Abbott's TDxFLx FPIA assays (100, 25, 30 and 100 ng/ml for

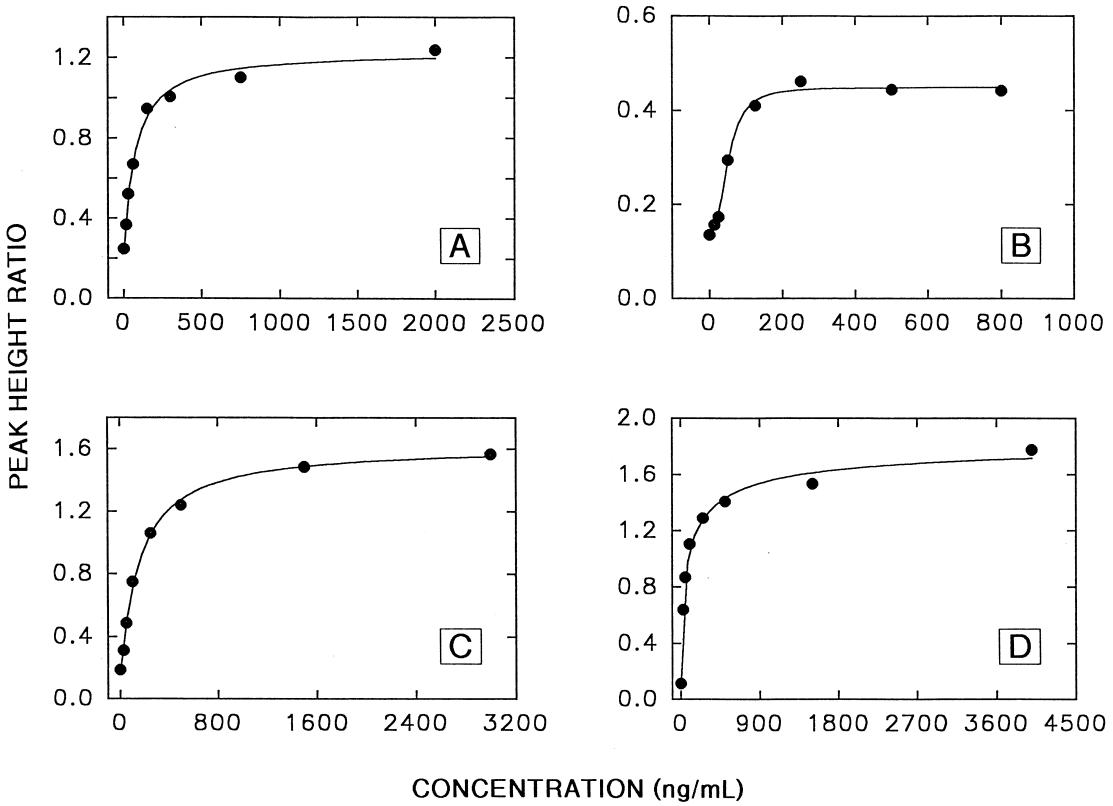

Fig. 4. Calibration graphs of the MOCA multianalyte immunoassay for (A) methadone, (B) morphine, (C) benzoylecgonine and (D) D-amphetamine. Data presented are based upon peak height ratios, i.e. the monitored peak heights divided by the peak height of the internal standard.

methadone, morphine, benzoylecgonine and D-amphetamine, respectively [38]).

Reproducibility was assessed by analysis ($n=6$, reaction mixtures prepared individually) of two-fold diluted low control urine (dilution with blank urine, for final concentrations see Table 2). RSD values of peak height ratios of methadone, morphine, benzoylecgonine and D-amphetamine were found to be 7.9, 5.8, 2.9 and 8.5%, respectively. Data evaluation based upon peak heights of the free tracers only, i.e. without internal standard, revealed significantly higher (twice or higher) RSD values. The same was found when peak areas instead of peak heights were employed as the basis for data evaluation. Similar data were obtained for analysis of patient urines. RSD values in the 3–9% range are also typical for assays based upon FPIA (cf. Section 2.2 and Ref. [38]).

3.3. Analysis of external quality control urines with the MOCA immunoassay

MOCA data obtained with external quality control urine 106 and urine blank are depicted in Fig. 5A. For all four analytes, small peaks for the free tracers were noted when the urine blank was analyzed. Significantly increased responses for the free tracers were observed with the undiluted and diluted external quality control urine. Thus, this urine was classified as being positive for all four parameters. The same was found to be true in the departmental drug assay laboratory using EMIT and when analyzing this urine by FPIA. This quality control urine was reported to have been prepared from urine of two polydrug abusers and to contain compounds of all four investigated classes of drugs, THC, benzodiazepines and PCP (UKNEQAS for drugs of abuse in urine, description of sample 106, November 1996). Chromatographic data reported revealed the presence of significant amounts of methadone (2.29 μg/ml), free morphine (5.64 μg/ml), morphine after hydrolysis (25.3 μg/ml), benzoylecgonine (2.46 μg/ml) and amphetamine (2.38 μg/ml). Using the MOCA assay with undiluted urine, peak height ratios were determined to be 1.25, 0.42, 1.66 and 2.30, respectively (Table 3). According to the calibrations presented in Fig. 4, urinary concentrations could be estimated as being >2.0, >0.2, >2.5 and >4 μg/

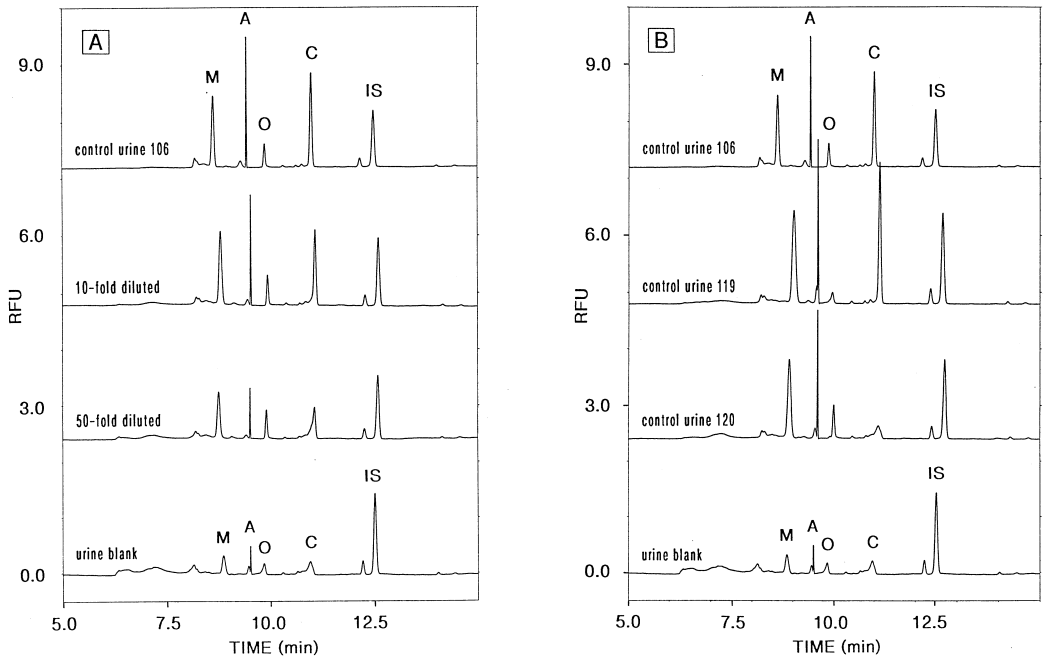

Fig. 5. MOCA multianalyte immunoassay data of urine blank and quality control urines (A) urine 106 (native and two dilutions with water) and (B) urines 106, 119 and 120. The *y*-scale offset is 2.4 RFU units.

Table 3
MOCA assay data obtained with quality control urine 106

Method	Methadone		Opiates		Cocaine metabolite		Amphetamines	
Reported conc.[a] (μg/ml)	2.29		5.64 (25.3)		2.46		2.38	
FPIA[b] (μg/ml)	2.95		High (18.1)		3.23		High (3.2)	
MOCA[c] urine dilution	Ratio	Conc. (μg/ml)	Ratio	Conc. (μg/ml)	Ratio	Conc. (μg/ml)	Ratio	Conc. (μg/ml)
Undiluted	1.250	High	0.418	0.12	1.659	High	2.295	High
10-Fold diluted	1.072	3.76	0.442	2.21	1.101	2.88	1.625	16.7
19-Fold diluted	0.834	2.13	0.465	High	0.875	2.97	1.284	4.86
50-Fold diluted	0.722	3.63	0.453	High	0.491	2.50	0.791	1.95

[a] Reported chromatographic data for methadone, free morphine (morphine after hydrolysis), benzoylecgonine and amphetamine, respectively.

[b] FPIA values determined in our laboratory. Values in brackets represent those obtained after 19-fold (opiates) and 10-fold (amphetamines) dilution of the urine.

[c] Ratio represents the peak height ratio. Concentrations are calculated using the calibration graphs presented in Fig. 4 and the dilution factor. High refers to a concentration above the calibration range shown in Fig. 4.

ml, respectively, with all four values being above the discriminating part of the calibration curve. Similarly, diluted urine provided peak height ratios that were found to be well within the calibration ranges (Table 3, Fig. 4). Not surprisingly, as these data are outside the uniform part of the calibration graphs, better agreement with chromatographic data was obtained after urine dilution. This is best seen for benzoylecgonine and methadone, the two single analyte parameters. For opiates, it was interesting to find that the peak height ratios did not change much whereas the calculated concentrations became higher with increased urine dilution. Similar effects were observed with patient urines (see below). Furthermore, the values obtained for amphetamines substantially decreased with increasing urine dilution. Thus, care should be taken in using the MOCA four-analyte immunoassay for quantitation of group responses. Nevertheless, the MOCA multianalyte immunoassay is shown to provide meaningful data for this complex urine specimen.

The data presented in panel B of Fig. 5 compares electropherograms of a blank urine together with those of the external quality control urines 106, 119 and 120. While sample 106 stemmed from urines of two polydrug abusers (see above), urine 119 originated from a subject taking salicylate (100 mg/day) and was fortified with methadone (0.8 μg/ml), D-amphetamine (0.4 μg/ml), benzoylecgonine (0.9 μg/ml), buprenorphine (0.5 μg/ml) and LSD (4.2 ng/ml). Sample 120 was prepared from a urine of a patient under simvastatin (20 mg/day) and salicylate (150 mg/day) pharmacotherapy that was fortified with methadone (0.4 μg/ml), morphine (0.8 μg/ml), D-amphetamine (0.4 μg/ml), oxazepam (0.4 μg/ml), buprenorphine (0.2 μg/ml) and LSD (1.0 ng/ml). Using the MOCA assay with cutoff values of 100 ng/ml (Fig. 4), urine 106 was correctly found to be positive for all four analytes, urine 119 was found to be positive for M, C and A and negative for O, and urine 120 was determined to be negative for the cocaine metabolite and positive for the three other analytes. EMIT provided the same classification. Furthermore, FPIA data of urines 119/120 were determined to be 0.95/0.46, 0.95/1.01, 1.34/0 and 0/0.703 μg/ml for M, A, C and O, respectively. Assuming cutoff values in the 200 to 300 ng/ml range, screening results are again in complete agreement with those of EMIT and MOCA. Thus, data obtained with the MOCA multianalyte assay are shown to be correct and the novel assay could be applied to the screening of patient urines for more than one class of drugs of abuse.

3.4. Analysis of patient samples with the MOCA immunoassay and confirmation by GC–MS

The MOCA immunoassay has been employed for

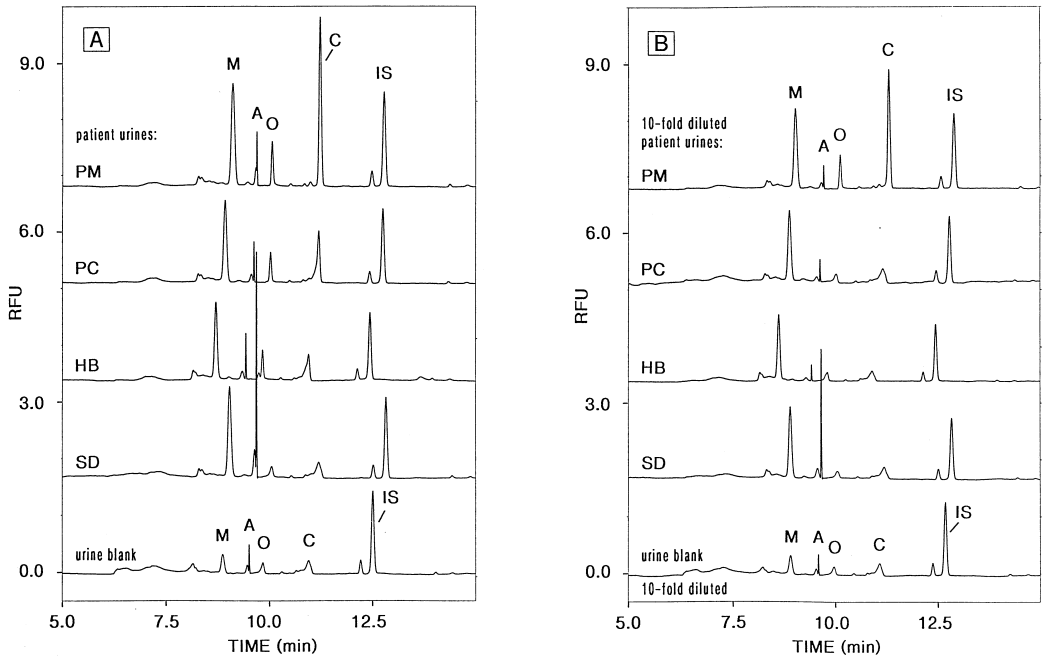

Fig. 6. MOCA multianalyte immunoassay data of selected (A) undiluted patient samples and urine blank and (B) patient samples and urine blank 10-fold diluted with water. The *y*-scale offset is 1.7 RFU units.

drug of abuse screening in eight patient urines, samples that were received from the emergency care unit. Immunoassay data of a urine blank and selected patient urines are presented in Fig. 6A, whereas the graphs depicted in Fig. 6B are those monitored with 10-fold diluted patient urines. Overall, good agreement between MOCA data and those obtained with other methods were noted (Tables 4 and 5). The results presented in Table 4 provide insight into the selection of the cutoff values for the MOCA assay in comparison with EMIT and FPIA data. Having undiluted urine, a 200 ng/ml cutoff does not classify all urines in the same way as EMIT and FPIA. Not surprisingly, improved agreement is obtained by lowering the cutoff to 100 or even 50 ng/ml. Furthermore, most parameters are still picked up after 10-fold dilution of the specimens. As mentioned above for urine 106, urine dilution provided higher values for opiates. In one case with a cutoff of 200 ng/ml, a false negative urine became positive

Table 4
Screening results of eight patient urines[a]

	EMIT[b]	FPIA (200 ng/ml cutoff)		MOCA (200 ng/ml cutoff)		MOCA (100 ng/ml cutoff)		MOCA (50 ng/ml cutoff)	
Urine dilution	None	None	10-fold	None	10-fold	None	10-fold	None	10-fold
Methadone	5	5	4	4	4	5	4	5	4
Opiates	6	7	5	4	5	5	5	7	5
Cocaine Metabolite	2	2	2	2	2	2	2	3	2
Amphetamines	1	2	1	1	1	1	1	1	1

[a] The number given indicates the number of specimens that tested positive.
[b] For cutoff values refer to Section 2.1.

Table 5
Comparative results of selected patient urines[a,b]

Sample	Method	Methadone	Opiates	Benzoylecgonine	Amphetamines
SD	MOCA	pos	neg	neg	pos
	FPIA[c]	pos (4.06*)	neg	neg	pos (30.9*)
	EMIT	pos	neg	neg	pos
	GC–MS	pos (methadone, EDDP)	neg	neg	pos (amphetamine)
HB	MOCA	pos	pos	(pos)	(pos)
	FPIA[c]	pos (17.0*)	pos (0.384)	neg	neg
	EMIT	pos	pos	neg	neg
	GC–MS	pos (methadone, EDDP)	pos (morphine)	neg	pos (MDE)
PC	MOCA	pos	(pos)	(pos)	neg
	FPIA[c]	pos (3.43)	pos (0.204)	neg	neg
	EMIT	pos	neg	neg	neg
	GC–MS	pos (methadone, EDDP	pos (morphine)	neg	neg
PM	MOCA	pos	pos	pos	neg
	FPIA[c]	pos (3.98)	pos (>10*)	pos (17.0*)	neg
	EMIT	pos	pos	pos	neg
	GC–MS	pos (methadone, EDDP)	pos (morphine, codeine, 6-MAM)	pos (cocaine, benzoylecgonine)	neg

[a] (pos) refers to traces only (<100 ng/ml for MOCA assay).
[b] For GC–MS, the compounds that were identified are listed in brackets.
[c] For FPIA, 0.2 μg/ml was chosen as cutoff. The results provided in brackets are in μg/ml and the asterisk refers to the analysis of 10-fold diluted urine.

after 10-fold dilution. Based on the data obtained thus far, a 100 ng/ml cutoff is recommended for classification of screening results. As multilevel calibration is possible, individual cutoffs can also be selected, a flexibility that is not given with EMIT screening and point of care tests (e.g. Triage and Testcup-5), but is typical for other instrumental screening procedures, including those based on FPIA and KIMS.

As is customary with all immunoassays, however, interferences providing false positive or insufficient sensitivity resulting in false negative results can never be excluded. Thus, data should be confirmed by another, non-immunological-based method that is at least as sensitive as the immunoassay employed. Four of the eight patient urines were therefore also analyzed by GC–MS. The data presented in Table 5 provide a comparison of the confirmation results with those obtained using the MOCA assay, FPIA and EMIT. The comparison is based upon analysis of undiluted urines and a cutoff of 100 ng/ml for the MOCA method. Using FPIA, responses ≥200 ng/ml were considered as being positive. For GC–MS, the compounds that were unambiguously identified are also listed in Table 5. Overall, good agreement was noted. EMIT classification for opiates of urine PC was determined to be correct, as MOCA, FPIA and GC–MS revealed only traces of opiates in this specimen. The same was found to be true for amphetamines in urine HB. The MOCA assay is demonstrated to be more sensitive than EMIT. Furthermore, the MOCA immunoassay appears to be more sensitive for benzoylecgonine than the GC–MS method (compare data of patients HB and PC).

4. Conclusions

As a continuation of our work characterizing competitive binding, electrokinetic capillary-based immunoassays for various drugs in human urine using reagents which were commercialized for FPIA, simultaneous analysis for two single analytes (methadone and benzoylecgonine) and two group of

analytes (opiates and amphetamines) was investigated. The multianalyte immunoassay is based upon incubation of equal amounts of urine with the reagents S and T (25 μl each) and 5 μl of the I.S. solution prior to injection of a small aliquot of the mixture onto the capillary and analysis of the fluorescein labelled, free tracers via on-column LIF detection. The paper reports the first validated multianalyte CE-based immunoassay for urinary drugs of abuse. The MOCA assay is quantitative (for the single-analyte format), detection limits being in the order of 30 ng/ml. Cutoff values can be set as low as 50 ng/ml, i.e. at values that are below those recommended by NIDA [39]. In the fused-silica capillary format (40 cm effective capillary length), the MOCA assay can be performed within about 13 min. Run times could be further reduced via employment of shorter capillaries. Moreover, the assay could also be performed in the chip-format, allowing for integrated reagent mixing, incubation and analysis as was recently described by Chiem and Harrison for analysis of theophylline in serum [32].

CE-based immunoassays are simple, rapid, sensitive and reliable approaches for multianalyte screening of drugs in body fluids. In principle, CE-based multianalyte immunoassays can be employed for any number of analytes. This methodology is limited by the CE separability of labelled antigens from each other and from the antibody–antigen complexes and by the detectability of the free tracer peaks. The principle employed lends itself to configure custom-made screening reagents that include the required antibodies and the labelled tracers. To increase versatility, but at the expense of instrumental complexity, strategies with different tagging of tracers together with multiple laser line LIF detection, with wavelength resolved or with time-resolved fluorescence detection could represent interesting approaches for simultaneous selective detection of many different types of solutes.

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References

[1] C.P. Price, D.J. Newman (Eds.), Principles and Practice of Immunoassay, 2nd ed., Macmillan Publishers, London, 1997.
[2] R. DeCresce, A. Mazura, M. Lifshitz, J. Tilson, Drug Testing in the Workplace, ASCP Press, Chicago, 1989.
[3] K.D. Eskridge, S.K. Guthrie, Pharmacotherapy 17 (1997) 497–510.
[4] H.H. Maurer, J.W. Arlt, T. Kraemer, C.J. Schmitt, A.A. Weber, Arch. Toxicol. Suppl. 19 (1997) 189–197.
[5] S.R. Binder, Adv. Chromatogr. 36 (1996) 201–271.
[6] W. Thormann, in: N. Guzman (Ed.), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, pp. 693–704.
[7] I.S. Lurie, in: J.A. Adamovics (Ed.), Analysis of Addictive and Misused Drugs, Marcel Dekker, New York, 1995, pp. 151–219.
[8] W. Thormann, in: S.H.Y. Wong, I. Sunshine (Eds.), Handbook of Analytical Therapeutic Drug Monitoring and Toxicology, CRC Press, Boca Raton, FL, 1997, pp. 1–19.
[9] W. Thormann, Y. Aebi, M. Lanz, J. Caslavska, Forensic Sci. Int. 92 (1998) 157–183.
[10] F. Tagliaro, S. Turrmna, P. Pisi, F.P. Smith, M. Mango, J. Chromatogr. B 713 (1998) 27–49.
[11] P. Wernly, W. Thormann, Anal. Chem. 63 (1991) 2878–2882.
[12] P. Wernly, W. Thormann, Anal. Chem. 64 (1992) 2155–2159.
[13] P. Wernly, W. Thormann, J. Chromatogr. 608 (1992) 251–256.
[14] M. Schafroth, W. Thormann, D. Allemann, Electrophoresis 15 (1994) 72–78.
[15] L. Steinmann, W. Thormann, J. Cap. Electrophoresis 2 (1995) 81–88.
[16] S. Molteni, J. Caslavska, D. Allemann, W. Thormann, J. Chromatogr. B 658 (1994) 355–367.
[17] A. Ramseier, J. Caslavska, W. Thormann, Electrophoresis 19 (1998) 2956–2966.
[18] W. Thormaun, M. Lanz, J. Caslavska, P. Siegenthaler, R. Portmann, Electrophoresis 19 (1998) 57–65.
[19] F.-T.A. Chen, S.L. Pentoney, J. Chromatogr. A 680 (1994) 425–430.
[20] F.-T.A. Chen, R.A. Evangelista, Clin. Chem. 40 (1994) 1819–1822.
[21] R.A. Evangelista, J.M. Michael, F.-T.A. Chen, Am. Clin. Lab. 14 (1995) 27–29.
[22] J. Choi, C. Kim, M.J. Choi, J. Chromatogr. B 705 (1998) 277–282.
[23] J. Choi, C. Kim, M.J. Choi, Electrophoresis 19 (1998) 2950–2955.
[24] D. Schmalzing, W. Nashabeh, X.-W. Yao, R. Mhatre, F.E. Regnier, N.B. Afeyan, M. Fuchs, Anal. Chem. 67 (1995) 606–612.
[25] D. Schmalzing, W. Nashabeh, M. Fuchs, Clin. Chem. 41 (1995) 1403–1406.
[26] L.B. Koutny, D. Schmalzing, T.A. Taylor, M. Fuchs, Anal. Chem. 68 (1996) 18–22.
[27] D. Schmalzing, L.B. Koutny, T.A. Taylor, W. Nashabeh, M. Fuchs, J. Chromatogr. B 697 (1997) 175–180.

[28] L. Steinmann, J. Caslavska, W. Thormann, Electrophoresis 16 (1995) 1912–1916.
[29] L. Steinmann, W. Thormann, Electrophoresis 17 (1996) 1348–1356.
[30] F. von Heeren, E. Verpoorte, A. Manz, W. Thormann, Anal. Chem. 68 (1996) 2044–2053.
[31] N. Chiem, D.J. Harrison, Anal. Chem. 69 (1997) 373–378.
[32] N. Chiem, D.J. Harrison, Clin. Chem. 44 (1998) 591–598.
[33] X. Liu, Y. Xu, M.P.C. Ip, Anal. Chem. 67 (1995) 3211–3218.
[34] T.M. Phillips, J.J. Chmielinska, Biomed. Chromatogr. 8 (1994) 242–246.
[35] K.F. Buechler, S. Moi, B. Noar, D. McGrath, J. Villela, M. Clancy, A. Shenhav, A. Colleymore, G. Valkirs, T. Lee, J.F. Bruni, M. Walsh, R. Hoffman, F. Ahmuty, M. Nowakowski, J. Buechler, M. Mitchell, D. Boyd, N. Stiso, R. Anderson, Clin. Chem. 38 (1992) 1678–1684.
[36] J. Towt, S.C. Tsai, M.R. Hernandez, A.D. Klimov, C.V. Cravec, S.L. Rouse, H.S. Subuhi, B. Twarowska, S.J. Salamone, J. Anal. Toxicol. 19 (1995) 504–510.
[37] R.G. Parsons, R. Kowal, D. LeBlond, V.T. Yue, L. Neargarder, L. Bond, D. Garcia, D. Slater, P. Rogers, Clin. Chem. 39 (1993) 1899–1903.
[38] TDx-TDxFLx Testanleitung Drogentests und Toxikologische Tests, No. B4D241 82-4441/R1, Abbott, Wiesbaden-Delkenheim, Germany.
[39] NIDA Guidelines, Fed. Reg. 53 No. 69, 1988, pp. 11970–11989.