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Stereoselective screening for and confirmation of urinary enantiomers of amphetamine, methamphetamine, designer drugs, methadone and selected metabolites by capillary electrophoresis

Data presented in this paper demonstrate that a competitive binding, electrokinetic capillary-based immunoassay previously used for screening of urinary amphetamine and analogs cannot be employed to distinguish between the enantiomers of amphetamine and methamphetamine. However, capillary zone electrophoresis with a pH 2.5 buffer containing (2-hydroxypropyl)- β -cyclodextrin as chiral selector is shown to permit the enantioselective analysis of urinary extracts containing methamphetamine, amphetamine, 3,4-methylenedioxymethamphetamine (Ecstasy) and other designer drugs, and methadone together with its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine. In that approach, enantiomer identification is based upon comparison of extracted polychrome UV absorption data and electropherograms obtained by rerunning of spiked extracts with spectra and electropherograms monitored after extraction of fortified blank urine. The suitability of the described chiral electrokinetic capillary method for drug screening and confirmation is demonstrated *via* analysis of unhydrolyzed quality control urines containing a variety of drugs of abuse. Furthermore, in a urine of a patient under selegiline pharmacotherapy, the presence of the *R*-(-)-enantiomers of methamphetamine and amphetamine could be unambiguously identified. Direct intake of an *R*-enantiomer or ingestion of drugs that metabolize to the *R*-enantiomers can be distinguished from the intake of *S*-(+)-enantiomers (drug abuse) or prescribed drugs that metabolize to the *S*-enantiomers of methamphetamine and amphetamine. The described approach is simple, reproducible, inexpensive and reliable (free of interferences of other major basic drugs that are frequently found in toxicological urines) and could thus be used for screening for and confirmation of urinary enantiomers in a routine laboratory.

Keywords: Capillary electrophoresis / Amphetamine / Methamphetamine / Designer drugs / Methadone / Enantiomers / Immunoassay / Confirmation testing / Multiwavelength detection / Chirality
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Abbreviations: **A**, amphetamine; **BE**, benzoylecgonine; **C**, antibody-tracer complex; **CO**, codeine; **CZEIA**, capillary zone electrophoresis immunoassay; **DD**, desmethyldiazepam; **DH**, diphenhydramine; **E**, ephedrine; **EDDP**, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; **EMIT**, enzyme-multiplied immunoassay technique; **7-F**, 7-aminoflunitrazepam; **FPIA**, fluorescence polarization immunoassay; **MA**, methamphetamine; **MDA**, 3,4-methylenedioxyamphetamine; **MDE**, 3,4-methylenedioxyethylamphetamine; **MDMA**, 3,4-methylenedioxymethamphetamine; **MET**, methadone; **MO**, morphine; **MQ**, methaqualone; **NE**, norephedrine; **NPE**, norpseudoephedrine; **OHP- β -CD**, (2-hydroxypropyl)- β -cyclodextrin; **PE**, pseudoephedrine

1 Introduction

Amphetamines, including amphetamine (A), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxyethylamphetamine (MDE), are chiral drugs that represent drugs of abuse, doping agents and, with decreasing popularity, also legal medication. Most are consumed as racemic mixtures. Typically, one of the two enantiomers of amphetamines is pharmacologically more active than the other. For example, the *S*-(+) enantiomers of A and MA have about five times more psychostimulant activity than the *R*-(-) enantiomers and are thus banned or controlled substances [1, 2]. *R*-(-)-MA is an allowed substance which is included in the Vick's Inhaler[®] sold in the United States [3] but is not part of the formulation of the Vick's Inhaler[®] in Switzerland.

Furthermore, a number of prescribed drugs are metabolized to the *R*(–) enantiomers of MA and A, *e.g.* selegiline: *R*(–)-deprenyl [4–6], a compound that acts as a selective monoamine oxidase inhibitor and is therefore often used for the treatment of Parkinson's disease [7]), to *S*(+) enantiomers of MA and A (example: benzphetamine prescribed as diet pill [4, 8, 9]) or to both enantiomers of MA and A (example: famprofazone, an analgesic, antipyretic drug that is even sold over-the-counter in some parts of the world [10]). This complicates the interpretation of MA/A urine drug testing results.

Urinary screening for drugs of abuse, including amphetamines, is usually performed with immunoassays, whereas gas chromatography – mass spectrometry (GC-MS) is the standard approach employed for confirmation of the presence or absence of a specific drug or metabolite. Liquid chromatography [11, 12] and capillary electrophoresis (CE) [13, 14] with multiwavelength solute detection represent other confirmatory methods. Independent of the method used, the customary assays are typically not enantioselective. Based on the MA/A urinary excretion patterns after intake of MA, A and various prescribed or over the counter (OTC) drugs (see above), stereoselective drug screening might be in order so that illegal consumption of MA/A can be assumed or excluded with higher certainty. Thus far, enantioselective separations employed for drug monitoring were mainly based upon gas or liquid chromatography with columns featuring expensive chiral stationary phases [1, 5]. Recently, CE has become a highly competitive tool for the separation of the enantiomers of a large number of compounds [15–18]. This approach is based upon the addition of a chiral selector to the electrophoresis buffer. Using CE for chiral separations has significant advantages compared to chiral chromatography. The main features of CE are high efficiency, short analysis times, low buffer consumption (and thus also low consumption of the chiral selector), low operation costs, short methods development and fast column equilibration after a buffer change.

To date, no specific chiral CE assay has been applied to routine clinical toxicology. However, the CE separation of the enantiomers of several drugs of interest in toxicology has been investigated. Using various cyclodextrins as chiral selectors, examples studied include the chiral differentiation of the optical isomers of racemethorphan and racemorphinan [19], of amphetamines, cathine, cathinone, cocaine and others [20], and of amphetamine and different analogs [21–28], to name but a few. Furthermore, CE-based urinary assays geared towards the assessment of stereoselective drug metabolism have been published, including those for methadone (MET) [29], MDMA [26] and MA [28]. Finally, Tagliaro *et al.* [27] elucidated

the use of chiral CE for analysis of MDMA and MDA enantiomers in hair of ecstasy users and Frost *et al.* [30] described the enantioselective determination of MET and its major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in serum, urine, and hair of individuals undergoing methadone therapy [30].

Only few reports found in the literature deal with the analysis of amphetamines and analogs in biological samples [13, 14, 26–28, 31–33]. This prompted us to continue working in this field. Recently, CE-based immunoassays suitable for the screening of amphetamines in urine and an achiral CE confirmatory assay based upon multiwavelength solute detection were developed and applied to the analysis of A, MA, MDMA and MDA in quality control and patient samples [32]. In the meantime, our laboratory was also engaged in investigating the use of the capillary zone electrophoresis immunoassay (CZEIA) for analysis of the enantiomers of A and MA and the development of a CE-based chiral assay for analysis of the urinary enantiomers of amphetamines in presence of MET and its major metabolite EDDP. The combination of amphetamines, designer drugs and MET is important as many drug abusers and individuals undergoing MET therapy often consume these drugs simultaneously. Chiral recognition is important, as certain enantiomers of A and analogs are not properly recognized by immunological screening or are metabolites from a therapeutic drug that the immunological assay might not be able to differentiate (see above and [1–6, 34]).

The purposes of this communication are (i) to present CZEIA data of single enantiomers of A and MA, (ii) to report the specifications of a CE assay for enantiomeric separation and analysis of urinary A, MA, MDMA, MDA, MDE, MET, and EDDP, (iii) to show the performance of the chiral assay for analysis of the enantiomers of these compounds in toxicological quality-control urines, (iv) to compare the chiral CE data with those obtained with an achiral CE method, and (v) to demonstrate the use of the chiral CE assay for identification of the urinary *R*(–) enantiomers of methamphetamine and amphetamine after intake of selegiline.

2 Materials and methods

2.1 Chemicals, reagents for immunoassays and origin of urine samples

All chemicals used were of analytical or research grade. *S*(+)-A (D-A), racemic MA (as sulfate and hydrochloride, respectively), codeine (CO), morphine (MO) and racemic MET were from the University Hospital Pharmacy (Bern, Switzerland). Racemic MDMA hydrochloride, (+)-nor-

pseudoephedrine (cathine, NPE) and benzoylecgonine (BE) were from Dr. R. Brenneisen (Department of Clinical Research, University of Bern, Switzerland). *R*-(–)-A (L-A), *S*-(+)-A (D-A), *R*-(–)-MA (L-MA), *S*-(+)-MA (D-MA), racemic MDA, racemic MDE and racemic EDDP were from Alltech (State College, PA, USA). Desmethyldiazepam (DD) and 7-aminoflunitrazepam (7-F) were kindly obtained from F. Hoffmann-La Roche (Basel, Switzerland). Methaqualone (MQ) was extracted with methanol from a Toquilone tablet (Medichemie, Ettingen, Switzerland) that contained 200 mg of that compound. Diphenhydramine (DH) was obtained from the laboratory of G. Streuli (Uznach, Switzerland), (+)-pseudoephedrine (PE), D-(+)-norephedrine (NE), both as hydrochlorides, and (2-hydroxypropyl)- β -cyclodextrin (OHP- β -CD) were from Fluka (Buchs, Switzerland), and ephedrine (E) hydrochloride was from the pharmacy of Dr. A. K  pfer (Herzogenbuchsee, Switzerland). KH_2PO_4 and $\text{Na}_2\text{B}_4\text{O}_7$ were from Merck (Darmstadt, Germany). The TDxFLx FPIA amphetamine/methamphetamine II assay reagent pack (No. 1A99-60) with the human urine calibrators (1A99-06), the TDxFLx FPIA methadone assay reagent pack (No. 9676-60) with the human urine calibrators (9676-02) and multiconstituent controls in human urine (9687-12) were kindly donated by Abbott (Baar, Switzerland). The reagent packs comprise separate vials for antibody-containing solution (solution S; <25% sheep antiserum) and fluorescein tracer solution (solution T with tracer concentration <0.01%) whose exact compositions are not disclosed. All TDxFLx products contain 0.1% sodium azide as a preservative. External quality control urines were purchased from Cardiff Bioanalytical Services (UKNE-QAS for drugs of abuse, Cardiff, UK). The urine of a patient under seligiline pharmacotherapy was kindly donated by the geriatric station of the Zieglerspital, Bern, Switzerland. Urines were received in the departmental drug assay laboratory where they were routinely screened for amphetamines using the EMIT d.a.u. Monoclonal Amphetamine/Methamphetamine Assay (Syva, San Jose, CA, USA) and for methadone using the EMIT d.a.u. Methadone Assay (Syva) performed on a Cobas Fara II Analyzer (Roche, Basel, Switzerland). The amphetamine/methamphetamine assay employs the response of 1000 ng/mL D-(+)-methamphetamine as cutoff and also recognizes D-(+)-amphetamine, MDA and MDMA at concentration levels ≥ 400 , 1000 and 3000 ng/mL, respectively [35]. The methadone assay is based upon a cutoff produced by 300 ng/mL methadone (no specification of chirality of calibrator). The samples were stored at -18°C until analysis. Our own urines were used as blank matrix. Before use, all urine samples 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 Fluorescence polarization immunoassay (FPIA)

Urines were analyzed on the TDxFLx (Abbott) using the TDxFLx FPIA amphetamine/methamphetamine II assay kit (Abbott). In this assay, *S*-(+)-A is employed as calibrator substance (range: 0–8000 ng/mL). MET was monitored with the methadone assay (calibration range: 0–4000 ng/mL). The assays were executed according to the manufacturer's instructions. For three concentration levels of *S*-(+)-A (500, 1500 and 4000 ng/mL), imprecisions ($n = 6$) were determined to be 5.01, 5.78 and 4.18%, respectively. Corresponding values for MET (300, 750 and 2000 ng/mL) were 1.73, 1.75 and 3.66%, respectively.

2.3 Electrophoretic instrumentation and running conditions for CZEIA

CE-based immunoassays were performed on an automated P/ACE 5510 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with an untreated 75 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The effective capillary length was 40 cm (total length, 47 cm). A constant voltage of 13 kV (current, 87–92 μA) was applied and the anode was on the sampling side. Sample injection occurred by positive pressure at 0.5 psi for 1 s. The capillary temperature was maintained at 20°C . The sample carousel was at ambient temperature. Solute detection was effected by a laser-induced fluorescence (LIF) detector assembly (Beckman) that was powered by an air-cooled argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) at 488 nm (operated with 5 mW power at the tip of the fiber optics which connects to the detector unit) and was equipped with a 520 nm emission filter. The photomultiplier tube gain was set to a maximum RFU of 100. Data were evaluated using the P/ACE Station Software (Beckman). Capillaries were conditioned by application of positive pressure (5 psi) employing 0.1 M NaOH (6 min), water (6 min) and running buffer (12 min). Between runs, the capillary was flushed with running buffer only (5 min). The running buffer vial was replenished every 5–6 runs and was composed of 50 mM sodium tetraborate (pH 9.3). Aliquots of solution S and urine (25 μL each) were pipetted into a 600 μL Eppendorf plastic vial and vortexed for about 10 s prior to addition of 25 μL of solution T. The whole mixture was then vortexed for about 10 s and incubated at room temperature for 10–15 min. Next, the reaction mixture was transferred into a P/ACE microvial (Beckman) which was inserted into a glass vial containing the microvial spring (Beckman).

2.4 Extraction

Urine samples (2 mL) were extracted at pH 9 using Toxi-Tubes A (Analytical Systems, Laguna Hills, CA, USA). This commercial liquid/liquid extraction system comprises an organic solvent mixture composed of CH_2Cl_2 and $\text{C}_2\text{H}_4\text{Cl}_2$. After vigorous shaking for about 30 s and centrifugation (3500 rpm, 10 min), the organic phase was transferred into a clean glass tube, two drops of 2 M acetic acid in ethyl acetate were added, and the organic solvent was evaporated under a gentle stream of nitrogen in a water bath at 35°C. The residue was reconstituted in 100 μL water.

2.5 Electrophoretic instrumentation and running conditions for chiral CE with multiwavelength UV absorption detection

A BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) was employed. If not otherwise stated, it was equipped with an untreated 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 60 cm total length (55.4 cm to the detector) which was mounted in a user-assembled cartridge (Bio-Rad); sample was injected by applying positive pressure of 2 or 4 psi $\times$ s, a constant voltage of 22 kV (current about 45 μA) was applied, the temperatures of cartridge and carousel were maintained at 20°C, and detection was effected *via* fast scanning at 5 nm resolution in the range between 195 and 320 nm. The single wavelength electropherograms depicted are those registered for 195 nm. Before each experiment the capillary was rinsed with 0.1 M NaOH for 2 min, water for 2 min, and running buffer for 4 min. BioFocus Integration software (Version 6.0; Bio-Rad) was employed for data conversion and evaluation. The buffer employed was composed of 75 mM KH_2PO_4 at pH 2.5 containing 8.3 mM OHP- β -CD. The chiral selector was added fresh on a daily basis.

2.6 Electrophoretic instrumentation and running conditions for chiral CE at a single wavelength

Analyses were performed with the BioFocus 3000 (see above) at a preselected wavelength or using the P/ACE 5510 capillary electrophoresis system (Beckman Instruments) with solute detection at 200 nm and with an untreated 50 μm ID fused-silica capillary (Polymicro Technologies) of 50 cm effective capillary length (total length, 57 cm). The buffer was the same as described above (*cf.* Section 2.5). Sample injection in the P/ACE 5510 occurred by positive pressure at 0.5 psi for 8 s. The capillary temperature was maintained at 20°C. The sample

carousel was at ambient temperature. If not stated otherwise, a constant voltage of 22 kV (current about 55 μA) was applied and the anode was on the sampling side. Data were evaluated using the P/ACE Station Software (Beckman). Capillaries were conditioned between runs by application of positive pressure (5 psi) employing 0.1 M NaOH (2 min), water (2 min) and running buffer (4 min).

2.7 Electrophoretic instrumentation and running conditions for achiral CE

A BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories) was employed. It was equipped with an untreated 50 μm ID fused-silica capillary (Polymicro Technologies) of 100 cm total length (95.4 cm to the detector). The sample was injected by applying positive pressure of 10 psi $\times$ s; a constant voltage of 20 kV (current between 40–50 μA) was applied, the temperatures of cartridge and carousel were maintained at 20°C, and detection was effected *via* fast scanning at 5 nm resolution in the range between 195 and 360 nm. The single wavelength electropherograms depicted are those registered for 195 nm. Before each experiment the capillary was rinsed with 0.1 M NaOH for 2 min, water for 2 min, and running buffer for 4 min. BioFocus Integration software (Version 6.0; Bio-Rad) was employed for data conversion and evaluation. The buffer employed was composed of 75 mM KH_2PO_4 buffer and 1% ethanolamine and was adjusted to pH 9.2 with 1 M HCl [32].

3 Results and discussion

3.1 Immunoassays for analysis of enantiomers of amphetamine, methamphetamine and methadone

As a continuation of our work characterizing competitive binding, electrokinetic capillary-based immunoassays for urinary amphetamines [32] using reagents which were commercialized for FPIA, analysis of enantiomers of A and MA by CZEIA in 50 mM borate buffer at pH 9.3 was investigated. This approach is based upon incubation of equal amounts of urine with the reagents S and T (25 μL each) prior to injection of a small aliquot of the mixture onto the capillary and analysis of the fluorescently labeled tracer *via* on-column LIF detection. Previously, the FPIA reagent kit was found to contain two immunoassay systems (two antibodies and two tracer molecules), one that recognizes mainly MA (A to a much lower degree only) and one that is geared towards monitoring of A (no apparent interaction with MA). In the monitored electropherograms, the peak size of the first tracer (peak MA in Fig. 1A) was observed to change in an MA dose-dependent manner. As the urinary MA concentration increased, an increase in the peak size of the free tracer was observed.

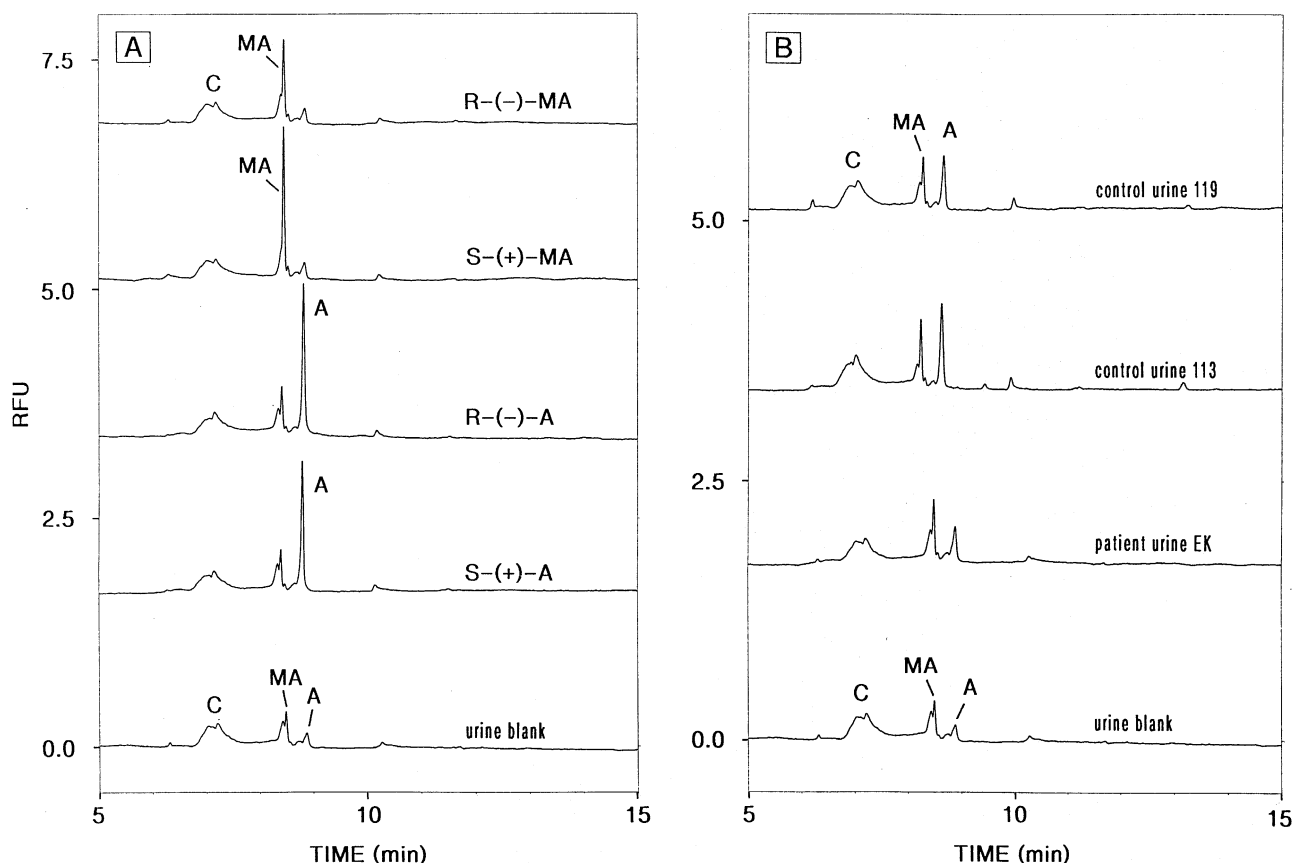


Figure 1. CZEIA data depicting the insufficient stereoselectivity associated with the monitoring of urinary enantiomers of A and MA. Data are presented with a y-scale offset of 1.7 RFU. The electropherograms shown in (A) represent those obtained with urine blank, and urine blank fortified with about 10 $\mu\text{g/mL}$ of *S*-(+)-A, about 10 $\mu\text{g/mL}$ of *R*-(-)-A, about 5 $\mu\text{g/mL}$ of *S*-(+)-MA and about 5 $\mu\text{g/mL}$ of *R*-(-)-MA (from bottom to top). Data of (B) are those of urine blank, patient EK who is under selegiline pharmacotherapy, control urine 113 and urine 119 (from bottom to top). A and MA denote the peaks associated with the fluorescing free tracers of A and MA, respectively. C marks the antibody tracer complexes. Experimental conditions as in Section 2.3.

The same was found to be true for the second tracer (peak A in Fig. 1A), except that it mainly reacted in the presence of A. In the CZEIA mode, detection limits for *S*-(+)-A and racemic MA were determined to be about 80 ng/mL [32].

The TDxFLx reagents employed are reported to contain *S*-(+)-A as calibrator substance and an amphetamine/methamphetamine antiserum with cross reactivities to *R*-(-)-A (cross-reactivity of 66% for a 10 $\mu\text{g/mL}$ drug level), *S*-(+)-MA (86% for 5 $\mu\text{g/mL}$) and *R*-(-)-MA (9.2% for 5 $\mu\text{g/mL}$) [34]. Furthermore, for urinary drug levels of 1 $\mu\text{g/mL}$, cross-reactivities for racemic A, MA, MDA, MDE and MDMA were reported to be 165, 62, 148, 43 and 97%, respectively [34]. Using CZEIA, reactivities of both immunoassay systems can easily be studied in the same experiment [32]. This prompted us to investigate the impact of the single enantiomers of A and MA on the two

competitive reaction systems. The data presented in Fig. 1A were obtained with blank urine (bottom electropherogram) and blank urine spiked with the enantiomers of A and MA at the 10 $\mu\text{g/mL}$ (for A) and 5 $\mu\text{g/mL}$ (for MA) concentration levels. Not surprisingly, the presented data show that both MA enantiomers produce a significantly elevated response of the MA tracer and no change in the A tracer peak. Furthermore, both enantiomers of A show much enhanced peaks of the A tracer and a small increase in the MA tracer peak. The data for MA indicate that there is stereoselectivity in the sense that the cross-reactivity for *R*-(-)-MA is smaller than that for *S*-(+)-MA. FPIA responses for the two MA urines were determined to be 343 ng/mL and 4394 ng/mL, respectively, values that are in good agreement with those reported by Cody and Schwartzhoff [34]. The same was found to be true for the A samples (6474 ng/mL) and high (> 8000 ng/mL) for urines containing about 10 $\mu\text{g/mL}$ *R*-(-)-A and *S*-(+)-A, re-

Table 1. Characteristic data of analyzed urines

Sample	Amphetamine and analogs				Methadone			
	Drug ^{a)} (concentration, ng/mL)	EMIT screening	FPIA (ng/mL)	Enantiomers found by chiral CE	Drug ^{a)} (concentration, ng/mL)	EMIT screening	FPIA (ng/mL)	Enantiomers found by chiral CE
113	S-(+)-A (1600)	Positive	2872	S-(+)-A	–	Negative	ND	none
106	rac. A (1500)	Positive	High (>8000)	R-(–)-A, S-(+)-A	Present	Positive	2953	R-(–)-MET, S-(+)-MET both EDDP enantiomers
122	rac. MDMA (3000) rac. MDA (300)	Positive	6690	R-(–)-MDMA, S-(+)- MDMA both MDA enantiomers	Present	Positive	1523	R-(–)-MET, S-(+)-MET both EDDP enantiomers
123	S-(+)-A (200) MA (1200)	Positive	5199	S-(+)-A, S-(+)-MA	Present	Positive	3766	R-(–)-MET, S-(+)-MET both EDDP enantiomers
119	S-(+)-A (400)	Positive	954	S-(+)-A	rac. MET (800)	Positive	949	R-(–)-MET, S-(+)-MET
Selegiline patient urine	–	Negative	108	R-(–)-MA, R-(–)-A	–	–	–	none

a) Values reported by UKNEQAS for Drugs of Abuse in Urine, Cardiff Bioanalytical Services, Cardiff CF1 6 DP, UK

spectively. As the concentration of the A samples is rather high, peak size differences in the monitored CZEIA electropherograms do not directly show the difference in reactivities (for calibration data refer to [32, 36]).

CZEIA data obtained from urine of a patient under selegiline pharmacotherapy and from two external quality control urines (specimens 113 and 119) are depicted in Fig. 1B. The electropherograms of these urines show elevated tracer peaks compared to those found with blank urine. FPIA data of the three samples were 108, 2872 and 954 ng/mL, respectively. Furthermore, according to the enzyme-multiplied immunoassay technique (EMIT) amphetamine/methamphetamine screening, these urines were found to test negative, positive, and positive, respectively (Table 1). The urine of a patient under selegiline pharmacotherapy is expected to comprise the *R*-enantiomers of A and MA whereas urines 113 and 119 are reported to contain *S*-(+)-A at concentrations of 1600 and 400 ng/mL, respectively. Based on these data, the conclusion can be drawn that the three urines contain substances that react with the antibodies involved. However, with the immunoassay data, no identification of the compounds involved, particularly their stereochemical nature, is possible. The same applies to the urine screening for methadone. FPIA performed in our laboratory for the analysis of *R*-(–)-MET, *S*-(+)-MET and a 1:1 mixture of the two fortified urines (racemic MET) at a MET concentration of about 1 µg/mL revealed responses of 566, 695, and 835 ng/mL, respectively. These data indicate that both enantiomers can be detected with similar cross-reactivity. Data confirming these findings could neither be found in the literature nor obtained from the manufacturer of the assay reagents. Furthermore the pamphlet pro-

vided by the manufacturer does not reveal the exact nature of the calibrator substance involved. Using chiral CE (see below), it could be identified as *S*-(+)-MET.

3.2 Separation of urinary enantiomers of amphetamine and analogs, MET and EDDP by chiral CE

For enantiomeric separation of basic drugs, acidic phosphate buffers containing different types of cyclodextrins as chiral selectors are typically employed [14, 20–23, 26, 29, 30]. In our previous investigations, urinary enantiomers of MDMA and two of its metabolites [14, 26] as well as urinary enantiomers of MET and its major metabolite EDDP [29] were determined by chiral CE using an acidic KH₂PO₄ buffer and OHP-β-CD. The same buffer was found to be suitable for the simultaneous separation of the enantiomers of A, MA, MDA, MDMA, MDE, EDDP and MET (in the order of detection; Figs. 2A and 3). The *R*-(–)-enantiomers of A, MA, MDMA and MET were detected before the corresponding *S*-(+)-enantiomers. Unfortunately, no pure enantiomers were available for MDA, MDE and EDDP. However, due to the structure similarity of the compound pairs A/MA (same propylamine residue on the aromatic ring and difference of a methyl group [32]) and MDA/MDMA (same methylenedioxy residue on the aromatic ring and difference of a methyl group only [26, 32]), it can be assumed that *R*-(–)-MDA is also detected ahead of *S*-(+)-MDA. The same should be true for the enantiomers of MDE. Furthermore, in analogy to the work of Frost *et al.* [30] in which EDDP enantiomers were analyzed in a similar buffer and assignment was possible *via* urinary EDDP analysis after ingestion of *R*-MET, *R*-EDDP most likely migrates faster than

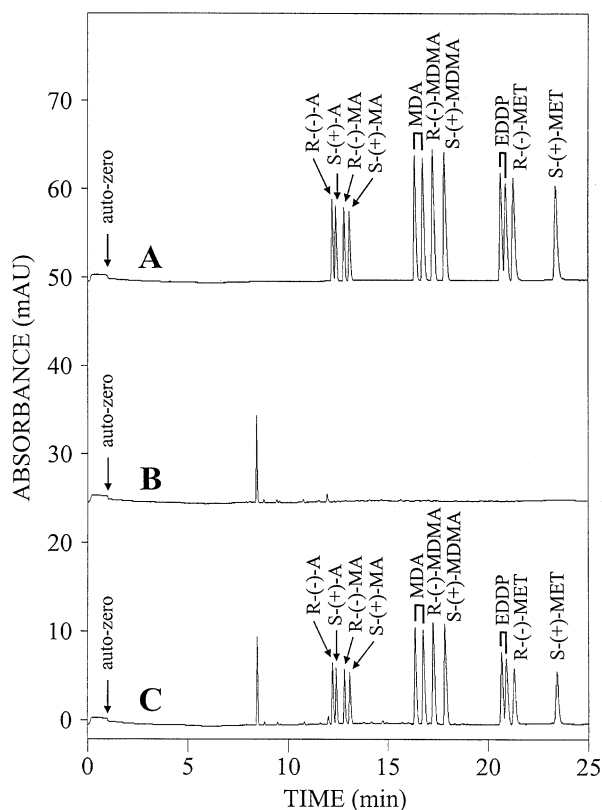


Figure 2. Electropherograms obtained with the chiral buffer of (A) A, MA, MDA, MDMA, MET and EDDP (about 20 $\mu\text{g/mL}$ of each enantiomer) dissolved in water, (B) after liquid/liquid extraction of blank urine, and (C) after liquid/liquid extraction of urine blank spiked with about 1 $\mu\text{g/mL}$ of each enantiomer. The apparatus used was the P/ACE 5510 and the y-scale offset was set to 25 mAU. Other conditions as in Sections 2.4 and 2.6.

S-EDDP. For A, MA, MDA, MDMA, MDE and MET (Fig. 3), an increase in enantioresolution as a function of the detection order was observed. It appears that larger residues around the chiral center are capable of forming stronger inclusion complexes, this resulting in enhanced enantioresolution.

The data presented in Fig. 2B indicate that analysis of extracts of urine blank results in clean electropherograms. Data obtained with a urine blank that was fortified with racemic drugs at about 1 $\mu\text{g/mL}$ of each enantiomer are presented in Fig. 2C. Using the same liquid/liquid extraction scheme, we previously determined that extraction recoveries for A, MA, MDA and MDMA were about 80% or slightly more [32]. Comparison of the data presented in panels A and C reveals that EDDP and MET are recovered as well. On the assessed drug level, no endogenous compounds appear to interfere with the compounds of interest. Furthermore, peak height ratios of enantiomers analyzed as racemic standards and in the extract appear to be equal. Commencing with 2 mL of urine and with extract reconstitution in 100 μL water (*cf.* Section 2.4), spectral identification limits for the enantiomers of A, MA, MDA, MDMA, EDDP and MET were found to be between 100 and 200 ng/mL. Detection limits ($S/N = 3$) were three to five times lower. Reproducibility was assessed on the 1000 ng/mL enantiomer concentration level *via* extraction of four 2 mL aliquots of fortified urine blank (conditions as for Fig. 2C) and consecutive analysis of the four reconstituted extracts. Data obtained are summarized in Table 2. Without internal standard, imprecision values for detection times were noted to increase with an increase in detection time and were all well below 0.5%. RSD values

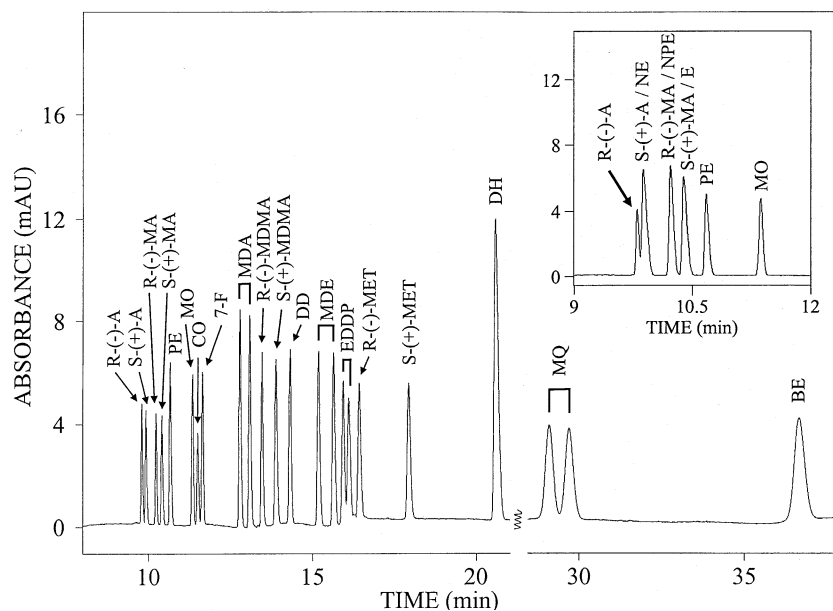


Figure 3. Electropherogram obtained with a model mixture comprising racemic A, MA, MDA, MDMA, MDE, EDDP and MET together with PE, MO, CO, 7-F, DD, DH, MQ and BE (about 10 $\mu\text{g/mL}$ of each enantiomer and about 20 $\mu\text{g/mL}$ of each achiral compound). The inset shows part of an electropherogram for a sample with about 8.5 $\mu\text{g/mL}$ of each enantiomer that was fortified with NE, NPE and E (about 17 $\mu\text{g/mL}$ of each). The apparatus used was the P/ACE 5510 with an applied voltage of 27 kV (current about 75 μA).

Table 2. Typical precision data of the chiral CE assay^{a)}

Enantiomer	RSD values of 4 repeats (%)										
	Without internal standard					With <i>S</i> (+)-A as internal standard			With <i>S</i> (+)-MDA as internal standard		
	Detection time	Peak area	Peak height	Relative peak area	Relative peak height	Relative detection time	Relative peak area	Relative peak height	Relative detection time	Relative peak area	Relative peak height
<i>R</i> (-)-A	0.22	9.47	8.50	9.27	8.30	0.010	0.16	0.41	0.107	4.48	4.63
<i>S</i> (+)-A	0.22	9.51	8.22	9.30	8.01	–	–	–	0.100	4.47	4.39
<i>R</i> (-)-MA	0.23	9.06	8.45	8.85	8.24	0.005	1.38	1.42	0.098	4.61	5.06
<i>S</i> (+)-MA	0.24	8.62	7.90	8.39	7.67	0.012	2.11	1.36	0.088	4.63	4.73
<i>R</i> (-)-MDA	0.31	4.85	4.55	4.65	4.32	0.086	5.63	4.27	0.015	1.24	0.42
<i>S</i> (+)-MDA	0.32	5.54	4.79	5.29	4.57	0.100	4.45	4.39	–	–	–
<i>R</i> (-)-MDMA	0.32	5.26	4.28	5.02	4.06	0.106	5.03	4.70	0.007	0.62	0.51
<i>S</i> (+)-MDMA	0.33	4.67	4.34	4.53	4.11	0.114	6.87	4.62	0.014	2.57	0.45
<i>R</i> -EDDP	0.35	8.79	8.69	8.47	8.38	0.138	1.71	1.60	0.040	3.22	4.05
<i>S</i> -EDDP	0.36	8.80	8.23	8.49	7.93	0.138	1.89	1.82	0.039	3.26	3.57
<i>R</i> (-)-MET	0.35	12.9	11.6	12.5	11.3	0.131	3.65	3.89	0.034	7.45	7.12
<i>S</i> (+)-MET	0.39	16.5	15.0	16.1	14.6	0.177	6.45	6.29	0.078	10.6	10.1

a) Conditions as for data of Fig. 2C.

for peak areas, peak heights and relative peak properties (*i.e.*, peak property divided by detection time) were determined to be between 4.1 and 16.5%. With use of an internal standard, greatly reduced RSD values were noted (Table 2).

Urine stemming from polydrug abusers typically contain a large number of different substances. Thus, the chiral CE assay described was tested for interferences with a range of compounds that are also extracted at alkaline pH. Figure 3 presents a typical electropherogram that was obtained with the compounds of interest (Fig. 2), racemic MDE, and other basic drugs that are expected to show up in extracts of unhydrolyzed urine. PE, MO, CO and 7-F (main urinary metabolite of flunitrazepam) were found to separate well and migrate between *S*(+)-MA and *R*(-)-MDA. Furthermore, DD (active metabolite of diazepam) appeared fully resolved between *S*(+)-MDMA and the MDE enantiomers, whereas DH and BE (urinary metabolite of cocaine) were detected significantly after the enantiomers of MET. Finally, MQ was found to produce a double peak. MQ represents an atropisomeric compound without a chiral C atom, steric hindrance of the rotation of the nitrogen-carbon bond to the tolyl group being responsible for the selective interaction with the chiral selector [37]. Although the data presented in Fig. 3 show high resolution, interferences of certain ephedrine with enantiomers of A and MA were observed (inset of Fig. 3). NE, NPE (cathine) and E were found to comigrate with *S*(+)-A, *R*(-)-MA and *S*(+)-MA, respectively. In

order to be able to properly distinguish between A and analogs with ephedrine, the achiral assay as described previously [32] could be employed.

3.3 Confirmation testing for enantiomers of amphetamine and analogs, MET and EDDP, in quality control urines by chiral CE with multiwavelength detection

A number of external quality control urines were analyzed by chiral CE. The data presented in Fig. 4 were obtained *via* analysis of the basic extract of urine 113, a urine that was reported to stem from a subject who was treated with simvastatin (20 mg/day) and salicylate (150 mg/day) and to have been fortified with *S*(+)-A (1600 ng/mL), quinalbarbitone (800 ng/mL), oxazepam (400 ng/mL), morphine (800 ng/mL) and phencyclidine (120 ng/mL) [38]. Data obtained by EMIT screening and FPIA are presented in Table 1. Using chiral CE, a relative simple electropherogram was monitored (Fig. 4). According to the detection time and the three-dimensional data presented in Fig. 4B, the peak detected after 13.4 min was suspected to be A or MA. Spectral confirmation with *S*(+)-A, *i.e.*, comparison of the normalized spectrum of the peak with that monitored separately *via* injection of a standard of *S*(+)-A (inset in panel A), suggested that the monitored peak could indeed represent this enantiomer of A, a banned substance in many countries. However, as enantiomers of A and MA have identical spectra, complete confirmation was only possible by spiking the extract and reanaly-

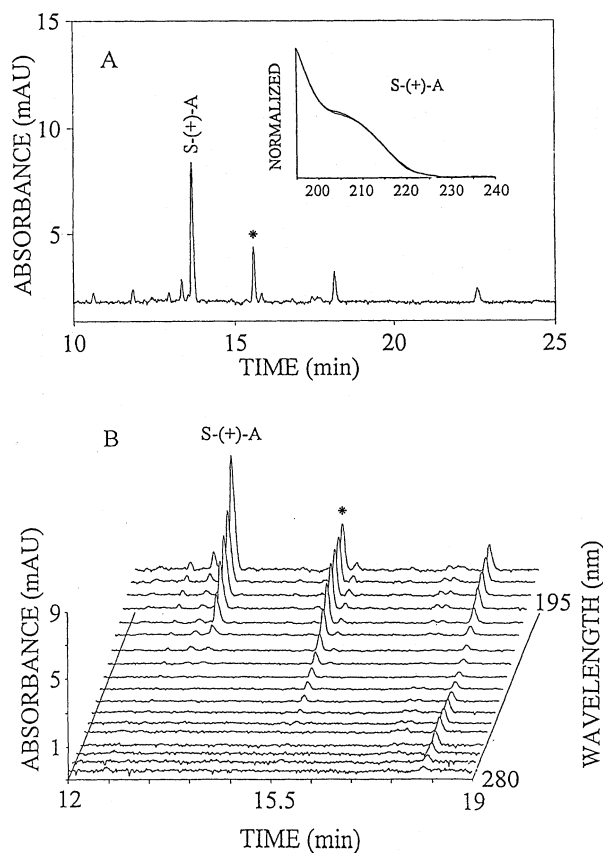


Figure 4. Chiral CE data of quality control sample 113; (A) depicts single-wavelength data at 195 nm whereas (B) shows the three-dimensional electropherogram between 195 and 280 nm (same run). The inset in (A) depicts spectral identity proof of A. The apparatus used was the BioFocus 3000 and the injection time was 4 psi $\times$ s. The peak marked with an asterisk represents MO. Other conditions as in Sections 2.4 and 2.5.

sis (data not shown). Although the CZEIA data presented in Fig. 1B revealed a clear response for A, enantiomeric identification is only possible with the chiral assay. Furthermore, the presence of MO (peak marked with asterisk in Fig. 4) could be confirmed *via* spectral analysis.

Single wavelength electropherograms obtained with the extracts of four other quality control urines are shown in Fig. 5. Immunoassay data obtained with these urines are presented in Table 1. The data depicted as graph A of Fig. 5 were monitored for urine 106, a specimen that stemmed from two polydrug abusers ingesting heroin, phenobarbitone, methadone and diazepam, and was (i) blended with a urine containing cannabinoids and (ii) fortified with racemic A (1500 ng/mL), buprenorphine (0.5 ng/mL) and phenylclidine (80 ng/mL). Compared to urine 113, a more complex electropherogram was obtained. Comparison of detection times and spectra revealed the

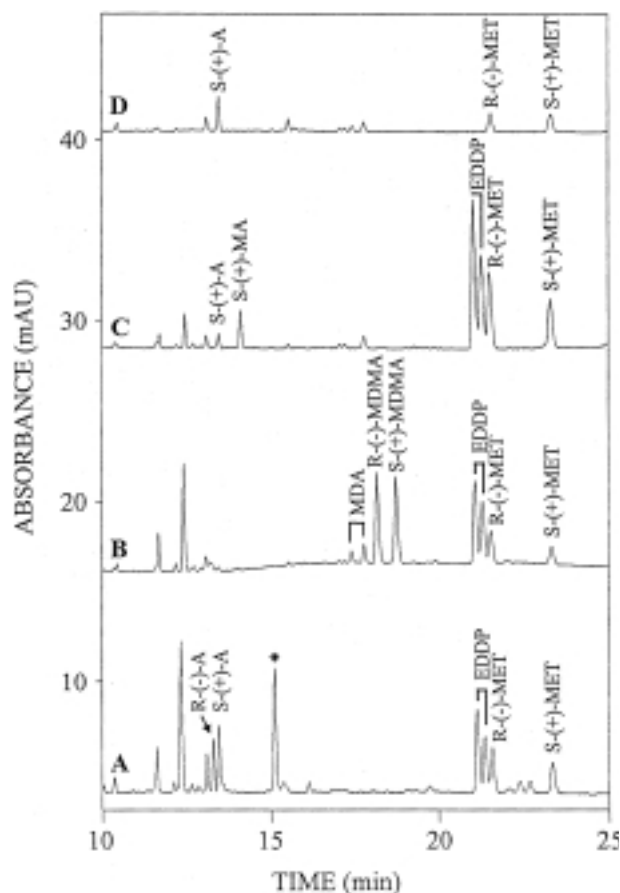


Figure 5. Single wavelength chiral CE data of quality control samples (A) 106, (B) 122, (C) 123, and (D) 119 presented with a y-scale offset of 13 mAU. The BioFocus 3000 was used. Experimental conditions were as in Fig. 4 except that the electropherograms were monitored at 195 nm only and the injection time was 2 psi $\times$ s. The peak marked with an asterisk (graph A) represents MO.

presence of the enantiomers of MET and EDDP, as well as free MO (marked with an asterisk). Furthermore, the identity of *R*-(-)-A and *S*-(+)-A could be revealed by spectral analysis and spiking of the extract prior to reanalysis (data not shown). Similarly, enantiomers of various compounds could easily be identified for urines 122, 123 and 119 (Fig. 5 B–D). Urine 122 stemmed from a patient undergoing treatment in a locked drug abuse ward. The urine contains cannabinoids, MET and EDDP, diazepam, temazepam and carbamazepine and was spiked with phenylclidine (40 ng/mL), racemic MDMA (3000 ng/mL) and racemic MDA (300 ng/mL). The presence of the enantiomers of MET, EDDP, MDMA and MDA was confirmed with our chiral CE assay (Fig. 5B). Sample 123 was reported to be a mixture of two patient urines – one patient being treated with methadone and the other with simvastatin (20 mg/day) and salicylate (150 mg/day) – that was spiked with MA (1200 ng/mL), *S*-(+)-A (200 ng/

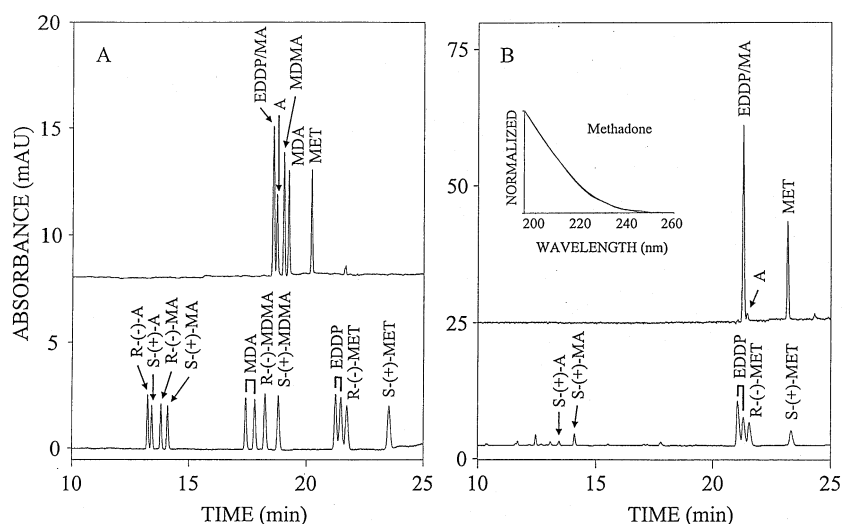


Figure 6. Achiral (top graphs, experimental conditions as in Sections 2.4 and 2.7) and chiral (bottom graphs, experimental conditions as in Sections 2.4 and 2.6 with a 2 psi $\times$ s injection) CE data of (A) a mixture of standard compounds, and (B) the extract of quality control urine 123. The inset in (B) depicts the spectral identity proof of MET monitored in the achiral assay. For the data of (A), standard compounds were dissolved in water at a concentration of about 5 μ g/mL (achiral data) and 10 μ g/mL (chiral data) of each enantiomer. The BioFocus 3000 was used.

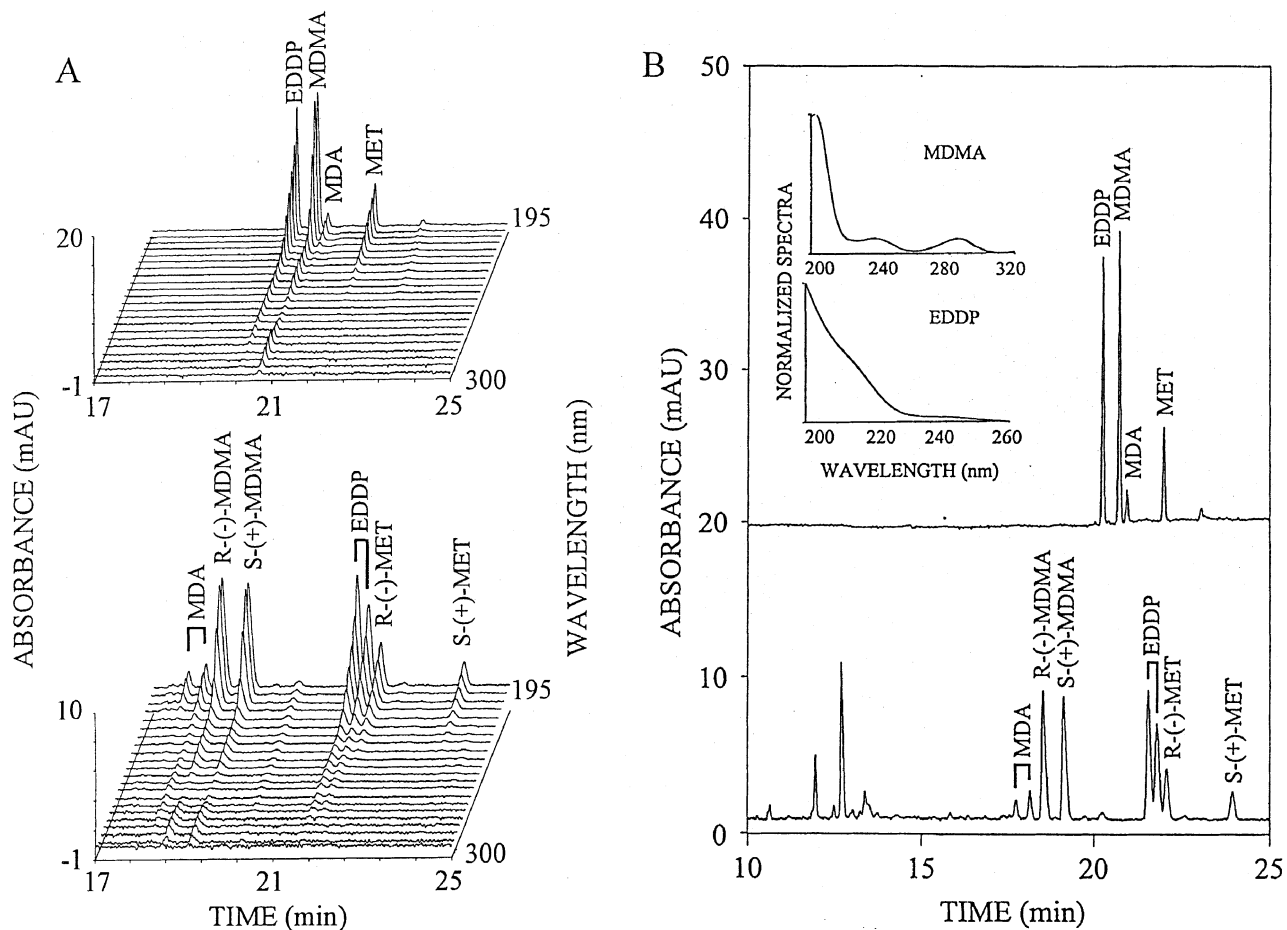


Figure 7. (A) Three-dimensional electropherograms and (B) single wavelength data at 195 nm of urine 122 obtained in the achiral (top, experimental conditions as in Section 2.7) and chiral (bottom, experimental conditions as in Section 2.5 with a 4 psi $\times$ s injection) buffers. The insets in (B) represent spectral identity proof of MDMA and EDDP. The BioFocus 3000 was used.

mL), buprenorphine (1 ng/mL) and LSD (7 ng/mL). Using our chiral CE assay, this specimen was found to contain *S*(+)-MA and *S*(+)-A and the enantiomers of MET and EDDP (Fig. 5C). No *R*(-)-enantiomer of MA was monitored, this suggesting that the sample was spiked with *S*(+)-MA rather than racemic MA (the nature of the added MA was not specified on the reported data sheet). Finally, analysis of the extract of urine 119 revealed the presence of equal amounts of the enantiomers of MET and *S*(+)-A (Fig. 5D). This urine was reported to have been spiked with *S*(+)-A (400 ng/mL), benzoylecgonine (900 ng/mL), MET (800 ng/mL), buprenorphine (0.5 ng/mL) and LSD (4.2 ng/mL) and did not stem from a drug abuser. Thus, our assay was again shown to provide correct data for that specimen. The MET profile of urine 119 is distinctly different from that of the others (compare with Fig. 5A–C) and there was no EDDP detected. After MET consumption, EDDP (major metabolite of MET) is typically excreted at a higher concentration than MET (see Figs. 5A–C), a pattern that is typical for patients undergoing racemic methadone therapy [29, 39]. Furthermore, the peak heights of *R*(-)-MET and the first eluting enantiomer of EDDP (*R*-EDDP) are higher than those of *S*-enantiomers. These observations are also in agreement with those reported for urines of patients under racemic MET pharmacotherapy [29, 30].

The chiral CE assay for urinary A and analogs, MET and EDDP is shown to provide insight into the enantiomeric composition of urine specimens. With the previously published achiral assay [32], this information was not accessible. Furthermore, in order to be able to properly distinguish between A and analogs with ephedrine, the achiral assay was operated at alkaline pH. Using a capillary of almost 100 cm effective length, resolution of the various basic compounds of interest was somewhat limited and MA and EDDP were typically found to comigrate (top panels in Figs. 6A and B). With the chiral assay, distinction between MA and EDDP is much easier (bottom panels in Figs. 6A and B). Not surprisingly, in absence of MA, EDDP can be determined properly using the achiral buffer (Fig. 7). Note that, as the data presented in Fig. 7 clearly indicate, chiral analysis provides much increased information at no expense to analysis time. Furthermore, the small amount of chiral selector required for that analysis increases the overall cost of the determination by only a negligible amount.

3.4 Determination of the *R*(-)-enantiomers of A and MA in a urine of a patient under selegiline pharmacotherapy

Chiral discrimination is an important aspect, as certain enantiomers of A and analogs are metabolites of a thera-

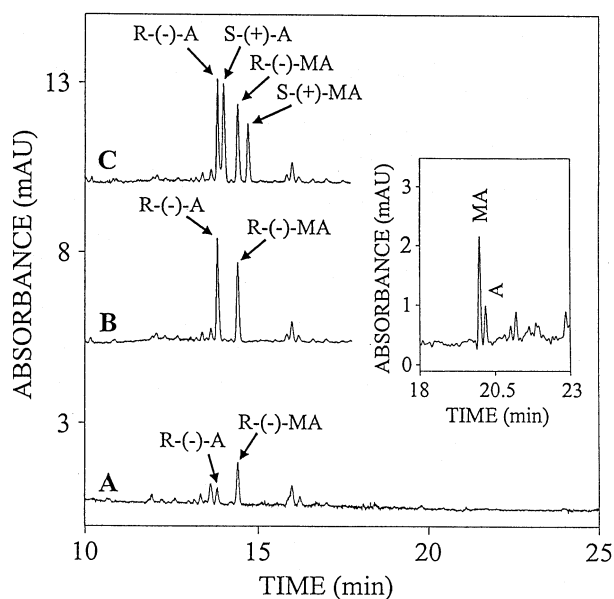


Figure 8. Chiral electropherograms of (A) the extracted urine of a patient under selegiline treatment, (B) the same urine extract spiked with about 10 µg/mL *R*(-)-A and *R*(-)-MA, and (C) the same urine extract spiked with about 10 µg/mL of each A and MA enantiomer. Injection conditions were 2 psi $\times$ s and the y-scale offset was set to 4 mAU. The BioFocus 3000 was used. Other conditions as in Sections 2.4 and 2.5. The inset depicts data obtained with the achiral buffer (for conditions refer to Section 2.7).

peutic drug which the immunological assay is unable to differentiate. As an example, *R*(-)-MA (the L-enantiomer of MA) and *R*(-)-A (the L-enantiomer of A) are metabolites of selegiline, a drug used to treat Parkinson's disease [4–6]. The data presented in Fig. 8A were obtained for analysis of a urine sample of a patient under selegiline treatment (5 mg twice daily). Spiking the urinary extract with the *R*(-)-enantiomers of A and MA and reanalysis under the same conditions (Fig. 8B) revealed assignment of the two selegiline metabolites. Finally, to make the picture complete, data obtained after spiking with the *R*(-)-enantiomers and the *S*(+)-enantiomers are depicted in Fig. 8C. As the *R*(-)-A peak was too small, spectral identity could not be assessed for that compound. Based upon peak heights, this patient urine was estimated to contain about 110 ng/mL *R*(-)-A and about 270 ng/mL *R*(-)-MA. FPIA analysis on the TDxFLx revealed a response corresponding to 108 ng/mL of the calibrator substance. The EMIT screening result was negative. Using the CZEIA, an increased response compared to that monitored for the urine blank was noted (Fig. 1B). The achiral CE protocol (inset of Fig. 8) recognized the presence of urinary A and MA but could not differentiate between *R*- and *S*-enantiomers. The chiral CE assay is shown to permit differentiating between a urine of a pa-

tient under pharmacotherapy with a drug that metabolizes to the *R*-enantiomers of MA and A and a urine of a drug abuser who typically ingests racemic or the more potent *S*(+)-enantiomers of MA and/or A (Figs. 4, 5C, and 6).

4 Concluding remarks

Chiral CE is shown to be a highly efficient technique for analysis of urinary enantiomers of amphetamines and analogs together with those of EDDP and MET. The assay is demonstrated to be capable of identifying the enantiomers of A, MA, MDA, MDMA, EDDP and MET in alkaline extracts of unhydrolyzed toxicological quality control urines. Furthermore, the chiral CE method can be employed for the determination of the characteristic urinary metabolites of selegiline, namely *R*(-)-A and *R*(-)-MA. Chiral determination of these compounds is important in clinical and forensic analysis. It can be employed for confirmatory testing as well as exclusion of false positive results and recognition of false negative data produced by immunological screening techniques. However, care should be taken in interpreting data associated with A and MA. Without identification of a metabolic precursor of MA/A (parent drug and/or specific metabolite other than MA/A) or enantioselective analysis of the MA/A excretion pattern, illegal consumption of MA/A cannot be confirmed. Following administration of a drug such as fenproporex [1, 40], the parent compound is typically detected only for a few hours after drug administration and A can be found for several days. Furthermore, the urinary enantiomeric ratio of A/MA can be of help in the identification of a parent drug. For example, A/MA enantiomeric ratios after selegiline intake were found to be between 0.37–0.42 ([6], Fig. 8), whereas in urines of users of Vick's Inhaler A/MA, enantiomeric ratios of 0–0.12 were found [6, 41].

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