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Journal of Chromatography A, 875 (2000) 27–41

JOURNAL OF CHROMATOGRAPHY A

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Review

Enantioselective determination of drugs in body fluids by capillary electrophoresis

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Abstract

During the past decade, chiral capillary electrophoresis (CE) emerged as a promising, effective and economic approach for the enantioselective determination of drugs in body fluids, hair and microsomal preparations. This review discusses the principles and important aspects of CE-based chiral bioassays, provides a survey of the assays developed and presents an overview of the key achievements encountered. Applications discussed encompass the pharmacokinetics of drug enantiomers, the elucidation of the stereoselectivity of drug metabolism and bioanalysis of drug enantiomers of toxicological and forensic interest. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Reviews; Pharmacokinetics; Stereoselective metabolism; Drugs

Contents

1. Introduction

About one fourth of all therapeutic agents is administered to man as mixtures of isomeric substances whose biological activity may well reside predominantly in one form. The majority of these are racemic mixtures of synthetic chiral drugs. It is well known that receptors and enzymes which are the targets of drug action are able to discriminate between stereoisomers. The use of racemic mixtures typically results in stereoselective drug metabolism and may also contribute to the toxicity or adverse effects encountered with drugs. Thus, the significance of stereochemical considerations in drug metabolism and pharmacokinetics has become an issue for both the pharmaceutical industry and the regula-

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PII: S0021-9673(99)01042-0

tory authorities. This new trend produced an increase in the demand for stereoselective separation techniques and analytical assays for the detection of enantiomeric purity in connection with drug synthesis and with biological, pharmacological, pharmacokinetic and clinical investigations. Furthermore, as certain enantiomers represent illicit or banned substances, chiral discrimination represents also an important topic in forensic analysis and doping control [1–4].

Stereospecific drug monitoring is widely accomplished via use of chromatographic methods which require rather expensive chiral stationary phases [4]. During the past few years chiral separations by capillary electromigration methods have been studied extensively and shown to provide high-resolution at low cost [5–10]. For enantiomeric separation under electrokinetic conditions, a chiral selector [such as a cyclodextrin (CD), a crown ether, a protein or a bile acid, to name but a few] and proper buffer conditions (pH, ionic strength, micelles, additives etc.) are required. Two methods have mainly been employed, namely capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). CZE is conducted within a plain buffer in which mainly charged solutes can be separated only. MECC is an electrokinetic separation technique that includes micelles. Micelles and surrounding buffer are being transported at different velocities which also permits separation and analysis of neutral solutes. In these approaches, the chiral selector is typically a buffer additive. Furthermore, Snopek et al. [11] reported high-resolution capillary isotachophoretic separations of drug enantiomers having various CDs in the leading electrolyte and Thormann et al. studied the separation of methadone enantiomers by capillary and recycling isotachophoresis [12], as well as continuous flow and interval flow electrophoresis [13]. Finally, chiral selectors incorporated into packed or gel-filled capillary columns lead to chiral capillary electrochromatography (CEC) [14,15] and bond to the capillary surface to open tubular electrochromatography (OTEC) [16]. Using enantioselective electrokinetic separation technology, applications include purity control of drugs [17], analysis of enantiomers of forensic interest and identification of the origin of drugs of abuse [18], preparation of enantiomerically enriched or pure chiral drug standards [12,13,19] and bioanalytical drug monitoring [20].

In this paper, capillary electrophoresis (CE) based assays for analysis of enantiomers of drugs and their major metabolites in biological samples [19,21–68] are comprehensively reviewed. For the sake of providing a deeper insight into the achievements made, distinction between enantiomer analysis in fortified body fluids (Table 1) and real world samples (Tables 2–5) is made. Furthermore, work dealing with the enantioseparation of drug standards is mentioned only if required for illustration of important features and to make a case for ongoing or future work. Examples discussed in more detail and illustrations comprise data from our laboratory.

2. Principles

All chiral drugs and their enantiomers analyzed thus far in body fluids, hair and microsomal preparations (Tables 1–5) are regular chiral compounds which do not have chiral heteroatoms. Enantiomers of drugs have been determined mainly in blood (plasma and serum) and urine (Tables 1–3, 5). Furthermore, enantiomers of zopiclone have been analyzed in saliva [29], of isoproterenol in microdialysates [42], of methadone in hair [25] and of amphetamine and analogs in hair [39,40]. No report dealing with the enantiomeric monitoring of a drug in a tissue could be found. Finally, for a number of drugs and metabolites, enantiomers in microsomal preparations have been determined (Table 4, [43–47]). As of today, all work dealing with the enantioselective determination of drugs in body fluids, hair and microsomal preparations is associated with the use of chiral CZE and MECC methods (Tables 1–5). Although there is a wide variety of chiral selectors available [5–10], most assays are based on the use of native or modified CDs (Tables 1–5) and two papers report the employment of linear maltooligosaccharides [56,65]. Furthermore, urinary ephedrine enantiomers have been determined in presence of (*S*)-*N*-dodecoxycarbonylvaline [54], urinary etodolac and two metabolites have been analyzed with vancomycin as chiral selector [34], and various applications report the combined use of a CD and another chiral selector [27,30,45]. Most of

Table 1
Papers reporting work with body fluids that were fortified with drug enantiomers[a]

Drug	Body fluid	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Amphetamine and analogs	Urine	CZE	OHP-β-CD	2.5	DSI, ultrafiltration, SPE, LLE	UV/200	[48]
Atropine	Serum	CZE	TM-β-CD	2.8	Protein precipitation with acetonitrile	UV/200	[49]
Bambuterol	Plasma	CZE	DM-β-CD	2.5	Supported liquid membrane (SLM) extraction	UV/205	[50]
Bunitrolol	Plasma	CZE	β-CD-phosphate	5.0	DSI	UV/210	[51]
Bupivacaine (mepivacaine as I.S.)	Serum	CZE	DM-β-CD	2.9	LLE	UV/220	[52]
Clenbuterol	Urine	CZE	DM-β-CD	2.5	DSI	UV/214	[53]
Ephedrine	Urine	MECC	(*S*)-*N*-dodecoxycarbonylvaline	8.8	DSI (filtration)	UV/214	[54]
Hexobarbital	Rat plasma	MECC	β-CD	7.0	DSI	UV/214	[55]
Ibuprofen	Serum	CZE	Maltrin MO40 (linear maltoologosaccharide)	7.8	LLE	UV/220	[56]
Leucovorin, 5-methyl-tetrahydrofolate	Plasma	CZE	γ-CD	7.0	Protein denaturation with urea+ ultrafiltration	UV/289	[57]
Mepivacaine	Serum	CZE	DM-β-CD	2.5	LLE	UV/215	[58]
Ondansetron	Serum	CZE	DM-β-CD	2.5	SPE	UV/254	[59]
Pentobarbital	Serum	CZE	OHP-γ-CD	9.0	SPE	UV/254	[60]
Prilocaine	Serum	CZE	DM-β-CD	2.5	SPE	UV/215	[61]
Primaquine	Plasma	CZE	β-CD-phosphate	5.0	DSI	UV/210	[51]
Secobarbital	Serum	CZE	OHP-γ-CD	9.0	SPE	UV/254	[62]
Terbutaline	Urine	CZE	DM-β-CD	2.5–3.0	DSI (filtration)	UV/200, ion spray MS	[63]
Terbutaline	Plasma	CZE	DM-β-CD	2.5	Coupled column liquid chromatography (CLC)	UV/210	[64]
Verapamil	Serum	CZE	TM-β-CD	2.7	LLE	UV/215	[52]
Warfarin	Plasma	CZE	Glucidex 2 (linear maltooligosaccharide)	7.0	LLE	UV/185	[65]

[a] All assays are based upon hydrodynamic sample injection.

the employed CDs are neutral. Charged CDs were used for enantiomeric analysis of bunitrolol and primaquine enantiomers in plasma ([51]; β-CD-phosphate), tramadol in urine { [32]; carboxymethyl-β-CD (CM-β-CD) at pH 10.1}, thalidomide in rat liver microsomal preparation ([46]; CM-β-CD at pH 6.0) and praziquantel in rat liver microsomal preparation ([47]; sulfobutylether-β-CD). It is important to realize that in chiral bioanalytical drug monitoring selection of a suitable chiral buffer additive is not only dependent on the drug administered. In many cases, enantioselective determination of a drug together with one or several metabolites is encountered. Thus, for use of a single assay, a chiral selector capable of resolving the enantiomers of the drug and those of the metabolites is required. Enantiomeric resolution in presence of large concentration differences between compounds of interest represents another challenging goal (for an example refer to Fig. 1 and Ref. [38]). Optimized conditions are obtained via careful adjustment of the concentration of the chiral selector, buffer pH, ionic strength and amount of additives.

Most chiral assays are based on the use of untreated fused-silica capillaries. Polyacrylamide-coated capillaries were employed for the enantiomeric analysis of tramadol [66], etodolac [34], bunitrolol and primaquine [51], bupivacaine [52] and amphetamine and analogs [39]. In some assays hexadecyltrimethylammonium bromide (HTAB) is used as buffer additive [52,58,59,61]. Hexadecyltrimethylammonium covers the negatively charged silica capillary wall and thereby prevents adsorption of positively charged endogenous compounds (e.g., proteins) and reverses electroosmosis. The use of HTAB or a similar ammonium salt can be important in CZE with direct injection of proteinaceous samples, but is not required in presence of dodecyl

Table 2
Monitoring of enantioselective pharmacokinetics and elimination kinetics in samples of patients, volunteers and animals

Drug	Chiral metabolite	Sample	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Cicletanine	–	Plasma, urine	MECC	γ-CD	8.6	LLE, H-LLE	UV/214	[21]
Dimethindene	*N*-Demethyldimethindene	Urine	CZE	OHP-β-CD	3.3	LLE	UV/200	[23]
Oxprenolol	CGP 6423, CGP 27905	Urine	CZE	OHP-β-CD	2.5	H-LLE	UV/200	[26]
Tramadol	*O*-Demethyltramadol	Urine	CZE	CM-β-CD	10.1	LLE	UV/214	[32]
Tramadol	*O*-Demethyltramadol, *N*-demethyltramadol, *O*-demethyl-*N*-demethyltramadol	Urine	CZE	CM-β-CD	2.5	LLE	UV/195	[66]
MDMA	MDA, HMMA	Urine	CZE	OHP-β-CD	2.5	DSI, SPE, H-SPE	UV/195	[38]
Zopiclone	*N*-Desmethylzopiclone, zopiclone-*N*-oxide	Urine, saliva	CZE	β-CD	2.75	LLE	LIF/325/450	[29]
Cicletanine	Cicletanine glucuronide, cicletaninesulfate	Rat urine	MECC	γ-CD	8.6	H-LLE	UV/214	[22]
Isoproterenol	–	Microdialysis samples from jugular vein of rats	CZE	M-β-CD	4.75	DSI	Amperometric detection, UV/220	[42,68]

Table 3
Assessment of the enantioselective drug metabolism in samples of patients, volunteers and drug abusers

Drug	Chiral metabolite	Sample	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Methadone	EDDP	Urine	CZE	OHP-β-CD	3.0	DSI, SPE	UV/195, 195–320/5	[24]
Methadone	EDDP	Serum, urine, hair	CZE	DM-β-CD	2.3	LLE	UV/200	[25]
Thiopental	Pentobarbital	Serum	MECC	γ-CD and (+)-campher-10-sulfonic acid	9.2	LLE	UV/200–320/5	[27]
Thiopental	Pentobarbital	Plasma	CZE	OHP-γ-CD	8.5	LLE	UV195–360/5, UV/200/245/300	[19]
Warfarin	–	Plasma	CZE	DM-β-CD	8.35	LLE	UV/310	[28]
Ibuprofen	2-Hydroxyibuprofen, 2-carboxyibuprofen and glucuronides	Urine	CZE	Dextrin 10 and TM-β-CD	5.26	(H)-SPE	UV/214	[30]
Clenbuterol	–	Urine	CZE	OH-ethyl-β-CD	3.3	LLE	UV/210	[31]
Verapamil	Norverapamil	Plasma	CZE	TM-β-CD	2.5	LLE	UV/200	[33]
Etodolac	7-Hydroxyetodolac, 8-(1′-hydroxyethyl)-ethodolac	Urine	CZE	Vancomycin	4.8	DSI	ESI-MS	[34]
Selegiline (not measured)	*R*-(−)-Amphetamine *R*-(−)-Methamphetamine	Urine	CZE	OHP-β-CD	2.5	LLE (Toxitube A)	UV/195 UV/195–320	[41]
Haloperidol[a]	Reduced haloperidol	Plasma	CZE	DM-β-CD	2.5	LLE	UV/200	[67]
Debrisoquine[a]	4-Hydroxydebrisoquine	Urine	CZE	TM-β-CD	2.5	SPE	UV/195	[35]
Mephenytoin	4-Hydroxymephenytoin	Urine	MECC	β-CD	9.1	H-DSI, LLE	UV/192, 192–297/5	[36]
Phenytoin[a]	4-Hydroxyphenytoin	Urine	MECC	β-CD	9.1	H-LLE	UV/192, 192–297/5	[36]

[a] Achiral drug.

Table 4
In vitro assessment of the stereoselectivity of drug metabolism using microsomal preparations

Drug	Chiral metabolite(s)	Sample matrix	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Flezelastine	*n*-Dephenethylflezelastine	Human, rat, bovine, porcine liver microsomes	CZE	β-CD	3.75	LLE	UV/210	[44]
Chrysene[a] (not measured)	*trans*-1,2-Dihydro-1,2-dihydroxychrysene	Rat liver microsomes	MECC	γ-CD	7.8	LLE	UV/220, 195–300	[43]
Mephenytoin	4-Hydroxymephenytoin	Human hepatic microsomes	MECC	Taurodeoxycholic acid and β-CD	7.2	Extraction	UV/214	[45]
Praziquantel	*trans*-4-Hydroxypraziquantel, *cis*-4-hydroxypraziquantel	Rat liver microsomes	CZE	Sulfobutylether-β-CD	5.25	LLE	UV/210	[47]
Thalidomide	4-Hydroxythalidomide, 5-hydroxythalidomide	Rat liver microsomes	CZE	CM-β-CD	6.0	LLE	UV/225	[46]

[a] Achiral substance.

sulfate micelles. In MECC with direct injection of plasma or serum, proteins become solubilized by dodecyl sulfate, drugs non-covalently bound to serum proteins are liberated and total drug concentrations can be determined [69]. MECC with direct sample injection (DSI) has been shown to allow analysis of hexobarbital enantiomers in rat plasma [55]. Alternatively, coated capillaries do also permit DSI analyses of drugs in plasma or serum [51]. Finally, DSI of urine [24,38,53,63], hydrolyzed urine [36] or microdialysates [42] can be used without special precaution in relation to capillary conditioning. All reported assays are performed in aqueous buffer systems that might contain low amounts (up to about 20%) of an organic solvent. No paper was found describing the use of a binary system ([70], organic solvent content>20%). Furthermore, despite the recent interest in nonaqueous CE (NACE) for achiral drug analysis in biofluids [71] and chiral separations of drug standards [72], no paper was found describing the use of NACE for enantioselective drug analysis in biological samples.

Not unlike in the monitoring of drugs in an achiral environment, sample preparation and detection are

Table 5
Determination of drug enantiomers of toxicological and forensic interest in samples of patients and drug abusers

Drug	Chiral metabolite	Sample	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Dextromethorphan, levomethorphan	Dextrophan, levorphanol	Urine	MECC	β-CD	9.05	H-SPE	UV/200	[37]
Methadone	EDDP	Urine	CZE	OHP-β-CD	3.0	DSI SPE	UV/195 195–320/5	[24]
Methadone	EDDP	Serum, urine, hair	CZE	DM-β-CD	2.3	LLE	UV/200	[25]
MDMA, MDA, MDE, ephedrine, amphetamine, methamphetamine		Urine, hair	CZE	β-CD	2.5	LLE (Toxitube A)	UV/200 UV/190–400	[39,40]
Amphetamine, methamphetamine, MDA, MDMA, methadone	EDDP	Urine	CZE	OHP-β-CD	2.5	LLE (Toxitube A)	UV/195 UV/195–320	[41]
Selegiline (not measured)	*R*-(−)-Amphetamine, *R*-(−)-Methamphetamine	Urine	CZE	OHP-β-CD	2.5	LLE (Toxitube A)	UV/195 UV/195–320	[41]

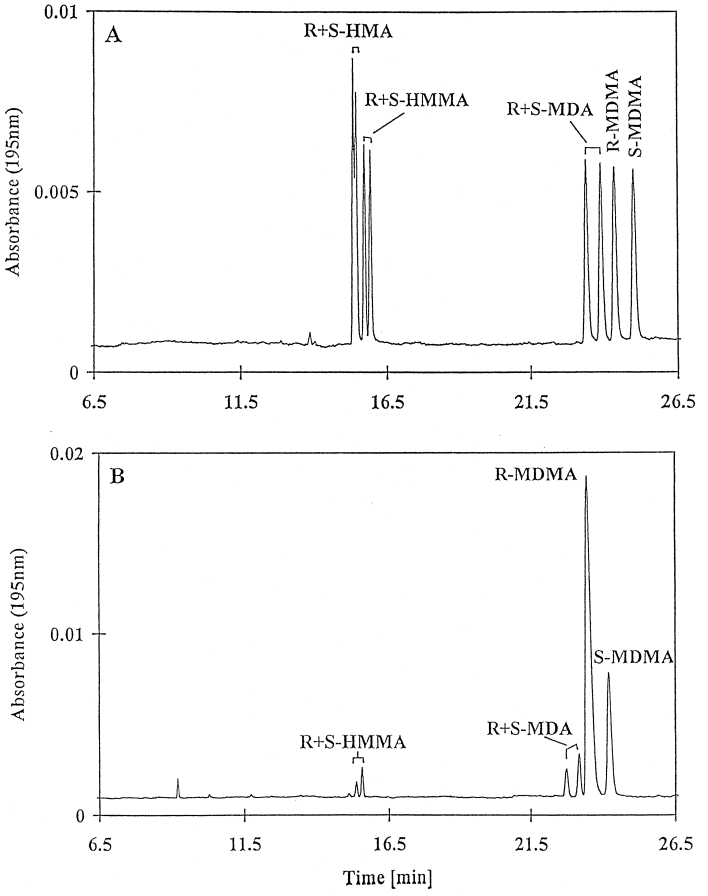

Fig. 1. Chiral separation of enantiomers of MDMA and metabolites. Panel A depicts CZE separations of the enantiomers of MDMA and its metabolites MDA, HMMA and HMA (all racemic, about 20 μg/ml each). Data shown in panel B were obtained with a hydrolyzed (β-glucuronidase from bovine liver) and extracted (solid-phase extraction) urine of a patient who was given 1.5 mg racemic MDMA/kg body mass and whose urine was collected 12 h after drug administration. The data reveal that the MDMA metabolism is stereoselective. The buffer was composed of 30 m*M* OHP-β-CD and 75 m*M* potassium dihydrogenphosphate (pH 2.5). The data were collected on a BioFocus 3000 capillary electrophoresis system (Bio-Rad Labs., Hercules, CA, USA) that was equipped with an untreated fused-silica capillary of 60 cm (55.4 cm effective length)×50 μm I.D. The sample was pressure injected, a constant voltage of 22 kV (current about 60 μA) was applied, the temperatures of cartridge and carousel were maintained at 20°C and detection was effected at 195 nm. Key: MDMA=3,4-methylenedioxymethamphetamine (Ecstasy); MDA=3,4-methylenedioxyamphetamine; HMMA=4-hydroxy-3-methoxymethamphetamine; HMA=4-hydroxy-3-methoxyamphetamine. From Ref. [77].

also key issues for the enantioselective determination of drugs in body fluids, hair and microsomal preparations. For analysis of drug enantiomers in body fluids, strategies for sample preparation ranging from DSI and injection after simple pretreatments [dilution, filtration, ultrafiltration, hydrolysis (H) and protein precipitation] to complete schemes for selective extraction, including solid-phase extraction (SPE) and liquid–liquid extraction (LLE) (Tables 1–5) have been described. The use of a high-performance liquid chromatography (HPLC) cleaning step [64] and an extraction device comprising a liquid membrane [50] have also been described. On-column UV absorbance detection is currently the most popular method employed (Tables 1–5). Because of the short optical pathlength within the detection cell, the lowest detectable concentration (without preconcentration of solutes, see below) is in

the 1–10 μ*M* (low μg/ml) range. This concentration sensitivity is one- to two-orders of magnitude lower than that encountered in HPLC. Other detection principles employed include laser induced fluorescence (LIF, [29]), amperometry [42] and mass spectrometry (MS, [34,63]). Compared to UV absorption detection, sensitivity enhancement when using LIF or electrochemical detection can be up to 1000-fold [73]. Compared to UV absorption, the latter three detection modes also provide increased selectivity which is useful for identification of solutes. Furthermore, MS detection in the MS^n ($n \geq 2$) format provides a structural proof. Quantitation is typically performed by single level or multi-level internal calibration using peak heights or peak areas, and by running the samples only once. For analysis by direct injection of the body fluid, no internal standard has to be included as long as sample introduction and transport are reproducible. Intra- and inter-day imprecisions are typically on the 3–10% and 4–15% levels, respectively. The assay for verapamil is claimed to have been validated for enantioselective drug monitoring with more than 100 quality control samples and to have been employed for over thousand human plasma samples [33].

Having UV absorbance detection and hydrodynamic injection of a sample that was not preconcentrated, ppm (μg/ml) detection limits are obtained. This applies to DSI and to the use of simple pretreatments, such as filtration, ultrafiltration and, to a somewhat lesser degree, also to hydrolysis. Furthermore, using protein precipitation with acetonitrile and hydrodynamic injection of the acetonitrile containing supernatant has also been shown to provide solute stacking and improved detection limits [74]. With extraction, solutes can be preconcentrated. Practical reasons, including the amount of body fluid available (typically 0.5 to 2 ml plasma, up to 5 ml urine) and the sample volume required in the sample vial (50 to 100 μl), concentrations enhancements up to about 50-fold can be reached. This leads to detection limits in the order of 20 to 50 ng/ml. Using very high volumes of body fluids (e.g., 10 ml urine and LLE [23]) concentrations around 1 ppb can thereby also be assessed. Furthermore, on-column preconcentration can also be attained by various electrokinetic injection and stacking procedures. Electrophoretic mass transport is highly regulated, this allowing charged solutes to be concentrated (stacked) across an electrolyte discontinuity, including that produced initially between sample and running buffer. This inherent and exclusive feature of electrophoresis may take place when the conductivity of the sample is smaller than that of the buffer (field amplified sample stacking). After hydrodynamic sample introduction, stacking is not only dependent upon sample composition, but also on the sample volume injected and thus limited by the capillary volume. Experimentally determined enhancement factors associated with in-column stacking typically does not exceed 100. Not surprisingly, sample clean up combined with in-column stacking has been shown to provide ppb detection limits for analysis of drug enantiomers in blood [21,50,59,61,64] and urine [21]. Head-column field amplified sample stacking associated with electrokinetic sample introduction takes place at the tip of the column and has no limited injection volume. It is typically performed with a sample extract of low conductivity and was reported to provide ppb detection limits for various drug enantiomers [25,31,33,42]. In that approach, analytes are stacked at the interface between the low-conductivity zone and the running buffer. Furthermore, little sample solvent is co-injected because the net electroosmotic velocity is typically much smaller than the local electrophoretic transport. Head column field amplified sample stacking has also been shown to provide a ppb concentration sensitivity for extracts of microliter amounts of serum or plasma [75,76]. It is important to realize that detection limits in CE are not only dependent on the type of detector used, but also on the matrix of the sample and the injection procedure employed.

3. Applications

3.1. Assessment of stereoselective pharmacokinetics and drug metabolism

One of the most challenging analytical goals is the simultaneous enantioselective determination of a drug together with its metabolites in real world samples. Recently, chiral CZE and chiral MECC

were found to be attractive methods for the elucidation of the stereoselective pharmacokinetics and metabolism of a fair number of drugs (Tables 2–4). There are several reasons why one would have or want to assess stereoselective aspects associated with drugs, including research dealing with the evaluation of the pharmacokinetic and activity differences of enantiomers (Tables 2–4), pharmacogenetics [35,36] and bioavailability of different drug formulations [33].

Elimination or excretion kinetics were determined for the enantiomers of various drugs (Table 2), including cicletanine [21,22], dimethindene [23], oxprenolol [26], tramadol [32,66], 3,4-methylenedioxymethamphetamine (MDMA or Ecstasy) [38], isoproterenol [42,68] and zoplicone [29]. In the CE methods developed, a chiral selector added to the running buffer permitted discrimination between the drug's enantiomers and in most cases also the enantiomers of one or more metabolites. After single dose drug administration to healthy volunteers [21,23,26,29,32,66] or patients [38], enantiomers of cicletanine were analyzed in plasma and urine [21] and of isoproterenol in microdialysates [42,68], whereas the enantiomeric excretion kinetics of all other drugs listed in Table 2 and their metabolites were assessed via analysis of urine samples. As an example, the work performed on the elucidation of the stereoselectivity of the MDMA metabolism [38] is briefly discussed. Using CZE with a phosphate buffer at pH 2.5 containing 30 m*M* (2-hydroxypropyl)-β-cyclodextrin (OHP-β-CD) as chiral selector, the simultaneous separation of the enantiomers of MDMA and its two metabolites 4-hydroxy-3-methoxymethamphetamine (HMMA) and 3,4-methylenedioxyamphetamine (MDA) was achieved. In the same configuration, 4-hydroxy-3-methoxyamphetamine (HMA) enantiomers could not be resolved (Fig. 1A). The chiral assay applied to the determination of urinary MDMA, MDA and HMMA is based upon enzymatic hydrolysis of conjugated HMMA and solid-phase extraction (Fig. 1B). Via analysis of the urines of two patients, the metabolism of MDMA could be demonstrated to be enantioselective, with significantly higher urinary amounts of *R*-(−)-MDMA being excreted compared to *S*-(+)-MDMA. The metabolism of the enantiomers of the two metabolites showed interindividual differences (Fig. 2). The work with cicletanine in rats represents an investigation of the activity of the cicletaninesulfate metabolite [22].

The assays summarized in Table 3 were applied to the enantiomeric analysis of drugs and/or metabolites in single samples of patients [19,24,27,28,31,33,35,67], healthy volunteers [30,34–36] and drug abusers [25]. The list of drugs also includes three examples in which an achiral drug is administrated and chiral metabolites are produced and monitored [35,36,67]. Stereoselectivity was assessed via enantiomeric *S*/*R* ratios. Body fluids analyzed comprised plasma, serum and urine. Additionally, methadone was determined in hair extracts [25] and zopliclone enantiomers but not their metabolites were measured in saliva [29]. For the sake of illustration, the following examples are briefly discussed. Chiral CE was successfully employed for the characterization of the well known aromatic hydroxylation stereoselectivity of mephenytoin [36] and debrisoquine [35] and thus to the determination of two hydroxylation polymorphisms in man. Data obtained with urines that were received for routine phenotyping with mephenytoin and debrisoquine confirmed the almost exclusive formation of the *S*-enantiomers of 4-hydroxymephenytoin and 4-hydroxydebrisoquine, respectively. Using a chiral MECC assay at alkaline pH for analysis of urinary mephenytoin and 4-hydroxymephenytoin enantiomers (0–8 h urine collected after ingestion of 100 mg mephenytoin) and injection of enzymatically hydrolyzed urine, extensive metabolizer phenotypes (EM) and poor metabolizer phenotypes (PM) could be recognized by the presence or absence of *S*-4-hydroxymephenytoin, respectively (Fig. 3). Furthermore, application of an extract of non-hydrolyzed urine permitted phenotyping via both the urinary *S*/*R* enantiomeric ratio of mephenytoin and the hydroxylated metabolite (Fig. 4) [36]. Likewise, after administration of 10 mg of achiral debrisoquine, 0–8 h urine collection and solid-phase extraction and analysis of the extract by chiral CZE, no *R*-4-hydroxydebrisoquine could be detected and differentiation between EMs and PMs for debrisoquine was unambiguously possible by the presence of a significant and no (or minor) peak for *S*-4-hydroxydebrisoquine, respectively [35]. Data obtained for 10 EM subjects and five PM subjects were found to agree with those

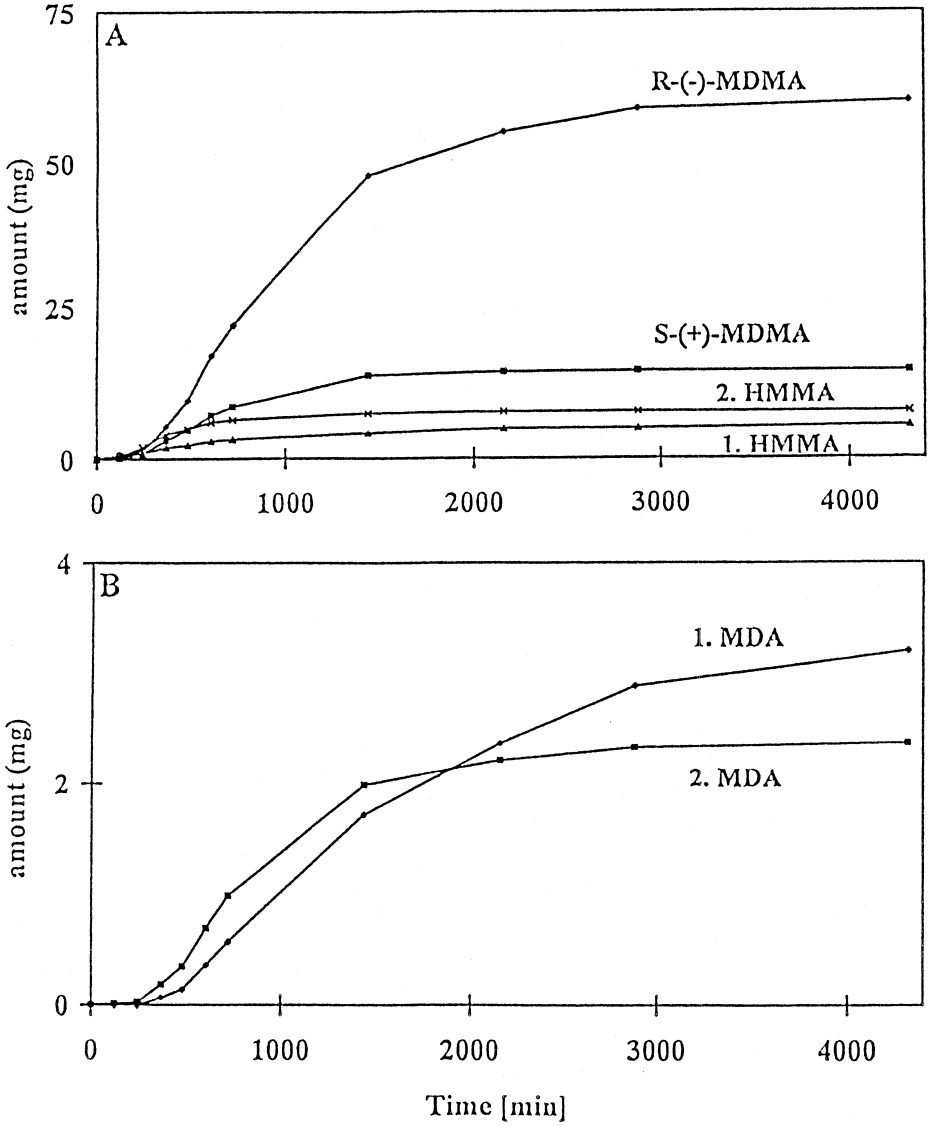

Fig. 2. Stereoselectivity of MDMA metabolism. Cumulative urinary excretion of MDMA, MDA and HMMA enantiomers of a patient over a period of 72 h after administration of 1.5 mg racemic MDMA/kg body mass and assessed by chiral CZE. An electropherogram of a urine specimen of the same patient is presented in Fig. 1B. From Ref. [38].

generated by the routine assay based on gas chromatography.

Finally, in vitro metabolic studies of xenobiotics with animal or human hepatic microsomes is an often employed approach for the investigation of drug metabolism. Examples studied with chiral CE are presented in Table 4 [43–47].

3.2. Clinical and forensic toxicology: bioanalysis of enantiomers of illicit and banned substances

Chiral CE has proven to be a simple, inexpensive and effective approach for the separation of enantiomers of toxicological, doping and forensic interest [18]. Bioanalytical examples studied with use of

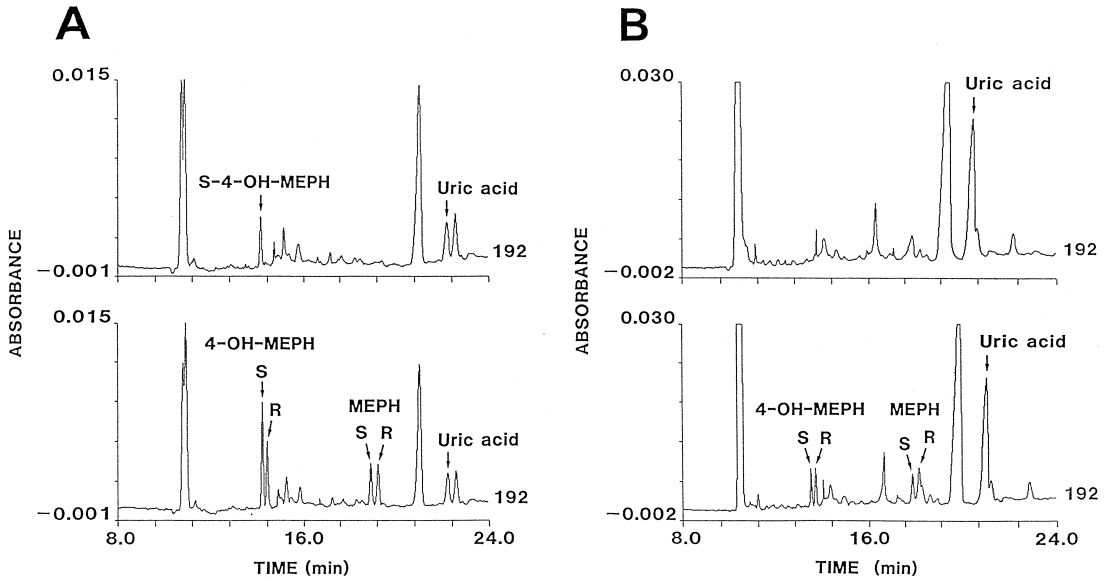

Fig. 3. Stereoselectivity of aromatic hydroxylation of mephenytoin. Chiral MECC electropherograms obtained with enzymatically hydrolyzed urines of (A) an extensive metabolizer and (B) a poor metabolizer phenotype. Top graphs represent those monitored after direct injection of the hydrolyzed urine whereas lower graphs are reruns with the hydrolyzed urine samples spiked with racemic mephenytoin and 4-hydroxymephenytoin. The running buffer was composed of 5.6 m*M* sodium tetraborate and 8.4 m*M* disodium hydrogenphosphate (titrated to pH 9.10 with phosphoric acid), 95 m*M* SDS, 40 m*M* β-CD and 8% (v/v) 2-propanol. Experiments were performed in an untreated capillary of 91 cm (54 cm effective length)×75 μm I.D. and sample was injected by vacuum. A constant voltage of 20 kV (48 μA) was applied and detection was effected at 192 nm. Key: MEPH=mephenytoin; 4-OH-MEPH=4-hydroxymephenytoin. From Ref. [36].

various CDs include the chiral differentiation of the optical isomers of racemethorphan and racemorphan in urine [37], of amphetamine and analogs in urine [38–41] and hair [39,40], as well as of methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in urine [24,25,41], serum [25] and hair [25] (Table 5). A chiral assay comprising a pH 2.5 buffer with 8.3 m*M* OHP-β-CD as chiral selector was shown to permit the enantioselective analysis of basic drugs in urinary extracts, including methamphetamine, amphetamine, MDMA and other designer drugs, and methadone together with its major metabolite EDDP. The suitability of this assay for drug screening and confirmation was demonstrated via analysis of alkaline extracts of unhydrolyzed quality control urines [41] and patient urines (Fig. 5). In that approach, enantiomer identification is based upon comparison of extracted polychrome UV absorption data and comparison of electropherograms with those obtained by rerunning of spiked extracts. The assay is simple, reproducible, inexpensive and reliable (free of interferences of other major basic drugs that are frequently found in toxicological urines [41]) and could thus be used to screen for and confirm of urinary enantiomers in a routine laboratory.

For clinical and forensic toxicology, chiral discrimination has a number of relevant applications. For example, the antitussive dextromethorphan (allowed drug) and the narcotic analgesic levomethorphan (banned drug, not commercially available) are the D-(+) and L-(−) isomers of 3-methoxy-*N*-methylmorphinan. Aumatell and Wells [37] demonstrated that these enantiomers can easily be distinguished with a chiral MECC assay that was developed for urinary analysis of the optical isomers of racemethorphan and racemorphan. Distinction of these compounds is not only of interest in forensic science (such as the elucidation of the cause of death after intake of levomethorphan), but also for the treatment of intoxicated patients. Furthermore, it has

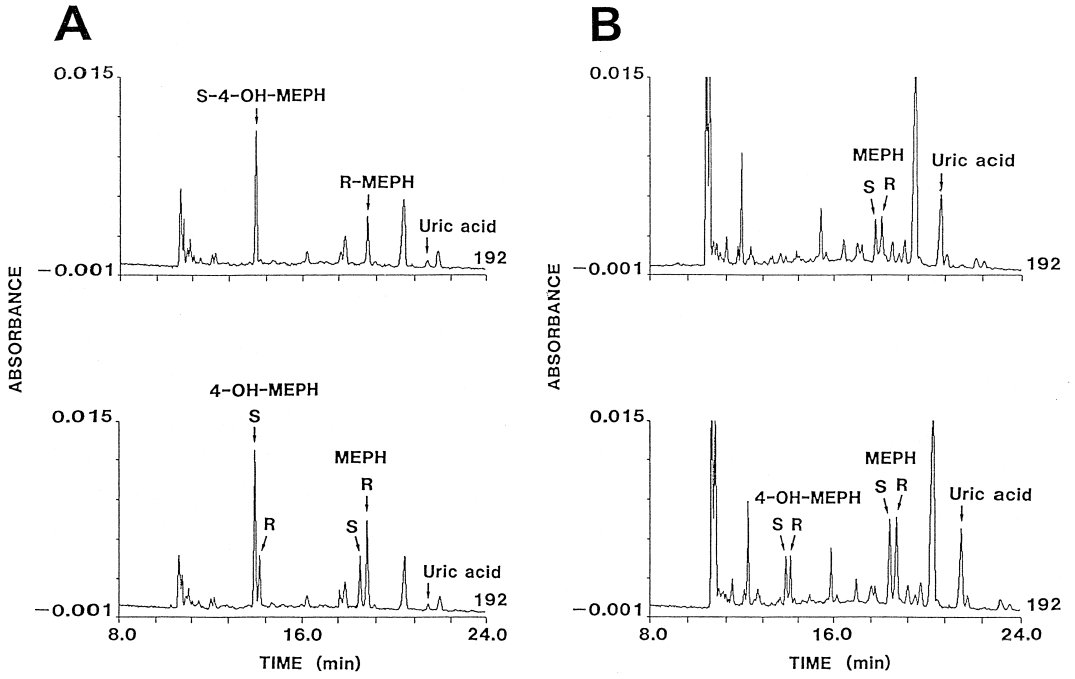

Fig. 4. Stereoselectivity of aromatic hydroxylation of mephenytoin assessed via urinary *S*/*R* ratios of mephenytoin and 4-hydroxymephenytion. Chiral MECC data obtained with extracted (liquid–liquid extraction), unhydrolyzed urines of (A) an extensive metabolizer and (B) a poor metabolizer phenotype. Other conditions as in Fig. 3. From Ref. [36].

been shown that their respective 3-hydroxy-*N*-methylmorphinan metabolites dextrorphan (allowed substance) and levorphanol (a powerful narcotic analgesic), respectively, can be simultaneously analyzed with the same assay [37]. The use of preparations containing dextromethorphan by athletes is allowed, whereas the use of levorphanol is expressly banned by the International Olympic Committee. Thus, the chiral CE assay can be applied in doping control.

There are numerous other examples which merit enantiomeric analysis, including (+)-propoxyphene (a narco-analgetic, controlled substance) and (−)-propoxyphene (an antitussive, allowed compound), (+)-/(−)-norpseudoephedrine [(+)-norpseudoephedrine (cathine) is controlled substance under international conventions], and (+)-/(−)-cocaine [(−)-cocaine is natural whereas the presence of (+)-cocaine indicates an illicit synthetic preparation]. These and others have been separated by chiral CE [18] but have not yet been monitored in body fluids.

Finally, distinction between the enantiomers of amphetamine and methamphetamine has some relevant applications as well. The *S*-(+) enantiomers (D enantiomers) of amphetamine and methamphetamine have about five-times more psychostimulant activity than the *R*-(−) enantiomers (L enantiomers) and are thus banned or controlled substances. *R*-(−)-Methamphetamine is included in the Vicks Inhaler sold in the United States. Furthermore, selegiline (a prescribed drug administrated to treat Parkinson patients) is known to metabolize to the *R*-(−) enantiomers of methamphetamine and amphetamine. Using a pH 2.5 chiral assay with OHP-β-CD as chiral selector for analysis of the extract of a urine of a patient under selegiline pharmacotherapy, the presence of the *R*-(−) enantiomers of methamphetamine and amphetamine could be unambiguously identified [41]. 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

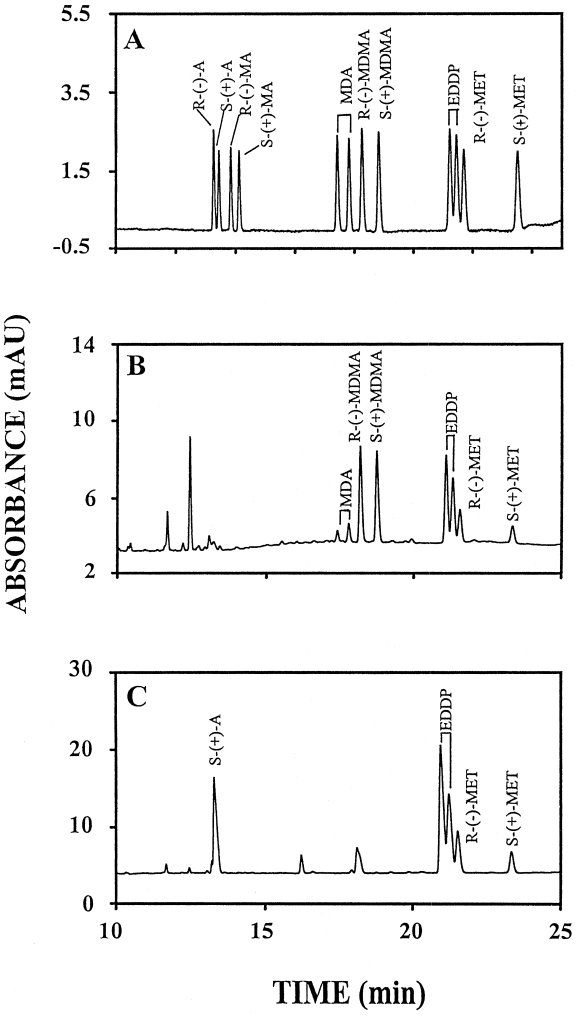

Fig. 5. Analysis of drug enantiomers of toxicological and forensic interest. Chiral CZE electropherograms of (A) standard compounds (about 10 μg/ml each), (B) an alkaline extract (liquid/liquid extraction using Toxitube A) of quality control urine 122, a urine of a person under methadone therapy that was fortified with MDMA (3 μg/ml) and MDA (0.3 μg/ml), and (C) an alkaline extract of a patient whose urine was found to contain rather large amounts of *S*-(+)-amphetamine, EDDP enantiomers and methadone enantiomers. Experiments were performed on a BioFocus 3000 as described in Ref. [41], using an untreated fused-silica capillary of 60 cm (55.4 cm to the detector)×50 μm I.D., pressure injection (2 p.s.i.×s), a constant voltage of 22 kV (current about 45 μA) and solute detection at 195 nm. The buffer employed was composed of 75 m*M* KH_2PO_4 at pH 2.5 containing 8.3 m*M* OHP-β-CD. Key: A=amphetamine; MA=methamphetamine; MDA=3,4-methylenedioxyamphetamine; MDMA=3,4-methylenedioxymethamphetamine (Ecstasy); MET=methadone; EDDP=2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine.

that metabolize to the *S* enantiomers of methamphetamine and amphetamine.

4. Achievements and outlook

Chiral CE has proven itself as being a powerful tool for enantioselective determination of drugs and metabolites in body fluids, hair and microsomal preparations. It represents an accurate technology which has found its place in a cost and environment controlled age. Compared to chiral HPLC, chiral CE provides higher efficiency and is simpler, faster and cheaper, and consumes a much smaller amount of organic solvents. It can handle small sample volumes, including those associated with microdialysis. Strategies for sample handling (direct injection, extraction), application (hydrodynamic vs. electrokinetic injection) and detection (absorbance vs. fluorescence vs. electrochemical) have been carefully worked out, its result being a mature technique with a ppb concentration sensitivity. Excellent examples represent those reported for enantioanalysis of cicletanine ([21], the first paper on this topic!), dimethindene [23], methadone [24,25], zopiclone [29], verapamil [33], clenbuterol [31] and MDMA [38], to name but a few, together with some of their metabolites. The paper on MDMA [38] comprises the first discussion of the stereoselective MDMA metabolism in man, the report on the stereoselective determination of clenbuterol in human urine [31] appears to be the first enantioselective description of the metabolism of this drug, and the work on the chiral haloperidol metabolite is claimed to be the first approach for enantioselective determination of this compound in a biological sample [67]. Furthermore, the report on the stereoselective in vitro metabolism of flezelastine (a new antiasthmatic/antiallergic drug) comprises first data about the stereoselectivity of the phase I metabolism of the enantiomers of flezelastine [44]. In that work, chiral CE is shown to be more suitable than chiral HPLC. In the context of bioanalysis of drug enantiomers of toxicological and forensic interest, the CE assay for chiral differentiation of the optical isomers of racemethorphan and racemorphan is claimed to be the only method that can simultaneously separate the enantiomers of 3-methoxy-*N*-methylmorphinan and 3-hydroxy-*N*-

methylmorphinan and thereby distinguish between the intake of a cough syrup and a banned substance [37]. Furthermore, the chiral assay capable of distinguishing between enantiomers of basic drugs in urine provides a simple approach for characterization of the enantiomeric pattern of amphetamine and analogs, methadone and EDDP. In some cases isomers that are metabolites of prescribed drugs can thereby be recognized and distinguished from illegal drug consumption [41]. Thus, one can confidently conclude and expect that chiral CE technology will be widely applied (i) to follow stereoselective pharmacokinetics and metabolic pathways of old and new drugs in the body and (ii) to monitor enantiomers of illicit drugs present in the body. Chiral CE does not only represents a complementary tool to the widely applied chromatographic methods, it also offers the possibility of bringing chiral separations and analyses into the routine arena.

5. Definition list

CE	Means capillary electrophoresis
CEC	Means capillary electrochromatography
CD	Means cyclodextrin
CM-β-CD	Means carboxymethyl-β-cyclodextrin
CZE	Means capillary zone electrophoresis
DM-β-CD	Means 2,6-dimethyl-β-cyclodextrin
DSI	Means direct sample injection
EDDP	Means 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EM	Means efficient metabolizer phenotype
ESI	Means electrospray ionization
H	Means hydrolysis
HMA	Means 4-hydroxy-3-methoxyamphetamine
HMMA	Means 4-hydroxy-3-methoxymethamphetamine
HPLC	Means high-performance liquid chromatography
HTAB	Means hexadecyltrimethylammonium bromide
LIF,	Means laser-induced fluorescence
LLE,	Means liquid–liquid extraction
M-β-CD,	Means methyl-*O*-β-cyclodextrin
MDA,	Means 3,4-methylenedioxyamphetamine
MDE,	Means 3,4-methylenedioxyethylamphetamine
MDMA,	Means 3,4-methylenedioxymethamphetamine
MECC,	Means micellar electrokinetic capillary chromatography
MS,	Means mass spectrometry
NACE,	Means nonaqueous capillary electrophoresis
OHP-β-CD,	Means (2-hydroxypropyl)-β-cyclodextrin
OHP-γ-CD,	Means hydroxypropyl-γ-cyclodextrin
OTEC,	Means open tubular electrochromatography
PM,	Means poor metabolizer phenotype
SPE,	Means solid-phase extraction
TM-β-CD,	Means (2,3,6-tri-*O*-methyl)-β-cyclodextrin

Acknowledgements

The authors gratefully acknowledge the loan of a BioFocus 3000 from its manufacturer, Bio-Rad Labs., Life Science Group, Hercules, CA, USA. The data presented in Fig. 5 were kindly generated by A. Ramseier. This work was supported by the Swiss National Science Foundation.

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