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Review

Stereoselective determination of drugs and metabolites in body fluids, tissues and microsomal preparations by capillary electrophoresis (2000–2010)

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ABSTRACT

During the past two decades, chiral capillary electrophoresis (CE) emerged as a promising, effective and economic approach for the enantioselective determination of drugs and their metabolites in body fluids, tissues and *in vitro* preparations. This review discusses the principles and important aspects of CE-based chiral bioassays, provides a survey of the assays developed during the past 10 years and presents an overview of the key achievements encountered in that time period. Applications discussed encompass the pharmacokinetics of drug enantiomers *in vivo* and *in vitro*, the elucidation of the stereoselectivity of drug metabolism *in vivo* and *in vitro*, and bioanalysis of drug enantiomers of toxicological, forensic and doping interest. Chiral CE was extensively employed for research purposes to investigate the stereoselectivity associated with hydroxylation, dealkylation, carboxylation, sulfoxidation, N-oxidation and ketoreduction of drugs and metabolites. Enantioselective CE played a pivotal role in many biomedical studies, thereby providing new insights into the stereoselective metabolism of drugs in different species which might eventually lead to new strategies for optimization of pharmacotherapy in clinical practice.

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 [1]. 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. The significance of stereochemical considerations in drug metabolism and pharmacokinetics is an issue for both the pharmaceutical industry and the regulatory authorities, and is important for optimization of pharmacotherapy, including therapeutic drug monitoring, and for research. This requires stereoselective separation techniques and analytical assays for the detection of enantiomeric purity and distribution in connection with drug synthesis and with biological, pharmacological, toxicological, pharmacokinetic and clinical investigations in man and animals. Furthermore, as certain enantiomers represent illicit or banned substances, chiral discrimination represents also an important topic in forensic analysis and doping control [1–10].

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Stereospecific drug monitoring is widely accomplished via use of chromatographic methods which require rather expensive chiral stationary phases [1–10]. During the past 20 years chiral separations by capillary electromigration methods have been studied extensively and shown to provide high-resolution at low cost [11–20]. 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. Differential interaction between analytes and selector provides the basis for enantiomeric separation. Various capillary electrophoresis (CE) modes are distinguished. Capillary zone electrophoresis (CZE) is conducted within a buffer in which mainly charged solutes can be separated only. The same is true for capillary isotachophoresis (CITP) in which the sample ions form consecutive zones in a discontinuous buffer system comprising a leading and a terminating component. Micellar electrokinetic capillary chromatography (MECC) is an electrokinetic separation technique that includes micelles. Micelles and surrounding buffer are being transported at different velocities and solutes become separated based upon differential partitioning. In all these approaches, the chiral selector is typically a buffer additive. Finally, chiral selectors bound to a packing material or attached to the inner wall of a capillary provide the basis of chiral packed capillary electrochromatography (CEC) and open tubular CEC, respectively [11–20]. Enantioselective electrokinetic separation applications include principal investigations, such as those associated with the determination of thermodynamic parameters for binding of drug enantiomers to the chiral selector [21] and drug enantiomerization [22], purity control of drugs [23], analysis of enantiomers of forensic interest and identification of the origin of drugs of abuse [24] and bioanalytical drug monitoring [25–29].

Stereoselective biomedical drug and metabolite analysis by CE has been dealt with for the past two decades. It comprises drug and metabolite analysis in body fluids, tissues and *in vitro* after incubation with microsomal preparations [25–29]. Milestones include the first MECC-based paper (determination of cicletanine enantiomers in human plasma [30]), the first CZE-based paper (monitoring of warfarin enantiomers in human plasma [31]), and the first CEC paper (analysis of venlafaxine and O-demethylvenlafaxine enantiomers in plasma [32]). Other first reports of this technology encompass work dealing with stereoselective drug metabolism *in vitro* (hydroxylation of mephenytoin in an incubation with human liver microsomes [33]), determination of drug stereoisomers of toxicological and forensic interest (racemethorphan and racemorphan in urine [34]), analysis of phase-II metabolites (ciprofibrate glucuronides in urine [35]), and monitoring of drug enantiomers in tissues rather than fluids (3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine in hair of ecstasy users [36]). Early work with CE-based chiral assays provided the first data on the stereoselective metabolism of flezelastine [37], clenbuterol [38], MDMA [39] and haloperidol [40]. Our interest in that field commenced with a collaborative effort in which the stereoselectivities of the aromatic hydroxylation of mephenytoin and phenytoin were assessed via MECC analysis of the enantiomers of the hydroxylated metabolites in human urine [41]. Other projects with analysis of urine samples included CZE investigations dealing with the enantioselective metabolism of methadone [42], MDMA [39], and debrisoquin [43], as well as the enantiomeric analysis of amphetamine, methamphetamine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and designer drugs in toxicological samples [44]. Another CZE-based effort focused on the selectivity of the thiopental to pentobarbital biotransformation assessed via analysis of patient plasma samples [45]. These

Table 1
Papers reporting work with body fluids that were fortified with drug enantiomers.

Drug	Body fluid	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
(+)-2-(4,5-Dihydro-1H-imidazo-2-yl)-2′-methoxy-5-fluoro-benzo-[1,4]-dioxan	Plasma	CZE	OHP-β-CD	4.0	SPE	UV/200	[49]
Amphetamine derivatives	Plasma	CZE	HS-γ-CD	2.5	LLE	ESI-MS, SIM	[50]
Amphetamine derivatives, methadone, tramadol	Plasma	CZE	HS-γ-CD	2.5	LLE	ESI-MS, SIM	[51]
Amphetamine, p-chloroamphetamine, 2-amino-1,2-diphenylethanol	Urine	CZE	Crown ether $18C6H_4$	4.0	SDME	UV/200	[52]
Arotinolol	Serum	MECC	STC	2.5	SPE	UV/220	[53]
Baclofen	Plasma	CZE	α-CD	9.5	LLE + derivatization	LIF/442/500	[54]
Carvedilol	Serum	CZE	OHP-β-CD	2.5	PP + LLE	UV/200	[55,56]
Carvedilol, metaproterenol, methoxamine, terbutaline	Serum	CZE	β-CD	2.5	PP	UV/210	[57]
Cetirizine	Plasma	CZE	S-β-CD	8.7	LLE	UV/200	[58]
Clenbuterol	Plasma	CZE	DM-β-CD	2.5	SPE	ESI-MS/MS, MRM	[59]
Disopyramide	Plasma	CZE	S-β-CD	4.5/6.5 separation/ detection	PP + LLE	ECL	[60]
Epinephrine, norepinephrine	Serum, urine	CZE	OHP-β-CD + DM-β-CD	2.9	PP	UV/205	[61]
Flurbiprofen	Plasma	NACE (MeOH)	PA-β-CD	–	PP + SPE	UV/250	[151]
Gemifloxacin	Urine	CZE in capillary CZE in chip	Crown ether $18C6H_4$	4.0 4.0	Dilution	UV/270 LIF/325/405	[62]
Hydroxymebendazole	Plasma	CZE	S-β-CD	7.0	LLE	UV/290	[63]
Labetalol	Plasma	CZE	ODAS-γ-CD	2.5	SPE	UV/228	[64]
Mirtazapine + metabolites	Urine	CEC	Vancomycin	6.0	SPE	UV/200	[65]
Pseudoephedrine	Urine	MECC	Poly-L-SUCLS	2.0	Filtration	ESI-MS, SIM	[66]
Salbutamol	Urine	CZE	Dermatan sulfate	5.3	SPE	UV/220	[67]
Salbutamol	Urine	NACE (MeOH)	HDAS-β-CD	Acidic	SPE	ESI-MS, SIM	[68]
Sulpiride, eticlopride	Serum	CZE	S-β-CD	2.9	SPE	UV/220	[69]
Thioridazine 5-sulfoxide	Plasma	CZE	OHP-β-CD + S-β-CD	3.0	LLE	UV/274	[70]
Vesamicol	Serum	CZE	S-β-CD	5.0	SPE	UV/260	[71]
Warfarin	Serum	CZE	HS-β-CD	2.5	LLE	UV/200	[72]
Warfarin	Plasma	CEC	(3R,4S)-Whelk-O1	4.0	PP + SPE	ESI-MS, SIM	[73]

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.
Amlodipine	–	Urine	ITP-CZE	OHP-β-CD	4.75–3.2	Dilution	Conductivity-UV/238	[74]
Anisodamine	–	Rabbit plasma	CZE	CM-γ-CD	2.5	LLE	UV/200 nm	[75]
Azelastine	Demethylazelastine, 6-hydroxyazelastine	Rat plasma	CZE	CM-β-CD + β-CD	5.0	LLE	UV/214	[76]
Carvedilol	–	Plasma	CZE	SUC-β-CD + M-α-CD	3.0	LLE	LIF/325/366	[77]
Carvedilol	–	Plasma	CZE	OHP-β-CD	4.0	LLE	UV/200	[78]
Ciprofibrate	Ciprofibrate glucuronide	Urine	CZE	γ-CD	6.0	SPE	UV/230	[35]
Disopyramide	Mono-N-desalkyldisopyramide	Plasma	CZE	S-β-CD	5.0	LLE	UV/214	[79]
Hydroxychloroquine	Desethylchloroquine, desethylhydroxychloroquine,	Urine	CZE	S-β-CD + OHP-β-CD	9.0	LPME	UV/220 + 343	[80]
Ibafloxacine	8-Hydroxyibafloxacine and glucuronides	Cat plasma, urine	CZE	Not given	7.5	H + SPE	UV/254	[81]
Ibuprofen	2′-Hydroxyibuprofen, 2′-carboxyibuprofen	Plasma, urine	CZE	TM-β-CD	5.0	SPE	UV/220	[82]
Ibuprofen	–	Plasma	CZE	S-β-CD	2.6	LLE	UV/220	[83]
Indobufen	–	Serum	CZE	TM-β-CD	5.0	LLE	UV/282	[84]
Ketoprofen	–	Serum	CZE	TM-β-CD	5.0	LLE	UV/253	[85]
Metoprolol	O-demethylmetoprolol, metoprolol acidic metabolite	Urine	CZE	CM-β-CD	4.0	LLE, DSI	UV/210/280	[86]
Mirtazapine	Demethylmirtazapine, 8-hydroxymirtazapine	Urine	CZE	CM-β-CD	2.5	H, PP, LPME	UV/205	[87]
Ofloxacine	Demethylofloxacine, ofloxacine N-oxide	Urine	CZE	SBE-β-CD	2.0	Filtration	LIF/325/520	[88]
Salbutamol	–	Urine	NACE (MeOH)	HDAS-β-CD	Acidic	SPE	UV/230	[152]
Terbutaline	–	Urine	CZE	OHP-β-CD	2.5	SPE	UV/205	[89]
Tramadol	O-demethyltramadol glucuronide	Urine	CZE	CM-β-CD + M-β-CD none – diastereomer sep.	10.0 3.0	DSI	LIF/257/290–320	[90]
Trans-tramadol	Trans-O-demethyltramadol	Serum	CZE	SBE-β-CD	2.5	LLE	UV/214	[91]
Trans-tramadol	Trans-O-demethyltramadol	Rat urine, kidney perfusate	CZE	SBE-β-CD	2.5	LLE	UV/214	[92]
Trans-tramadol	Trans-O-demethyltramadol	Plasma, urine	CZE	SBE-β-CD	2.5	LLE	UV/214	[93]
Venlafaxine	O-demethylvenlafaxine	Plasma	CZE	PH-γ-CD	2.5	LLE	UV/195	[94]

and many other applications were discussed in our previous review [26].

In this paper, CE-based assays for analysis of enantiomers of drugs and their major metabolites in biological samples published between 2000 and 2010 are comprehensively but not exhaustively reviewed. Selected papers which were not mentioned before are also included. On the other hand, work dealing with the CE monitoring of the enantiomers of amino acids and acids in body fluids and tissues, including aspartic acid [46], tryptophan [47] and lactic acid [48], is not included in this review. As with our previous compilation 10 years ago [26], insight into the achievements is presented via distinction between enantiomer analysis in fortified body fluids (Table 1), body fluids and tissues of humans and animals (Tables 2 and 3), *in vitro* preparations (Table 4) and in forensic biosamples (Table 5 [49–153]). Furthermore, the stereoselective CE work associated with a multidisciplinary ketamine project is presented separately (Table 6 [154–169]). Major focus is given on systems in which stereoisomers of parent drug and metabolites are analyzed together in one run. An example is presented in Fig. 1 in which the enantiomers of ketamine and many of their metabolites could be separated anionically in presence of randomly sulfated β-cyclodextrin (S-β-CD). Work dealing with the enantioseparation of drug standards is mentioned only if required for illustration of important features. Examples discussed in more detail and illustrations comprise data from our laboratory.

2. Principles

All drugs and/or their metabolites analyzed in body fluids, tissues and microsomal preparations during the past 10 years (Tables 1–6) are not only regular chiral compounds with one or multiple chiral C atoms, but also molecules with chiral heteroatoms, such as sulfoxides [70,95–97] and N-oxides [104], as well as rotamers with hindered rotation around a bond between two moieties [112–114]. Samples comprising body fluids and tissues of humans, rats, cats, dogs, guiney pigs, equines and rabbits have been analyzed. Enantiomers of drugs have been determined mainly in

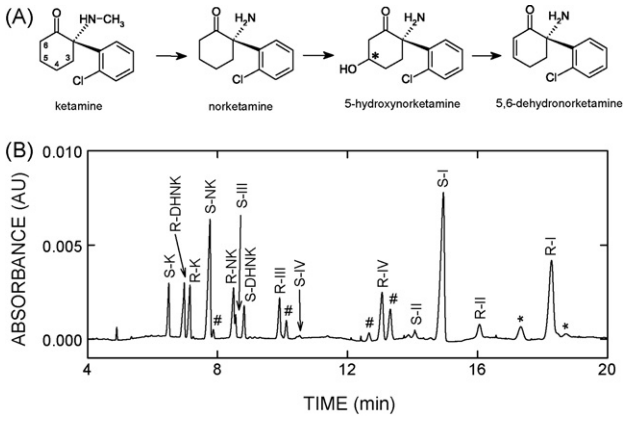

Fig. 1. CE separation of stereoisomers of ketamine and its metabolites. Panel A depicts selected chemical structures together with the main metabolic pathways in the liver and panel B depicts a typical electropherogram obtained with an alkaline extract prepared from a pony plasma that was collected 30 min after cessation of a 2 h target-controlled infusion of racemic ketamine with a maintained S-ketamine concentration in plasma of 1 μg/mL [157]. The peaks marked with asterisks are peaks found after analysis of blank plasma and the peaks marked with # are unidentified ketamine metabolites. The analysis was performed in a 50 μm I.D. fused-silica capillary of 45 cm total length at −20 kV and a pressure driven flow towards the anode (0.1 psi on inlet side) using a phosphate–Tris buffer at pH 2.5 containing 10 mg/mL sulfated β-cyclodextrin (blend of two lots). Key: K, ketamine; NK, norketamine; DHNK, dehydronorketamine; I–IV, four hydroxylated norketamine metabolites with the hydroxy group at different positions of the cyclohexanone ring of ketamine. Adapted from Ref. [156].

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

Drug	Chiral metabolite	Sample	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Albendazole[a]	Albendazole sulfoxide	CSF	CZE	S-β-CD	7.0	LLE	UV/290	[95]
Albendazole[a]	Albendazole sulfoxide, albendazole sulfone[a]	Plasma	CZE	(R)-DNBPG	9.0	LLE	UV/295, 250–360	[96]
Albendazole[a]	Albendazole sulfoxide, albendazole sulfone[a]	Plasma, saliva	CZE	S-β-CD	7.0	LLE	UV/225 F/280/320	[97]
Catechin, epicatechin	–	Urine	CZE	β-CD	8.35	Filtration and dilution	UV/240	[98]
Celiprolol	–	Urine	CZE	S-β-CD	4.0	LLE	UV/220	[99]
Chloroquine	Desethylchloroquine	Plasma	CZE	OHP-γ-CD + CM-γ-CD	9.65	LLE	LIF/325/405	[100]
Citalopram	Demethylcitalopram	Plasma	CZE	S-β-CD	2.5	LPME	UV/200	[101]
Citalopram	Demethylcitalopram, didemethylcitalopram, citalopram N-oxide	Urine	CZE	CM-γ-CD	5.0	SPE	UV/205	[102]
(−)-Deprenyl, (+)-deprenyl	Demethyldeprenyl, methamphetamine, amphetamine	Rat plasma, liver, kidney, heart	CZE	DM-β-CD	2.7	LLE	UV/190	[103]
Deprenyl	Deprenyl N-oxide and other deprenyl metabolites	Rat urine	CZE	DM-β-CD + CM-β-CD	2.7	LLE	UV/200	[104]
Dimethindene	6-Hydroxydimethindene 6-hydroxy-N-demethyldimethindene	Human urine, rat urine and serum, guinea pig urine	CZE	OHP-β-CD	3.3	(H)-LLE	UV/254/260	[105]
Dimethindene	6-Hydroxydimethindene glucuronide, 6-hydroxy-N-demethyldimethindene glucuronide	Rat urine and serum, guinea pig urine	CZE	CM-β-CD	6.0	LLE + SPE DSI	UV/254/260	[105]
Fluoxetine	Norfluoxetine	Serum, plasma	CZE	DM-β-CD + PH-γ-CD	2.5	LLE	UV/195	[106]
Hydromorphon	Hydromorphol, norhydromorphol	Urine	CZE	HDAS-β-CD	2.0	H + SPE	UV/210, 195–320	[107]
Itraconazole	Hydroxyitraconazole	Serum, plasma	CZE	S-β-CD	2.0	LLE	UV/214	[108]
Lorazepam	Lorazepam 3O-glucuronide	Urine	MECC	OHP-β-CD	9.1	SPE	UV/200, 195–320	[109]
Mebendazole[a]	Hydroxymebendazole, hydroxyaminomebendazole, aminomebendazole[a]	Plasma	CZE	S-β-CD	4.2	LLE	UV/214	[110]
Methadone	EDDP	Plasma	CZE	HS-β-CD	5.0	LLE	UV/200	[111]
Methaqualone	–	Urine	CZE	OHP-β-CD	2.5	LLE	UV/200	[112]
Methaqualone	Monohydroxylated metabolites	Urine	CZE	OHP-β-CD	2.1	H + SPE, H + LLE	UV/200	[113,114]
Mianserin	N-demethylmianserin	Plasma	CZE	OHP-β-CD	3.0	LPME	UV/200	[115]
Mianserin	Demethylmianserin, 8-hydroxymianserin	Plasma	CZE	OHP-β-CD	3.0	LLE	UV/211	[116]
Mirtazapine	N-demethylmirtazapine	Plasma	CZE	CM-β-CD	2.5	SPE	UV/205	[117]
Ofloxacin	–	Urine	CZE	S-β-CD	2.5	Dilution	UV/291	[118]
Oxycodone, noroxycodone	Oxycodol, noroxycodol	Urine	CZE	HDAS-β-CD	2.0	H + SPE	UV/210, 195–320	[119]
Pheniramine	Pheniramine metabolites (not identified)	Urine	ITP-CZE	CE-β-CD	4.75–4.50	Dilution	UV/265	[120,121]
Phenprocoumon	–	Urine	CZE	α-CD	5.4	DSI	LIF/325/405	[122]
Praziquantel	Trans-4-hydroxypraziquantel	Plasma	MECC	S-β-CD	10	LLE	UV/210	[123]
Quinidine, quinine	Quinidine metabolites	Urine, serum, saliva	CZE	β-CD	2.5	LLE	LIF/325/450	[124]
Trans-tramadol	Trans-O-demethyltramadol	Rat CSF, brain tissue, serum	CZE	SBE-β-CD	2.5	LLE	UV/214	[125]
Venlafaxine	O-demethylvenlafaxine	Serum	CZE	PH-γ-CD	2.5	LLE	UV/195	[126]
Venlafaxine	O-demethylvenlafaxine	Plasma	CEC	Vancomycin	6.0	LLE	UV/195	[32]
Warfarin	–	Plasma	MECC	Poly-L,L-SULV	6.0	PP + SPE	ESI-MS, SIM	[127]

[a] Achiral compound.

blood (plasma and serum) and urine (Tables 1–3, 5 and 6). Furthermore, enantiomers of albendazole sulfoxide [97] and methadone and EDDP [144] have been analyzed in saliva, of albendazole sulfoxide [95] and trans-tramadol and trans-O-demethyltramadol [125] in cerebrospinal fluid (CSF), of trans-tramadol and trans-O-demethyltramadol in kidney perfusate [92], of deprenyl and metabolites in rat liver, kidney and heart [103] and of trans-tramadol and trans-O-demethyltramadol in rat brain [125]. Finally, for a number of drugs and metabolites, enantiomers in microsomal preparations have been determined (Table 4).

Most of the work dealing with the enantioselective determination of drugs in body fluids, tissues and microsomal preparations is associated with the use of chiral CZE methods (Tables 1–6). A few assays are based upon MECC [53,66,109,123,127,132,143], CEC [32,65,73] and ITP-CZE [74,120,121]. Nonaqueous CE (NACE) with methanol as solvent was used as well [68,130,151,152]. Although there is a wide variety of chiral selectors available [11–20], most assays are based on the use of native or modified CDs (Tables 1–6). Other selectors used include the crown ether (+)-(18-crown-6)-tetracarboxylic acid (18C6H$_4$) [52,62], sodium taurocholate (STC) [53], vancomycin [32,65], polysodium N-undecenoxycarbonyl-L-leucine sulfate (poly-L-SUCLS) [66], dermatan sulfate [67], (R)-(−)-(3,5-dinitrobenzoyl)-α-phenylglycine ((R)-DNBPG) [96], polysodium N-undecenoyl-L,L-leucyl-valinate (poly-L,L-SULV) [127], maltodextrin [133], and the 1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene based statio-

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

Drug	Chiral metabolite	Sample matrix	CE method	Chiral selector	Buffer pH	Sample preparation	Detection	Ref.
Cimetidine[a]	Cimetidine sulfoxide	FMO1, FMO3, FMO5	CZE	SBE-β-CD	2.5	PP for off-line EMMA for in-line	UV/220	[128]
Deprenyl, methamphetamine, amphetamine	N-oxydated metabolites	FMO3 + FMO1, HLM	CZE	OHP-β-CD	3.6	SPE	UV/200	[129]
Fenbendazole[a]	Oxfendazole, fenbendazole sulfone[a]	RLM	NACE (MeOH)	HDMS-β-CD + HDAS-β-CD	Acidic	PP + filtration	UV/230	[130]
Hydroxychloroquine	Desethylchloroquine, desethylhydroxychloroquine, bisdesethylchloroquine	RLM, MLM	CZE	S-β-CD + OHP-β-CD	9.0	LLE	UV/220	[131,153]
Itraconazole	Hydroxyitraconazole	CYP3A	CZE	S-β-CD	2.0	LLE	UV/214	[108]
Mephenytoin	4-Hydroxymephenytoin	CYP2C19	MECC	β-CD	9.1	LLE	UV/195, 195–360	[132]
Methadone	EDDP	HLM, CYP2D6, CYP3A4, CYP2C9, CYP2C19	CZE	DM-β-CD	2.3	SPE	UV/200, 195–360	[132]
Methaqualone	Monohydroxylated metabolites	HLM, CYP3A4, CYP2D6, CYP2C19	CZE	OHP-β-CD	2.1	LLE	UV/200, 200–320	[114]
Oxycodone, noroxycodone	Oxycodol, noroxycodol	S9 fraction, HLC	CZE	HDAS-β-CD	2.0	SPE	UV/210, 195–320	[119]
Primaquine	Carboxyprimaquine	Rat liver mitochondrial fraction	CZE	Maltodextrin	3.0	LLE	UV/264	[133]
Propafenone, S-propafenone, R-propafenone	5-Hydroxypropafenone, N-depropylpropafenone	CYP2D6, CYP3A4, CYP1A1, CYP1A2	CZE	S-β-CD	2.0	LLE	UV/195, 195–320	[134]
Propafenone	5-Hydroxypropafenone, N-depropylpropafenone	HLM, CYP2D6, CYP3A4, CYP2A6	CZE	S-β-CD	2.0	LLE	UV/195, 195–320	[135]
Thalidomide S-(−)-thalidomide R-(+)-thalidomide	5-Hydroxythalidomide 5′-hydroxythalidomide	RLM	CZE	β-CD + SBE-β-CD	4.5	LLE	UV/230	[136]
Thiobencarb[a]	Thiobencarb sulfoxide	Rat liver microsomal fraction (S9)	CZE	OHP-β-CD	8.5	SPE	UV/220	[137]
(+)-Trans-tramadol, (−)-trans-tramadol	(+)-Trans-O-demethyltramadol, (−)-trans-O-demethyltramadol	RLM	CZE	SBE-β-CD	2.5	LLE	UV/214	[138]
Verapamil	Norverapamil	CYP3A4	CZE	S-β-CD	6.5	Filtration + sonification + 2% DMSO added	UV/234/260	[139]

[a] Achiral compound.

nary phase ((3R,4S)-Whelk-O1) [73]. Various applications report the use of mixtures of CDs. Compared to the previous decade, during which mostly uncharched CDs were employed [26], we noted a strong increase of the use of charged CDs. S-β-CD, with 7–11 mol sulfate/mol β-CD, was most often used [58,60,63,69,71,79,80,83,95,97,99,101,108,110,118,123,131,134,135,139,154–169] whereas the better defined highly sulfated CD derivatives (HS-β-CD and HS-γ-CD [170]) were only used in a few assays [50,51,72,111,144,145]. These and other selectors, such as phosphated-γ-CD (PH-γ-CD) [94,106,126], carboxymethyl-β-CD (CM-β-CD) or carboxymethyl-γ-CD (CM-γ-CD) [75,76,86,87,90,100,102,104,105,117,149], carboxyethyl-β-CD (CE-β-CD) [120,121] and sulfobutylether-β-CD (SBE-β-CD) [88,91–93,125,128,136,138], are products comprising multiple isomers. Finally, single isomer anionic selectors, such as heptakis (2,3-di-O-acetyl-6-O-sulfo)-β-cyclodextrin (HDAS-β-CD) [68,107,119,130,146,148,152], octakis-(2,3-di-O-acetyl-6-O-sulfo)-γ-cyclodextrin (ODAS-γ-CD) [64], and heptakis (2,3-di-O-methyl-6-O-sulfo)-β-cyclodextrin (HDMS-β-CD) [130], and the single isomer cationic CD derivative 6-monodeoxy-6-mono(3-hydroxy)propylamino-β-cyclodextrin (PA-β-CD) [151], were employed. 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 administrated. 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. This is typically achieved having multiple chiral selectors, such as mixtures of completely different CDs and/or negatively charged CDs comprising multiple isomers, such as S-β-CD. A handicap with the latter product might be hampering lot-to-lot differences as were observed for the separation of ketamine enantiomers [155]. The use of mixtures of single isomer charged CDs, CDs which provide well defined conditions [171,172], would be an attractive alternative. Enantiomeric resolution in presence of large concentration differences between compounds of interest represents another challenging goal. Optimized conditions are obtained via careful adjustment of the concentration of the chiral selector, buffer pH, ionic strength, as well as the nature and amount of additives.

Most chiral CE assays are based on the use of untreated fused-silica capillaries. Polyacrylamide coated [76] and polyvinylalcohol coated [99] capillaries were employed for the enantiomeric analysis of azelastine and celiprolol, respectively. Dynamic coatings with buffer additives were used as well, including those formed

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.
Methamphetamine and related compounds	Amphetamine	Urine	CZE	DM-β-CD + β-CD	2.5	LLE	UV/195	[140]
3,4-Methylenedioxymethamphetamine	4-Hydroxy-3-methoxymethamphetamine	Urine	CZE	OHP-β-CD	2.5	SPE	UV/204	[141]
3,4-Methylenedioxymethamphetamine	3,4-Methylenedioxyamphetamine	Urine	CZE	β-CD	3.0	LLE	F/280/320	[142]
Racemorphan (dextrorphan, levorphanol)	–	Urine	MECC	β-CD	9.4	SPE	UV/192	[143]
Methadone	EDDP	Saliva	CZE	HS-γ-CD	4.5	LLE	UV/200	[144]
3,4-Methylenedioxymethamphetamine, methadone	–	Plasma	CZE	HS-γ-CD	2.5	PP or LLE	ESI-MS, SIM	[145]
Methamphetamine and related compounds	Amphetamine, p-hydroxymethamphetamine	Urine	CZE	HDAS-β-CD	1.7	LLE	ESI-MS, SIM	[146]
Methamphetamine and related compounds	Amphetamine, p-hydroxymethamphetamine	Urine	CZE	β-CD + DM-β-CD	2.2	LLE, SPE	ESI-MS, SIM	[147]
Methamphetamine and related compounds	Metabolites	Urine	CZE	HDAS-β-CD	2.0	Dilution and filtration	ESI-MS, SIM	[148]
Selegiline	Amphetamine, methamphetamine	Rat and human urine	CZE	CM-β-CD	2.5	LLE	UV/205	[149]
Terbutaline	–	Urine	CZE	OHP-β-CD	2.5	SPE	UV/204	[150]

with polycationic spermine [96,100], various cellulose derivatives [74,102–104], polyvinylalcohol [101] and polyethylene glycol [108]. Not unlike in the monitoring of drugs in an achiral environment, sample preparation and detection are key issues for the enantioselective determination of drugs in body fluids, tissues and microsomal preparations. For analysis of drug enantiomers in body fluids, strategies for sample preparation ranging from direct sample injection (DSI) and injection after simple pretreatments (dilution, filtration, ultrafiltration, hydrolysis (H) and protein precipitation (PP)) to selective extraction, such as solid-phase extraction (SPE) and liquid/liquid extraction (LLE), have been described (Tables 1–5). In-line single drop microextraction (SDME) [52] and liquid-phase microextraction (LPME) [80,101] have also been used.

On-column UV absorbance detection is the most popular method employed for analyte detection (Tables 1–5). Because of the short optical path length within the detection cell, the lowest detectable concentration (without preconcentration of solutes, see below) is in the 1–10 μM (low μg/mL) range. This concentration sensitivity is 1–2 orders of magnitude worse than that encountered in high-performance liquid chromatography (HPLC) and can be somewhat improved via use of a capillary with an extended light path at the site of detection. For examples refer to Refs. [78,84,94,100,106]. Other detection principles employed include laser induced fluorescence (LIF) [54,62,77,88,90,100,122,124], lamp based fluorescence (F) [97,142], electrospray ionization mass spectrometry (ESI-MS) [50,51,59,66,68,73,127,145–148], and electrochemiluminescence (ECL) [60]. These four detection modes also provide increased selectivity which is useful for identification of solutes. Compared to UV absorption detection, sensitivity enhancement when using LIF can be up to 1000-fold such that ng/mL concentrations of enantiomers can be monitored in small volumes of sample (e.g. liquid/liquid extracts from 100 μL plasma [77]). Similar detection limits can be reached with MS detection [51,68,73,145,146]. Overall, MS offers the greatest potential to serve as a universal detector in chiral CE. All analytes can be identified and quantitated and this independent of their ability to absorb light, fluoresce or undergo electrochemical reactions at sensing electrodes. Quantitation is typically performed by multi-level internal calibration using peak heights or peak areas, and by running the samples only once. Intra- and interday imprecisions are typically on the 3–10% and 4–15% levels, respectively.

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 hydrolysis. PP with acetonitrile and hydrodynamic injection of the acetonitrile containing supernatant can provide solute stacking and improved detection limits [173]. With extraction, solutes can be preconcentrated. Practical reasons, including the amount of body fluid available (typically 0.5–2 mL plasma, up to 5 mL urine) and the sample volume required in the sample vial (50–100 μL), concentrations enhancements up to about 50-fold can be reached. This leads to detection limits in the order of 20–50 ng/mL. Furthermore, on-column preconcentration can also be attained by electrokinetic injection (for assay examples see Refs. [51,54,57,73,75,89,103,108,116,145]) and stacking procedures [49,71,75,79,87,100,108,116]. 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 lower 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

Table 6
Studies with determination of stereoisomers of ketamine and its metabolites in biosamples by CE.[a,b]

Applied drug	Metabolite(s)	Sample	Purpose of study	Ref.
rac-K, S-K	NK	Horse and pony plasma	Assay development and characterization of ketamine demethylation	[154]
rac-K, S-K	NK, DHNK	Pony plasma and urine	Assay modification, DHNK identification, enantioselectivities in plasma and urine	[155]
rac-K, S-K, rac-NK	NK, DHNK, OH-NK	Pony plasma and urine, equine liver microsomes	Identification of hydroxylated NK metabolites, stereoselectivity of NK hydroxylation	[156]
rac-K	NK	Pony plasma	Antinociceptive effects and ketamine demethylation under target-controlled infusion, PBPk modeling[c]	[157]
rac-K	NK	Pony plasma	Pharmacokinetic-based algorithm for traget-controlled infusion of ketamine in ponies	[158]
rac-K	NK	Pony plasma	Effect on limp withdrawal reflex evoked by transcutaneous electrical stimulation	[159]
rac-K, S-K	NK	Pony plasma	Pharmacokinetics after single i.v. bolus drug administration under isoflurane anesthesia	[160]
rac-K, S-K	NK	Pony plasma	Pharmacokinetics after single i.v. bolus drug administration and sedation with xylazine	[161]
rac-K, S-K	NK	Pony plasma	Effects of low-dose infusion on nociceptive withdrawal reflex in standing ponies	[162]
rac-K, S-K	NK	Pony plasma	Pharmacokinetics after constant rate, low dose (subanesthetic) infusion in ponies	[163]
rac-K, S-K	NK	Horse plasma	Anesthesia recovery quality after low-dose infusion during anesthesia with isoflurane	[164]
rac-K	NK	Canine plasma	Effect on nociceptive stimuli after low-dose constant rate infusion in concious dogs	[165]
rac-K, S-K	NK	Equine liver and lung microsomes	Ketamine demethylation in liver and lung, modeling with two-phase model based on Hill kinetics	[166]
rac-K, S-K, R-K	NK, DHNK, OH-NK	Equine, canine and human liver microsomes	Hepatic ketamine metabolism in different species *in vitro*	[167]
rac-K, S-K	NK	Equine, canine and human liver microsomes	Ketamine demethylation in presence of other analgesics *in vitro*	[168]
rac-K, rac-NK	NK, DHNK, OH-NK	Single human CYP enzymes	Identification and characterization of CYP enzymes involved in metabolism	[169]

[a] Assay based upon alkaline liquid/liquid extraction and analysis in a phosphate-Tris buffer at pH 2.5 containing 10 mg/mL randomly sulfated β-cyclodextrin (product of Aldrich with 7–11 mol sulfate/mol β-CD) using reversed polarity and 195 nm detection [154].
[b] rac-K: racemic ketamine, rac-NK: racemic norketamine, S-K: S-ketamine, R-K: R-ketamine, NK: norketamine, DHNK: dehydronorketamine, OH-NK: hydroxylated norketamine.
[c] Physiologically based pharmakokinetic modeling.

associated with in-column stacking typically does not exceed 100. With electrokinetic injection, however, a 1000-fold increase can easily be reached. Thus, sample clean up combined with stacking has been shown to provide ppb detection limits for analysis of drug enantiomers in biosamples [49,75,116,145,162]. 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 in vivo

One of the most challenging analytical goals is the simultaneous enantioselective determination of a drug together with its metabolites in real world samples. During the past 10 years, chiral CZE, MECC and CEC were found to be attractive methods for the elucidation of the stereoselective pharmacokinetics and metabolism of a number of drugs (Tables 2 and 3). 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, pharmacogenetics, excretion and bioavailability.

Urinary excretion kinetics were determined for the enantiomers of various drugs and/or their metabolites (Table 2), including amlodipine [74], ciprofibrate [35], hydroxychloroquine [80], metoprolol [86], mirtazapine [87], ofloxacine [88], terbutaline [89] and tramadol [90]. Other investigations dealt with the complete elucidation of the pharmacokinetics via analysis of the drug enantiomers in plasma or serum. Examples studied included the drugs anisodamine [75], carvedilol [78], ibafloxacine [81], ibuprofen [82,83], indobufen [84], ketoprofen [85], trans-tramadol [91,93], venlafaxine [94] and ketamine [157,160,161,163]. For one drug, carvedilol, enantiomer plasma concentrations were found to be comparable to those determined by HPLC [78]. Except for the pharmacokinetics of ketoprofen enantiomers, for which no stereoselectivity was observed [85], enantioselective differences were noted for the metabolic steps of the drugs. In the CE methods, one or multiple chiral selectors added to the running buffer permitted discrimination between the drug's enantiomers and in many cases also the stereoisomers of one or several metabolites. The work performed on the elucidation of the stereoselectivity of the MDMA metabolism [39], via the cumulative urinary excretion of MDMA and two of its metabolites, was given as example in our previous review [26]. The example presented here is focused on the determination of the pharmacokinetic parameters of ketamine enantiomers in equine plasma [160]. The data presented in Fig. 2 were obtained after liquid/liquid extraction at alkaline pH using a pH 2.5 CE assay comprising 10 mg/mL sulfated-β-CD as chiral selector and having detection limits for all enantiomers of 10 ng/mL [154]. The assay is similar to that whose electropherogram is presented in Fig. 1 except that a different lot of the chiral selector was used and that no hydrodynamic co-flow was applied. The data depicted in Fig. 2 represent mean plasma concentrations of S-ketamine (filled circles), R-ketamine (open circles), S-norketamine (filled triangles) and R-norketamine (open triangles) after bolus administration of 2.2 mg/kg racemic ketamine to 7 ponies anesthetised with isoflurane in oxygen. The data reveal that plasma concentrations of S-ketamine and R-ketamine are essentially equal whereas plasma concentrations for S-norketamine are statistically

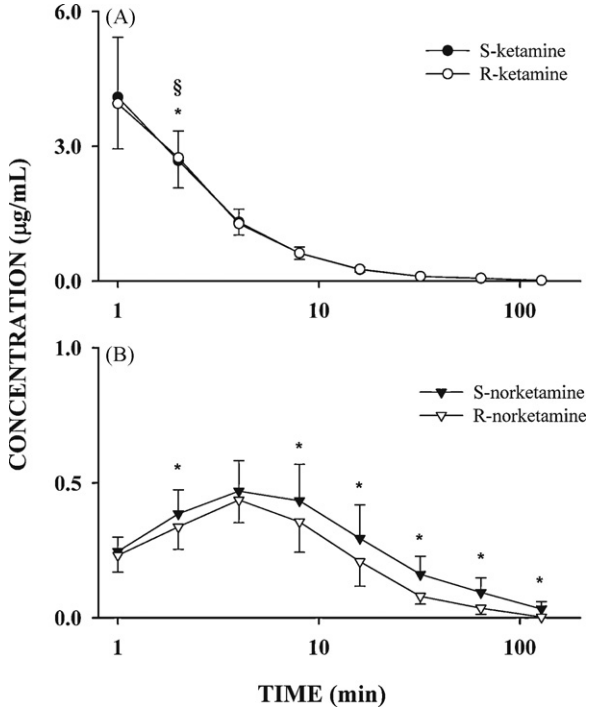

Fig. 2. Pharmacokinetic data of ketamine and norketamine in pony plasma. Mean and SD of plasma concentrations of (A) S-ketamine (filled circles) and R-ketamine (open circles) and (B) S-norketamine (filled triangles) and R-norketamine (open triangles) after bolus administration of 2.2 mg/kg racemic ketamine to 7 ponies anesthetised with isoflurane in oxygen. $^{*}P < 0.05$ within treatment group. $^{\S}P < 0.05$ between these data and those obtained after bolus administration of 1.1 mg/kg S-ketamine to the same 7 ponies (data not shown). Analyses were performed in a 50 μm I.D. fused-silica capillary of 38 cm total length at −20 kV. From Ref. [160].

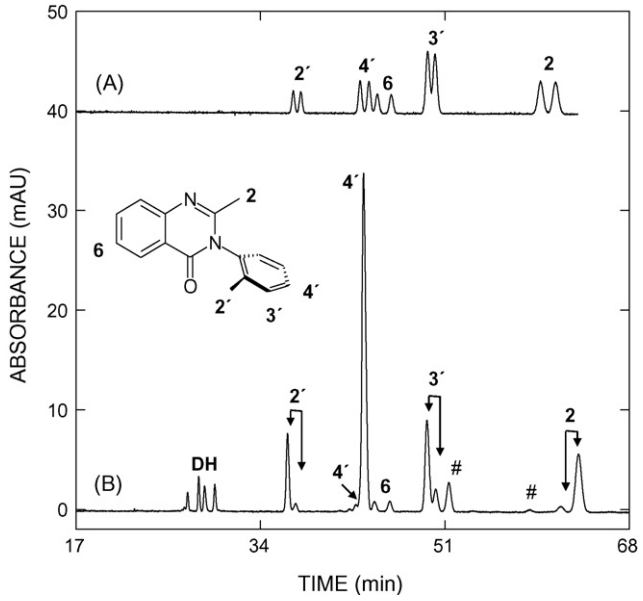

Fig. 3. Stereoselectivity of methaqualone hydroxylation in man. Panel A depicts the electropherogram obtained with the five monohydroxylated metabolites (about 20 μg/mL each) and panel B the data obtained with an enzymatically hydrolyzed and liquid/liquid extracted urine that was collected 8 h after intake of one tablet of Toquilone compositum containing 250 mg methaqualone and 25 mg diphenhydramine. Analyses were performed in a 50 μm I.D. capillary of 50 cm total length at 18 kV (current about 65 μA) employing a 75 mM phosphate buffer at pH 2.1 and 50 mM hydroxypropyl-β-cyclodextrin as chiral selector. The chemical structure of one methaqualone rotamer is presented as insert. The numbers indicate the sites of hydroxylation. Key: 2: 2-hydroxymethaqualone, 6: 6-hydroxymethaqualone, 2′: 2′-hydroxymethaqualone, 3′: 3′-hydroxymethaqualone, 4′: 4′-hydroxymethaqualone. Peaks marked with # are unidentified methaqualone metabolites.
Adapted from Ref. [113].

significantly higher than plasma concentrations of R-norketamine. Similar data were obtained under target-controlled infusion of racemic ketamine [157]. Furthermore, enantiomeric CE was also used to investigate the N-demethylation of ketamine to norketamine in ponies after bolus injection of racemic and S-ketamine under sedation with xylazine [161] and under subanesthetic constant rate infusion [163]. In all these investigations, previously unknown stereoselectivities were noted.

The assays summarized in Table 3 were applied to the enantiomeric analysis of drugs and/or metabolites in samples of patients, volunteers, and study animals. The list of drugs also includes examples in which an achiral drug is administrated and chiral metabolites are produced and monitored. These include two anthelmintic drugs, albendazole and mebendazole. Albendazole becomes oxidized to chiral albendazole sulfoxide [95–98], and mebendazole is hydroxylated [110]. Analysis of plasma from echinoccocosis patients undergoing pharmacotherapy with these drugs revealed that the two metabolic steps are enantioselective in man. Similarly, methaqualone, a hypnotic and anticonvulsive drug in which the rotation about the nitrogen-to-aryl bond between the planar 2-methyl-quinazolin-4(3H)-one structure and the o-tolyl moiety is sterically hindered (for structure see Fig. 3), becomes stereoselectively hydroxylated in man. This was assessed for the first time using enantioselective CE [113,114]. The pH 2.1 buffer used comprised hydroxypropyl-β-CD (OHP-β-CD) and was capable to resolve the enantiomers of the five major monohydroxylated metabolites of methaqualone found in hydrolyzed human urine. Typical electropherograms obtained with standards and a liquid/liquid extract of an enzymatically hydrolyzed urine are presented in Fig. 3. The latter data clearly reveal that hydroxylation of methaqualone is highly stereoselective. Except for the enantiomers of 6-hydroxymethaqualone, all other monohydroxylated metabolytes are preferentially formed as one enantiomer (Fig. 3B). Only very small amounts of unmetabolized methaqualone enantiomers are excreted [112] and are not detectable under the given conditions [113]. Another interesting study focused on the N-oxidation of deprenyl which is accompanied by the formation of a new stereogenic center on the quaternary nitrogen atom which leads to four diastereomers [104]. Using a pH 2.7 buffer with a dual CD system comprising 2 mM CM-β-CD and 4 mM dimethyl-β-CD (DM-β-CD), analysis of liquid/liquid extracts of rat urine revealed that the N-oxidation of the two deprenyl enantiomers occurs in a stereoselective manner. Other studies included the assessment of the impact of antiviral drugs on the metabolism of methadone for which monitoring of the R-enantiomer, the enantiomer with the much higher therapeutic effect compared to S-methadone, is of clinical interest [111], the ketoreduction of oxycodone and noroxycodone to oxycodol and noroxycodol, respectively, processes in which diastereoisomers are formed [119], and the hydroxylation of itraconazole [108]. In all these studies, stereoselectivity was assessed via a stereoisomeric ratio, e.g. an enantiomeric S/R ratio. Body fluids analyzed comprised plasma, serum and urine. Additionally, enantiomers of albendazole sulfoxide were determined in CSF [95] and saliva [97] of patients treated with albendazole, of deprenyl and its main metabolites in rat liver, kidney and heart tissues [103], and of trans-tramadol and trans-O-demethyltramadol in rat CSF and rat brain tissue of various locations [125].

3.2. Assessment of stereoselective pharmacokinetics and drug metabolism in vitro

During the past 10 years, a large number of *in vitro* metabolic studies of xenobiotics using stereoselective CE appeared in the

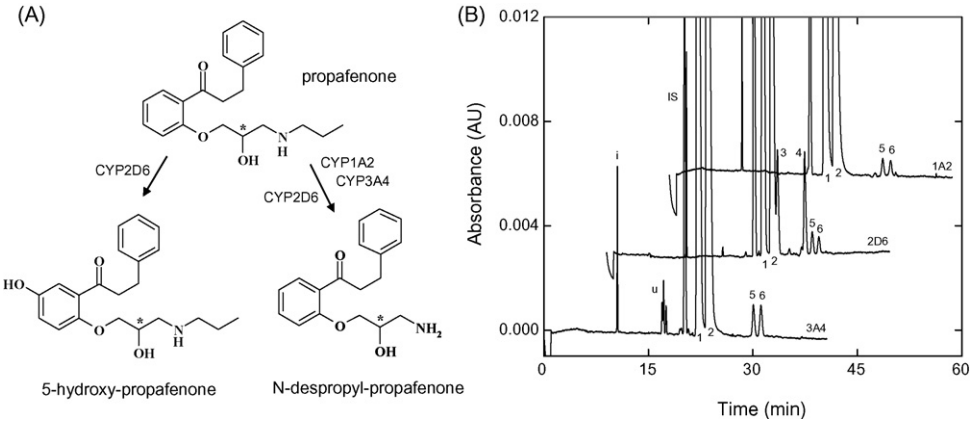

Fig. 4. Metabolism of propafenone *in vitro*. Panel A depicts chemical structures of propafenone together with the main metabolic pathways in the liver and panel B depicts electropherograms obtained with alkaline extracts prepared from 3 h *in vitro* incubations of 100 μM racemic propafenone with 25 pmol of three single CYP enzymes, namely CYP3A4, CYP2D6 and CYP1A2. Analyses were performed in a 50 μm I.D. fused-silica capillary of 36 cm total length at 13 kV using a 100 mM sodium phosphate solution, pH 2.0, 19% methanol and 0.6% sulfated β-cyclodextrin. Data are presented with *x*- and *y*-axis shifts of 9 min and 0.003 AU, respectively. Key: (1) S-propafenone; (2) R-propafenone; (3) S-5-hydroxypropafenone; (4) R-5-hydroxypropafenone; (5) S-N-depropylpropafenone; (6) R-N-depropylpropafenone; IS, internal standard (atenolol); u, unidentified metabolites produced by CYP3A4; i, peak unrelated to propafenone.
Adapted from Refs. [134,135].

literature (Tables 4 and 6). In most cases, the sample matrix was simplified prior to analysis and modified cyclodextrins or mixtures of cyclodextrins were used to obtain resolution of the target stereoisomers. Most CE configurations employed aqueous CZE-based conditions. One investigation was conducted in a micellar system (mephenytoin [132]) and one in methanol, a non-aqueous medium (fenbendazole [130]). Systems studied include sulfoxidation [128,130,137], N-oxidation [129], hydroxylation [108,114,131,132,134–136], dealkylation [131,134,135,138,139], carboxylation [133] and ketoreduction [119] reactions in presence of human or rat liver microsomes (HLM or RLM, respectively), human or rat liver S9 fraction, human liver cytosol (HLC), recombinant human single cytochrome P450 (CYP) enzymes or recombinant human single flavin monooxygenase (FMO) enzymes (Table 4). Not included in Table 4 are our investigations dealing with the metabolism of ketamine in different species which includes the N-demethylation to norketamine followed by hydroxylation of norketamine [156,166–169] (Table 6 and Section 3.4). Cimetidine [128], fenbendazole [130] and thiobencarb [137] are prochiral drugs which undergo metabolism by primary oxidation of the sulfide moiety to their sulfoxides, compounds that exhibit a sulfur stereogenic center, and by a second oxidation to the achiral sulfon derivative. The 6-ketoreduction of oxycodone and noroxycodone results in diastereomers which could not be separated without having a chiral selector [119].

The main topic of all investigations was to elucidate stereoselectivities in the metabolism of the drugs and metabolites. Except for the sulfoxidation of fenbendazole in rat liver microsomes [130], for which the reported data suggest that there might be no stereoselectivity, all systems revealed stereoselectivities of metabolic steps. These were either observed by analyzing (i) time-based incubations at one substrate concentration [108,114,119,129–133,135–137,156,166–169] or (ii) a range of substrate concentrations with incubation during a specified time interval followed by data fitting to a kinetic model which provides a complete characterization of a metabolic step for a given experimental configuration [128,134,138,139,166,167,169].

The use of recombinant single CYP or FMO enzymes instead of organ microsomes provides direct and unambiguous information about the involvement of a specific enzyme in a metabolic step together with its stereospecificity when an appropriate chiral assay is used. Using chiral CE, this was successfully shown for CYP enzymes involved in the metabolism of itraconazole [108], mephenytoin [132], methadone [132], methaqualone [114], propafenone [134,135], verapamil [139] and ketamine and norketamine [169], as well as for FMO enzymes involved in the sulfoxidation of cimetidine [128] and the N-oxidation of deprenyl, methampetamine and amphetamine [129]. While certain data were gathered to assess the stereoselectivity aspect only, a number of investigations revealed information that was previously unknown. Examples include the involvement of CYP2B6 and CYP2A6 as the only CYP enzymes which metabolize norketamine [169] and of CYP2D6 in the dealkylation of propafenone [135].

As an example, data obtained with propafenone are presented in more details. Propafenone becomes hydroxylated by CYP2D6 and dealkylated by CYP3A4, CYP2D6 and CYP1A2 (Fig. 4A). This could be nicely shown via incubation of racemic propafenone with single CYPs followed by enantioselective analysis of alkaline liquid/liquid extracts using a CE assay with S-β-CD as chiral selector (Fig. 4B). The kinetics of these reactions were also characterized [134,135]. The data presented in Fig. 5 depict kinetic data for the propafenone hydroxylation with CYP2D6. For that purpose, 9 different propafenone enantiomer concentrations between 0.8 and 25 μM were incubated for 30 min and 5-hydroxy-propafenone enantiomers were determined by CE. From the obtained data, metabolite formation rates were calculated and plotted against the initial propafenone enantiomer concentration in the incubation mixture (Fig. 5). The obtained data were fitted to two models, the Michaelis-Menten model and the Hill equation. The latter model was found to provide the better fit which indicates the occurrence of autoactivation. Furthermore, incubation of racemic propafenone vs. the single enantiomers revealed differences in the elucidated parameters, including the maximum formation rate and the maximal clearance due to autoactivation, for the enantiomers incubated as racemate or alone [134].

3.3. 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 [24,26,36,44]. During the past 10 years, bioanalytical examples studied with use of various CDs include the chiral differentiation of the optical isomers of racemorphan in urine [143], of metamphetamine and analogs in urine [140–142,146–149], of selegiline metabolites in urine [140,148,149], of methadone and its main metabolite EDDP in saliva [144], of MDMA and methadone

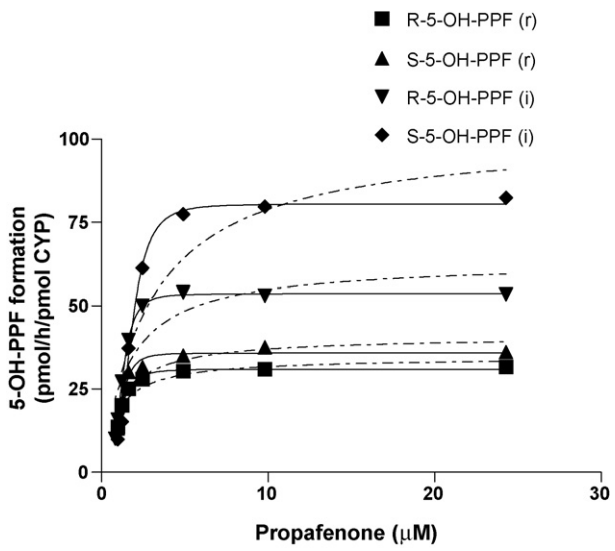

Fig. 5. Kinetics of propafenone hydroxylation catalyzed by human CYP2D6 *in vitro*. 5-Hydroxypropafenone (5-OH-PPF) formation rate vs. propafenone enantiomer concentration in the incubation mixture for the human CYP2D6 *in vitro* metabolism of racemic, R- and S-propafenone. 30 min incubations with 12.5 pmol CYP2D6 and 0.8–25 μM of each propafenone enantiomer were undertaken. The data were fit to the Hill equation (autoactivation model, solid lines, good fit) and the Michaelis–Menten model (broken lines, bad fit). Incubations with racemic and individual enantiomers are referred to as (r) and (i), respectively.
Adapted from Ref. [134].

in plasma [145], and of terbutaline in urine [150] (Table 5). These efforts essentially represent a continuation of the investigations reported in the nineties [26]. The work evolved towards the use of fluorescence detection for MDMA and its main metabolite 3,4-methylenedioxyamphetamine [142], as well as ESI-MS detection for MDMA and methadone in plasma [145] and for methamphetamine and related compounds in urine [147–149], detection principles that are more specific than the customary UV absorption detection.

For illicit and banned drugs, chiral discrimination has a number of relevant applications. E.g. 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. Dextrorphan and levorphanol, respectively, are their O-demethylated metabolites. Although the four compounds can be distinguished by MECC in presence of β-CD [34], urinary monitoring of the two metabolites is required only [143]. 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. The use of preparations containing dextromethorphan by athletes is allowed, whereas the use of levorphanol is banned by the International Olympic Committee. Thus, the chiral MECC 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 [24] 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. Thus, the only presence of the R-enantiomers of these compounds indicates the intake of drugs that metabolize to the R-enantiomers. Chiral CE has been successfully used to distinguish between the intake of S-(+) enantiomers (evidence for drug abuse) or R-enantiomers of methamphetamine and amphetamine (evidence for prescribed drug selegiline or non-prescription nasal inhalants) [44,140,148,149]. Furthermore, both urinary enantiomers are detected after consumption of methamphetamine of European origin, whereas only the S-(+) enantiomers are often found in association with this abused drug distributed in Korea and Japan [146,149].

The International Olympic Committee and the World Antidoping Agency restrict the use of β_2-agonists, such as terbutaline and salbutamol, and only the inhaled administration is allowed for therapeutic reasons. Thus, assays leading to the distinction between the routes of administration should be available. In the case of salbutamol, the rate of sulfation and the urinary S-(+)/R-(−) enantiomeric ratio depend on the route of administration. As the same might be true for terbutaline, a chiral CE assay for urinary terbutaline was developed. Using this assay, the S-(+)/R-(−) enantiomeric ratio of terbutaline after oral administration of terbutaline racemate was found to be lower than that obtained after inhalation of the drug but the difference was not statistically different [150]. More work is required to assess the suitability of this approach for doping analysis.

The assay for methadone and its main metabolite EDDP in saliva [144] was developed for the determination of the enantiomeric ratios of methadone and EDDP in oral fluid of addicts under maintenance program for narcotic dependence. Using electrokinetic sample injection from a methanol/water (50:50, v/v) based matrix obtained by liquid/liquid extraction at alkaline pH, LODs and LOQs for all enantiomers were 2.4 and 8 ng/mL, respectively. With this assay, data obtained with 60 patient specimens revealed EDDP data for 10 samples (concentration range: 9–22 ng/mL; R/S ratio between 0.70 and 0.94) and methadone data for all samples (concentration range: 18–622 ng/mL; R/S ratio between 1.00 and 3.13). These data may reflect the free fraction of methadone and EDDP enantiomers in plasma.

Not included in Table 5, but also of interest from a toxicological point of view, is ketamine and its main metabolite norketamine. S-ketamine ((+)-ketamine) and S-norketamine ((+)-norketamine) are active anesthetic agents, whereas R-ketamine ((−)-ketamine) and R-norketamine ((−)-norketamine) produce hallucinatory effects [7]. Due to the hallucinogenic effects at subanesthetic doses, ketamine is abused by medical personnel and ketamine (also known as special K) became popular among the European and other party scenes as a drug of abuse. Ketamine is usually taken as the racemate. There is a difference in efficacy and toxicity between the ketamine enantiomers and the norketamine enantiomers which calls for an assay that should be able to determine the stereoisomers in body fluids, including plasma and urine. For humans, such analyses were thus far performed by chiral chromatography only [7]. The CE-based chiral assay for analysis of ketamine and its metabolites in equine plasma and urine, however, should be suitable for that purpose as well (Fig. 1B, [155,156]).

3.4. The multidisciplinary ketamine project

Ketamine (for chemical structure see Fig. 1A) is an intravenous analgesic and anesthetic drug widely used in clinical practice of man and animals. Ketamine is administered as racemate (in most countries) or as S-ketamine (e.g. in Germany to humans and

Switzerland to cats). The S-enantiomer is more potent than the R-enantiomer and exhibits a greater clearance and faster anesthetic recovery compared to the racemate [7,174]. Although used extensively in horses and dogs, the pharmacokinetic data and clinical analgesic effects of S- and R-ketamine are poorly documented for these species, and in analogy to the findings in human medicine, application of S-ketamine instead of racemic ketamine could be of great clinical interest. Furthermore, the metabolic fate of ketamine is not well known. All this led to a multidisciplinary, comprehensive enantioselective ketamine study with the involvement of clinicians, pharmacologists and scientists from Vetsuisse (veterinary medicine of Bern and Zürich), US, Argentina and our laboratory [154–169]. This work comprises pharmacokinetic, clinical, infusion and drug metabolism aspects in which enantioselective CE was used to investigate the stereoselectivity of (i) the biotransformation of ketamine to norketamine via determination of their enantiomers in plasma, urine and incubations of ketamine with microsomal preparations of different species, (ii) norketamine hydroxylation and formation of dehydronorketamine in different species and (iii) the human CYP enzymes metabolizing ketamine and norketamine (Table 6).

Ketamine undergoes extensive hepatic first-pass metabolism, with the major pathways involving N-demethylation to norketamine, hydroxylation followed by glucuronidation of norketamine at positions 4–6 of the cyclohexanone ring and formation of 5,6-dehydronorketamine (for selected chemical structure see Fig. 1A). The enantioselective CE assay is based upon the use of randomly sulfated β-cyclodextrin (product of Aldrich with 7–11 mol sulfate/mol β-CD) in a phosphate-Tris buffer at pH 2.5. This medium was found to resolve the enantiomers of ketamine, norketamine and dehydronorketamine found in alkaline extracts of equine plasma and urine [154,155], dog plasma [165] and *in vitro* preparations [156,166–169]. The stereoisomers of the four most abundant hydroxynorketamine metabolites, all with hydroxylation at the cyclohexanone ring, were detected in plasma (Fig. 1B) and urine, whereas three of them were found *in vitro* [156]. The data presented in Fig. 1B illustrate the high resolution obtained with 10 mg/mL of the chiral selector together with the apparent stereoselectivities observed for most compounds of interest. It is important to mention that this project was hampered by lot-to-lot variations of the chiral selector which mostly influenced the resolution of ketamine enantiomers. A blend of two lots provided best data to resolve the compounds of interest [155].

The enantioselective ketamine CE assay was applied to the determination of the stereoselective biotransformation of ketamine after bolus [160,161], target control [157] and low dose, subanesthetic continuous infusion of racemic ketamine or S-ketamine to Shetland ponies [163] and of racemic ketamine to conscious dogs [165]. Investigations with Shetland ponies sedated with xylazine revealed that the elimination of S-ketamine was faster after S-ketamine compared with racemic ketamine administration and the time to standing position was significantly diminished [161]. Furthermore, compared with racemic ketamine, anesthesia recovery is improved with S-ketamine infusions in patient horses [164], and pharmacokinetics is dependent on comedication as was demonstrated with isoflurane anesthesia compared to sedation with xylazine [160,161]. *In vitro* work comprised incubation of S-ketamine and racemic ketamine with equine liver and lung microsomes [166], incubation of racemic ketamine, S-ketamine and R-ketamine with equine, canine and human liver microsomes [167], simultaneous incubation of ketamine and other analgesics (methadone, ibuprofen, diclofenac and xylazine) with equine, canine and human liver microsomes [168], and incubation of racemic ketamine with single human cDNA expressed CYP450 enzymes [169]. These efforts revealed that the N-demethylation of ketamine is stereoselective and not only occurring in the liver (e.g. also in the lung in equines), that CYP3A4, CYP2C19, CYP2B6, CYP2A6, CYP2D6 and CYP2C9 are involved in the metabolism of ketamine to norketamine, and that CYP2B6 and CYP2A6 (two enzymes with low abundance) were the only CYP enzymes found to transform norketamine to its hydroxylated metabolites and dehydronorketamine. Furthermore, enantioselective CE data were used for physiologically based pharmacokinetic modeling which allows the estimation of plasma and tissue drug levels [157], to predict the infusion rate of racemic ketamine to maintain a pre-determined plasma concentration of S-ketamine [158] and to establish a two-phase model based on Hill kinetics to describe the N-demethylation of ketamine to norketamine *in vitro* [166]. Using liver microsomes, the *in vitro* N-demethylation of ketamine to norketamine in equines, canines and humans could be described by Michaelis–Menten kinetics [167]. For single human CYPs, the kinetics could be best described by either the Michaelis–Menten equation (CYP3A4) or the Hill model (CYP2B6) [169]. All the performed work, in which enantioselective CE played a pivotal role, provided (i) new insights into the metabolism of ketamine in different species and (ii) may eventually lead to new strategies for optimization of ketamine use in clinical veterinary practice.

4. Achievements and outlook

Since its inception two decades ago, chiral CE has proven itself as being a powerful tool for enantioselective determination of drugs and metabolites in body fluids, tissues and microsomal preparations. It represents an accurate technology which has found its place in a cost and environment controlled age. This is manifested by the large number of investigations reported in the literature. Compared to chiral HPLC, chiral CE provides higher efficiency and is simpler, faster and cheaper, consumes a much smaller amount of organic solvents and handles small sample volumes. It permits chiral separations and analyses to be performed under completely different conditions, such as CZE, MECC and CEC. For the first two techniques, separation conditions can be changed rapidly via a simple buffer change, an approach that does not require lengthy column conditioning procedures. Furthermore, there is fully automated equipment available, namely instruments featuring single capillaries and setups with multiple capillaries for higher throughput. Strategies for sample preparation (direct injection, extraction, etc.), application (hydrodynamic vs. electrokinetic injection) and detection (absorbance vs. fluorescence vs. MS) have been carefully worked out, its result being a mature technique with a ppb concentration sensitivity. With the exception of MS detection and the CEC application, this status was almost completely established 10 years ago. Our previous review provides an almost complete list of all the work performed during the first decade [26].

During the past decade, first applications using CEC [32], MS enantiomer detection [50,51,59,66,68,73,127,145–148] and samples originating from organ tissues other than hair [103,125] appeared in the literature. Furthermore, compared to the previous decade, there was a tremendous increase in the use of charged CDs (sulfated CDs) and mixtures of CDs as chiral selectors. Many new CE assays were developed and applied to relevant investigations with the result that (i) the suitability of using CE could be further demonstrated with examples that were previously studied by chiral chromatography and (ii) new insights into the stereoselectivity of drug metabolism both *in vivo* and *in vitro* were gained for systems that were not previously investigated with another enantioselective method. Examples for the first group are the assays for ibuprofen [82,83], ketoprofen [85] and disopyramide [79]. The scientific literature was extended with previously unknown enantioselective drug metabolism aspects, including the stereoselectivity of the hydroxylation of itraconazole [108], mebendazole

[110] and methaqualone [113,114] in man, the ketoreduction of oxycodone and noroxycodone [119] in humans, and the ketamine metabolism in equines and canines [154–169]. Assays for simultaneous separation of enantiomers of a drug together with its metabolites, which could not be separated by HPLC in one run, evolved, including those for thalidomide and its hydroxylated metabolites [136], propafenone, depropylpropafenone and 5-hydroxypropafenone [135], mianserin, demethylmianserin and 8-hydroxymianserin [116], chloroquine and desethylchloroquine [100], and five monohydroxylated metabolites of methaqualone [113,114]. During the decade, CE was also successfully used to investigate the drug metabolism kinetics *in vitro*, including those of propafenone [134], cimetidine [128], verapamil [139] and ketamine [166,167,169]. Enantioselective CE played an important role in the multidisciplinary ketamine project. It provided new insights into the stereoselective metabolism of ketamine in different species, both *in vivo* and *in vitro*, and the obtained results might eventually lead to new strategies for optimization of pharmacotherapy with this drug in clinical practice.

Chiral CE was extensively employed for research purposes. As it does not only represent a complementary tool to the widely applied chromatographic methods, it should also offer the possibility of bringing chiral separations and analyses into the routine arena. To our knowledge, however, this has not yet occurred. A couple of critical aspects of chiral CE technology might be responsible for this, namely lack of (i) sufficient concentration sensitivity with UV detection in absence of stacking, (ii) peak detection time stability over long periods of time (e.g. due to lot-to-lot differences of chiral selectors, particularly with certain charged CDs), and (iii) insufficient distribution of CE-MS equipment. Peak detection stability is much improved via use of single isomer selectors. Ongoing efforts in research laboratories using novel and robust sheathless ESI interfaces [175,176], as well as the announcement of such interfaces from the key manufacturers of CE equipment, will lead to improved CE-MS setups and thus open the possibility for a better adoption of MS as universal detector in CE-based biomedical analysis. On the other hand, despite clear reasons to target single enantiomers of many chiral drugs, such as antidepressants [177], only very few enantioselective analyses are performed on a routine basis [7,8]. High costs of chiral chromatographic columns might have hindered an adoption of chiral separations in routine laboratories. Although chiral CE should be well suited for that purpose, CE instrumentation is not well distributed among clinical, pharmacological and forensic laboratories. The high costs of automated CE instrumentation and the reluctance to learn new technologies might be the major reasons for the rather slow adoption of chiral CE technology. It is hoped that this review is encouraging researchers and laboratory officials to introduce this technology into their stereoselective drug assay projects.

Nomenclature

18C6H$_4$	(+)-(18-crown-6)-tetracarboxylic acid
CE	capillary electrophoresis
CE-β-CD	carboxyethyl-β-cyclodextrin
CEC	capillary electrochromatography
CD	cyclodextrin
α-CD	α-cyclodextrin
β-CD	β-cyclodextrin
CITP	capillary isotachophoresis
CM-β-CD	carboxymethyl-β-cyclodextrin
CM-γ-CD	carboxymethyl-γ-cyclodextrin
CSF	cerebrospinal fluid
CYP	cytochrome P450
CZE	capillary zone electrophoresis
(R)-DNBPG	(R)-(−)-(3,5-dinitrobenzoyl)-α-phenylglycine
DM-β-CD	heptakis (2,6-di-O-methyl)-β-cyclodextrin
DSI	direct sample injection
ECL	electrochemiluminescence
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EMMA	electrophoretically mediated microanalysis
ESI	electrospray ionization
F	fluorescence
FMO	flavin monooxygenase
H	hydrolysis
HDAS-β-CD	heptakis (2,3-di-O-acetyl-6-O-sulfo)-β-cyclodextrin
HDMS-β-CD	heptakis (2,3-di-O-methyl-6-O-sulfo)-β-cyclodextrin
HLM	human liver microsomes
HLC	human liver cytosol
HPLC	high-performance liquid chromatography
HS-β-CD	highly sulfated-β-cyclodextrin
HS-γ-CD	highly sulfated-γ-cyclodextrin
LIF	laser induced fluorescence
LLE	liquid/liquid extraction
LPME	liquid-phase microextraction
M-α-CD	methyl-O-α-cyclodextrin
M-β-CD	methyl-O-β-cyclodextrin
MDMA	3,4-methylenedioxymethamphetamine
MECC	micellar electrokinetic capillary chromatography
MLM	mouse liver microsomes
MRM	multiple reaction monitoring
MS	mass spectrometry
NACE	non-aqueous capillary electrophoresis
ODAS-γ-CD	octakis-(2,3-di-O-acetyl-6-O-sulfo)-γ-cyclodextrin
OHP-β-CD	(2-hydroxypropyl)-β-cyclodextrin
OHP-γ-CD	(2-hydroxypropyl)-γ-cyclodextrin
PA-β-CD	6-monodeoxy-6-mono(3-hydroxy) propylamino-β-cyclodextrin
PH-γ-CD	phosphated-γ-cyclodextrin
Poly-L-SUCLS	polysodium N-undecenoxycarbonyl-L-leucine sulfate
Poly-L,L-SULV	polysodium N-undecenoyl-L,L-leucyl-valinate
PP	protein precipitation
RLM	rat liver microsomes
S-β-CD	sulfated-β-cyclodextrin
SBE-β-CD	sulfobutylether-β-cyclodextrin
SDME	single drop microextraction
SIM	single ion monitoring
SPE	solid-phase extraction
STC	sodium taurocholate
SUC-β-CD	succinyl-β-cyclodextrin
TM-β-CD	heptakis 2,3,6,-tri-O-methyl-β-cyclodextrin
(3R,4S)-Whelk-O1	1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene based stationary phase

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