Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 1000–1008

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Therapeutic drug monitoring of seven psychotropic drugs and four metabolites in human plasma by HPLC–MS

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ARTICLE INFO

Article history:
Received 20 April 2009
Accepted 5 July 2009
Available online 15 August 2009

Keywords:
Method development
Quantification
Atypical antipsychotics
Antidepressants
SPE
LC–MS
Validation

ABSTRACT

A simple and sensitive LC–MS method was developed and validated for the simultaneous quantification of aripiprazole (ARI), atomoxetine (ATO), duloxetine (DUL), clozapine (CLO), olanzapine (OLA), sertindole (STN), venlafaxine (VEN) and their active metabolites dehydroaripiprazole (DARI), norclozapine (NCLO), dehydrosertindole (DSTN) and O-desmethylvenlafaxine (OVEN) in human plasma. The above mentioned compounds and the internal standard (remoxipride) were extracted from 0.5 mL plasma by solid-phase extraction (mix mode support). The analytical separation was carried out on a reverse phase liquid chromatography at basic pH (pH 8.1) in gradient mode. All analytes were monitored by MS detection in the single ion monitoring mode and the method was validated covering the corresponding therapeutic range: 2–200 ng/mL for DUL, OLA, and STN, 4–200 ng/mL for DSTN, 5–1000 ng/mL for ARI, DARI and finally 2–1000 ng/mL for ATO, CLO, NCLO, VEN, OVEN. For all investigated compounds, good performance in terms of recoveries, selectivity, stability, repeatability, intermediate precision, trueness and accuracy, was obtained. Real patient plasma samples were then successfully analysed.

1. Introduction

Therapeutic drug monitoring is a useful tool for the clinical management of patients receiving a pharmacotherapy, particularly in psychiatry. Recommended target plasma concentrations for psychoactive drugs have already been published [1]. Aripiprazole, clozapine, olanzapine, and sertindole are so-called second generation or atypical antipsychotics. Compared to first generation antipsychotics, they have greatly improved the response to treatment of schizophrenia spectrum disorders by their efficacy on negative symptoms [2]. Venlafaxine and duloxetine are second generation antidepressants, with a selective inhibitory activity on serotonine and noradrenaline reuptake, which present a safer profile than the tricyclic antidepressants. Atomoxetine is a new noradrenaline reuptake inhibitor used for the treatment of attention deficit and hyperactivity disorder. The chemical structures and calculated physico-chemical properties of these seven studied psychotropic drugs and their active metabolite are presented in Fig. 1 and Table 1, respectively.

Most of the published methods allow quantification of a single compound, sometimes with their related metabolite [3–6]. Simultaneous quantification of various psychotropic drugs have also been published [7], mostly clozapine in combination with olanzapine [8–12], or the antidepressant venlafaxine with other drugs in the same therapeutic class [13–16]. For a monitoring service aiming to cover a large panel of psychotropic drugs, the opportunity of simultaneous quantification is very attractive also in terms of practical aspect and labour time. Although some methods have been published using gas chromatography [17–19], separation was mainly performed by HPLC coupled with UV [20–24], MS [5,11,25] and MS–MS detection [22,26–30]. For mass spectrometry, electrospray ionization in the positive mode (ESI+) was mainly used, rather than atmospheric pressure chemical ionization [31,32]. Therefore, a development on a LC–MS in ESI+ is considered suitable.

ESI mode has been reported to be particularly sensitive to the matrix effects when biological matrix such as plasma are analysed [33–35]. This matrix effects may influence the quality of a quantitative bioanalysis. They are reported as being the origin of possible co-elution of endogenous matrix components which can lead to unpredictable alteration of the MS signal [33–35]. Therefore, a clean sample extraction process such as LLE and SPE is mandatory to reduce this undesirable effect.

Liquid–liquid extraction (LLE) remains an attractive approach for extracting the molecules of interest from complex matrix such as plasma [3]. Recently, methods with on-line [36] or off-line solid-phase extraction (SPE) procedures [19] have been proposed. In this paper, the development and validation of a method is reported for

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0731-7085/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.jpba.2009.07.007

Table 1
Relevant LC–MS characteristics.

Compound	pK_a basic[a]	log P[a]	log D pH 8[a]	$[M+H]^+$	Voltage [V]	t_R [min][b]
VEN	9.26	2.91	1.63	278	160	6.8
OVEN	9.33	2.26	0.91	264	160	4.7
OLA	7.77	3.29	3.05	313	140	8.1
DUL	10.02	3.73	1.74	298	80	8.4
CLO	7.14	3.47	3.42	327	40	9.7
NCLO	7.94	3.08	2.81	313	140	7.5
STN	9.06	5.26	4.18	441	150	10.7
DSTN	8.74	6.62	5.82	439	150	11.3
ARI	6.71	5.59	5.57	446	150	12.7
DARI	6.71	5.63	5.61	448	150	11.7
ATO	10.12	3.28	1.2	256	140	7.9

[a] pK_a basic, log P and log D at pH 8 were calculated using Advanced Chemistry Development Software V8.14 for Solaris (ACD/Labs, Toronto, Canada).
[b] t_R: Retention time.

the simultaneous determination of new psychotropic drugs ($n = 7$) and their respective active metabolites ($n = 4$), using SPE prior to LC–MS analysis.

2. Experimental

2.1. Chemicals and reagents

The drugs were kindly provided by their manufacturers: aripiprazole (ARI) and dehydroaripiprazole (DARI) by BMS (New Brunswick, USA); atomoxetine (ATO), duloxetine (DUL), and olanzapine (OLA) by Eli Lilly (Indianapolis, USA); clozapine (CLO) and norclozapine (NCLO) by Novartis (Basel, Switzerland); sertindole (STN) and dehydrosertindole (DSTN) by Lundbeck (Copenhagen, Denmark); venlafaxine (VEN) and O-desmethylvenlafaxine (OVEN) by Wyeth Ayerst (Princeton, USA); and the internal standard (IS) remoxipride (RMO) by Astra-Zeneca (Södertälje, Sweden). Hydrochloric acid (HCl 37%) was purchased from Merck (Darmstadt, Deutschland), and acetonitrile (ACN) and methanol (MeOH) both in gradient HPLC grade from J.T. Baker (Deventer, Holland). Ammonium hydroxide (25%), ammonium acetate for MS, and citric acid monohydrate 99–102% were bought from Sigma–Aldrich (Steinheim, Deutschland). Ultrapure water was obtained from a Milli-Q® RG with a QPAQ2 column system (Millipore, MA, USA). Other chemicals were of analytical grade. For the preparation of calibration and validation standards, more than 10 different batches of human plasma were obtained from the Hospital's blood transfusion center (CHUV, Lausanne, Switzerland).

2.2. Standard solution, working solution

Standard stock solutions of each analyte at 1 mg/mL were prepared by dissolving the adequate amount of pure analyte in MeOH and stored for a maximum of 1 year at −20 °C. Two working solutions were obtained by diluting the stock solutions with 0.01N HCl to 10 ng/μL for DUL, OLA, STN, DSTN and to 50 ng/μL and subsequently to 10 ng/μL for ATO, CLO, NCLO, ARI, DARI, VEN and OVEN according to their plasma concentration ranges. These solutions were divided into aliquots (0.5 mL) and stored at −20 °C. The two working solutions were then both diluted to 1.0 and 0.2 ng/μL to prepare calibration standards or validation standards at the

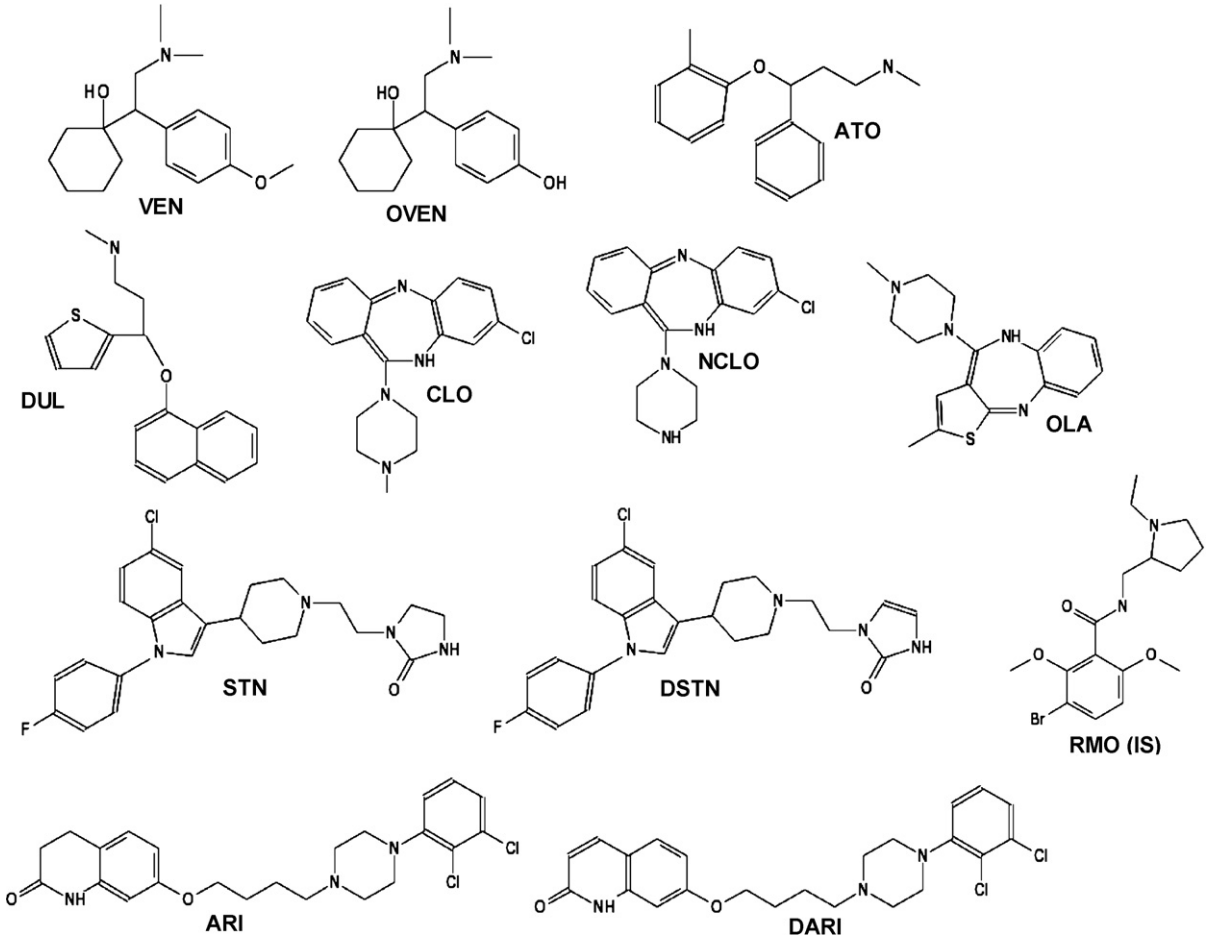

Fig. 1. Structure of molecules of interest.

appropriate concentration in plasma. Two different batches were prepared, one for preparation of the calibration standards and the other one for the validation standards. Finally, IS solution was prepared at 2 ng/mL in MeOH and stored at −20 °C.

2.3. Equipment

The liquid chromatography system consisted of an Agilent HP1100 binary pump equipped with a 100-vial autosampler (Agilent Technologies, Walldbronn, Deutschland), with a measured dwell volume of 1.15 mL. The system was coupled to a single quadrupole mass spectrometer (Agilent MSD), with electrospray ionization in the positive mode. Data acquisition, data handling and instrument control were performed by Chemstation 8.01.01 (Agilent Technologies). The whole system was maintained at 22 °C in an air conditioned room. The best chromatographic parameters were determined using HPLC modeling software (Osiris 4.1.1.2, Datalys, Grenoble, France), on the basis of two generic gradients that only differed in slope. The buffer capacity was carefully checked using PHoEBuS software version 1.3 (Analis, Namur, Belgium).

Separation was carried out on a Xbridge C18 column (2.1 × 100 mm; 3.5 μm) (Waters, Milford, MA, USA) equipped with a guard cartridge (2.1 × 10 mm; 3.5 μm) containing the same packing material. A 5 μL sample was injected into the system at a flow rate of 300 μL/min. Ammonium acetate 20 mM adjusted to pH 8.1 with ammonium hydroxide 25% (A) and ACN (B) was used as the mobile phase with the following gradient program: 16% of B at 0 min, 33.5% of B at 1.31 min, 60% of B maintained from 7.51 to 10.9 min, followed by a washing step at 85% of B from 11 to 13 min and finally, a 5 min reconditioning step at the initial conditions. The stability of the buffer solution was checked and found to remain stable for at least 2 weeks.

Analytes were quantified in the single ion monitoring (SIM) mode. All results were based on the peak area ratio between the

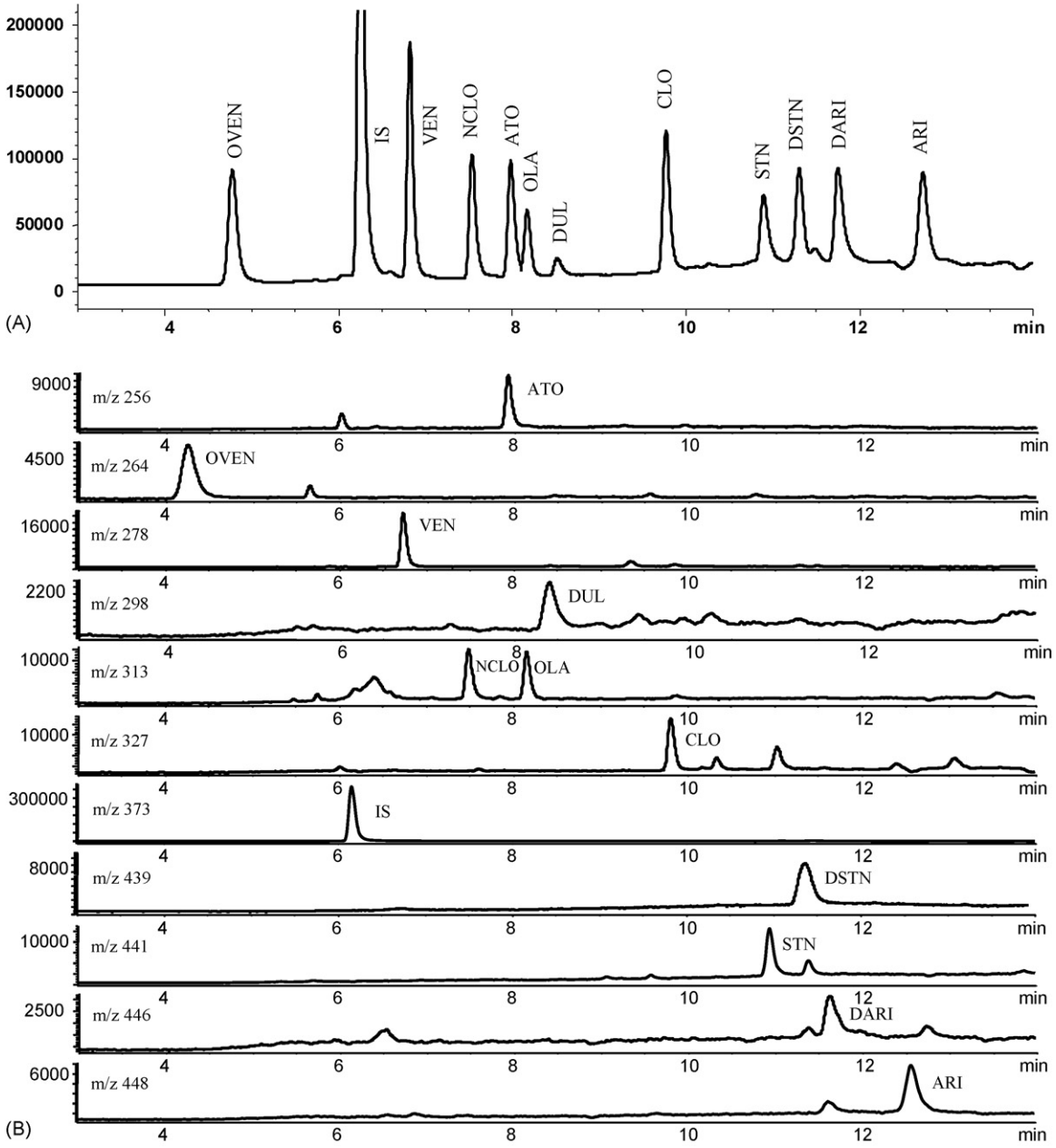

Fig. 2. Total ion current chromatogram of a plasma extract containing drugs at 50 ng/mL (OVEN, VEN, NCLO, ATO, CLO, DARI, ARI) or 20 ng/mL (OLA, DUL, STN, DSTN) and 100 ng/mL IS. Extracted ion chromatograms at 5 ng/mL. Note the isotopic contribution peak of DSTN and DARI on STN and ARI, respectively.

Table 2
Recoveries, matrix effect and process efficiency at 10 ng/mL except for the IS at 100 ng/mL.

Compound	Process efficiency (%)	Matrix effect (%)	Extraction recovery (%)
VEN	105	99	106
OVEN	106	99	107
OLA	131	118	111
DUL	107	116	92
CLO	103	101	102
NCLO	91	94	97
STN	126	133	95
DSTN	97	102	95
ARI	131	122	108
DARI	147	120	123
ATO	97	95	102
RMO (IS)	91	91	100

drug and the IS. The MS conditions were set as followed: drying gas flow 8 L/min, nebulizer pressure 40 psi, drying gas temperature 350 °C, capillary voltage 2000 V, dwell time 24 ms. Table 1 lists the m/z ratios measured, as well as the optimal fragmentor voltages for each $[M+H]^+$.

2.4. Sample preparation

Plasma calibration standards and plasma validation standards were extracted by SPE. The loading, washing, elution, evaporation and reconstitution steps of the sample preparation used during the SPE process were systematically investigated and the final process is reported below. First, 100 ng IS was added to 500 μL of plasma sample. The mixture was then diluted with 500 μL 1 M citric acid in water, vortexed and 1000 μL was loaded onto a SPE 96-well plate Oasis MCX support 10 mg (Waters, Milford, MA, USA), previously conditioned with 500 μL MeOH followed by 500 μL 1 M citric acid in water. A washing step consisting of 1 mL of 1 M citric acid in water followed by 1 mL MeOH was performed prior to elution. The compounds were then eluted with 500 μL MeOH–ammonium hydroxide 25% (94:6, v/v). After each step, a slow vacuum was applied until the wells were dry. The extracted samples were evaporated to dryness (40 °C, N_2 flow), and the residue was reconstituted in 250 μL mobile phase, i.e. ammonium acetate (pH 8.1; 20 mM)–ACN (84:16, v/v) and 5 μL was injected into the LC–MS system.

2.5. Method validation

The validation of this method was based on the guidelines of the Food and Drug Administration (FDA) and the recommendations of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [37]. The conference report of Viswanathan et al. from the workshop held on the same topic was also considered [38]. Three validation series were assessed to determine selectivity, repeatability and intermediate precision, trueness and accuracy on the basis of peak area ratio of drug and IS.

Method selectivity was ascertained for each validation series by analysing two different blank plasmas extracted and injected at the beginning of the HPLC analysis. In addition, after the highest validation standard, blank plasma was injected to determine any possible carryover effect. Drugs usually prescribed and/or taken in combination with the molecules of interest as well as some metabolites were tested. Plasma spiked with these potential interfering drugs were extracted and analysed. In case of similar retention factors, the potential signal suppression was carefully assessed by comparing peak area of the analyte alone and with two increasing concentrations of the potential interference.

Matrix effects were qualitatively estimated by simultaneously post-infusing a standard solution of the analytes and the IS [39]. Different batches of extracted blank plasma ($n = 6$) and blank plasma containing IS ($n = 6$) were analysed. An alteration of the m/z ratio baseline of the studied analytes at its retention time was considered as a matrix effect [33]. Matrix effects were quantitatively investigated at low (10 ng/mL) and high concentrations (150 ng/mL for DUL, OLA, STN, DSTN and 800 ng/mL for ATO, CLO, NCLO, VEN, OVEN, ARI, DARI) on the basis of the procedure proposed by Matuszewski et al. [40].

The variability of the peak areas was evaluated by calculation of the relative standard deviation (RSD) value on an assay done in triplicate for all analytes with a pool of five different sources of blank plasma. The process efficiency was obtained as the ratio between peak area of the plasma spiked before extraction and a standard solution at the corresponding level directly injected. Matrix effect was established using the peak area ratio between a plasma spiked after extraction and a standard solution directly injected. Extraction recoveries were defined as the ratio between plasmas spiked before and after the extraction.

Table 3
Drugs tested for selectivity assays.

Therapeutic class	Compound	t_R (min)[a]
Analytes and IS	ODVenlafaxine	4.71
	Remoxetine	6.17
	Venlafaxine	6.75
	Norclozapine	7.43
	Atomoxetine	7.98
	Olanzapine	8.05
	Duloxetine	8.45
	Clozapine	9.62
	Sertindole	10.71
	Dehydro-Sertindole	11.14
	Dehydro-Aripiprazole	11.58
	Aripiprazole	12.58
Antidepressants	Clomipramine	nd
	Desmethy-clomipramine	nd
	Imipramine	nd
	NODDVenlafaxine	3.35
	NDVenlafaxine	6.02
	Desmethyl-mirtazapine	6.34
	Nortriptyline	8.44
	Desmethyl-citalopram	7.12
	Citalopram	7.67
	Reboxetine	8.02
	Mirtazapine	8.39
	Desipramine	8.65
	Desmethyl-trimipramine	8.65
	Norfluoxetine	8.66
	Trazodone	8.86
	Fluoxetine	8.91
	Amitriptyline	9.83
	Sertraline	10.38
	Trimipramine	10.89
	Mianserine	11.29
Anxiolitics-hypnotics	Midazolam	8.88
Antipsychotics	Chlorpromazine	nd
	Sulpiride	nd
	Amisulpride	4.65
	9-OH Risperidone	6.90
	Risperidone	7.40
	Loxapine	7.54
	Haloperidol	8.69
	Quetiapine	8.97
	Ziprasidone	10.08
	Norsertindole	10.30
	Clopenthixol	10.55
Pro cognitifs	Galanthamine	4.21
	Memantine	6.38
	Rivastigamine	6.39
	Donepezil	8.77
Other drug	Methadone	9.05

[a] t_R: Retention time.

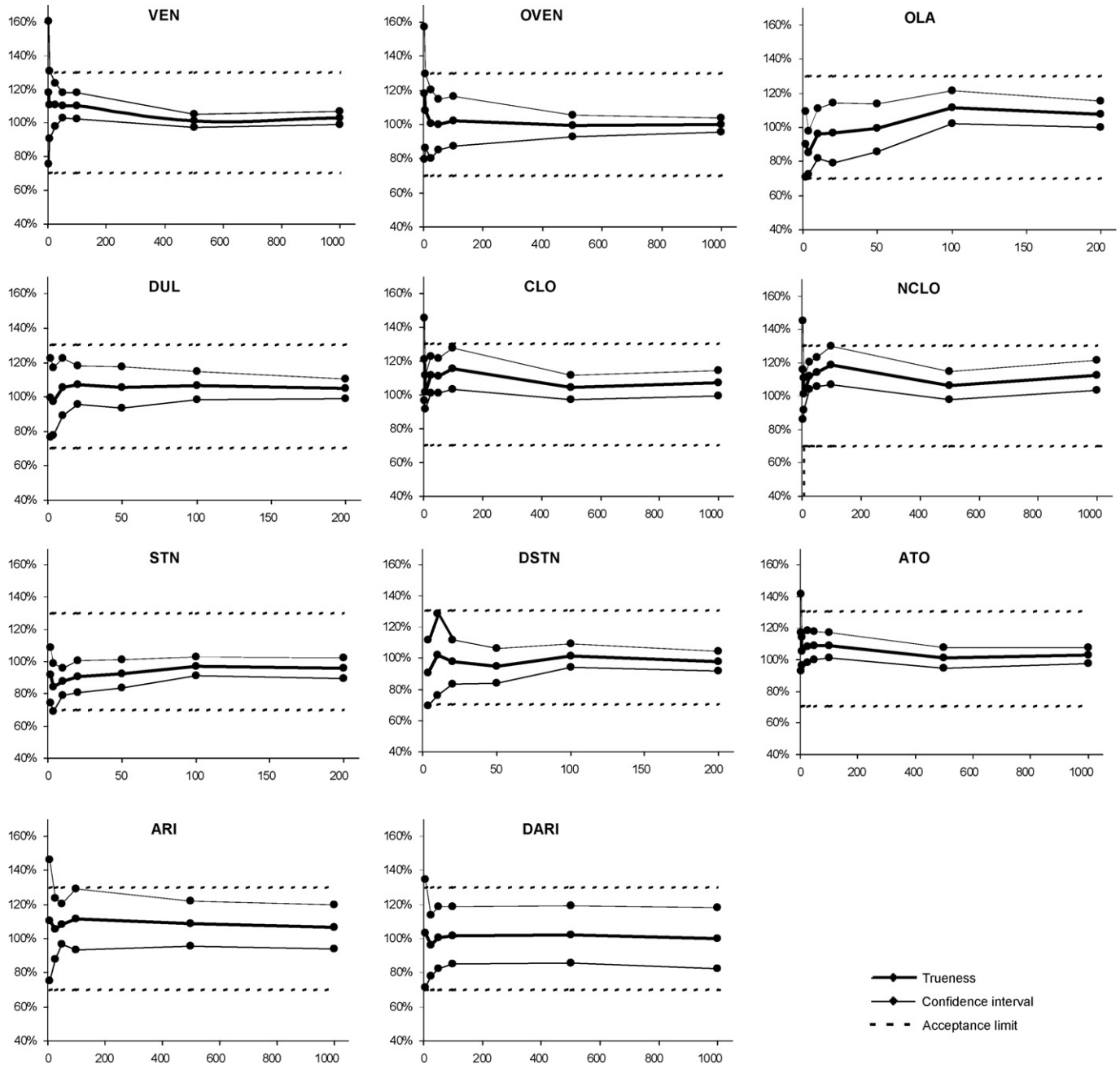

Fig. 3. Accuracy profiles within the acceptance limit ($\lambda = \pm 30\%$), and with confidence interval ($\alpha = 0.05$) calculated for each target in the dosing range.

Calibration standards (CS) were set in duplicate at the following concentrations to cover the plasma therapeutic range and expected patient's plasma values [1,21,41–43]: 2, 4, 20, 200 ng/mL for OLA, DUL, STN, DSTN and 2, 5, 500, 1000 ng/mL for ARI, DARI, ATO, CLO, NCLO, VEN, OVEN and validation standards (VS) were performed in quadruplicate at seven concentrations: 2, 4, 10, 20, 50, 100, 200 ng/mL for OLA, DUL, STN, DSTN and 2, 5, 25, 50, 100, 500, 1000 ng/mL for ARI, DARI, ATO, CLO, NCLO, VEN, OVEN. These solutions were independently prepared for each series using a pool of 6 different plasmas. The lowest and highest levels included in the VS, with respect to an acceptable repeatability, intermediate precision and trueness were considered as the lower limit of quantification (LLOQ) and the upper limit of quantification, respectively.

A 2-fold dilution with water of VS containing the analytes at twice the concentration of the highest CS was also included in the process to evaluate the plasma dilution effect with water in case of concentrations above the determined range of quantification. For stability tests, five blank plasmas were spiked at a low and high concentration with respect to their calibration ranges: 6 and 850 ng/mL for ATO, CLO, NCLO, VEN, OVEN, 10 and 850 ng/mL for ARI, DARI, 6 and 170 ng/mL for DUL, OLA, STN, and 8 and 170 ng/mL for DSTN. Freshly spiked plasmas were divided into 1 mL aliquots. One set of aliquots ($n = 5$) from each concentration was analysed immediately and the determined concentration was defined as the nominal level. Sets of aliquots were quantified after storage at room temperature for 24, 72 h and at +4 °C for 72 h, and after 1 and 3 freeze-thaw cycles.

Post-preparative stability in the autosampler was also assessed by leaving the extracted sample at room temperature and at +4 °C for 36 h. Finally, the long term stability was also assessed by keeping one set of aliquots at −20 °C for 2 months. For

Table 4
Assay validation parameters.

	Concentration (ng/mL)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
VEN	2	118.2	16.2	19.5
	5	110.9	9.1	9.9
	25	110.9	2.5	6.4
	50	110.5	1.7	3.8
	100	110.4	1.7	3.9
	500	101.4	2.1	4.2
	1000	102.8	2.1	4.0
	2000/2	100.7	2.4	5.5
OVEN	2	118.4	12.3	17.8
	5	108.2	7.3	10.9
	25	100.4	3.2	10.9
	50	99.9	2.3	8.1
	100	102.0	1.3	7.7
	500	99.4	3.4	5.3
	1000	99.8	2.2	4.1
	2000/2	99.1	3.4	6.0
OLA	2	90.1	8.4	11.6
	4	85.1	5.4	8.2
	10	96.4	6.1	8.2
	20	96.8	7.9	9.9
	50	99.7	6.5	7.8
	100	111.7	4.7	4.9
	200	107.7	4.0	4.2
	400/2	100.8	5.5	5.2
DUL	2	99.6	11.0	12.5
	4	97.3	6.9	10.9
	10	105.6	7.2	8.6
	20	107.1	4.9	5.7
	50	105.4	4.6	6.2
	100	106.6	4.1	4.2
	200	104.8	3.0	3.9
	400/2	103.0	3.5	3.5
CLO	2	121.4	11.0	11.0
	5	101.6	5.3	5.5
	25	111.9	3.5	5.2
	50	111.1	2.8	5.0
	100	115.4	3.0	5.7
	500	104.3	3.8	5.4
	1000	107.1	3.8	8.2
	2000/2	105.5	3.4	8.7
NCLO	2	115.8	13.8	14.0
	5	101.1	5.4	5.1
	25	112.2	2.8	4.0
	50	114.5	2.8	4.1
	100	118.5	3.6	5.4
	500	106.2	4.4	6.9
	1000	112.7	4.4	4.9
	2000/2	105.5	8.7	8.6
STN	2	91.8	7.5	10.2
	4	84.0	10.0	3.8
	10	87.6	4.4	5.3
	20	90.6	5.7	5.8
	50	92.2	5.4	5.2
	100	97.1	3.4	3.7
	200	96.0	3.6	4.6
	400/2	98.8	4.5	4.5
DSTN	2	–	–	–
	4	90.5	7.8	12.8
	10	102.2	9.2	14.0
	20	97.6	4.8	7.8
	50	94.9	4.8	6.4
	100	101.5	4.0	4.4
	200	98.0	3.5	4.6
	400/2	98.5	3.8	4.4
DARI	2	–	–	–
	5	103.1	10.4	16.8
	25	96.1	9.1	10.1
	50	100.7	7.8	9.8
	100	101.8	7.5	9.0
	500	102.3	9.0	7.8
	1000	100.3	9.8	9.7
	2000/2	103.5	11.0	10.9
ARI	2	–	–	–
	5	110.8	10.3	17.5
	25	105.6	7.4	9.2
	50	108.5	4.9	5.9
	100	111.4	6.7	8.7
	500	108.9	6.6	7.0
	1000	106.7	6.6	6.0
	2000/2	108.0	7.6	6.8
ATO	2	117.1	12.9	11.4
	5	105.4	4.1	4.6
	25	108.3	3.5	5.0
	50	108.9	3.1	4.5
	100	109.0	2.9	4.0
	500	101.1	3.4	3.7
	1000	102.7	2.6	4.0
	2000/2	101.9	2.9	3.9

all experiments, difference in analyte concentration was determined as the ratio between the obtained level after storage and the nominal level. The RSDs of the set of 5 samples were also calculated.

3. Results and discussion

3.1. Choice of the experimental conditions in HPLC and MS

Due to the limited mass resolution (around *m/z* 1 FWHM) of the single quadrupole analyser, special attention was paid to the chromatographic separation of compounds with close *m/z* ratios. For instance, a baseline separation of OLA and NCLO by RPLC is mandatory, as the difference of *m/z* ratios (around 0.4 units) is not sufficient for an unambiguous differentiation. In addition, an isotopic peak contribution of DSTN (*m/z* 439) and DARI (*m/z* 446) could interfere with STN (*m/z* 441) and ARI (*m/z* 448), respectively. In agreement with other studies [44,45], improvement of the peak shape, better selectivity, higher retention of basic polar metabolites and superior signal responses, leading to a better chromatographic profile, could be observed at alkaline pH. A signal enhancement in basic conditions for basic drugs was attributed to a better desolvatation and spray stability in ESI when the drug is eluted with a higher ACN content [44,45]. Therefore, the chromatographic separation was investigated at different alkaline pH. For this purpose, various pH buffers between 8.1 and 10, with ammonia concentration ranging from 10 to 50 mM, were evaluated. According to PHoEBuS software calculation, each prepared buffer had a capacity at least equal to 6 mM/pH units. Thanks to the HPLC modeling software, the optimal separation was obtained at pH 8.1, in agreement with a study at pH 8 reported elsewhere for STN [21].

No influence on the separation was observed for a pH range between 7.6 and 8.2 and ammonium buffer concentration between 15 and 25 mM. In addition, the separation was not affected by a change of the temperature in a range of ±5 °C. With the selected LC conditions, the run time was 13 min with a final 2 min flush step at 85% ACN, followed by a re-equilibration time of 5 min at the initial buffer composition. The average retention times of all analytes are listed in Table 1.

A typical total ion current (TIC) chromatogram and the respective extracted ion current (XICs) of the 11 investigated compounds

Table 5
Stability testing of drugs analysed in this study ($n = 5$).

Drug	**Venlafaxine**				**O-desmethylvenlafaxine**				**Olanzapine**			
Nominal conc. (ng/mL)	6		850		6		850		6		170	
	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %
Room temperature, 24 h	2	0	1	1	1	−3	1	2	1	−2	2	−1
Room temperature, 72 h	3	1	2	2	5	6	2	5	6	−14	2	1
4 °C, 72 h	1	2	1	2	1	6	2	3	3	−15	5	5
Freeze/thaw												
1 cycle	2	−1	2	3	4	2	2	2	2	−2	2	−6
3 cycles	2	−4	2	4	2	−3	2	3	2	−17	1	−4
Stability in storage (−20 °C, 2 months)	2	−3	2	9	2	−1	1	8	8	−8	5	7
Autosampler												
Room temperature, 36 h	4	1	3	−4	3	−17	4	−5	3	44	14	1
4 °C, 3 6h	3	0	2	−1	2	0	2	1	2	37	5	1

Drug	**Duloxetine**				**Clozapine**				**Norclozapine**			
Nominal conc. (ng/mL)	6		170		6		850		6		850	
	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %
Room temperature, 24 h	5	−2	1	−2	1	−1	1	4	3	−5	2	−1
Room temperature, 72 h	5	−17	1	−15	2	−3	1	−1	5	−13	2	−7
4 °C, 72 h	2	−15	3	−13	2	−4	2	−2	2	−12	3	−6
Freeze/thaw												
1 cycle	2	−14	2	−15	2	−9	3	0	3	−10	3	−2
3 cycles	2	−12	2	−13	1	−10	2	2	2	−14	2	0
Stability in storage (−20 °C, 2 months)	11	13	4	4	5	−10	1	−6	4	−10	4	8
Autosampler												
Room temperature, 36 h	11	10	4	−5	3	8	7	−8	5	−6	7	−3

Drug	**Sertindole**				**Dehydrosertindole**				**Atomoxetine**			
Nominal conc. (ng/mL)	6		170		8		170		6		850	
	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %
Room temperature, 24 h	7	5	4	7	4	2	4	3	3	−1	1	0
Room temperature, 72 h	5	2	8	8	8	11	8	11	2	−7	1	−3
4 °C, 72 h	7	6	7	8	4	13	7	12	1	−7	2	−4
Freeze/thaw												
1 cycle	3	−3	4	0	8	0	3	−3	0	1	3	2
3 cycles	4	−2	2	2	3	6	2	−2	2	0	2	3
Stability in storage (−20 °C, 2 months)	12	5	4	−9	3	−18	3	−11	4	−15	1	11
Autosampler												
Room temperature, 36 h	6	13	6	−6	2	17	8	−4	4	−10	3	−4

Drug	**Aripiprazole**				**Dehydroaripiprazole**			
Nominal conc. (ng/mL)	10		850		10		850	
	RSD %	var. %	RSD %	var. %	RSD %	var. %	RSD %	var. %
Room temperature, 24 h	4	6	3	5	1	−2	5	−3
Room temperature, 72 h	3	7	4	−6	4	−28	3	5
4 °C, 72 h	8	−1	4	−6	2	−12	5	17
Freeze/thaw								
1 cycle	3	2	4	−4	4	−2	4	11
3 cycles	3	3	3	−1	3	−21	4	14
Stability in storage (−20 °C, 2 months)	13	−4	2	−16	5	−16	2	−20
Autosampler								
Room temperature, 36 h	5	6	4	1	8	−9	8	−8

are presented in Fig. 2. Although stable isotope labelled IS are highly recommended, in this method, all of the compounds of interest are simultaneously analysed during the same run and the choice of a stable isotope labelled IS for each drug was thus not considered. Therefore, special attention was paid to the matrix effects. Different analogs, which may also compensate for matrix effects [35,46], were investigated during the development. Remoxipride, an antipsychotic agent, which presents the advantage that it cannot be present in the blood of patients as it was withdrawn from the market several years ago, was chosen as IS.

3.2. Development of a solid-phase extraction procedure

An easily automatable SPE process was selected as the sample preparation procedures. Since the compounds of interest possess a basic function, the SPE sorbent chosen combines cation exchange and hydrophobic interactions and leads to an enhancement of selectivity. However an endogenous plasma compound interfered with NCLO and OLA (m/z 313) during the development. To overcome this problem, different washing steps were tested with ACN, MeOH, different ratios of MeOH/isopropanol, 2% formic acid and an increasing

Table 6
Levels of psychotropic drugs determined in human plasma in ng/mL.

	n	Calibration range validated	Therapeutic range	Real samples	
				Active drugs	Metabolites
Venlafaxine	31	2–1000	200–400[a]	5–413	37–783
Olanzapine	34	2–200	20–80	2–76	–
Duloxetine	8	2–200	20–80	13–93	–
Clozapine	58	2–1000	350–600	14–895	6–566
Sertindole[b]	3	2–200	50–100	2–8	4–15
Aripiprazole	30	5–1000	150–300	9–441	6–492

[a] The therapeutic range is the sum of VEN and its metabolite OVEN.
[b] The validated range is 4–200 ng/mL for metabolite DSTN.

percentage of alkaline solution in MeOH. An attempt was made to add a protein precipitation prior to the SPE process as already reported elsewhere [47]. A satisfactory step was obtained with citric acid as already reported for OLA [48]. A better recovery was obtained using MeOH rather than ACN in the elution step. Good extraction performance in terms of plasma purity, recoveries and repeatability were hence obtained. Extraction recovery are shown in Table 2.

3.3. Validation

No interference was observed in the different blank plasmas as well as no carryover between injections during all analyses. The 2 min flush at a high percentage of ACN at the end of the run was hence found satisfactory. In order to assess the selectivity, the method was applied to plasmas spiked with antidepressants, anxiolitics, hypnotics, antipsychotics, pro-cognitive, some metabolites and other drugs susceptible to be used as co-medication. Four substances co-eluted with the compounds of interest, namely risperidone with NCLO, reboxetine with OLA, amisulpride with OVEN and nortryptiline with DUL but were distinguished by the MS detection. In addition, no clinically significant signal suppression effect was found, as the effect was below 4.5% for OLA, OVEN and DUL and below 11% for NCLO (data no shown). Therefore, no interferences of drugs usually associated with the studied analytes were observed (Table 3).

The post-infusion tests were applied to all analytes and the IS. No qualitative interferences were observed on the different sources of blank plasma since no alteration of the MS signal at the retention time of the analyte was observed (data no shown). The results of quantitative assessment of process efficiency, matrix effect and extraction recovery are reported in Table 2. Consistent results were obtained at high concentration but for sake of clarity, only low concentration is shown. They were repeatable (CV < 15% for $n = 3$) in all the quantitative matrix effects observations.

The process efficiency, which represents the combined effects of the extraction recoveries and the matrix effect [35], generally ranged from 91% to 107%. Four values were observed above 120%, namely STN (126%), OLA (131%), ARI (131%) and DARI (147%). A greater response of OLA in biological matrix was previously reported [7]. The signal response enhancement leads to an overestimation of a maximum of 14.7 ng/mL instead of 10 ng/mL for DARI, the drug with the highest effect, which is not of clinical significance. The matrix effect ranged between 91% and 120% except for STN (133%) and ARI (122%). Finally extraction recoveries ranged from 92% to 111% with a 123% for DARI.

The validation process was initially performed with 7 calibration standard levels ($n = 2$): 2, 5, 25, 50, 100, 500, 1000 ng/mL for ATO, CLO, NCLO, VEN, OVEN, ARI, DARI and 2, 4, 10, 20, 50, 100, 200 ng/mL for DUL, OLA, STN, DSTN. Several regression models were tested, and the most suitable model was obtained with 4 calibration levels. Thus, the calibration curve from 2 to 200 ng/mL was suitably fitted by a linear regression forced through 0 for DUL, OLA, STN, DSTN. A quadratic regression response model was mandatory as calibration for ATO, CLO, NCLO, VEN, OVEN, ARI, DARI. The accuracy profiles within the acceptance limit ($\lambda = \pm 30\%$), and with confidence interval ($\alpha = 0.05$) calculated for each target in the dosing range, are shown in Fig. 3. The LLOQ was established at 2 ng/mL for all drugs except 4 ng/mL for DSTN, and 5 ng/mL for ARI and its metabolite DARI.

As reported in Table 4, trueness, repeatability and intermediate precision were in the acceptance criteria over the evaluated assay range. A 2-fold dilution of twice the highest concentration was found to be in the accepted range of the accuracy profile. Therefore, a plasma dilution with water could be performed if required. The room temperature stability test demonstrated that all compounds were stable up to 72 h storage with the exception of DUL and DARI, which were stable only up to 24 h storage (Table 5).

At low concentrations of DUL and DARI, a difference of 17% and 28% respectively, were calculated at 72 h. The stability after 1 freeze-thaw cycle was confirmed for all compounds. For 3 cycles, DARI and OLA presented some degradation (−21%, −17%, respectively). The instability of OLA is consistent with another stability report [49]. Extracted plasmas were all stable except for OLA, which presented a significant signal enhancement at low concentration (6 ng/mL), at room temperature and at 4 °C. In the case of an HPLC–MS analysis performed 36 h after extraction (following a technical failure for example), OLA requires re-extraction prior to LC–MS analysis. Some discrepant results have been published concerning the stability of OLA [7,26,49], and the option of adding some ascorbic acid was proposed to decrease OLA degradation and tested without success [11,23]. The metabolites DARI and DSTN presented a decreased amount after 2 months at −20 °C. All the details of stability tests are presented in Table 5.

4. Application to samples from psychiatric patients

The developed method was used to quantify the plasma drug level of 177 patients (Table 6). CLO, VEN, ARI, OLA were the most prescribed drugs most prescribed drugs. ATO has only just been introduced on to the Swiss market and therefore no patient plasma could be collected at the time of these analysis. The plasma levels were within the calibration ranges. In two cases, they were prediluted 2-fold for concentration of CLO 1380 ng/mL and 1620 (not accounted for in Table 6). No concentration above 2000 ng/mL was observed. Therefore, the method was suitable for therapeutic drug monitoring. Integrated external quality controls samples from 2 providers (Health Control Therapeutic Drug Scheme, Cardiff Bioanalytical Services Ltd., United Kingdom and UTAK, Radolfzell-Bohringen, Germany) as well as patient plasma samples from another hospital lab were successfully quantified.

5. Conclusions

A simple HPLC–MS method was developed and validated according to FDA guidelines and SFSTP protocols for the quantification of ATO, ARI, DARI, DUL, CLO, NCLO, OLA, STN, DSTN, VEN and OVEN in human plasma. The SPE process allows to efficiently remove endogenous interfering substances from the matrix while the HPLC–MS method permits the quantification of seven substances and their metabolites over the wide concentration range usually measured in psychiatric patients. Finally, this method has been successfully used for quantification of real plasma samples.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

We thank Mr. I. Marchi for helpful discussions during the development of the method. We are also grateful to Dr. M. Fathi (Geneva, Switzerland) for supplying the control plasma samples of patients with pre-measured drug concentrations. We also thank the editorial assistance of Mrs. K. Powell Golay, V. Sari and, M. Brawand and the bibliographical help of Mrs. E. Ponce. This work was supported in part by a grant from Eli Lilly and from the Swiss National Research Foundation (project no.: 120686). We also thank Waters Company for supplying SPE plates during the development process.

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