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Development of a solid phase extraction for 13 'new' generation antidepressants and their active metabolites for gas chromatographic–mass spectrometric analysis

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Abstract

A solid phase extraction procedure (SPE) for 13 'new' antidepressants (venlafaxine, fluoxetine, viloxazine, fluvoxamine, mianserin, mirtazapine, melitracen, reboxetine, citalopram, maprotiline, sertraline, paroxetine and trazodone) together with eight of their metabolites (*O*-desmethylvenlafaxine, norfluoxetine, desmethylmianserine, desmethylmirtazapine, desmethylcitalopram, didesmethylcitalopram, desmethylsertraline and *m*-chlorophenylpiperazine) from plasma is optimized using HPLC-DAD as monitoring system. Special attention has been paid to the choice of washing and eluting solvent, resulting in a highly concentrated, clean and moisture free extract, also suitable for GC–MS. A total number of 10 sorbents (apolar, polymeric, ion-exchange and mixed mode) was evaluated. Based on recovery, reproducibility and absence of interfering substances the strong cation exchanger gave the best results. Recoveries were determined at low and high therapeutic and toxic levels and ranged between 70 and 109% for all compounds, except for trazodone (39%).

Keywords: Solid phase extraction; Antidepressants; GC–MS; Metabolites

1. Introduction

Depression is a common mental disorder that affects about 121 million people worldwide. According to the World Health Organisation this mood disorder will be the second leading contributor to the global burden of disease, calculated for all ages and both sexes by the year 2020. Depression is a chronic or recurrent illness that affects both economic and social functions of the patient and can eventually lead to suicidal behaviour [1,2]. Depression is assumed to be involved in 50% of all suicide attempts in the Western World, while 25% of severe depressed patients attempt at least one suicide [3].

Between 1960 and 1980, depression was treated with tricyclic antidepressants, monoamine oxidase inhibitors and lithium. The side-effects, toxicity and severe drug–drug interactions of these compounds in combination with the remarkable advances in the understanding of the central nervous system lead to the introduction of several "new" antidepressants (ADs) [4,5]. These new generation antidepressants work more selectively on the noradrenalin and/or serotonin pathways. An important group of new ADs consists of the selective serotonin reuptake inhibitors (SSRI), which include fluoxetine, fluvoxamine, sertraline, paroxetine and citalopram. Another group consists of the selective noradrenalin reuptake inhibitors (NRI) including reboxetine, viloxazine and maprotiline. Venlafaxine is a selective inhibitor of serotonin as well as noradrenalin reuptake (SNRI), while mirtazapine and mianserin are noradrenergic and specific serotonergic antidepressants (NSSA). Trazodone is a serotonin antagonist and reuptake inhibitor [3–6].

These compounds, however, also show considerable adverse drug reactions, side-effects and can have a delayed therapeutic effect, which could lead to poor patient compliance. Nowadays, psychiatric medication is prescribed in numerous combinations, leading to more possible drug–drug interactions, while the dose is largely based on trial-and-error [7]. Therapeutic drug monitoring (TDM) is under-utilized in the field of psychiatry as the

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therapeutic ranges of these compounds seem quite broad, leading to the generally accepted notion of low toxicity for these new ADs. On the other hand, the relationship between a blood concentration and the therapeutic effects is not always fully understood. TDM, though, could be of interest for monitoring patient compliance. In other situations, such as liver and kidney impairment, poor metabolism by CYP450 isoenzymes and comedication with inhibitors and inducers of those enzymes, and in the elderly population, TDM could provide valuable information for a cost-effective and more rational use of psychiatric drugs [7–12].

Analytical methods for the detection of ADs are not only of interest in the field of clinical toxicology, but also in forensics as they are often involved in intoxications [13–18]. Recently, the metabolite of trazodone, *m*-chlorophenylpiperazine has entered the illicit drug market, because it mimics the psychoactive effects of MDMA [19,20].

An important step in an analytical method is the extraction of the compounds of interest from the biological matrix. The standard procedure for extracting ADs is based on a liquid–liquid extraction after alkalinization (pH = ±9) with potassium borate, sodium carbonate, NaOH or KOH. A variety of organic solvents is used such as heptane–isoamylalcohol, *n*-butyl chloride, diethyl ether or *n*-heptane–ethylacetate [3,16,21–27]. Sometimes a back extraction under acidic conditions (HCl) is applied, followed by a direct injection on the HPLC system [23,25]. For GC-purposes the ADs are extracted as above followed by an additional extraction step into an organic solvent after alkalinization [22,24].

Recently, several SPE-methods for either one or a mixture of several ADs were developed. Although some authors suggest that differences in batch of columns, not standardized use of vacuum, and variable intensity of drying steps can lead to irreproducible recovery [3], SPE has several advantages. High selectivity, cleaner extracts, no emulsions, reduced solvent usage and higher throughput by automatisation are such advantages. In addition, a large variety of sorbents (polar, apolar, mixed-mode, ion-exchange and polymeric sorbents or combinations) allows the development of extraction procedures for specific needs. The sorbents used are the apolar C_8 [28,29], 3M-Empore high-performance extraction disk [30] or C_{18} [31] and the mixed modes Bond Elut Certify [32,33] or Oasis MCX [34]. Oasis HLB, a hydrophilic–lipophilic balanced column, was also used in sample preparation before HPLC analysis of ADs [35,36].

In this paper a SPE procedure for a unique mixture of 13 compounds (venlafaxine, fluoxetine, viloxazine, fluvoxamine, mianserin, mirtazapine, melitracen, reboxetine, citalopram, maprotiline, sertraline, paroxetine and trazodone) and eight of their active metabolites from plasma is developed (Fig. 1). HPLC-DAD was used during the development and the optimization as both aqueous and organic fractions (after evaporation) could be analysed. Because the metabolites are active, it is relevant to determine both the parent compound and the demethylated metabolites as this can contribute to the overall therapeutic and toxic effects. Metabolites can also give extra information about the moment of ingestion, the metabolic capacity and compliance [3]. Special attention has been paid to the choice of the washing and eluting solvent in the SPE procedure, resulting in an extract that is highly concentrated, clean, and free from moisture, so that it can be used also for GC–MS purposes.

2. Experimental

2.1. Reagents

2.1.1. Chemicals

Venlafaxine·HCl (Ven) and *O*-desmethylvenlafaxine maleate (ODMV) were kindly provided by Wyeth (New York, NY, USA). Mianserin·HCl (Mia), desmethylmianserin·HCl (DMMia), mirtazapine (Mir) and desmethylmirtazapine maleate (DMMir) were a gift from Organon (Oss, The Netherlands). Sertraline·HCl (Ser), desmethylsertraline maleate (DMSer) and reboxetine methanesulphonate (Reb) were a gift from Pfizer (Groton, CT, USA). Citalopram·HBr (Cit), desmethylcitalopram·HCl (DMC), didesmethylcitalopram tartrate (DDMC) and melitracen·HCl (Meli) were kindly provided by Lundbeck (Valby, Denmark). ACRAF (Roma, Italy) donated trazodone·HCl (Traz) and its metabolite *m*-chlorophenylpiperazine·HCl (mCPP), while paroxetine·HCl hemihydrate (Par) was donated by GlaxoSmithKline (Erembodegem, Belgium). Viloxazine·HCl (Vil) was a kind gift from AstraZeneca (Brussels, Belgium). Novartis Pharma (Basel, Switzerland) donated maprotiline·HCl (Map). Fluvoxamine maleate (Fluv) was donated by Solvay Pharmaceuticals (Weesp, The Netherlands). Fluoxetine·HCl (Fluox) and norfluoxetine·HCl (NorFl) were purchased from Sigma–Aldrich (Steinheim, Germany). Fluoxetine D6 oxalate (FD6), mianserin D3 (MD3) and paroxetine D6 maleate (PD6) (100 μg/ml MeOH) were purchased from Promochem (Molsheim, France).

Methanol, acetonitrile and water were all of HPLC grade (Merck, Darmstadt, Germany).

Ammonia-solution 25%, *ortho*-phosphoric acid (85%), NaOH (5 M), ammonium chloride, sodium dihydrogen phosphate monohydrate and glycine were also from Merck. Formic acid was purchased from Riedel-de Haën (Seelze, Germany). Toluene (Suprasolv quality, Merck, Darmstadt, Germany) and 1-(heptafluorobutyryl) imidazole (HFBI) (Fluka, Bornem, Belgium) were used for derivatization in case of GC–MS analysis.

Glycine HCl-buffer was made by adding 4.1 ml 0.2 M HCl to 50 ml of 0.1 M glycine solution (0.75 g/100 ml) and then diluting with water till 100 ml. Phosphate buffer (25 mM) pH 2.5 was made by adding approximately 6.7 g of $NaH_2PO_4{\cdot}H_2O$ to 2.7 l of HPLC-water and adjusting the pH by adding phosphoric acid. The phosphate buffer (25 mM) pH 6.5 was made by dissolving 2.8 g of $NaH_2PO_4{\cdot}H_2O$ in 1 l of HPLC grade water and adjusting the pH with 5 M NaOH.

2.1.2. Stock solutions

Stock solutions were prepared in methanol at a concentration of 1 mg/ml for each compound individually and stored at about −20 °C. Two mixtures of compounds were made due to the overlap of some compounds in the

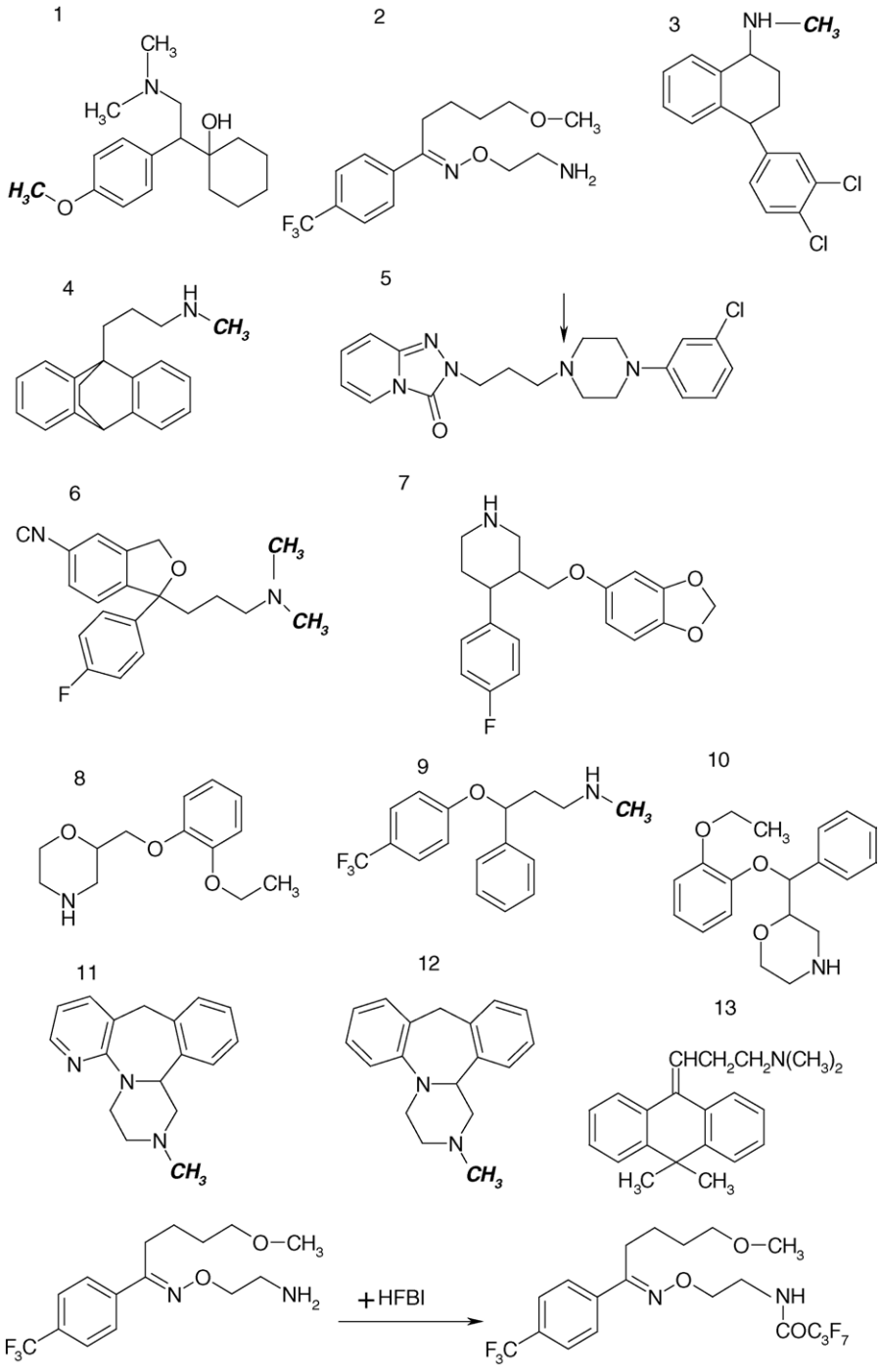

Fig. 1. Structures of the 'new' generation antidepressants. The highlighted functions are those that are demethylated in the metabolization process. The arrow indicates the N-dealkylation of the piperazinyl nitrogen resulting in the formation of *m*-chlorophenylpiperazine. The derivatization using HFBI is demonstrated using fluvoxamine (2) as an example. (1) Venlafaxine, (2) fluvoxamine, (3) sertraline, (4) maprotiline, (5) trazodone, (6) citalopram, (7) paroxetine, (8) viloxazine, (9) fluoxetine, (10) reboxetine, (11) mirtazapine, (12) mianserine and (13) melitracen.

HPLC-method. Mixture 1 contained desmethylmirtazapine, *O*-desmethylvenlafaxine, desmethylcitalopram, didesmethylcitalopram, reboxetine, paroxetine, maprotiline, fluoxetine, norfluoxetine and *m*-chlorophenylpiperazine. Mixture 2 contained mirtazapine, viloxazine, desmethylmianserin, citalopram, mianserin, fluvoxamine, norsertraline, sertraline, melitracen, venlafaxine and trazodone.

Mixtures for GC were different due to different overlaps under these conditions. Mixture 1 contained venlafaxine, viloxazine, fluvoxamine, melitracen, desmethylmianserin, reboxetine, citalopram, sertraline, desmethylcitalopram and trazodone, while mixture 2 contained *m*-chlorophenylpiperazine, norfluoxetine, *O*-desmethylvenlafaxine, fluoxetine, mianserin, mirtazapine, desmethylmirtazapine, norsertraline, maprotiline,

Table 1
Therapeutic and toxic range of 'new' generation antidepressants and their active metabolites in plasma together with characteristics relevant for SPE development

Compound	ActMet	Fb (%)	pK_a	log P	Ther.C. (μg/l)	Tox.C. (μg/l)	Reference
1. Venlafaxine	*O*-Desmethylvenlafaxine	30	9.24 (9.74)[a]	0.43	200–400	1000–1500	[47]
2. Fluvoxamine		77	8.7	0.04	50–250	650	[46]
3. Sertraline	Desmethylsertraline	98	9.48	5.29	50–250	290/1600	[46]
4. Maprotiline	Desmethylmaprotiline	90	10.5	4.5	75–250	300–800	[46]
5. Trazodone	*m*-Chlorophenylpiperazine	90	6.7	3.2	500–2500	4000	[46]
6. Citalopram	Desmethylcitalopram Didesmethylcitalopram	50	9.5	3.74	20–200	(L) 500	[46]
7. Paroxetine		95	9.9	3.95	10–75	350–400	[46]
8. Viloxazine		85–90	8.1	1.8	130		
9. Fluoxetine	Desmethylfluoxetine	94.5	8.7 (9.37)[a]	4.05	150–500 (100–500)[a]	1000 (900)[a]	[47] [46]
10. Reboxetine		97			50–160		
11. Mirtazapine	Desmethylmirtazapine	85		7.1	20–100 (50–300 sum)	1000–2000	[47]
12. Mianserine	Desmethylmianserine	90	7.05	3.36	15–70	500–5000	[46]
13. Melitracen					10–100		[46]

ActMet: active metabolite in plasma; Fb: fraction bound; pK_a: dissociation constant; log P: partition coefficient (octanol/water); Ther.C.: therapeutic concentration range; Tox.C.: toxic concentration; Ref.: reference.
[a] Information between parentheses concerns the metabolite.

didesmethylcitalopram and paroxetine. The concentrations spiked are the low and high therapeutic concentrations, and the toxic levels as can be seen in Table 1.

2.1.3. Stability

Stock solutions in methanol (1 mg/ml) are stable for at least 3 months. According to Pépin and coworkers these new ADs seem to be stable in blood and plasma samples [12,37]. Although Uges and Conemans [3] describe that most ADs are not stable under alkaline conditions in daylight and several ones are described to adsorb to glass test tubes, no degradation was noticed when eluting the ADs from the SPE tubes using an alkaline eluent. However, in our procedure the contact time with the base was kept to a minimum. Compounds derivatised with HFBI are stable for at least 4 days at room temperature and can indure three thaw–freeze cycles.

2.2. SPE sorbents

The SPE sorbents applied in this study can be divided in four different categories. *The apolar sorbents* studied were Bond Elut C_{18} (Varian, Middelburgh, The Netherlands), Empore HD C_8 (Chrompack-Varian, Middelburg, The Netherlands) and RP-select B Lichrolut (Merck, Darmstadt, Germany). *Polymeric sorbents* consisted of Focus (Varian), Strata X (Phenomenex, Bester, Amstelveen, The Netherlands) and Oasis HLB (Waters, Milford, MA, USA). Strong and weak cation exchangers (Phenomenex) were evaluated as *ion-exchange sorbents*. Bond Elut Certify (Varian) and Strata XC (Phenomenex) were two *mixed modes* combining ion-exchange properties with, respectively, C_8 or a styrene–divinylbenzene polymer. Most of the sorbents contained 200 mg of sorbent mass, except for Bond Elut Certify (130 mg), Focus (50 mg) and the Empore HD C_8 (6 ml, 10 nm).

2.3. Instrumentation and chromatographic conditions

2.3.1. HPLC

A LaChrom Elite HPLC (Merck-Hitachi, Darmstadt, Germany), consisting of a L2100 micro-pump, a L2200 autosampler, a L2300 column oven and a L2450 DAD, was used to monitor the SPE optimization. A LiChroCART C18 5 μm 4-4 guard column combined with a C18 endcapped Purospher Star (Merck, Darmstadt, Germany) LiChroCART 125-3 (5 μm) column was used. The oven was set at 40 °C and the gradient run started at 85% phosphate buffer (25 mM, pH 2.5) and 15% acetonitrile. At 20 min the organic phase contribution was 40%, and at 25 min 50%. From 25.1 min until 35 min the column equilibrated under starting conditions. The flow rate of the mobile phase was held at 0.5 ml/min. The DAD measured from 210 to 380 nm, and the chromatograms were integrated at 220 nm, except for mirtazapine and desmethylmirtazapine (300 nm).

2.3.2. GC–MS

A HP 6890 GC system equipped with a HP 5973 mass-selective detector and a HP 7683 split/splitless auto injector was used (Agilent Technologies, Avondale, PA, USA). A splitless single-tapered deactivated inlet liner with glass wool and a 30 m × 0.25 mm i.d., 0.25 μm Varian factorFOUR VF-5ms column (Varian, Middelburg, The Netherlands) were used.

The GC conditions were as follows: the initial temperature was set at 90 °C for 0.5 min, ramped at 50 °C/min to 180 °C for 10 min, whereafter the temperature was ramped again at 10 °C/min to 300 °C, where the temperature was held for 10 min. The splitless injection temperature was 300 °C, the purge time was 1 min and the helium flow was held constant at 1.3 ml/min. The injection volume was 1 μl. The mass-selective detector conditions were set at 300 °C for the transferline, 230 °C for the

Table 2
Monitored ions in SIM mode

Compounds	Start time (min)	Ion 1	Ion 2	Ion 3
mCPP	7.0	**166.1**	394.1	392.1
Venlafaxine		**58.1**	259.2	473.0
Norfluoxetine	9.5	**117.0**	330.0	472.0
Viloxazine		240.0	296.1	**433.0**
Fluvoxamine		226.0	**258.1**	514.0
ODMV		**58.0**	245.0	
Fluoxetine	11.4	**117.0**	344.0	486.0
Fluoxetine D6		**123.0**	350.0	492.0
Mianserin	14.0	193.2	249.2	**264.2**
Mianserin D3		193.2	220.1	**267.2**
Mirtazapine	16.5	**195.1**	208.1	265.2
Melitracen		**58.1**	202.1	291.0
DMMia	17.8	193.0	249.0	**446.0**
DMMir		**195.0**	250.0	447.0
Reboxetine		138.0	**371.0**	509.0
DMSer		**274.0**	487.0	489.0
Citalopram		**58.0**	208.0	324.0
Maprotiline	19.4	191.0	**445.0**	473.0
Sertraline		**274.0**	501.0	503.0
DMC		208.1	**238.1**	488.0
DDMC		208.0	**238.1**	474.0
Paroxetine	21.1	138.0	388.2	**525.0**
Paroxetine D6		138.0	394.0	**531.2**
Trazodone	25.0	**205.1**	356.1	371.2

Ions used for quantitation are given in bold face.

source and 150 °C for the quadrupole. The electron-impact mode with an electron voltage of 70 was used. The spectra obtained were measured in the SIM mode. The dwell time was 50 ms and the ions monitored are shown in Table 2. The highlighted ions are the quantifiers.

2.3.3. Centrifuge, evaporator and vacuum manifold

The centrifuge was a Mistral MSE 200 BRS (Drogenbos, Belgium).

For evaporation of the samples, a TurboVap LV Zymark (Teralfene, Belgium) was used. The water bath was held at 40 °C. A Visiprep TM Disposable liner vacuum manifold (Supelco, Bornem, Belgium) was used for controlling the flow in the SPE-procedure.

2.4. Human plasma sampling

To have blank plasma, blood was taken from healthy volunteers in dipotassium EDTA Vacutainers (Novolab, Geraardsbergen, Belgium). Within 2 h these tubes were centrifuged at 1200 × *g* for 10 min and the plasma was removed and stored at −20 °C.

2.5. SPE development

HPLC grade water was spiked with mixture 1 or 2 (1 μg/ml for each compound). The spiked samples were loaded on the SPE phases and tested on retention behaviour, ease of elution and of enduring wash-steps. First, the retention onto the column was examined (Fig. 2A). The column was conditioned with 1 ml of eluent, 2 ml of methanol, followed by 3 ml of an aqueous solution (water, water–HCOOH pH 2.89 or water–ammonia pH 10.80) for the non-ionic sorbents or a phosphate buffer (pH 6.5 or 2.5) for the ionic sorbents. Then the spiked water sample was loaded followed by 2 ml of the same aqueous solution as used in the conditioning phase. This solution was collected and analysed by HPLC. After a drying period of ±5 min the compounds were eluted with methanol–2% formic acid (except for SCX and Strata XC). The eluent was also analysed after evaporation under nitrogen at 40 °C and redissolution in 1 ml of mobile phase (starting conditions) of the HPLC (50 μl were injected). Secondly (Fig. 2B), several possibilities for eluting the compounds were also studied. Conditioning and loading of the samples were done as described above. Then, after drying, two times 1.2 ml (five bed volumes) of eluent were added, collected separately and analysed. The tested eluting solvents were methanol, methanol–2% formic acid, methanol–2% ammonia and methanol–5% ammonia. At last (Fig. 2B), methanol and methanol/water (90/10, 70/30, 50/50, by volume) were tested as wash solution by washing with 5 ml after conditioning the column and by analysing the washing solvent (5 ml) as well as the elution solvent (3 ml) after evaporation. A schematic overview of these experiments can be seen in Fig. 2.

2.6. Protein binding

One millilitre of plasma was spiked at therapeutic concentrations of the drugs. A mixture of 100 ng desmethylmirtazapine, 150 ng *O*-desmethylvenlafaxine, 30 ng desmethylcitalopram, 10 ng didesmethylcitalopram, 80 ng reboxetine, 75 ng paroxetine, 125 ng maprotiline, 250 ng fluoxetine, 500 ng norfluoxetine and 10 ng *m*-chlorophenylpiperazine (mixture 1) or a mixture of 100 ng mirtazapine, 100 ng viloxazine, 20 ng desmethylmianserin, 100 ng citalopram, 35 ng mianserin, 125 ng fluvoxamine, 125 ng desmethylsertraline, 125 ng sertraline, 50 ng melitracen, 375 ng venlafaxine and 100 ng trazodone (mixture 2) was spiked by evaporating the mixtures at 40 °C with nitrogen and adding the plasma afterwards. The solutions were equilibrated overnight at 4 °C, as low temperature stimulates protein binding.

Afterwards the spiked plasma was submitted to SCX SPE-tubes directly or after a deproteinization with different reagents. Standard mixtures were also analysed. Acid (2% H_3PO_4), glycine-buffer, methanol and acetonitrile were tested for their capacity to break the protein bond. Dilution of the sample in combination with slow pass-through of the sample was also tested.

The procedures for the acid/buffer and for the organic solvents involved addition of 3 ml of the substances to the plasma, a vortex step followed by centrifugation for 10 min at 1121 × *g*. The top layer was then removed and, respectively, 4–6 ml of phosphate buffer was added to the acid/buffer top layer and the organic top layer. The glycine-buffer required an extra 10 min equilibration-stirring time before centrifuging. The diluting procedure was achieved by adding 4 ml of buffer to the plasma, a vortex and centrifugation step.

The final SPE procedure consisted of a conditioning step with 3 ml of eluent, 2 ml of methanol and 3 ml of buffer (pH 6.5 or

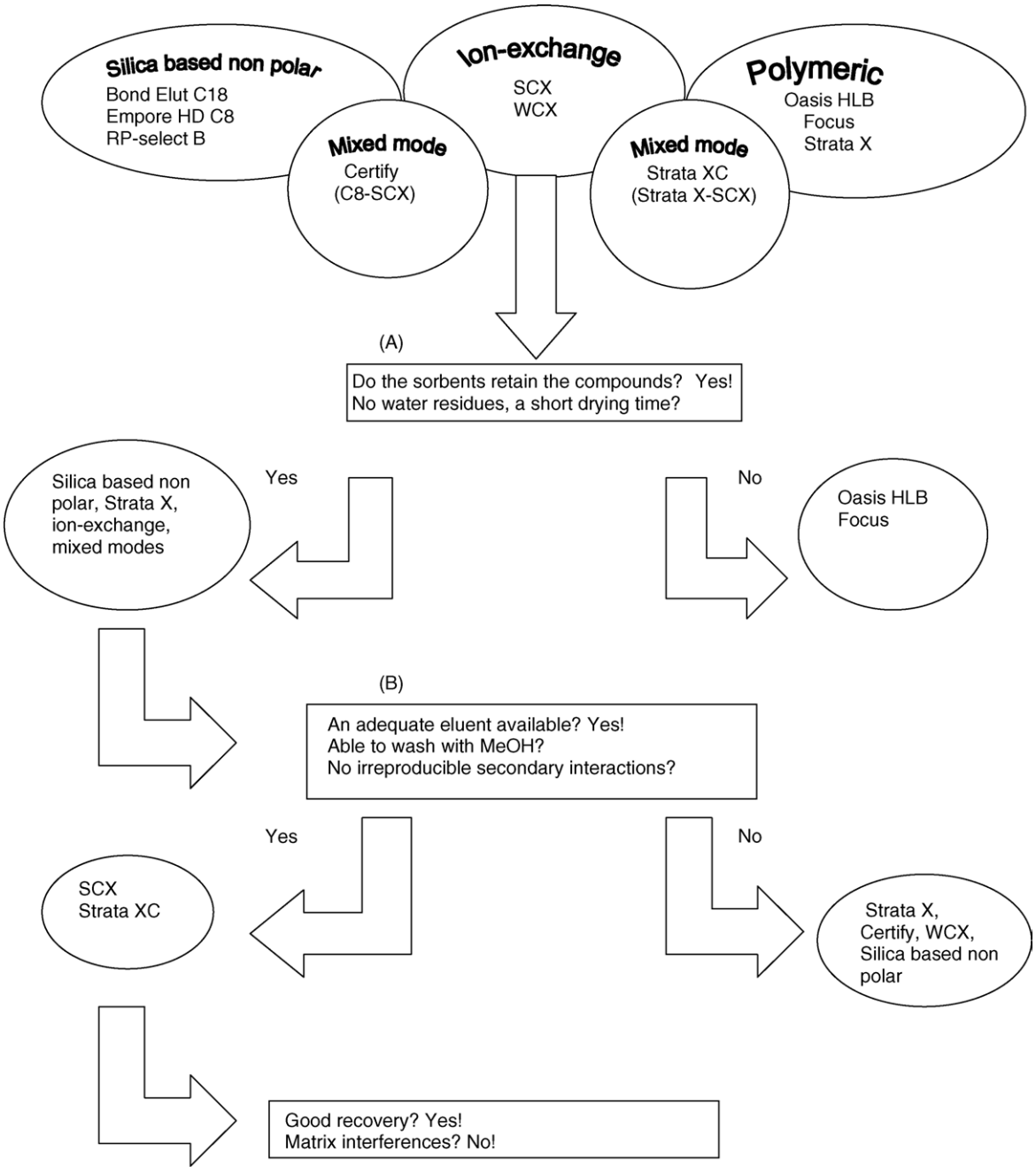

Fig. 2. Decision scheme for the SPE development.

2.5), and a sample load step followed by a wash step of 4 ml methanol using −20 kPa vacuum. After drying the column for 2 min at −50 kPa, the compounds were eluted with 2 ml of 5% ammonia in methanol. The solid phase tubes were again dried for 1 min using −50 kPa vacuum. The eluent was evaporated to dryness under nitrogen at 40 °C and redissolved in 0.5 ml of the acetonitrile (15%)–phosphate buffer mixture. A 50 μl aliquot was injected on the HPLC-column.

2.7. SPE and GC–MS

Plasma samples (1 ml) were spiked with mixture 1 or 2 of the ADs (2.1.2.) in their low therapeutic, high therapeutic or toxic concentrations (Table 1). If the relevant level of the metabolite was not found in literature, a concentration ratio metabolite/main compound of 0.5 was chosen, except for desmethylmianserin (1) and *O*-desmethylvenlafaxine (0.2). The samples were then extracted after dilution of the sample, according to the SPE-procedure described in 2.6. Before evaporating the extracts, 50 μl of fluoxetine D6 (10 ng/μl in MeOH), 35 μl of paroxetine D6 (10 ng/μl in MeOH) and 15 μl of mianserin D3 (1 ng/μl in MeOH) were added as ISTDs. Because of the large differences in concentration between low- and high therapeutic and toxic concentrations, and thus peak area, the recoveries were determined using three different internal standards added in a different amount. In this way for the low- and high therapeutic

and toxic concentrations, respectively, mianserin D3, paroxetine D3 and fluoxetine D6 were used.

After evaporation of the extracts, 100 μl of HFBI was added and heated at 90 °C for 1 h. Thereafter, 0.5 ml of HPLC-water and 1 ml of toluene were added. After vortexing, the sample was centrifuged at 1121 × *g* for 10 min and stored at −20 °C for approximately 1 h. The toluene layer was separated from the ice and was evaporated at 40 °C. The residue was redissolved by adding 50 μl of methanol and 1 μl was injected onto the GC–MS.

3. Results and discussion

3.1. Solid phase extraction development

When developing a SPE method, one has to select the proper SPE sorbent, the proper washing and eluting solvents and the final extract should fit for the final determination procedure (HPLC or GC). Several sorbents were selected because of their potential interactions with the antidepressants. As the antidepressants can have a positive charge at adequate pH, cation exchangers such as SCX, WCX, Strata XC and Bond Elut Certify could be of interest. Silica based apolar columns are interesting for the extraction of apolar compounds from a polar matrix such as plasma, although they tend to have polar and secondary ionic characteristics as well. Polymeric sorbents, such as Oasis HLB, could be of interest because of their combined polar (metabolites) and apolar properties.

Conditioning or solvation of the sorbent is necessary for reproducible interaction with the compounds. Before conditioning, eluent was passed through the column, to elute possible contaminants of the column. All of the phases tested retained the antidepressants, but two of the polymeric sorbents, Focus and Oasis HLB also retained a lot of water, probably due to their hydrophilic character, resulting in a longer drying step. These two phases were not used for further experiments, as we intended to analyse the extracts by GC–MS, thus requiring moisture-free extracts (Fig. 2A).

When loading the aqueous samples on the silica based non polar phases, three different pH values were used. For the C_{18}, C_8 and RP Select B, a difference was observed in retention at different pH's. Silica based apolar sorbents may still contain a limited number of unreacted or 'free' silanols. These silanols provide polar, acidic patches on the column surface capable of binding amines through hydrogen bonding and cation exchange mechanisms. Since the ionization of the ADs depends on the loading conditions, interactions with these residual silanols may cause an enhanced retention. These secondary interactions could be interesting, but are not reproducible as the number of free silanol groups can change from batch to batch.

When evaluating ion-exchange phases, the pH during the loading and eluting step is again very important. The pH during the loading step should be two pH units lower than the pK_a of the compounds and two pH units higher than the sorbent. At this pH, approximately 99% of the groups are charged. A loading pH of 6.5 or 2.5 was chosen, respectively, according to the choice of a weak cation- (carboxyl pK_a is 4.8) or a strong cation exchanger (sulphonic acid pK_a < 1). At this loading pH the compounds are retained on the sorbent.

The sorbent beds were eluted with two times 1.2 ml (five bed volumes) of different solvents (Fig. 2B). A fast, reproducible elution with a limited volume of solvent is the most interesting. Therefore, it is advantageous if elution happens with the first 1.2 ml of the eluent. Especially methanol 2% formic acid and methanol with 2% ammonia gave good results for most of the sorbents, although they required more than five bed volumes of eluent for complete elution. For the strong-cation exchangers methanol–ammonia (5%) was necessary. Methanol–acid and methanol–base works on the secondary interactions of the silica based phases. Under acidic conditions the silanol functions are not charged, while under basic conditions the antidepressants are not. For the strong cation exchangers acidic conditions were not successful, as even at low pH the sulphonic acid groups remain negatively charged.

A good eluent should be strong enough to elute the compounds of interest in a limited volume, but on the other hand the stronger the eluent, the more interfering (matrix) compounds are also eluted. Therefore, an optimized wash step is necessary. When examining the eluting solvent, methanol was not suited, because it did not elute all compounds from the phases and in addition no compounds were eluted from the strong ion-exchangers. However, it could thus serve as an interesting wash solvent. The use of organic solvents for washing is also interesting as it also shortens the drying time before elution. Methanol did elute several compounds (especially trazodone) from C_{18}, C_8, RP Select B, WCX, Certify and Strata X. Certify is a mixed mode of C_8 and SCX, perhaps the slightly lower bed mass and the ratio between those two phases are important. Consequently these phases were all left out for further investigation, due to their irreproducible secondary interactions (silica based) and/or loss of compounds during the methanol wash. Most of the polymeric phases were left out due to water remains after the loading step. Only the Strata XC and the SCX sorbent were tested for the final GC–MS application (Fig. 2B).

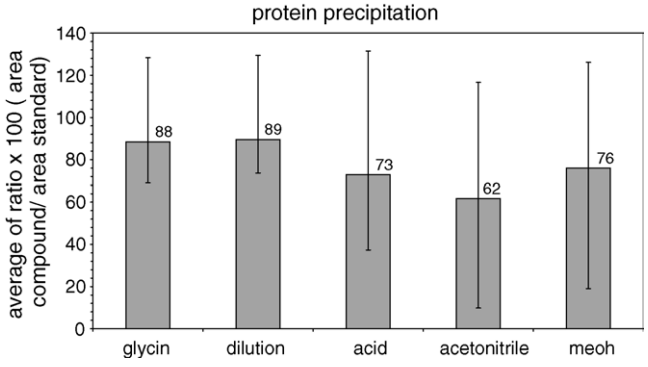

Fig. 3. Comparison of protein precipitation methods using HPLC as monitoring method. The average of all ratios × 100 (peak area compound/peak area standard) for each compound was calculated for the different protein precipitation methods. The lowest and highest value is indicated.

3.2. Protein precipitation

Most new antidepressants are highly bound to proteins, mainly to alpha1-glycoprotein and to a lesser extent to albumin and lipoproteins [38–42]. When using SPE as sample preparation, protein binding can lower the analyte recovery, as the active sites of the compounds that would normally interact with the sorbent are not available for this interaction. Another problem is the fact that most proteins are large molecules prohibiting penetration of the sorbent pores. As a result, the drug is carried through the sorbent bed by the protein instead of being retained [48].

Sonication, centrifugation [30,32] and dilution in combination with a slow sorbent pass-through of the sample seem to be appropriate to demolish the protein binding of drugs [31,33,43]. This protein bond depends on temperature, pH, protein content and molecules that compete for the same sites on the protein. Thus, addition of salt or change of pH and dilution can also modify the protein binding. Denaturation of the protein by adding organic solvents to the sample is another method used. As an

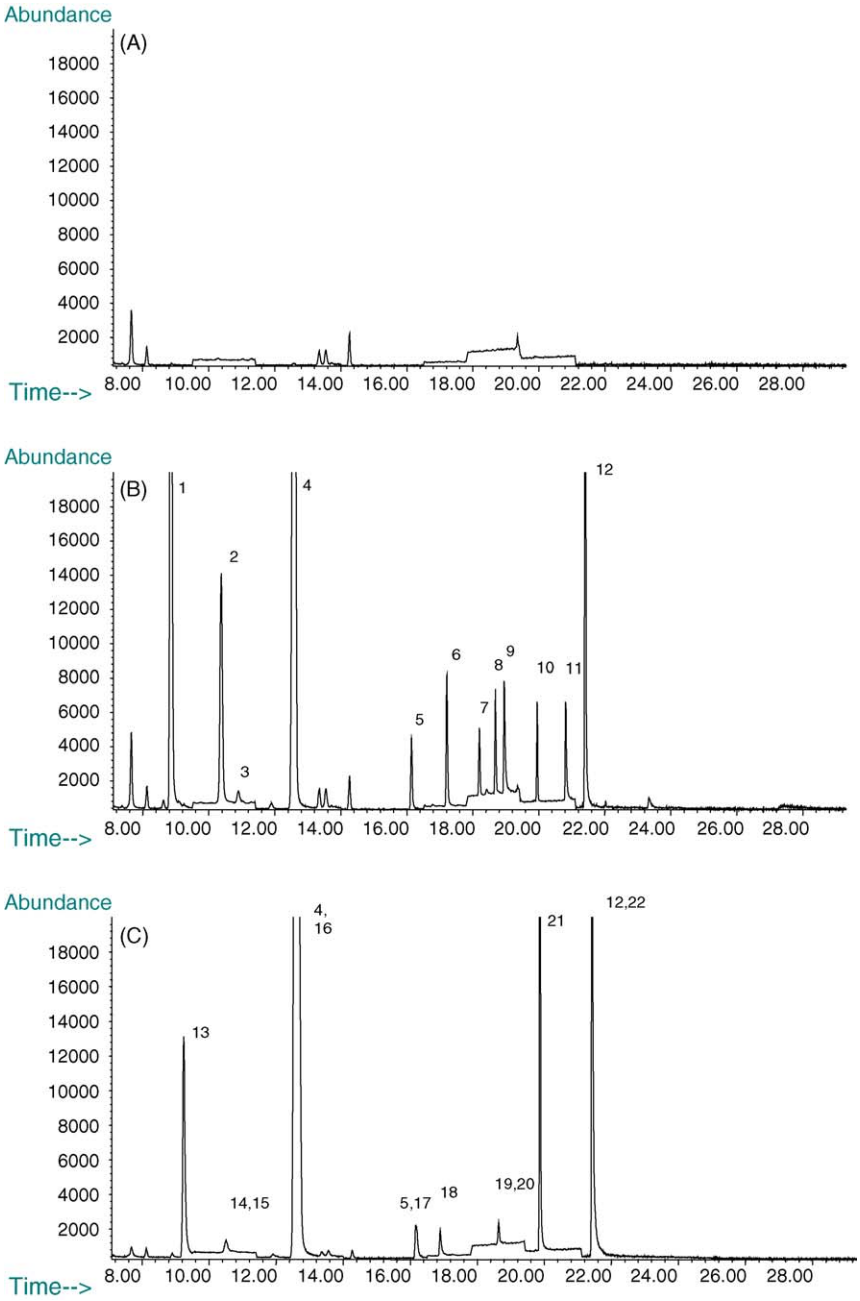

Fig. 4. GC–MS trace of a blank plasma sample (A); mixture 1 at low therapeutic concentrations (B); mixture 2 at low therapeutic concentrations (C) after SPE with SCX. Compounds: venlafaxine (1), viloxazine (2), fluvoxamine (3), fluoxetine D6 (4), mianserin D3 (5), melitracen (6), desmethylmianserin (7), reboxetine (8), citalopram (9), sertraline (10), desmethylcitalopram (11), paroxetine D6 (12), *m*-chlorophenylpiperazine (13), norfluoxetine (14), *O*-desmethylvenlafaxine (15), fluoxetine (16), mianserin (17), mirtazapine (18), desmethylmirtazapine (19), norsertraline (20), maprotiline (21) and paroxetine (22). All chromatograms are given at the same sensitivity setting.

ion-exchange procedure is used, addition of salts was not tested as they could interact with the SPE sorbent, leading to lower recovery of the compounds of interest. When testing the different methods, at first, it seemed that the glycine HCl buffer and addition of an acid gave the best results. Dilution also gave acceptable results as it weakens the protein–drug binding and increases the time of contact of the drugs with the adsorbent. Although addition of organic solvents results in precipitation and denaturation of the protein, acetonitrile gave the worst results, probably due to coprecipitation of the ADs. The results were explained because the loading pH was lower (2.5 instead of 6.5) when using the glycine-buffer or an acid which had an impact on the retention on the strong cation exchanger of the compounds, especially for trazodone and mirtazapine. Therefore, when diluting the sample using a phosphate buffer of pH 2.5, the results were better than with the phosphate buffer at pH 6.5 and even better than the glycine/acid results (Fig. 3). A significant difference in peak area was seen between the glycine, acid or dilution method when using an ANOVA-test ($p < 0.02$, except for DMSer and DDMC). The pH of the solution not only influenced the retention on the sorbent as fractional precipitation of proteins can be achieved by a varying pH of the plasma sample. At the iso-electric point there is no net charge and thus the solubility of the protein decreases and leads to precipitation. At pH 3 the proteins (iso-electric point of alpha1-glycoprotein: 3.53) [44] will carry less negative charges than under physiological conditions, thus the ADs that are positively charged in those conditions, will show less ionic interactions [41].

Binding to proteins is not only due to ionic interactions. For alpha1-glycoprotein the lipophilicity is also relevant. An increase in lipophilicity of a compound results in higher protein binding [41,44,45]. Albumin binds best the hydrophobic and anionic compounds [39], thus less the positively charged antidepressants.

3.3. SPE and GC–MS

The recoveries and absence of interferences were evaluated for the SCX and the Strata XC SPE column. Because the 'new' generation ADs have an amine-function a cation exchange mechanism was plausible (Fig. 1). Retention on both the SCX and Strata XC phases is based on this mechanism, but Strata XC being a mixed-mode phase combining ion-exchange and a styrene–divinylbenzene polymer shows hydrophobic and aromatic interactions as well. Combining different interaction mechanisms can be interesting to extract a variety of compounds, but can also lead to co-extraction of matrix compounds that are not of interest, leading to more background in the final analysis. GC-traces for blank plasma after extraction from a SCX column were slightly cleaner than with the Strata XC phase. No interfer-

Table 3
Percent Recovery for the 13 'new' generation ADs and eight of their active metabolites from the SCX and Strata XC column, determined at low and high therapeutic and toxic levels in GC–MS mode and in HPLC mode

Compounds	Recovery (%) (CV%)						
	GC						HPLC
	SCX			Strata XC			SCX
	Low concentration	High concentration	Toxic concentration	Low concentration	High concentration	Toxic concentration	Therapeutic concentration[a]
mCPP	92 (8)	101 (8)	88 (5)	78 (4)	83 (1)	80 (5)	89 (2)
Venlafaxine	100 (6)	86 (6)	96 (5)	84 (4)	85 (10)	83 (5)	88 (1)
Norfluoxetine	71 (17)	87 (6)	74 (4)	53 (15)	63 (5)	72 (5)	81 (2)
Viloxazine	114 (8)	105 (6)	108 (2)	83 (9)	100 (9)	89 (4)	105 (2)
Fluvoxamine	76 (10)	81 (10)	105 (4)	54 (16)	77 (9)	85 (6)	96 (2)
ODMV							113 (1)
Fluoxetine	110 (8)	96 (7)	92 (1)	89 (4)	92 (1)	85 (5)	80 (2)
Fluoxetine D6							
Mianserin	80 (5)	77 (4)	82 (2)	82 (8)	83 (2)	80 (5)	79 (4)
Mianserin D3							
Mirtazapine	93 (9)	78 (4)	83 (3)	92 (9)	81 (1)	81 (6)	78 (8)
Melitracen	93 (7)	81 (8)	93 (2)	73 (4)	94 (4)	82 (5)	80 (3)
DMMia	92 (7)	94 (4)	99 (4)	67 (9)	88 (3)	87 (5)	83 (7)
DMMir	94 (12)	90 (2)	95 (2)	90 (5)	80 (4)	87 (2)	
Reboxetine	94 (10)	97 (3)	104 (4)	73 (13)	97 (7)	94 (5)	110 (2)
DMSer	66 (15)	84 (10)	103 (8)	52 (7)	62 (3)	77 (4)	74 (2)
Citalopram	101 (6)	71 (6)	91 (9)	68 (10)	98 (12)	74 (6)	97 (2)
Maprotiline	105 (9)	95 (4)	94 (2)	89 (5)	85 (1)	86 (2)	102 (12)
Sertraline	65 (12)	75 (6)	67(7)	70 (7)	90 (10)	78 (10)	76 (3)
DMC	104 (10)	92 (6)	106 (3)	71 (14)	96 (1)	92 (5)	98 (3)
DDMC		88 (5)	83 (9)		61 (4)	78 (9)	128 (7)
Paroxetine	102 (14)	89 (7)	101(5)	70 (13)	79 (1)	85 (4)	106 (3)
Paroxetine D6							
Trazodone		30 (55)	48 (25)		90 (38)	46 (30)	95 (2)

[a] Therapeutic concentrations as described in Section 2.6.

ences on the compounds of interest, though, were seen for both phases in the final GC–MS analysis (Fig. 4A).

The recoveries for both columns were reproducible as demonstrated in Table 3. In addition the recoveries were constantly lower using Strata XC as compared to SCX. Perhaps, this can be explained by the fact that the ion-exchange mechanism is dominating the retention. When using a mixed-mode, the ion-exchange groups are less numerous. A larger bed volume could lead to more interaction with the compounds of interest, but of course also with unwanted matrix constituents. On the other hand, methanol is not a good disruptor of hydrophobic and dipolar interactions. Therefore, a small percentage of acetonitrile in the methanol–ammonia elution solvent would probably neutralize these non-ionic interactions during elution, leading to enhanced recovery yields for the Strata XC.

The recovery of trazodone was quite low and not reproducible. Trazodone analysis showed multiple problems. First of all, the chromatography of trazodone is not optimal. Trazodone is a tertiary amine and therefore it can not be derivatized with HFBI, leading to a bad peak shape and possible adsorption to the glass wool in the inlet liner. In addition, the compound has a high melting point, leading to the need for a high injection and eluting temperature, which probably enhances degradation and thus bad peak shape. On the other hand the protein precipitation method, optimal SPE washing- and cleaning solvents and loading pH were always a compromise for trazodone. During the method development, trazodone was most sensitive for the MeOH wash step and results seemed better when using a more acidic loading pH. When looking at the recovery of trazodone when analysed with HPLC-DAD (Table 3), it is clear that the main problem is the GC–MS analysis.

The recovery value for the metabolite of venlafaxine, i.e. *O*-desmethylvenlafaxine (ODMV) was not obtained for the GC–MS analysis, due to overlap with norfluoxetine and an aspecific fragmentation pattern compromizing identification and quantification (quantifier m/z 58).

4. Conclusion

A reproducible SPE method for 13 'new' generation ADs and some of their metabolites was developed for GC–MS analysis. Based on recovery, reproducibility and absence of interfering substances the strong cation exchanger gave the best results.

One millilitre of plasma was diluted with 4 ml phosphate buffer (pH 2.5; 25 mM). The final SPE consisted of a conditioning step with 3 ml of eluent, 2 ml of methanol and 3 ml of the eluting phosphate buffer followed the sample load. After a wash step (4 ml of methanol) the compounds were eluted with 2 ml of 5% ammonia in methanol. The mean recoveries at low and high therapeutic and toxic levels ranged between 70 and 109% for all compounds, except for trazodone (39%).

References

[1] WHO: http://www.who.int/mental_health.
[2] S.M. Sampson, Mayo Clin. Proc. 76 (2001) 739.
[3] D.R.A. Uges, J.M.H. Conemans, Handbook of Analytical Separations, Elsevier Science, 2000, p. 229.
[4] P. Pacher, E. Kohegyi, V. Kecskemeti, S. Furst, Curr. Med. Chem. 8 (2001) 89.
[5] J.M. Kent, Lancet 355 (2000) 911.
[6] J.M. Kent, Lancet 355 (2000) 2000.
[7] J. Lundmark, F. Bengtsson, C. Nordin, M. Reis, J. Walinder, Acta Psychiatr. Scand. 101 (2000) 354.
[8] M.J. Burke, S.H. Preskorn, Clin. Pharmacokinet. 37 (1999) 147.
[9] P.B. Mitchell, Br. J. Clin. Pharmacol. 49 (2000) 303.
[10] P.B. Mitchell, Clin. Chem. Lab. Med. 42 (2004) 1212.
[11] C.B. Eap, E.J. Sirot, P. Baumann, Ther. Drug Monit. 26 (2004) 152.
[12] S. Heller, C. Hiemke, G. Stroba, A. Rieger-Gies, E. Daum-Kreysch, J. Sachse, S. Hartter, Ther. Drug Monit. 26 (2004) 459.
[13] A. de Meester, G. Carbutti, L. Gabriel, J.M. Jacques, Acta Clin. Belg. 56 (2001) 258.
[14] T. Azaz-Livshits, A. Hershko, E. Ben-Chetrit, Pharmacopsychiatry 35 (2002) 112.
[15] K.E. Goeringer, I.M. McIntyre, O.H. Drummer, Forensic Sci Int. 121 (2001) 70.
[16] K.E. Goeringer, L. Raymon, G.D. Christian, B.K. Logan, J. Forensic Sci. 45 (2000) 633.
[17] C.A. Kelly, N. Dhaum, W.J. Laing, F.E. Strachan, A.M. Good, D.N. Bateman, J. Toxicol. Clin. Toxicol. 42 (2004) 67.
[18] D.E. Adson, S. Erickson-Birkedahl, M. Kotlyar, Ann. Pharmacother. 35 (2001) 1375.
[19] M.H. Baumann, R.D. Clark, A.G. Budzynski, J.S. Partilla, B.E. Blough, R.B. Rothman, Neuropsychopharmacology 30 (2005) 550.
[20] F.T. Peters, S. Schaefer, R.F. Staack, T. Kraemer, H.H. Maurer, J. Mass Spectrom. 38 (2003) 659.
[21] K.M. Kim, B.H. Jung, M.H. Choi, J.S. Woo, K.J. Paeng, B.C. Chung, J. Chromatogr. B 769 (2002) 333.
[22] C.B. Eap, G. Bouchoux, M. Amey, N. Cochard, L. Savary, P. Baumann, J. Chromatogr. Sci. 36 (1998) 365.
[23] K. Titier, N. Castaing, E. Scotto-Gomez, F. Pehourcq, N. Moore, M. Molimard, Ther. Drug Monit. 25 (2003) 581.
[24] E. Lacassie, J.M. Gaulier, P. Marquet, J.F. Rabatel, G. Lachâtre, J. Chromatogr. B 742 (2000) 229.
[25] C. Duverneuil, G.L. de la Grandmaison, P. de Mazancourt, J.C. Alvarez, Ther. Drug Monit. 25 (2003) 565.
[26] U. Gutteck, K.M. Rentsch, Clin. Chem. Lab. Med. 41 (2003) 1571.
[27] R.N. Gupta, J. Chromatogr. 576 (1992) 183.
[28] L. Kristoffersen, A. Bugge, E. Lundanes, L. Slordal, J. Chromatogr. B 734 (1999) 229.
[29] M.A. Raggi, R. Mandrioli, G. Casamenti, F. Bugamelli, V. Volterra, J. Pharm. Biomed. Anal. 18 (1998) 193.
[30] C. Frahnert, M.L. Rao, K. Grasmader, J. Chromatogr. B 794 (2003) 35.
[31] A. Bakkali, E. Corta, J.I. Ciria, L.A. Berrueta, B. Gallo, F. Vicente, Talanta 49 (1999) 773.
[32] M.A. Martinez, C.S. de la Torre, E. Almarza, J. Anal. Toxicol. 28 (2004) 174.
[33] C.K. Lai, T. Lee, K.M. Au, A.Y.W. Chan, Clin. Chem. 43 (1997) 312.
[34] M. Lenz, W.R. Bork, Toxichem. + Krimtech. 69 (2002) 99.
[35] M.A. Raggi, R. Mandrioli, G. Casamenti, V. Volterra, S. Pinzauti, J. Chromatogr. A 949 (2002) 23.
[36] C. Sabbioni, F. Bugamelli, G. Varani, L. Mercolini, A. Musenga, M.A. Saracino, S. Fanali, M.A. Raggi, J. Pharm. Biomed. Anal. 36 (2004) 351.
[37] G. Pépin, G. Duffort, V. Dumestre, M.H. Ghysel, P. Kintz, M. Villain, G. Lachâtre, P. Mura, M. Perrin, Y. Ricordel, The International Association of Forensic Toxicologists (TIAFT) 41st International Meeting, Melbourne, Australia, 2003 (Abstract book, paper 36).
[38] C. Bertucci, E. Domenici, Curr. Med. Chem. 9 (2002) 1463.
[39] N.A. Kratochwil, W. Huber, F. Muller, M. Kansy, P.R. Gerber, Biochem. Pharmacol. 64 (2002) 1355.
[40] E. Pike, B. Skuterud, P. Kierulf, J.E. Bredesen, P.K.M. Lunde, Clin. Pharmacokinet. 9 (1984) 84.

[41] T. Fournier, N. Medjoubi-N, D. Porquet, Biochim. Biophys. Acta Protein Struct. Mol. Enzym. 1482 (2000) 157.
[42] K.M. Piafsky, Clin. Pharmacokinet. 5 (1980) 246.
[43] P. Molander, A. Thomassen, L. Kristoffersen, T. Greibrokk, E. Lundanes, J. Chromatogr. B 766 (2002) 77.
[44] D.G. Ferry, N.B. Caplan, L.X. Cubeddu, J. Pharm. Sci. 75 (1986) 146.
[45] Z.H. Israili, P.G. Dayton, Drug Metab. Rev. 33 (2001) 161.
[46] TIAFT therapeutic and toxic drug concentration list: http://www.tiaft.org/.
[47] M. Schulz, A. Schmoldt, Pharmazie 58 (2003) 447.
[48] Varian, Handbook of Sorbent Extraction Technology, 1998, 138 pp.