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Simultaneous determination of citalopram, fluoxetine, paroxetine and their metabolites in plasma and whole blood by high-performance liquid chromatography with ultraviolet and fluorescence detection

L. Kristoffersen[a,*], A. Bugge[a], E. Lundanes[b], L. Slørdal[a,c]

[a]*National Institute of Forensic Toxicology, P.O. Box 495 Sentrum, N-0105 Oslo, Norway*
[b]*Department of Chemistry, University of Oslo, P.O. Box 1033 Blindern, N-0315 Oslo, Norway*
[c]*Department of Pharmacology and Toxicology, Faculty of Medicine, Norwegian University of Science and Technology, N-7005 Trondheim, Norway*

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Abstract

A method for the simultaneous determination of the three selective serotonin reuptake inhibitors (SSRIs) citalopram, fluoxetine, paroxetine and their metabolites in whole blood and plasma was developed. Sample clean-up and separation were achieved using a solid-phase extraction method with C_8 non-endcapped columns followed by reversed-phase high-performance liquid chromatography with fluorescence and ultraviolet detection. The robustness of the solid-phase extraction method was tested for citalopram, fluoxetine, paroxetine, Cl-citalopram and the internal standard, protriptyline, using a fractional factorial design with nine factors at two levels. The fractional factorial design showed two significant effects for paroxetine in whole blood. The robustness testing for citalopram, fluoxetine, Cl-citalopram and the internal standard revealed no significant main effects in whole blood and plasma. The optimization and the robustness of the high-performance liquid chromatographic separation were investigated with regard to pH and relative amount of acetonitrile in the mobile phase by a central composite design circumscribed. No alteration in the elution order and no significant change in resolution for a deviation of ±1% acetonitrile and ±0.3 pH units from the specified conditions were observed. The method was validated for the concentration range 0.050–5.0 μmol/l with fluorescence detection and 0.12–5.0 μmol/l with ultraviolet detection. The limits of quantitation were 0.025 μmol/l for citalopram and paroxetine, 0.050 μmol/l for desmethyl citalopram, di-desmethyl citalopram and citalopram-*N*-oxide, 0.12 μmol/l for the paroxetine metabolites by fluorescence detection, and 0.10 μmol/l for fluoxetine and norfluoxetine by ultraviolet detection. Relative standard deviations for the within-day and between-day precision were in the ranges 1.4–10.6% and 3.1–20.3%, respectively. Recoveries were in the 63–114% range for citalopram, fluoxetine and paroxetine, and in the 38–95% range for the metabolites. The method has been used for the analysis of whole blood and plasma samples from SSRI-exposed patients and forensic cases.

Keywords: Citalopram; Fluoxetine; Paroxetine

*Corresponding author. Fax: +47-2238-3233.
E-mail address: lena.kristoffersen@labmed.uio.no (L. Kristoffersen)

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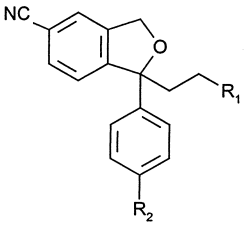
1. Introduction

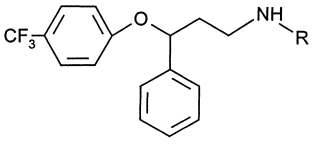
The antidepressants fluoxetine (FLU), paroxetine (PAR) and citalopram (CIT) (Fig. 1a–d) belong to the class of selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs). The SSRIs have a different, and generally better tolerated, adverse effect profile when compared to the tricyclic antidepressants (TCAs) with approximately equivalent antidepressant efficacy [1]. Additionally,

(a)



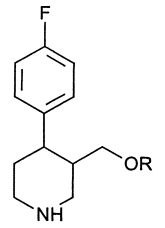
Compound	R_1	R_2	M_r
Citalopram (CIT)	$-CH_2N(CH_3)_2$	F	324.4
Desmethylcitalopram (DCIT)	$-CH_2NHCH_3$	F	310.4
Didesmethylcitalopram (DDCIT)	$-CH_2NH_2$	F	296.4
Citalopram propionic acid (CITPA)	-COOH	F	311.3
Citalopram N-oxide (CITNO)	$-CH_2\overset{\uparrow}{N}(CH_3)_2$ (O)	F	340.4
Cl-Citalopram (CITCl)	$-CH_2N(CH_3)_2$	Cl	340.9

(b)

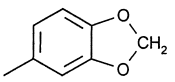
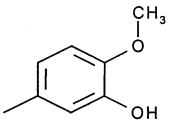
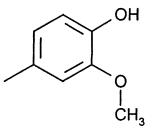


Compound	R	M_r
Fluoxetine (FLU)	$-CH_3$	309.3
Norfluoxetine (NFLU)	-H	295.3

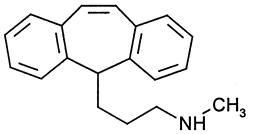
Fig. 1. (a) Structures and molecular masses (M_r) of citalopram and its metabolites. (b) Structures and molecular masses (M_r) of fluoxetine and norfluoxetine. (c) Structures and molecular masses (M_r) of paroxetine and its metabolites. (d) Structure and molecular masses (M_r) of the internal standard protriptyline.



(c)

Compound	R	M_r
Paroxetine (PAR)		329.4
(-)Trans 4-(4-fluorophenyl)-3-(3-hydroxy-4-methoxyphenoxymethyl)piperidine-hydrochloride (BRL 36583A)		331.4
(-)Trans 4-(4-fluorophenyl)-3-(4-hydroxy-3-methoxyphenoxymethyl)piperidine-hydrochloride (BRL 36610A)		331.4

(d)



M_r: 263.4

Fig. 1. (*continued*)

some SSRIs are indicated for obsessive–compulsive, panic and anxiety disorders [2,3]. Therefore, SSRIs are currently widely prescribed medications.

Patients with depression are at high risk of suicide attempts [1,4]. Although the toxicity of the SSRIs is comparatively low, there have been case reports of deaths in which CIT [5,6] or FLU [7,8] were strongly suspected as causative agents. Deaths related to a combination of a SSRI with other drugs or ethanol have been reported [5,9–13]. The blood concentrations which are reported to be toxic or lethal are >3.1 μmol/l for CIT [4], >9.1 μmol/l for PAR [4] and >4.2 μmol/l for FLU [13]. In the treatment of depressive disorders with FLU, CIT and PAR, respective plasma concentrations in the approximate ranges 0.16–1.5 μmol/l (0.05–0.45 μg/ml) [14], 0.12–0.6 μmol/l (0.04–0.2 μg/ml) [15], and 0.061–0.58 μmol/l (0.020–0.19 μg/ml) [14] have been observed. The concentrations of the metabolites are generally lower than that of the parent compound.

FLU, PAR and CIT are metabolized by the cytochrome P450 system (CYP) in the liver. The metabolites of PAR are unlikely to exert significant inhibitory activity with respect to 5-HT or noradrenaline uptake mechanisms [16]. A metabolite of PAR, BRL 36583A (Fig. 1c), has been demonstrated in vitro to inhibit CYP2D6 [17]. CIT and FLU are demethylated to desmethylcitalopram (DCIT) and norfluoxetine (NFLU), respectively. DCIT is further N-demethylated to di-desmethylcitalopram (DDCIT). NFLU has similar potency and selectivity with regard to 5-HT reuptake and liver enzyme inhibition as the parent compound [17,18]. The CIT metabolites, DCIT and DDCIT, have SSRI properties, but are unlikely to contribute substantially to the therapeutic effect of CIT since they are present in lower concentrations and are less potent serotonin reuptake inhibitors compared to CIT [15]. Nevertheless, in forensic analysis and therapeutic drug monitoring it is beneficial to quantify the metabolites as this information may be useful to shed light on time relations, including the time lapse between drug ingestion and subsequent events, and unusual metabolizing patterns. An example of the latter include the report that a high level of DDCIT and CIT can cause fatal cardiac arrhythmias in dogs [6].

Analytical methods for the determination of SSRIs and their metabolites are of obvious use for pharmacokinetic studies and in situations where the patients do not respond as expected due to drug interactions, non-compliance or other causes. Several high-performance liquid chromatography (HPLC), gas chromatography (GC), and thin-layer chromatography (TLC) methods have been published for the determination of CIT, PAR, FLU, sertraline (SER) and fluvoxamine (FLUV) and their metabolites in plasma or serum [19–23,25]. A few methods which allow determination of SSRIs in whole blood have also been published [24]. Little has been published on analytical methods for the simultaneous determination of several compounds of the SSRI class.

A common situation in forensic toxicology is the paucity of information concerning which drugs have been ingested prior to death. For screening of antidepressants in forensic blood samples, the methods should be applicable for simultaneous determination of as many antidepressants as possible. In addition, the methods should be able to quantify SSRIs in whole blood which is the most common sample matrix in forensic analysis. Eap et al. [26] reported that simultaneous determination of CIT, PAR, SER and their N-demethylated metabolites, and possibly FLU, NFLU and FLUV, in plasma could be achieved by liquid–liquid extraction and GC–mass spectrometry (MS). To our knowledge, this is the only published method which allows the simultaneous determination of at least three SSRIs. The ability of this method to determine SSRIs in biological samples other than plasma is not reported.

In this paper, we present an automated and rapid solid-phase extraction (SPE) and isocratic HPLC method with fluorescence and ultraviolet (UV) detection which simultaneously determines the SSRIs CIT, PAR, FLU and their metabolites DCIT, DDCIT, citalopram-*N*-oxide (CITNO), NFLU, BRL 36610A and BRL 366583A in either plasma or whole blood at therapeutic and overdose concentrations. In contrast to most published methods, we present an assay which is optimized and robustness tested by systematically searching for satisfactory conditions by multivariate techniques including fractional factorial design and response surface methods [27].

2. Experimental

2.1. Reagents

PAR hydrochloride hemihydrate, (−)-*trans* 4-(4-fluorophenyl) - 3 - (3 - hydroxy - 4 - methoxyphenoxymethyl)piperidine hydrochloride (BRL 36583A) and (−)-*trans* 4-(4-fluorophenyl)-3-(4-hydroxy-3-methoxyphenoxymethyl)piperidine hydrochloride (BRL 36610A) were kindly provided by SmithKline Beecham (Worthing, UK). CIT hydrobromide, DCIT hydrochloride, DDCIT tartrate monohydrate, CITNO hydrochloride, CIT propionic acid (CITPA) and Cl-citalopram oxalate (CITCl) were gifts from H. Lundbeck (Copenhagen, Denmark). Eli Lilly (Indianapolis, IN, USA) kindly supplied FLU hydrochloride and NFLU hydrochloride. Protriptyline hydrochloride (PRO) was purchased from Sigma (St. Louis, MO, USA).

Potassium dihydrogenphosphate, dipotassium hydrogenphosphate trihydrate and formic acid, all analytical grade, and gradient grade methanol were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was purchased from Mallinchrodt Baker (Deventer, The Netherlands), while gas phase sequencer grade trifluoroacetic acid (TFA) was purchased from Rathburn (Walkerburn, UK). Ammonium formate was obtained from BDH (Poole, UK). Deionised water from a Milli-Q station (Millipore, Bedford, MA, USA) was used for all procedures.

2.2. Preparation of standard solutions

Stock solutions of PAR, CIT, FLU, BRL 36610A, BRL 36583A, DCIT, DDCIT, CITNO, CITPA, CITCl and NFLU as well as stock solution of the internal standard PRO were prepared in methanol at a concentration of 2500 μmol/l using 20-ml volumetric flasks. The stock solutions were stored at −78°C.

For robustness testing of the SPE procedure HPLC calibration solutions of CIT, PAR, FLU and CITCl were prepared in HPLC mobile phase at the concentrations 0.5, 2.5 and 5.0 μmol/l which corresponds to 0.1, 0.5 and 1.0 μmol/l in whole blood or plasma when correcting for the times five volume reduction during sample preparation. The HPLC calibration solutions of PRO contained 0.1, 0.25 and 0.5 μmol/l PRO which corresponds to 0.02, 0.05 and 0.1 μmol/l in whole blood or plasma. Drug-free citrated plasma and sodium fluoride whole blood from healthy donors were supplied by The Blood Centre at Ullevaal Hospital (Oslo, Norway). Whole blood and plasma samples were spiked with CIT, PAR and FLU to a final concentration of 0.5 μmol/l of each and stored at −78°C. Before the SPE analysis, 50 μl of an aqueous solution containing 0.50 μmol/l PRO and 5.0 μmol/l CITCl was added to the whole blood and plasma samples.

For method validation experiments HPLC calibration solutions including all the analytes were prepared in HPLC mobile phase at the concentration levels 0.25 (0.60), 0.50 (1.25), 2.5, 5.0, 12.4 and 25.0 μmol/l which corresponds to 0.05 (0.12), 0.1 (0.25), 0.5, 1.0, 2.5 and 5.0 μmol/l in whole blood or plasma, and where the figures in parentheses represent the concentration of FLU, NFLU, BRL 36610A and BRL 36583A. The concentration of the internal standard PRO was 0.25 μmol/l in the HPLC calibration solutions. Drug-free plasma and whole blood were spiked with appropriate aliquots of the stock solutions to give concentrations in the range 0.050 to 5.0 μmol/l for each of the analytes. The spiked plasma and whole blood samples were stored at −78°C. Before SPE 50 μl of an aqueous 0.50 μmol/l internal standard solution of PRO was added to the whole blood and plasma samples.

2.3. Instrumentation

The automatic SPE procedure was performed on two RapidTrace SPE Workstation modules from Zymark (Hopkinton, MA, USA).

The HPLC system consisted of a GT-104 online degasser, a FCV-10AL low-pressure gradient flow control valve, an LC-10AT solvent delivery module, a SIL-10AXL automatic sample injector, a CTO-10A column oven, a SPD-10A spectrophotometric detector, a RF-10AXL spectrofluorometric detector, a CBM-10A communication module and a CLASS-LC10 integrator, all from Shimadzu (Kyoto, Japan).

2.4. Chromatographic conditions

HPLC separation was performed on a Symmetry C_{18} column (150×3.9 mm, 5 μm) which was protected by a Sentry guard column (20×3.9 mm), both from Waters (Milford, MA, USA). The mobile phase consisted of 45 m*M* ammonium formate (pH 4.0)–acetonitrile (70:30, v/v). Formic acid (5.0 *M*) was used to adjust the pH of the mobile phase. The mobile phase flow-rate was 1.2 ml/min and the column temperature was maintained at 45±0.1°C. UV detection of FLU and NFLU was carried out at 230 nm. Two separate conditions were used for fluorescence detection. CIT, its metabolites and CITCl were detected at an excitation wavelength of 245 nm and an emission wavelength of 295 nm. PAR, its metabolites and the internal standard PRO were detected at an excitation wavelength of 295 nm and an emission wavelength of 350 nm. The fluorescence detector settings were set to low sensitivity and a gain of 4. The injection volume was 15 μl.

2.5. SPE procedure

A 0.5-ml volume of whole blood or plasma was mixed with 50 μl of the aqueous 0.50 μmol/l internal standard solution and sonicated in an ultrasonic bath for 15 min at ambient temperature. The sample was diluted with 5.0 ml of 0.1 *M* KH_2PO_4 (pH 7.0)–methanol (90:10, v/v), mixed on a Whirlimixer and centrifuged at 2000 rpm (780 *g*) for 10 min. The supernatant was applied to a 3-ml, 100 mg IST C_8 non-endcapped extraction column (International Sorbent Technology, Mid Glamorgan, UK). The SPE procedure is described in Table 1. The eluate was evaporated to dryness under a stream of N_2 at 40°C (Turbovap, Zymark) and redissolved in 100 μl mobile phase before HPLC analysis.

2.6. Quantification

Peak height was used for all quantifications. The recovery of the analytes from spiked samples were obtained by external standard quantification with HPLC mobile phase standards.

HPLC mobile phase standards were also used for the internal standard quantification based on peak height ratios which was used for the method validation and for determination of SSRIs in patient samples. Due to the difference in recovery of the analyte and the internal standard, recovery correction was performed. The recovery factor (RF) was determined from the uncorrected concentrations obtained from the between-day results as described in Eq. (1)

$$\text{Recovery factor (RF)} = \frac{\text{Added concentration}}{\text{Uncorrected concentration}} \quad (1)$$

The corrected concentrations were obtained by multiplying the uncorrected concentrations with the recovery factor.

Table 1
SPE procedure for the determination of PAR, CIT, FLU and their metabolites in human plasma and whole blood using RapidTrace SPE workstation

Step	Process	Reagent	Flow rate (ml/min)
1	Condition	2 ml methanol	2.0
2	Condition	1 ml water	2.0
3	Condition	1 ml 0.1 *M* KH_2PO_4, pH 7.0	2.0
4	Load	5.7 ml diluted whole blood or plasma	1.5
5	Rinse	2.0 ml water	2.0
6	Purge cannula	6.0 ml water	30
7	Rinse	2.0 ml 0.1 *M* KH_2PO_4, pH 7.0	2.0
8	Rinse	1.0 ml acetonitrile–water (50:50, v/v)	2.0
9	Purge cannula	6.0 ml 2% (v/v) trifluoroacetic acid in methanol	30
10	Elute	0.75 ml 2% (v/v) trifluoroacetic acid in methanol	1.5
11	Purge cannula	6.0 ml water	30

2.7. Samples

The method has been used for the determination of PAR in whole blood and plasma samples from patients who were treated with 20 mg/day and 40 mg/day Seroxat (Novo Nordisk, Copenhagen, Denmark). In addition, we used the assay to measure PAR in two autopsy samples. The ingested dose was unknown. Two sample replicates were used.

3. Experimental design

The recovery of the analytes related to the average recovery obtained for the center points was used as response in the SPE optimization and robustness experiments. The resolution, retention time and alteration in retention order were used as responses for the optimization and robustness testing of the HPLC method. The resolution was generally calculated by the integrator system according to the equation

$$\text{Resolution } (R_s) = 2\frac{(t_R - t_{Rp})}{W + W_p} \quad (2)$$

where t_R and t_{Rp} are the retention times of the analyte and the previous peak, while W and W_p are the peak width of the analyte and the previous peak, respectively. The peak width is the time width between two intersections by tangent line at the inflection point right and left of the peak and baseline. Second-order regression models were developed by means of multiple linear regression (MLR)

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_1^2 + b_4x_2^2 + b_5x_1x_2 \quad (3)$$

where Y is the resolution or the retention time, $b_0 \ldots b_5$ represent the regression coefficients, and x_1 and x_2 are the coded levels for the pH and relative amount of acetonitrile (Table 2).

Regression coefficients which were non-significant for all the responses were excluded from the models. All the experimental design experiments were carried out in a randomized order. Three center points were used. MODDE 4.01 software from Umetri (Umeå, Sweden) was used for design and evaluation of the chemometric studies.

4. Results and discussion

4.1. Chromatographic separation

The Symmetry C_{18} HPLC column was chosen because of minimal batch-to-batch variation. Columns from three different batches have been tested, and gave highly reproducible results (not shown). The chromatographic separation of the SSRIs and their metabolites is shown in Fig. 2a–f. Fluorescence detection was used for the determination of CIT, PAR and their metabolites. FLU and NFLU were detected with UV, however at high concentrations fluorescence detection could be used. The compounds were separated isocratically in less than 15 min. The resolution of each pair was ≥ 1.5.

To establish the HPLC method, the effect of different mobile phase parameters was investigated in screening designs with regard to resolution, alteration in retention order and retention time. The factor levels which were found to be satisfactory in the screening design experiments were set as center points values in a central composite design circumscribed (CCC). The aims were; firstly, to investigate the factors that from screening designs had showed to be significant, and, secondly, to determine the method specifications which would ensure a satisfactory performance of the HPLC separation, and thereby recommend factor settings where robustness could be assured. Satisfactory performance criteria were identified by $R_s \geq 1.4$, no alteration in

Table 2
The two-factor central composite design circumscribed used for the evaluation of the HPLC method

Factor	$-\alpha$	-1	0	$+1$	$+\alpha$
x_1: pH	3.4	3.6	4.0	4.4	4.6
x_2: Relative amount of acetonitrile (%)	23	25	30	35	37

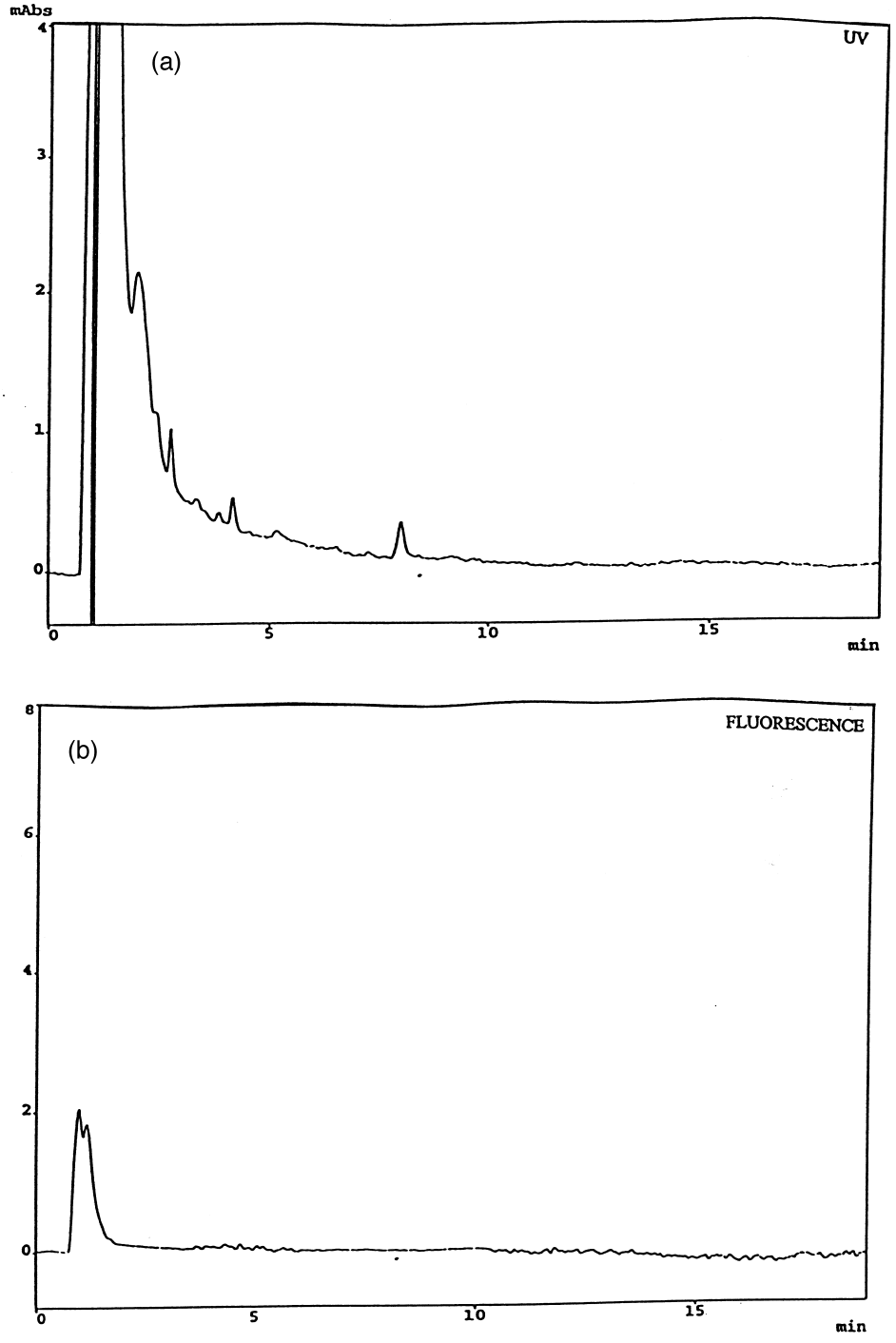


Fig. 2. Chromatograms of a drug free human whole blood sample (a) and (b), a whole blood sample spiked with 0.050 μmol/l PRO (IS) and 0.50 μmol/l BRL 36610A, BRL 36583A, DDCIT, DCIT, CIT, CITNO, PAR, NFLU and FLU (c) and (d), and a whole blood sample from a forensic case (autopsy) after PAR exposure (e) and (f).

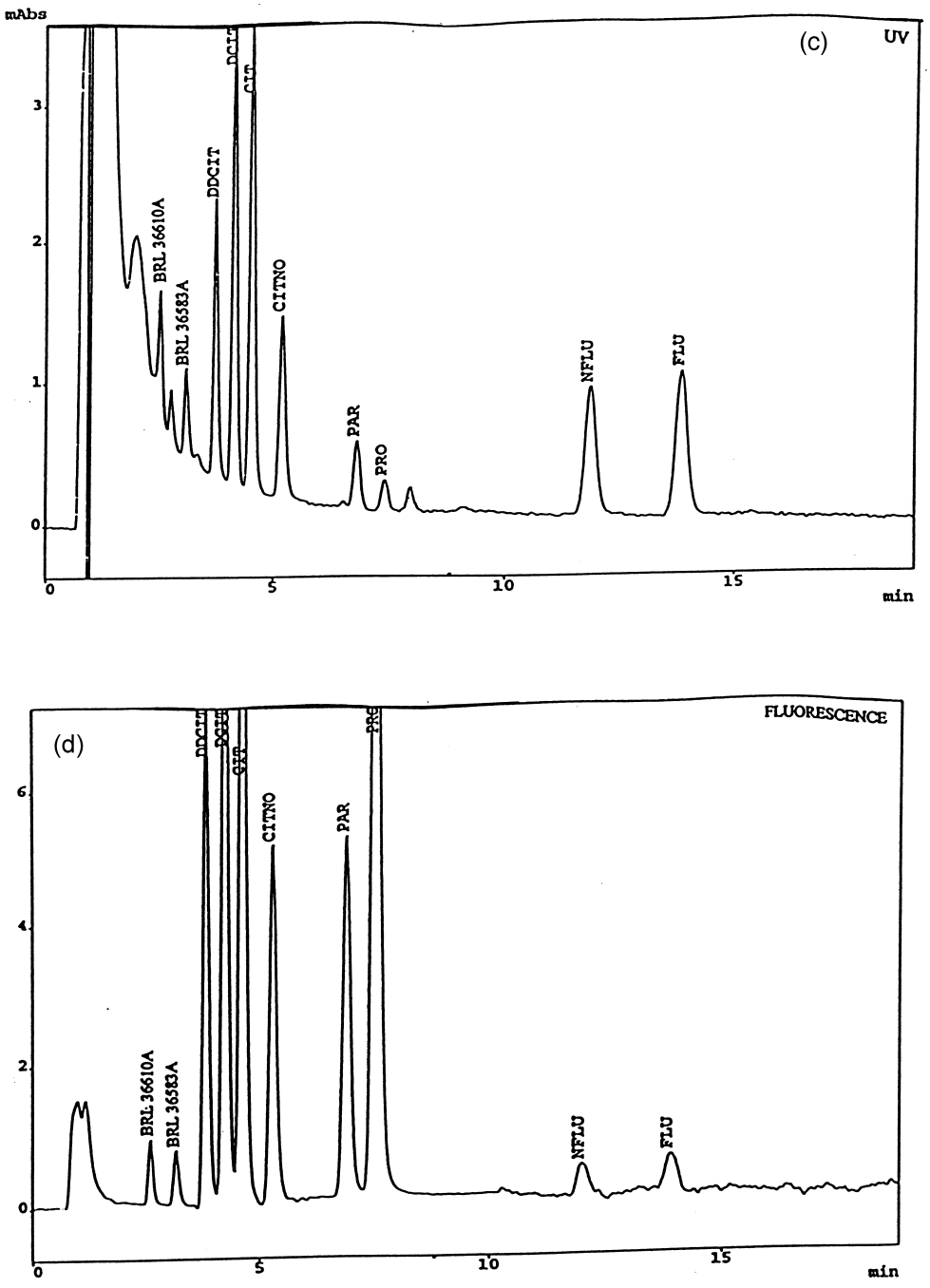


Fig. 2. (*continued*)

retention order, and a retention time of maximum 25 min.

In the CCC 11 experiments were performed in which the relative amount of organic solvent was varied from 23% to 37% and the pH from 3.4 to 4.6 (Table 2). A summary of the statistical evaluation of

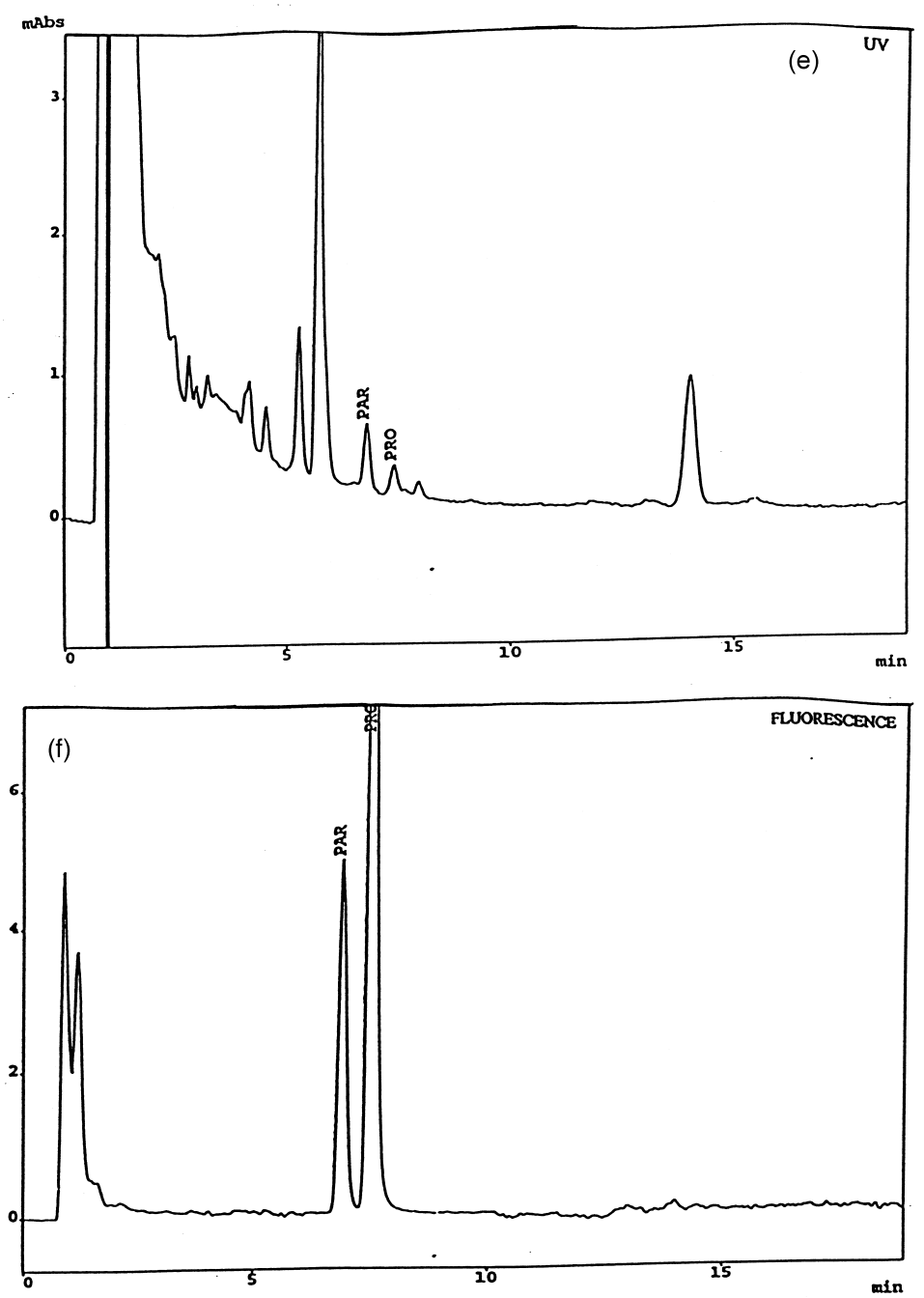


Fig. 2. (*continued*)

the model is given in Tables 3 and 4. The MLR regression analysis gave an acceptable summary of fit $R^2 \geq 0.936$ and $Q^2 \geq 0.741$. All models were significant ($P_{reg} \leq 0.001$).

As expected, a decrease of the relative amount of acetonitrile in the mobile phase increased the resolution and retention time of all the analytes except the resolution between PAR and PRO. The resolution

Table 3
Model equations for the resolution of the drugs based on scaled and centered coefficients[a]

Drug	Equation	R^2	Q^2	P_{reg}	$P_{lack\ of\ fit}$
PAR–PRO	$1.97-0.010x_1-0.030x_2+0.009x_1^2-0.29x_2^2$	0.944	0.741	<0.001	0.017
BRL 36610A–BRL 36583A	$2.46+0.052x_1-1.98x_2+0.012x_1^2+0.76x_2^2$	0.998	0.991	<0.001	0.22
DCIT–CIT	$1.51+0.071x_1-0.63x_2+0.018x_1^2+0.009x_2^2$	0.994	0.977	<0.001	0.35
DDCIT–DCIT	$1.70+0.058x_1-0.77x_2+0.009x_1^2+0.045x_2^2$	0.995	0.978	<0.001	0.39
CIT–CITNO	$2.47-0.36x_1-1.43x_2-0.19x_1^2+0.21x_2^2$	0.991	0.972	<0.001	0.43
NFLU–FLU	$3.96+0.028x_1-0.52x_2+0.046x_1^2-0.25x_2^2$	0.936	0.747	0.001	0.059

[a] The fraction of variation of the response explained by the model (R^2), the fraction of variation of the response predicted by the model (Q^2) and the *P*-values for regression and lack of fit obtained in the ANOVA for the second-order models are also shown. All the models were significant ($\alpha=0.05$). x_1: pH, x_2: relative amount of acetonitrile in the mobile phase.

between PAR and PRO was only influenced by the quadratic term of relative amount of acetonitrile in the mobile phase, and show a maximum ridge which passes through the experimental domain, Fig. 3. Furthermore, the resolution between CIT and CITNO was dependent of the linear and quadratic terms of pH and relative amount of acetonitrile in the mobile phase. As seen from the response surface, Fig. 3, the reduction in resolution was most pronounced at high pH, independently of the amount of acetonitrile. In addition, the retention of CIT was dependent of pH, while the retention of CITNO was not affected by a pH change. This may account for the decrease in resolution between CIT and CITNO at higher pH, and can be explained by the fact that the amount of protonated CIT decreases by increasing pH and in reversed-phase chromatography this is a known effect which prolongs the retention time of basic analytes.

No alteration in retention order was observed for the analytes studied. However, a metabolite of CIT, CITPA, showed alteration in elution order with regard to FLU. The alteration, i.e., elution of CITPA prior to FLU, was only seen at conditions which deviated from the specifications. CITPA was not extracted under the validated SPE conditions (Table 1).

The CCC was used to determine the method specifications which would ensure satisfactory performance of the HPLC separation. The results revealed that the HPLC method was robust with regard to resolution ($R_s \geq 1.4$), retention time (<23 min) and consistency in retention order when the relative amount of acetonitrile in the mobile phase is kept in the range 29–31% and the pH in the range 3.7–4.4.

4.2. Choice of internal standard

PRO was chosen as internal standard because this drug showed no interference with endogenous compounds in whole blood and plasma samples and was baseline separated from the other analytes. Furthermore, PRO is not used therapeutically in our country and problems caused by co-medication are thus not of major concern. For the determination of CIT, PAR and their metabolites with fluorescence detection, an analog of CIT, CITCl, can also be used as internal standard. In this work PRO was used since this

Table 4
Model equations for the retention time of the drugs based on scaled and centered coefficients[a]

Drug	Equation	R^2	Q^2	P_{reg}	$P_{lack\ of\ fit}$
CIT	$4.9+0.17x_1-3.7x_2-0.006x_1^2+1.6x_2^2$	0.998	0.992	<0.001	0.15
CITNO	$5.6+0.082x_1-4.6x_2-0.073x_1^2+2.0x_2^2$	0.997	0.988	<0.001	0.16
PAR	$7.3+0.30x_1-7.25x_2-0.020x_1^2+3.4x_2^2$	0.996	0.984	<0.001	0.13
PRO	$8.1+0.30x_1-7.7x_2-0.026x_1^2+3.5x_2^2$	0.997	0.988	<0.001	0.11
FLU	$14.7+0.63x_1-17.5x_2-0.10x_1^2+8.5x_2^2$	0.995	0.981	<0.001	0.079
NFLU	$12.6+0.52x_1-14.9x_2-0.066x_1^2+7.2x_2^2$	0.996	0.981	<0.001	0.089
CITPA	$18.0-1.7x_1-15.9x_2-0.84x_1^2+6.8x_2^2$	0.994	0.976	<0.001	0.047

[a] See text to Table 3.

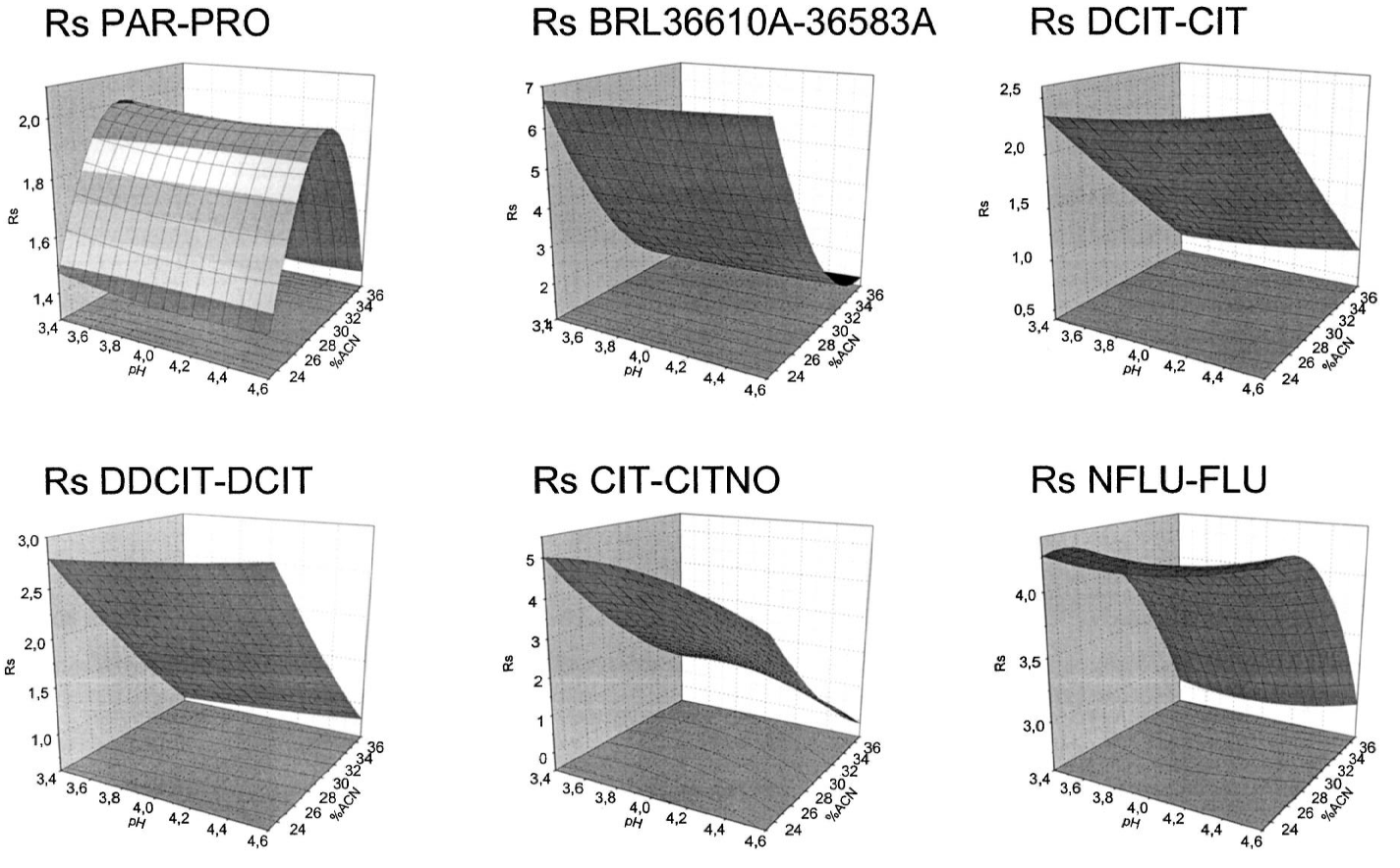


Fig. 3. Response surface of the resolution between adjacent peaks in the HPLC separation obtained by the central composite design circumscribed.

internal standard could be used both with UV and fluorescence detection.

4.3. Solid-phase extraction

FLU, CIT and PAR are highly protein-bound. To obtain the desired interaction between analytes and sorbent, the analytes must be in an unbound form. Low flow-rates and dilution of the sample are reported to increase the concentration of unbound drug available for sorbent interaction. Biological samples are generally viscous and dilution will also ease their passage through the sorbent bed. When a large volume of aqueous sample is loaded, the silica based material may no longer be wetted and a reduction in recovery and poor reproducibility might be observed. The addition of methanol to the sample before SPE analysis may help to maintain the equilibrium between the stationary and mobile phases and hence ensure a wetted sorbent. In the SPE procedure non-endcapped C_8 columns were used. The interaction between the basic analytes and residual silanol groups of the sorbent was enhanced at pH 7.0. Elution was achieved with acidified methanol, thus suppressing the secondary interactions. The SPE procedure is listed in Table 1.

Low recovery of the CIT metabolites DDCIT and CITNO was observed when the SPE conditions were as specified in Table 1. The recovery of these metabolites could easily be increased by reducing the amount of acetonitrile in the washing solution to 30%. Unfortunately, at these conditions interfering peaks in the UV chromatograms were observed.

CITPA was not extracted at the validated SPE conditions (Table 1). Preliminary screening design experiments used in the optimization of the method showed that the extraction recovery of the compounds was dependent on the pH of the washing buffer. When reducing the pH to 3.0 and using 30% acetonitrile in the washing solution, the recovery of CITPA was approximately 85% (results not shown). The marked increase in the recovery of CITPA may be due to increased hydrophobic interactions and possibly also enhancement of polar interaction between the protonated analyte and the protonated silanol groups. However, the recovery of the basic analytes decreased at these acidic conditions. These observations indicate that the secondary interactions between the ionized silanol groups and the ionized basic analytes are important for the retention and elution of the analytes. Nevertheless, the recovery of CIT, its metabolites DCIT, DDCIT, CITNO and CITPA and an analog of CIT, CITCl, was $\geq$70% at these SPE conditions. This SPE procedure was only valid when employed with fluorescence detection. Due to reduced recovery and interferences in the UV chromatograms at these conditions, the pH and relative amount of acetonitrile in the washing steps of the final SPE method were set to 7.0 and 50%, respectively.

In order to investigate the robustness of the sample clean-up, the SPE procedure was tested at modest deviations from the conditions specified in the method (Table 1). A fractional factorial design 2^{a-5} III was performed (Table 5). The factor levels that were satisfactory in the preliminary screening design

Table 5
The factor levels used in the fractional factorial design, 2^{a-5} III, for the robustness testing of the SPE procedure

Factor	Abbreviation	Units	−1	+1	0
pH of dilution buffer	(A)	pH	6.5	7.5	7.0
% Methanol in the dilution buffer	(B)	% methanol	8	12	10
Application rate	(C)	ml/min	1	2	1.5
Elution rate	(D)	ml/min	1	2	1.5
Wash solution composition[a]	(E)	% acetonitrile	45	55	50
Elution volume	(F)	ml	0.65	0.85	0.75
Elution solution composition	(G)	% TFA[b]	1	3	2
SPE column	(H)		Batch 1	Batch 2	Batch 1
Drying[c]	(I)	s	0	30	0

[a] This level was set to 35 (−1), 40 (0) and 45 (+1) in the robustness experiment for the plasma samples.
[b] TFA = Trifluoroacetic acid.
[c] The drying was performed before the elution.

experiments were set as center point values in the robustness experiment. The response was the recovery of CIT, FLU, PAR, PRO and CITCl related to the average recovery obtained for the center points.

The fractional factorial design showed two significant effects ($\alpha = 0.05$), i.e., elution solution composition (G) and drying (I), for PAR in whole blood. With respect to CIT, FLU, PRO and CITCl the robustness testing showed no significant main effects in either whole blood or plasma. The descriptive statistics for the experimental design showed minimal variation in the response for all the analytes, with relative standard deviation (RSD) ≤ 5.6. The effects of the factors, for all the compounds of interest, were less than or equal to 6% of the average response (data not shown). Hence, the SPE method showed satisfactory robustness to small (and inevitable) changes in the procedure.

4.4. Method validation

Since the method was intended for both plasma and whole blood samples, we chose to develop a method based on HPLC calibration in combination with control samples of the appropriate type carried through the whole procedure. The HPLC calibration curves were found to be linear over the investigated range with coefficients of correlation (r^2) > 0.999. The HPLC calibration curves combined with recovery factors were used for quantification. In addition, we sought a method that could be used for therapeutic drug monitoring of SSRIs as well as forensic analysis after SSRI overdoses. Hence, the method was validated for a wide concentration range, 0.050–5.0 μmol/l, in whole blood and plasma. The validated concentration range exceeds those reported previously [19,20,24].

The recovery correction results in increased standard deviation since both the recovery factor and the individual measurements are subjected to random errors. However, in our method the results are given as the average of two sample replicates, and hence the precision is comparable to the uncorrected results [28]. Control samples, carried through the whole procedure, is used to control the recovery factor.

The limit of quantification (LOQ) was determined using a signal to noise ratio of 10:1. The LOQ was 0.025 μmol/l for CIT and PAR, 0.050 μmol/l for DCIT, DDCIT and CITNO, and 0.12 μmol/l for BRL 36610A and BRL 36583A with fluorescence detection. For FLU and NFLU the LOQ was 0.10 μmol/l using UV detection. Thus, the method seems suitable for the therapeutic drug monitoring of the three SSRIs [14,15].

Recovery of PAR, CIT and FLU were in the range 63–114%, and in the 38–95% range for the metabolites (Tables 6 and 7). The calibration curve was forced through origo since this gave a more consistent recovery over the entire concentration range. As can be seen from the results, the recoveries of some of the analytes differed between plasma and whole blood. The recovery correction saves time and extra work since otherwise two calibration curves would have been necessary; one for plasma and one for whole blood.

The within- and between-day precision is shown in Tables 6–8. The between-day precision was estimated by performing the same determination during a period of one month by the same analyst. The validation was performed with two RapidTrace SPE Workstation modules and different batches of SPE columns, plasma and whole blood. RSD for the within-day and between-day precision were in the range 1.4 to 10.6% and 3.1 to 20.3%, respectively. The between-day precision was satisfactory at higher concentrations. At the 0.050 μmol/l and 0.12 μmol/l levels a comparatively high RSD was observed for most of the analytes, which can be expected as these concentrations were near the LOQ.

The selectivity of the HPLC determination was evaluated by injecting quinine, venlafaxine, clozapine, mianserin, doxepin, imipramine, alimemazine, nortriptyline, clonazepam, verapamil, fluvoxamine, amitriptyline, levomepromazine, clomipramine and nefazodone. No interference was observed from any of these compounds.

4.5. Application

A single RapidTrace SPE Workstation module can process up to ten samples sequentially. Each workstation can include up to 10 modules for a total

Table 6
Within-day precision, recovery and recovery factors (RFs) for the determination of PAR, CIT, FLU and their metabolites in human whole blood[a]

Added concentration (μmol/l)	Uncorrected concentration[b] (μmol/l)	RSD (%)	RF	Corrected concentration (μmol/l)	Recovery[c] (%)	RSD (%)
CIT						
0.050	0.074±0.0031	4.1	0.699	0.052	111±5	4.2
0.50	0.78±0.016	2.0	0.699	0.54	109±3	2.5
5.0	7.1±0.15	2.1	0.699	5.0	101±1	1.2
DCIT						
0.050	0.061±0.0033	5.4	0.861	0.052	89±5	5.7
0.50	0.59±0.019	3.2	0.861	0.50	82±3	3.6
5.0	5.4±0.13	2.3	0.861	4.6	77±1	1.8
DDCIT						
0.050	0.038±0.0034	8.8	1.435	0.055	54±4	7.8
0.50	0.31±0.024	7.7	1.435	0.44	43±3	7.2
5.0	2.62±0.078	3.0	1.435	3.8	38±1	3.6
CITNO						
0.050	0.033±0.0019	5.7	1.559	0.052	50±2	4.7
0.50	0.309±0.0044	1.4	1.559	0.48	43±1	2.1
5.0	2.75±0.053	1.9	1.559	4.3	39±0.6	1.6
PAR						
0.05	0.060±0.0034	5.7		n.c.[d]	71±5	7.0
0.5	0.46±0.034	7.5		n.c.	63±4	6.8
5.0	5.2±0.16	3.1		n.c.	74±2	2.1
BRL 36610A						
0.12	0.113±0.0053	4.7	1.338	0.15	59±3	5.5
0.50	0.35±0.010	3.0	1.338	0.47	54±2	3.2
5.0 a	3.2±0.11	3.3	1.338	4.3	55±2	3.6
BRL 36583A						
0.12	0.116±0.0034	3.0	1.381	0.16	67±3	4.1
0.50	0.37±0.019	5.0	1.381	0.51	59±3	4.6
5.0 b	3.4±0.19	5.6	1.381	4.7	58±3	5.5
FLU						
0.12	0.118±0.0050	4.2	0.956	0.11	87±4	4.1
0.50	0.61±0.019	3.1	0.956	0.59	85±2	2.3
5.0	5.5±0.30	5.5	0.956	5.3	80±0.7	0.8
NFLU						
0.12	0.108±0.0078	7.2		n.c.	75±4	5.4
0.50	0.51±0.024	4.6		n.c.	70±4	5.3
5.0	4.2±0.21	5.0		n.c.	62±0.7	1.2

[a] The corrected concentration is obtained by multiplying the uncorrected concentration with the recovery factor.
[b] Mean±SD, $n=6$, a: $n=5$, b: $n=4$.
[c] The curve was forced through origo.
[d] n.c.=No correction.

Table 7
Within-day precision, recovery and recovery factors (RFs) for the determination of PAR, CIT, FLU and their metabolites in human plasma[a]

Added concentration (μmol/l)	Uncorrected concentration[b] (μmol/l)	RSD (%)	RF	Corrected concentration (μmol/l)	Recovery[c] (%)	RSD (%)
CIT						
0.050	0.087±0.0030	3.4	0.724	0.063	114±4	3.8
0.50	0.70±0.014	1.9	0.724	0.51	111±2	2.2
5.0	6.7±0.54	8.1	0.724	4.9	109±4	3.6
DCIT						
0.050	0.079±0.0022	2.8	0.834	0.066	95±3	3.3
0.50	0.60±0.019	3.2	0.834	0.50	93±3	2.9
5.0	5.6±0.47	8.5	0.834	4.6	90±5	5.5
DDCIT						
0.050	0.060±0.0048	8.0	1.282	0.076	63±8	11.9
0.50	0.36±0.030	8.3	1.282	0.46	54±4	8.1
5.0	3.0±0.31	10.6	1.282	3.8	48±5	11.0
CITNO						
0.050	0.057±0.0057	9.9	1.301	0.075	61±9	14.5
0.50	0.36±0.020	5.4	1.301	0.47	55±3	5.6
5.0	3.2±0.27	8.4	1.301	4.21	52±3	6.3
PAR						
0.050	0.087±0.0021	2.4	0.829	0.072	90±3	3.7
0.50	0.57±0.015	2.7	0.829	0.47	87±2	2.6
5.0	5.9±0.46	7.9	0.829	4.9	95±3	2.8
BRL 36610A						
0.12	0.16±0.099	6.1		n.c.[d]	67±6	9.6
0.50	0.47±0.024	5.1		n.c.	66±4	5.6
5.0	3.8±0.33	8.7		n.c.	62±4	6.8
BRL 36583A						
0.12	0.132±0.0058	4.4		n.c.	63±4	6.0
0.5	0.44±0.018	4.1		n.c.	66±3	4.3
5.0	4.2±0.35	8.4		n.c.	67±4	6.5
FLU						
0.12	0.139±0.0035	2.5	0.910	0.13	97±4	4.0
0.50	0.60±0.014	2.3	0.910	0.55	92±2	1.7
5.0	5.9±0.6	10.2	0.910	5.4	93±3	2.9
NFLU						
0.12	0.133±0.0077	5.8	0.949	0.13	90±2	2.2
0.50	0.59±0.015	2.5	0.949	0.56	89±1	1.5
5.0	5.6±0.58	10.3	0.949	5.4	89±3	3.6

[a] The corrected concentration is obtained by multiplying the uncorrected concentration with the recovery factor.
[b] Mean±SD, $n=6$.
[c] The curve was forced through origo.
[d] n.c.=No correction.

Table 8
Between-day precision for the determination of PAR, CIT, FLU and their metabolites in human plasma and whole blood[a]

Added concentration (μmol/l)	Whole blood a[b]			Plasma e[c]		
	Uncorrected concentration (μmol/l)	RSD (%)	Corrected concentration (μmol/l)	Uncorrected concentration (μmol/l)	RSD (%)	Corrected concentration (μmol/l)
CIT						
0.050	0.079±0.0089	11.3	0.055	0.076±0.0093	12.2	0.055
0.50	0.72±0.043	6.0	0.50	0.69±0.051	7.5	0.50
5.0	6.6±0.39	5.9	4.6	6.5±0.47	7.3	4.7
DCIT						
0.050	0.067±0.010	15.1	0.057	0.068±0.0088	12.9	0.056
0.50	0.58±0.037	6.4	0.49	0.59±0.053	8.9	0.50
5.0	5.3±0.30	5.6	4.6	5.6±0.44	7.9	4.6
DDCIT						
0.050	0.046±0.0088	18.9	0.067	0.049±0.0081	16.7	0.063
0.50	0.35±0.038	10.9	0.50	0.39±0.054	13.8	0.50
5.0	2.9±0.24	8.2	4.2	3.4±0.45	13.3	4.3
CITNO						
0.050	0.039±0.0069	17.6	0.061	0.045±0.0075	16.7	0.059
0.50	0.32±0.025	7.8	0.50	0.38±0.047	12.5	0.49
5.0	2.8±0.23	8.3	4.4	3.6±0.35	9.8	4.6
PAR						
0.050	0.064±0.0089	14.0	n.c.[d]	0.070±0.010	14.4	0.058
0.50	0.49±0.032	6.5	n.c.	0.57±0.034	5.9	0.47
5.0	4.9±0.33	6.9	n.c.	5.7±0.33	5.7	4.7
BRL 36610A b						
0.12	0.12±0.014	12.2	0.15	0.14±0.021	14.9	n.c.
0.50	0.36±0.031	8.6	0.48	0.49±0.064	13.1	n.c.
5.0	3.2±0.23	7.2	4.34	4.0±0.55	13.7	n.c.
BRL 36583A c, f						
0.12	0.10±0.012	11.6	0.14	0.13±0.015	11.9	n.c.
0.50	0.35±0.051	14.4	0.49	0.48±0.053	11.1	n.c.
5.0	3.3±0.35	10.6	4.6	4.4±0.49	11.3	n.c.
FLU						
0.12 d, g	0.11±0.021	18.3	0.11	0.12±0.014	11.8	0.11
0.50 d, g	0.58±0.039	6.8	0.55	0.58±0.034	5.8	0.53
5.0 h	5.5±0.43	7.7	5.3	5.8±0.18	3.1	5.2
NFLU						
0.12 d, g	0.10±0.019	20.3	n.c.	0.12±0.017	14.1	0.12
0.50 d, g	0.49±0.025	5.1	n.c.	0.55±0.031	5.6	0.52
5.0 g	4.3±0.37	8.6	n.c.	5.4±0.20	3.8	5.2

[a] The corrected concentration is obtained by multiplying the uncorrected concentration with the recovery factor. The recovery factors are listed in Tables 6 and 7.

[b] Whole blood: a (mean±SD, $n=9$), b: $n=7$, c: $n=6$, d: $n=10$.

[c] Plasma: e (mean±SD, $n=12$), f: $n=11$, g: $n=15$, h: $n=15$.

[d] n.c.=No correction.

sample capacity of 100 samples. Twenty minutes were required to complete one SPE, and up to 30 samples could be processed in 1 h when using 10 modules. Hence the method is rapid and suitable for automated routine analysis.

The method has been used for the analysis of whole blood and plasma samples from PAR-exposed patients and forensic cases. Fig. 2e and f show chromatograms from an autopsy sample where 0.50 μmol/l PAR was measured in whole blood. The ingested PAR dose was unknown. It must be emphasised that the death was not related to PAR ingestion. In addition, we have used the assay to measure PAR in plasma and whole blood samples submitted for therapeutic monitoring of patients. The observed PAR concentration range was 0.048–0.25 μmol/l in plasma and whole blood, the metabolites BRL 36610A and BRL 36583A were not detected. These data were in agreement with those reported in the literature [14]. The results suggest that the method is well suited for use in both forensic and clinical settings.

5. Conclusion

This method allows automated and simultaneous quantification of CIT, DCIT, DDCIT, CITNO, PAR, BRL 36610A, BRL 36583A, FLU and NFLU over a wide concentration range in whole blood and plasma samples. Response surface and fractional factorial design were successfully used to optimize and test the robustness of the HPLC and SPE procedures. In addition, the combination of two detectors makes possible the screening of samples for a series of drugs, as well as selective detection of a few of these. Published methods have mainly been developed for therapeutic drug monitoring of single compounds of the SSRI class in either plasma, serum, urine or tissue samples. The present method is suitable for routine drug monitoring at therapeutic and overdose concentrations in plasma and whole blood samples and therefore may have a greater area of application.

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