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On-line extraction using an alkyl-diol silica precolumn for racemic citalopram and its metabolites in plasma Results compared with solid-phase extraction methodology

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Abstract

Sample preparation is usually the most critical and time consuming step when using HPLC for drug analysis in biological matrixes. Sample extracts have to be clean considering both chromatographic interferences and column maintenance. To meet some of these criteria a fully automated on-line extraction (OLE) analysis method was developed for the antidepressant drug citalopram and its two demethylated metabolites, using an RP-C4-ADS extraction column. A comparison between the new OLE method and an off-line solid-phase extraction method showed that the two methodologies were equal in analytical precision but that the OLE method was faster and therefore superior in sample capacity per day.

Keywords: Citalopram; On-line extraction; Column switching; Restricted access material

1. Introduction

In this study citalopram (1-[3-(dimethylamino)propyl] - 1 - (*p* - flourophenyl) - 1,3 - dihydro-5-isobenzofurancarbonitrile) (CT) was analysed. CT is one of the most frequently used antidepressant drugs. It is a selective serotonin reuptake inhibitor (SSRI) which potentiates serotonergic neurotransmission. CT appears to be effective in depression, anxiety, obsessional and control disorders [1]. CT is a racemic mixture of two enantiomers (*R* and *S*) with different pharmacological effects, *S*-citalopram being the more potent enantiomer. There are stereoselective chromatographic methods for the analysis of CT available [2]. The metabolism of CT is relatively well documented. In plasma non-metabolised CT dominates but the metabolites, which also have pharmacological effects but are less potent, occur as well [3]. The enzymatic systems in the first demethylation step are the cytochrome P450 (CYP) subsystems 3A4, 2C19 and 2D6 while the second demethylation is exclusively mediated by CYP2D6 [4,5] which may be of importance since approximately 7% of the Caucasian population are referred to as slow CYP2D6 metabolisers [6]. Taking into account all possible causes for variation in drug concentrations; drug–drug interactions, non-compliance and metabolic deviations etc. there is a need for a Therapeutic Drug Monitoring (TDM) routine.

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Routine TDM methodology has to fulfil the criteria of being cost effective, easily conducted in a routine clinical laboratory setting and robust in bioanalytical performance but still of high quality in both identification and quantification.

Usually the most time consuming and critical step in a drug analysis in biological matrixes is the sample preparation. Sample extracts have to be clean considering both possible interferences in the actual chromatography and column maintenance. Injection of unclean samples e.g. protein-containing extracts, will indisputably shorten the column life due to protein precipitation on the column frits and packing material, resulting in decrementing chromatographic performance [7].

The most widely used sample preparation techniques are solid-phase extraction (SPE) and liquid–liquid extraction (LLE). To get the sample preparation procedure automated, on-line extraction (OLE) is a used technique: However when this is done with a regular reversed-phase packing material the lifetime of the extraction precolumn is very limited (a total volume of ~6 ml plasma injected) due to protein precipitation causing increased back pressure [8].

The last decade a variety of new on-line extraction columns, among them the reversed-phase alkyl-diol silica (RP-ADS) have been introduced. Terms associated with the packing material in these columns are ''internal phase'', ''internal surface'', ''shielded'', ''semipermeable'', ''mixed functional'' and ''mixed mode columns'' [9]. The columns are also referred to as RAM-columns, (restricted access materials) in which the packing material in most types has a hydrophilic outer surface and a hydrophobic inner surface. During injection of untreated biological samples, (hemolysed blood, plasma, serum, cell culture and tissue homogenates) the packing material excludes the macro molecules from the inner hydrophobic surface by size exclusion. The matrix is washed out with the loading mobile phase while smaller hydrophobic molecules are extracted by the hydrophobic retentive capability of the inner surface. These RAM columns have a longer lifetime, (~50 ml plasma) than their conventional reversed-phase counterpart [10,11].

It has not been shown that RAM on-line extraction gives more clean sample extracts than SPE, LLE or OLE with regular reversed-phase packing material, but it is still, in terms of cost efficiency, an attractive alternative as it often can be handled as a fully automated and HPLC integrated sample preparation with long life time (total injection volume) [12–17].

The aim of this study was to develop a fully automated, HPLC integrated, sample preparation and analysis of citalopram (CT) and two of its metabolites, desmethylcitalopram (DCT) and didesmethylcitalopram (DDCT). The method developed was then to be adopted on samples from patients treated with CT and compared with an already established analysis methodology using off-line solid-phase extraction and HPLC [18].

2. Experimental

2.1. Chemicals

Citalopram (Lu 10-171 B), desmethylcitalopram (Lu 11-109 C), didesmethylcitalopram (Lu 11-161-X) and Internal Standard (IS) (Lu 10-202-O) were kindly supplied by H. Lundbeck (Copenhagen, Denmark), disodium hydrogenphosphate from Merck KGaA (Darmstadt, Germany), potassium dihydrogenphosphate from Fluka (Buchs, Switzerland), HPLC-grade acetonitrile and methanol from LabScan Analytical Sciences (Dublin, Ireland). Water was obtained from a Milli-Q station, Millipore AB (Stockholm, Sweden). Human plasma for preparing spiked samples was delivered from the blood bank of Linköping University Hospital, (Linköping, Sweden).

2.2. Instrumentation

The instrumentation used was a Varian 2510 HPLC pump (Varian, Harbour City, California, USA), a Gynkotek M300 HPLC pump (Dionex, Sunnyvally, USA). An Aspec XL was used as autosampler and switching valves module (Gilson, Middleton Wisconsin, USA). The detection was performed with a Waters 474 Scanning fluorescence detector (Waters Corporation, Milford USA). The columns in the analytical system was a NovaPak Phenyl Guard column and NovaPak Phenyl 100×8 mm analytical column with a particle size of 4 μm

(Waters Corporation, Milford USA). The on-line extraction was performed on a 4×25 mm LiChrospher RP-ADS column with an inner surface covered with either C_4, C_8 or C_{18} chains (Merck KgaA, Darmstadt, Germany). The chromatograms were recorded and processed with EZ Chrom chromatography data system Version 6.5 (Scientific Software, Pleasanton, USA).

2.3. Statistics

Kolmogorov Smirnovs test for normal distribution, Paired *t*-test, Wilcoxon signed rank test and regression tests were computed using StatView for Windows, version 5.0 (SAS Institute, Cary NC, USA).

The relative difference between the two analytical methods, (the concentration of the SPE method minus the concentration of the OLE method divided by the mean concentration of the two methods. (SPE [conc.]−OLE [conc.])/Mean [conc.]), the correlation diagrams and computations were made using Microsoft® Excel 97 (Microsoft Corp., USA)

2.4. Precolumn evaluation

Three different RP-ADS columns were tested during the development of this application. They differed in length of the carbon chain (C_4, C_8 and C_{18}) covering the inner pore surface of the packing material.

In order to establish the volume needed for matrix washout, the extraction column was connected directly to a UV detector measuring at a short UV wavelength (210 nm), which would detect most proteins. A plasma injection was then made and the time (volume) from injection to stable baseline was measured.

The recovery of the analytes was evaluated by measuring the peak height of CT, DCT and DDCT spiked in plasma ($n=3$) on each RP-ADS column and comparing it with the peak heights of a water solution with the same amount of analytes injected directly ($n=3$) without the extraction procedure.

2.5. Preparation of calibrators and controls

A stock solution containing CT, DCT and DDCT in the concentration 10, 5 and 1 μg/ml respectively, was made in Milli-Q water. Calibration curves in plasma with five individual concentrations of the analytes were made. The concentration intervals were CT 20–200 ng/ml, DCT 10–100 ng/ml and DDCT 2–20 ng/ml.

Two control samples were prepared. The first control was composed of CT, DCT and DDCT at low concentrations of 45, 22.5 and 4.5 ng/ml, respectively. The second control was composed of CT, DCT and DDCT at high concentrations of 180, 90 and 18 ng/ml respectively. A 500 μl aliquot of a 500 ng/ml internal standard was added to 5 ml volumes of each calibration standard and the two control samples. The solutions were mixed by vortex and then transferred into 1.1 ml aliquots in glass tubes fitting the Aspec XL sample tray.

2.6. The analytical cycle

The Aspec XL aspirates 220 μl sample mixture and 780 μl MQ water and delivers these volumes to the injection loop. Valve A is then switched and Pump 1 delivers the sample mixture to the extraction column. (see Fig. 1). CT, DCT and DDCT are then

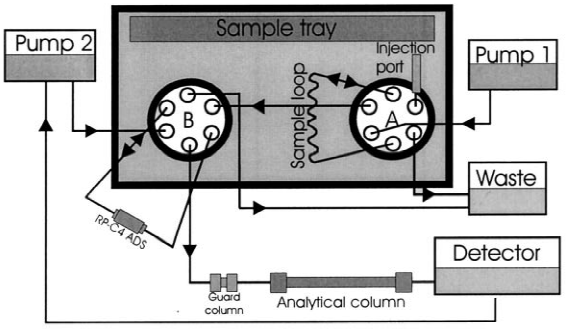


Fig. 1. The HPLC integrated sample preparation configuration and chromatographic conditions. Pump 1: Varian 2510 HPLC pump delivering the sample preparation mobile phase (MQ-water) Flow 1 ml/min. Pump 2: Gynkotek M300 HPLC pump delivering the analytical mobile phase (30% Acetonitrile/0.07 *M* phosphate buffer pH 4.5). Flow 3 ml/min. Sample tray: Aspec XL. Valve A: 6 port valve on Aspec XL for sample loading. Valve B: 6 port valve on Aspec XL for switching between sample preparation system and analytical system. Sample loop: 1000 μl. RP-C4 ADS: Licrosphere RP-C8 ADS on-line extraction column. Analytical column: NovaPak Phenyl 100×8 mm. Guard column: NovaPak Phenyl. Detector: Fluorescence detector excitation wavelength 235 nm, emission wavelength 290 nm.

extracted from the plasma by the RP-ADS column. A clean sample extract is achieved by washing the ADS-column with MQ-water for 5 min (5 ml). Valve B is then switched and Pump 2 is employed for elution of the analytes from the extraction column and transport of these analytes to the analytical column for separation.

During the analytical separation the RP-C4 ADS is washed with 1 ml of acetic acid in methanol (2:98, v:v) and reconditioned with MQ-water for 1.5 min. The system then starts with the next sample which allows the extraction to proceed during the analytical separation of the previous sample. (Table 1, Fig. 1)

2.7. Validation experiments

Day 1, three calibration curves were produced and analysed by the on-line extraction method. After each calibration curve a blank plasma sample and a high and a low control were analysed. Day 2, two new calibration curves were analysed, the second was injected twice and presented as a mean value of the duplicate injections.

2.8. Patient sample analysis

Blood samples were obtained from patients ($n=$ 13) on chronic oral treatment with CT for major depressive disorder. Samples were drawn as trough levels, i.e. immediately before administration of the next dose and the levels were considered to be obtained under therapeutic steady state conditions for the clinical comparison.

Blood sampling for drug analysis was made by cubital vein puncture and the blood was collected in glass tubes with heparin additive. The samples were centrifuged (~3000 rpm) and the plasma supernatant was transferred to another tube, frozen and kept at −20°C until analysis.

The patient samples, the control samples and the blank plasma were first analysed by the solid-phase extraction method [18] and then by our on-line extraction method. The volume of the patient plasma samples did not admit more than single injections in the on-line extraction method but in the solid-phase extraction the samples were analysed in duplicates and the results were presented as mean values of the two injections. The results for patient samples and controls were in the latter case calculated from the last, double injected calibration curve from the validation experiment.

3. Results

3.1. Precolumn evaluation

There were no differences in recovery for the three columns. However, the lowest volume needed (5 ml) for the washout of plasma was obtained with RP-C4-ADS. No interfering peaks were detected in the chromatogram after injection of blank plasma and the chromatograms from a patient plasma sample showed good retention time match with a spiked plasma sample e.g. calibrators and controls. (Fig. 2a–c).

Consequently the on-line extraction column used in this application was RP-C4-ADS.

3.2. Validation results

The recovery was 90% for CT and DCT and 85% for DDCT. The calibration curves showed excellent linearity. r^2 was >0.99 for all five calibration curves

Table 1
Schedule for the analytical cycle

Time (min)	Precolumn	Analytical column
0.0–5.0	Injection and preparation	(re)conditioning
5.0–6.5	Substances being transferred	Substances being transferred and separation starts
6.5–12	Wash and reconditioning	Separation

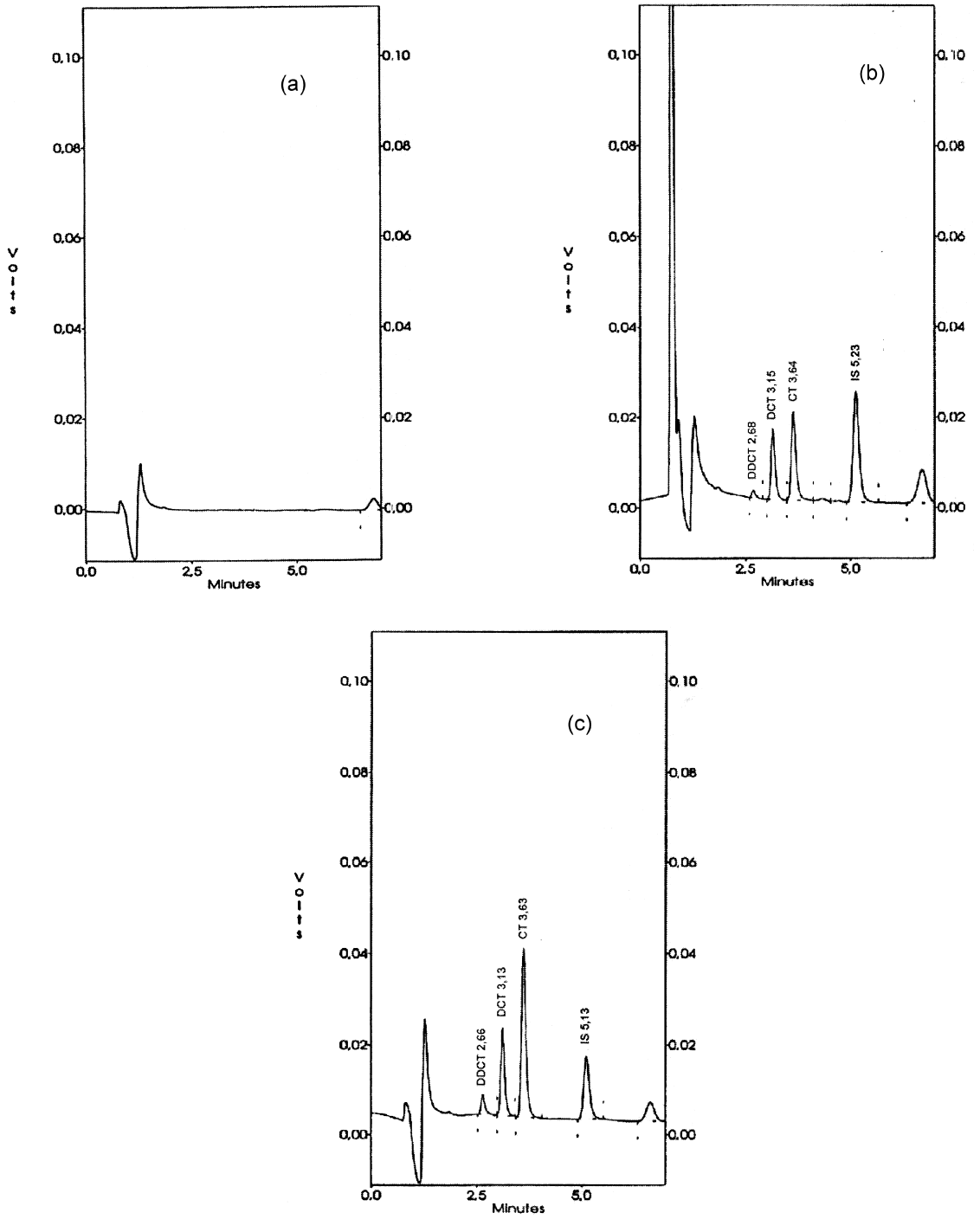


Fig. 2. (a) Chromatogram of blank plasma after on-line extraction by a RP-C4-ADS column, fluorescence detection (excitation at 235 nm and emission at 290 nm). (b) Chromatogram of a patient plasma sample after on-line extraction by a RP-C4-ADS column, fluorescence detection (excitation at 235 nm and emission at 290 nm) 1=DDCT, 2=DCT, 3=CT and 5=IS. (c) Chromatogram of a spiked plasma sample (calibrator 3) after on-line extraction by a RP-C4-ADS column, fluorescence detection (excitation at 235 nm and emission at 290 nm) 1=DDCT, 2=DCT, 3=CT and 4=IS.

for CT and DCT and for calibration curves 3, 4 and 5 for DDCT (Table 2).

Carryover between injections was eliminated by the extra precolumn washing step.

3.3. Comparison of patient samples concentration outcome

There was a good correlation between the results obtained with SPE and OLE (Fig. 3a–c). The concentration outcome was not normally distributed so the results obtained with the SPE methodology and our OLE methodology were compared with Wilcoxon signed rank test for non-parametric data. Plasma levels of CT, DCT and DDCT did not differ between the two analytical methodologies ($P=0.139$).

When the recovery of the three analytes was studied in detail the two metabolites had behaved differently in the two methods. A statistically significant correlation was found saying that at high concentrations of DCT the SPE method gave a higher recovery and that at low concentrations of DCT the OLE method gave a higher recovery. The opposite was found for DDCT (Fig. 4).

3.4. Comparison of the time consumption of the different analytical methods

The analytical time, and in particular the analyst participation was considerably lower in the OLE method. A sample set of 20 injections gave a total analysis time of 4 h and 15 min of which the analyst was participating during the first 15 min (centrifugation and adding internal standard). The SPE method which is run in batches of 20 samples per extraction gave an analysis time of 4.5 h of which the analyst participated approximately 2 h during the sample preparation.

Table 2
Results of the validation experiment[a]

	Conc. (ng/ml)	Day 1			Day 2		Std deviation	C.V. (%)	C.V. (1–2 excluded) (%)
		1 DDCT/IS	2 DDCT/IS	3 DDCT/IS	4 DDCT/IS	5 DDCT/IS duplicate inj.			
Cal 1	2	0.017	0.027	0.035	0.042	0.040	0.010	32	8
Cal 3	10	0.072	0.139	0.165	0.172	0.173	0.043	30	2
Cal 5	20	0.234	0.293	0.324	0.332	0.333	0.042	14	1
Blank									
K 1		0.046	0.068	0.072	0.072	0.072	0.011	17	0
K 2		0.222	0.272	0.293	0.307	0.302	0.034	12	2
Cal 1	10	0.187	0.190	0.179	0.198	0.185	0.007	4	
Cal 3	50	0.807	0.821	0.818	0.820	0.804	0.008	1	
Cal 5	100	1.632	1.574	1.561	1.557	1.537	0.036	2	
Blank									
K 1		0.351	0.358	0.366	0.353	0.346	0.008	2	
K 2		1.423	1.463	1.428	1.470	1.429	0.022	2	
Cal 1	20	0.354	0.354	0.341	0.379	0.358	0.014	4	
Cal 3	100	1.512	1.543	1.533	1.550	1.527	0.015	1	
Cal 5	200	3.049	2.943	2.898	2.918	2.899	0.063	2	
Blank									
K 1		0.621	0.626	0.655	0.638	0.630	0.013	2	
K 2		2.500	2.589	2.534	2.619	2.562	0.047	2	

[a] The figures are ratios of the peak height of the substance analysed and the peak height of the internal standard. Calibrator (Cal) for level 2 and 4 are excluded but the coefficient of variation, (C.V.) are in accordance with the other calibrator levels. K1=low control, K2=high control, DDCT=didesmethylcitalopram, DCT=desmethylcitalopram, CT=citalopram, I.S.=internal standard.

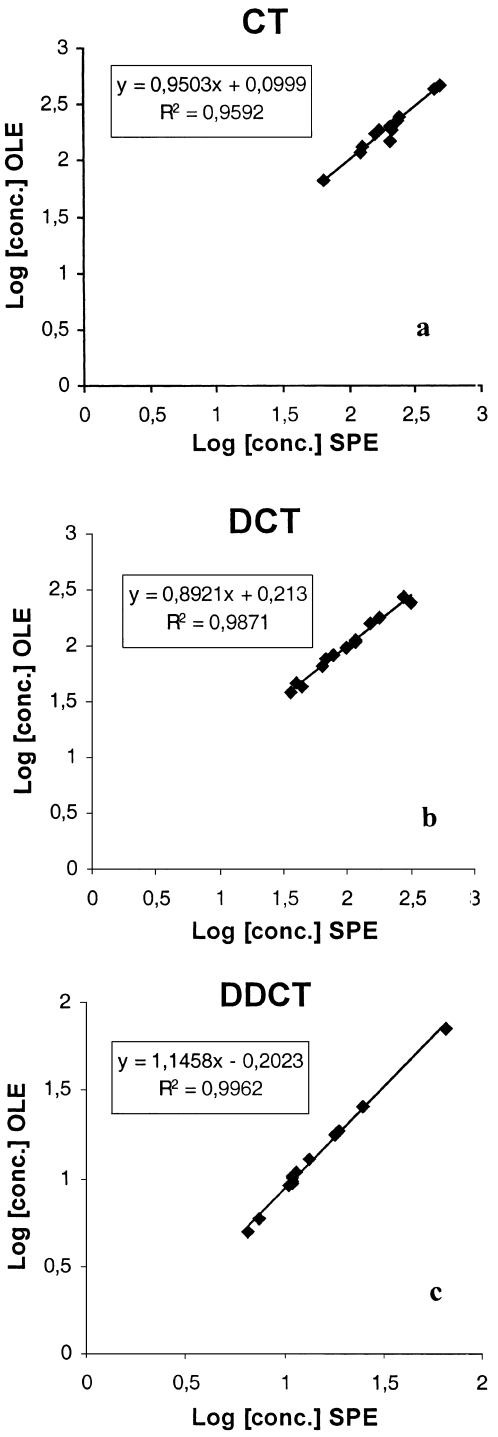


Fig. 3. The concentration outcome of the two methods for the patient samples (n=13). OLE=on-line extraction, SPE=solid-phase extraction, CT=citalopram, DCT=desmethylcitalopram, DDCT=didesmethylcitalopram.

4. Discussion

4.1. The RP-C4-ADS column

The packing material of RP-C4-ADS is a 25 μm polymeric silica with a pore size of 60 Å which gives a mass cut off at approximately 15 kD. The inner pore surface is in this application covered with a 4-carbon chain. The outer surface is covered with alkyl-diol groups. The retention mechanism is referred to as ''size exclusion reversed-phase chromatography''.

Under optimised conditions, the analytes of interest are concentrated at the beginning of the RP-ADS column whereafter they are eluted on to the analytical column in the back flush mode for best possible plate count in the chromatography.

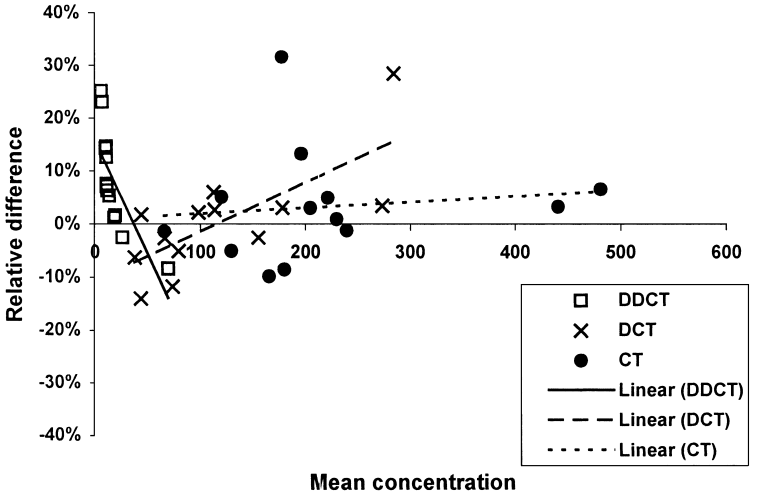
During the development of this method no degradation of the RP-C4-ADS column was observed but when a new RP-C4-ADS column was applied, at the beginning of the validation experiments, the column showed a change in behaviour for DDCT during the three first extracted calibration curves. However, after a total of 16 injections (two calibration curves and the controls and blanks belonging to them) the column showed excellent reproducibility.

It was not possible to give a clear explanation to this but one plausible explanation is that when extracting a more polar substance (i.e. DDCT) which has a lower affinity for the inner surface it is lost during the wash out step of the sample preparation. In a hydrophobic stationary phase the carbon chains tend to stick together if the surroundings are too hydrophilic. In this case, after the 16 injections and the conditioning steps between them the C4 chains of the inner surface finally reaches a stable, ''fluffy'' condition and gets reproducible.

Another possibility is that DDCT, (and other plasma components with H-binding properties) interfere very strong with free silanol groups on the packing material. The recovery would then improve during time because the active adsorptive sites of the silica surface gets saturated.

The problem wasn't studied any further but perhaps a general conditioning procedure when new columns are applied could be assembled.

Considering the patient sample analysis there are some possible explanations about the observed trend



	Slope	R^2	P-Value
DDCT	y = -0,0043x	O,528	0,0049
DCT	y = 0,0009x	0,544	0,0040
CT	y = 0,0001x	0,015	0,694

Fig. 4. The relative difference between the two methods, (SPE [conc.]−OLE [conc.])/Mean [conc.]. DDCT=didesmethylcitalopram, DCT=desmethylcitalopram, CT=citalopram.

that DCT has a higher recovery on SPE and DDCT a higher recovery in the on-line extraction methodology in the upper concentration interval.

Since there were several weeks between the two analysis one explanation might be that a spontaneous degradation from DCT to DDCT occurred and that the degradation was dependent of the concentration of DCT. This would then be most pronounced when the sample had a high DCT concentration in the first analysis (the SPE). However, when the samples were compared individually, we could not show that the samples with a high DCT concentration in the SPE method had a corresponding high DDCT concentration in the on-line extraction method, (measured several weeks later). The ratio DDCT/DCT for each sample did not differ when the two methods was compared by correlation analysis. (y=0.92x, r^2= 0.92). Besides, Carlsson et al. [18], have shown that spiked plasma samples are stable for 6 months stored at −20°C.

Another explanation would be that the calibration curves for DCT and DDCT were not linear in either the SPE method or the on-line extraction method. However, when looking at the calibration curves both methods yield excellent r^2-values on both DCT and DDCT. Neither could residual plots show any deviations from linearity.

4.2. Choice of methodology

In the comparison between the two analytical methods it was not possible to say whether one method was performing better than the other. Both methods have their advantages and disadvantages. The on-line extraction method is fast and fully automated. The possibility to inject as much as 50 ml plasma [11] on the same extraction column makes the capacity of the sample tray in the autosampler or the runtime of the chromatography the limit of how many samples that can be analysed per day. On the other hand on-line extraction requires an extra switching valve and an extra HPLC pump. Still, the

extra pump does not have to be of ‘‘top of the market quality’’ as it does not has to be pulse free and works at relatively low pressures, (below 10 bars).

SPE is a well established technique with an almost unlimited range of variation of packing materials. When handled correctly the extracts are very clean which vouch for a good analytical column maintenance and few chromatographic interferences. It is however time consuming and therefore expensive.

In conclusion it is apparent that in this application the on-line extraction method is superior in sample capacity per day and that it is more cost effective since the whole process is automated.

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