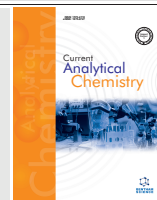


RESEARCH ARTICLE

Simultaneous Quantification of Citalopram and its Main Metabolite, Desmethylcitalopram, in Human Saliva by UHPLC



Ewelina Dziurkowska and Marek Wesolowski*

Department of Analytical Chemistry, Medical University of Gdansk, Gen. J. Hallera 107, 80-416 Gdansk, Poland

Abstract: Background: This study was designed to develop a reliable method for simultaneous quantification of Citalopram (CIT) and its main active metabolite, Desmethylcitalopram (DCIT), in saliva of patients undergoing treatment with CIT.

Methods: To compare two procedures of saliva purification, Solid-Phase (SPE) and Liquid-Liquid (LLE) extractions, saliva samples obtained from healthy volunteers were spiked with adequate quantities of CIT and DCIT. Different cartridges were used for SPE, while dichloromethane for LLE. Chromatographic separation and quantitation were carried out by UHPLC with DAD detector using a C-18 column and a mixture of acetonitrile and redistilled water (37:63, v:v) with the addition of formic acid (pH 3.5) as a mobile phase.

Results: A comparison of both purification procedures showed that the most satisfactory results were obtained by SPE using Discovery C18 cartridge and redistilled water with formic acid (pH 3.5) as a washing solvent. Dichloromethane proved to be the best extractant in LLE. Both procedures enabled the separation of analytes from human saliva with high precision and recovery.

Conclusions: Validation of the developed UHPLC procedure revealed that, regardless of how the sample was purified, the method was characterized by good linearity (between 10 and 1000 ng/mL), sensitivity, reproducibility, specificity and low values of limits of detection and quantitation. The limits of quantitation were 4.0 and 8.0 ng/mL for SPE and LLE, respectively. The efficiency of the method in therapeutic drug monitoring of CIT and DCIT in saliva of patients was confirmed.

Keywords: Citalopram, desmethylcitalopram, saliva, simultaneous UHPLC quantitation, solid-phase extraction, liquid-liquid extraction.

1. INTRODUCTION

Citalopram (CIT) is one of the most prevalent antidepressants and acts by the selective inhibition of serotonin reuptake. It is extensively metabolized in the liver by the cytochrome P450 isoenzymes – CYP3A4 (Cytochrome P450 3A4) and CYP2C19 (Cytochrome P450 2C19) into the active metabolites Desmethylcitalopram (DCIT) and didesmethylcitalopram. Although DCIT is approximately four times weaker as an inhibitor of serotonin reuptake when compared to CIT, it is a substrate for the same isoenzymes and is their potent inhibitor. The mean half-life elimination time of CIT for adults is 35 h and this occurs largely through the passing of urine, 10% as an unchanged drug. The mean half-life elimination time of the main metabolite of CIT, desmethylcitalopram is, by contrast 59 h [1]. The chemical structures of both substances are presented in Fig. (1).

Citalopram is considered a safe drug and in the majority of cases, application in polypragmasy with other drugs does

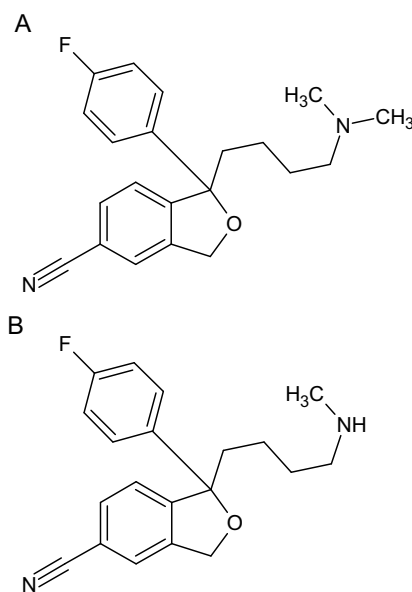


Fig. (1). Chemical structures of: (A) citalopram and (B) desmethylcitalopram.

*Address correspondence to this author at the Medical University of Gdansk, Department of Analytical Chemistry, Gen. J. Hallera 107, 80-416 Gdansk, Poland; Tel: +48 58 349 1096; Fax: +48 58 349 1524; E-mail: marwes@gumed.edu.pl

not require the monitoring or modification of therapy. However, CIT is a poor inhibitor of isoenzymes of Cytochrome P450, so there is a clear possibility of interaction, which may result in an increase of the concentration of CIT or its main metabolite, DCIT [1].

The renal clearance of CIT reduces with age, because of the decreased metabolic activity. In patients over 60 years of age, the elimination half-life is approximately 30% longer in comparison to younger subjects. This suggests the possibility of age-related changes in the activity of CYP2C19, which cause a significant decline in the ratio of DCIT to citalopram. Given potential pharmacokinetic differences in the elderly and middle-life patients, it is recommended that the levels of CIT and DCIT be monitored [2].

A literature review shows that CIT and DCIT have been determined in many human or animal biological materials. The most commonly used is whole blood [3], or more often plasma [4-10] or serum [11-14]. Although blood allows determination of the free and bounded fraction of the drug, but sampling involves physical intrusion into the patient, which may cause infection and entails additional stress. Another biological material often used for the analysis of CIT and DCIT is urine [3, 10, 15], but have also been determined in hair and nails from living bodies [16] and different tissues or organs such as muscle, brain and kidney procured from the dead [17, 18]. Saliva is an underrated biological material which can also be used for the monitoring of CIT and DCIT concentrations, but so far only CIT has been determined in oral fluid [4, 19, 20]. Saliva is readily available, indicates biological activity in the human organism and only allows the unbounded fraction of the drug to be determined, *i.e.* the active form. Saliva sampling is also straightforward and does not require specialist staff for collection. In spite of this, there is still a shortage of information about the determination of CIT and DCIT in saliva – only two papers discussing solid-phase extraction of CIT from saliva [4, 20] can be found in the literature, but this material contains no information on the isolation and determination of its main metabolite, DCIT.

Taking into account the long mean half-life elimination time of both CIT and DCIT, the decrease in metabolic activities of the enzymes, possible interactions with other prescribed drugs and the scarcity of data on simultaneous determination of CIT and DCIT in saliva, the aim of this study was to develop a quick, efficient and reliable method for extraction and determination of both analytes in saliva. Since chromatography is the most commonly used analytical technique for the determination of CIT and its metabolite, UHPLC with DAD detector was applied in this study. Thus far, various extraction procedures have been applied for the isolation of both CIT and DCIT from blood and urine, *i.e.* Solid-Phase Extraction (SPE) [5, 6, 12-14, 17, 18, 20], its modification such as SBSE (*Stir Bar Sorptive Extraction*) [10, 15], and Liquid-Liquid Extraction (LLE) [3, 8, 9, 16, 19, 21]. Biological samples have also been purified using deproteinization with organic solvents [7,11]. In view of this, the decision was made to evaluate the suitability of both SPE and LLE for purification of human saliva prior to simultaneous quantitation of CIT and DCIT. The effectiveness of the developed and validated procedure was verified using saliva

samples from women suffering from depression and under treatment with citalopram.

2. MATERIALS AND METHODS

2.1. Reagents and Equipment

Methanol solutions of citalopram hydrobromide (1 mg/mL) and desmethylcitalopram hydrobromide (1 mg/mL) were purchased from Sigma-Aldrich (St. Louis, USA). Venlafaxine hydrochloride (VEN), an Internal Standard (IS) was supplied as courtesy of Moehs Iberica SL (Barcelona, Spain). Acetonitrile, methanol and formic acid were obtained from POCh (Gliwice, Poland). All chemicals and reagents were of UHPLC grade. Water was purified by double distillation in a Destamat[®] Bi-18 system (Heraeus Quarzglas, Hanau, Germany).

A solid-phase equipment consisted of Chromabond 73015 (Macherey-Nagel, Duren, Germany) with vacuum pump NOZZ AT.18 (KNF Neuberger Inc, Trenton, USA). SPE columns Discovery C 18 (Supelco, Bellefonte, USA) were used for solid-phase extraction. High speed centrifuges, models EBA 20S (Hettich Lab Technology, Germany) and MPW-320 (MPW, Poland), a laboratory shaker 358 S (Elpin, Poland), and water bath LW-12 (Cabrolab Elektronik, Poland) were used for both SPE and LLE.

2.2. Sample Collection

Complete saliva samples were obtained from healthy volunteers (5 females and 5 males, aged 24 – 35 years) in the afternoon and immediately frozen to -20°C and stored until the time of analysis. Participants refrained from eating and drinking for at least 0.5 h and rinsed their mouths with water before taking the samples. The whole saliva was collected into plastic tubes without stimulation. Afterwards, the study protocol was approved by the ethical committee of the Medical University of Gdansk, Poland.

To verify the suitability of the method developed and validated, saliva procured from women suffering from depression and undergoing treatment with 20 mg/day of CIT was analyzed. Twenty patients were recruited at the Hospital for Nervous and Mental Diseases in Starogard Gdanski (Poland) based on psychiatric diagnosis, defined according to the International Classification of Diseases (ICD-10).

2.3. Solid-phase Extraction

Saliva was defrosted and 1 mL of oral fluid extracted with Discovery C18 SPE cartridges. Extraction was preceded by conditioning of cartridges with 1 mL of methanol and 1 mL of redistilled water. Saliva was then centrifuged at 8.000 g for 5 min and the supernatant loaded on extraction columns. After the samples passed through, the cartridges were rinsed with 2 mL of redistilled water with the addition of formic acid (pH 3.5). Once the cartridges were dried, the samples were eluted with 0.5 mL of methanol. Then, the extracts were evaporated to dryness in a water bath, the dry residue was dissolved in 100 μL mixture of acetonitrile and water (50:50, v:v) and transferred to an Eppendorf tube. Finally, the samples were centrifuged at 8.000 g for 2 min,

transported to autosampler vials and 20 μL of the solution was analyzed by UHPLC.

2.4. Liquid-liquid Extraction

Defrosted saliva (1 mL) was transferred into plastic tubes and 3 mL of dichloromethane was added. After shaking for 20 min, the samples were centrifuged at 8.000 g for 5 min and the dichloromethane layer was transferred to a glass tube and evaporated. The subsequent procedure was similar to that performed for samples after solid-phase extraction.

2.5. Extraction Recovery

To determine the efficiency of CIT and DCIT isolation from saliva, the extraction recovery was calculated. The twelve samples of blank saliva were divided into two equal groups. The first 6 samples were analyzed as described in section 2.3 or 2.4. The remaining 6 nonspiked samples were extracted. The dry residue was spiked with CIT and DCIT at two concentration of analytes, 50 and 500 ng/mL, and evaporated for the second time. The data obtained after UHPLC analysis were used to calculate the extraction recovery.

2.6. UHPLC Analysis

Chromatographic separation was carried out using a UHPLC apparatus Nexera XR (Shimadzu, Kyoto, Japan) with diode array detector. A C-18 column (Nucleosil 100-5, 125 \times 4 mm i.d., 5 μm) with a guard column C18 (Nucleosil 100-5, 5 \times 4 mm i.d., 5 μm) (both from Knauer, Berlin, Germany) was also employed. As a mobile phase, a mixture of acetonitrile and redistilled water (37:63, v:v) with the addition of formic acid (pH 3.5) was used at a flow rate of 2 mL/min. The analysis was performed at 25°C for a duration of 7 min.

2.7. Method Validation

Commercially available stock solutions of CIT and DCIT were diluted with methanol to the desired concentration. A stock solution of internal standard – VEN (1 mg/mL) was prepared by dissolving 10 mg of the reference substance in 10 mL of methanol. The working solutions thus obtained were refrigerated at 4°C in a stable condition for 6 months. Stability was monitored daily by UHPLC analysis.

To construct the calibration curves, 1 mL of saliva defrosted and centrifuged at 8.000 g for 5 min was transferred into plastic tubes, to which suitable amounts of working solutions of CIT and DCIT were added to obtain the desired concentrations (10, 50, 100, 500, 750 and 1000 ng/mL). Next, the 100 μL of IS at concentration of 100 $\mu\text{g}/\text{mL}$ was added and the samples of spiked saliva were extracted with SPE or LLE and analyzed by UHPLC. Each curve point was repeated four times.

The precision and accuracy of the method were established for CIT and DCIT at concentrations of 20, 200 and 750 ng/mL. Each concentration was determined four times on the same day for the intraday study. In the case of the interday study, the same concentration was analyzed once a day over four days.

Freezing and thawing cycles were used to determine the stability of analytes as the frozen saliva was in storage. Two concentrations of the analytes (50 and 500 ng/mL) were used in the experiment. The freezing and thawing cycle of the frozen saliva was run in triplicate and each concentration analyzed once a week over four weeks. Two batches of samples, each including six 4-mL samples of saliva obtained from one person, were spiked with 200 μL of CIT and DCIT, one at a concentration of 1 $\mu\text{g}/\text{mL}$ and the second at 10 $\mu\text{g}/\text{mL}$. Each sample (1 mL) was spiked with 100 μL of IS at concentration 100 $\mu\text{g}/\text{mL}$, extracted with SPE or LLE and analyzed by UHPLC. The remainder of the sample was frozen at -20°C and the procedure repeated the following week.

The stability of CIT and DCIT was also examined at 4°C. Six 4-mL samples of defrosted oral fluid were spiked with 200 μL of both analytes at a concentration of 1 $\mu\text{g}/\text{mL}$. Next, six samples were spiked with 200 μL of the solution at a concentration of 10 $\mu\text{g}/\text{mL}$. From each sample 1 mL of oral fluid was taken and an appropriate amount of IS were added. Then the samples were extracted by SPE or LLE and analyzed by UHPLC. The remaining saliva was refrigerated for one day. The following day, 1 mL of each sample was removed and analyzed in the same fashion.

3. RESULTS AND DISCUSSION

3.1. Chromatographic Separation

The first step in the optimization of the method was to determine the retention time of CIT and DCIT, which produced a result of 3.5 min for DCIT and 3.8 min for CIT. The UV spectra revealed that the maximum absorption for CIT and DCIT was observed at 238 nm.

3.2. Extraction Process

A literature review shows that both SPE and LLE can be used for isolation of CIT and DCIT from different biological materials, with the exception of saliva, for which only CIT has as yet been determined. In this study, two extraction procedures were compared in order that the better of the two could be employed. Therefore, a preliminary study on the optimization of CIT and DCIT extraction from human saliva was carried out to choose the most effective cartridge and the appropriate type of solvent or solvent mixture for washing the cartridge. The study on C8 and C18 cartridges using redistilled water and mixtures with the addition of small quantities of formic acid or phosphate buffer at pH 2.5 revealed that C18 cartridge and redistilled water with formic acid were found to be the most satisfactory combination for SPE of the compounds in question. Furthermore, the extraction yield reached a value at about 100% for each analyzed concentrations of CIT and DCIT. When considering LLE, a preliminary study showed that dichloromethane was the most effective solvent for extracting the analyte under study from saliva [22].

To determine the efficiency of CIT and DCIT isolation from saliva, the extraction recovery was calculated according to the formula:

$$\text{Extraction recovery (\%)} = \frac{\text{AUC of drug peak for saliva spiked before extraction}}{\text{AUC of drug peak ratio for saliva spiked after extraction}} \times 100$$

where: AUC is the area under the peak.

Table 1. Characteristic of SPE and LLE of citalopram and desmethylcitalopram from saliva spiked with standards.

Parameters	Citalopram				Desmethylcitalopram			
	SPE		LLE		SPE		LLE	
Nominal concentration (ng/mL)	50.0	500.0	50.0	500.0	50.0	500.0	50.0	500.0
Before extraction	-							
$\bar{x} \pm SD$ (ng/mL)	49.9±4.4	523.3±16.2	49.2±2.1	499.6±14.6	48.6±3.5	508.8±19.6	31.8±1.2	424.3±15.9
RSD (%)	8.8	3.1	4.3	2.9	7.3	3.9	3.9	3.8
Recovery (%)	99.8	104.7	98.4	99.9	97.1	97.1	63.7	63.7
After extraction	-							
$\bar{x} \pm SD$ (ng/mL)	52.2±3.7	534.0±12.6	53.4±2.9	517.2±13.1	51.9±2.5	514.9±16.9	43.5±2.0	476.7±5.1
RSD (%)	7.1	2.4	5.3	2.5	4.8	3.3	4.7	1.1
Recovery (%)	105.0	106.8	106.8	103.4	103.8	103.0	87.1	95.3
Extraction yield (%)	95.0	98.0	92.2	96.6	93.6	97.3	73.1	89.0

The results presented in Table 1 show that the extraction recovery of CIT from saliva spiked with 50 and 500 ng/mL of the drug was as follows: 95 and 98% for SPE, and 92 and 97% for LLE. The recovery of DCIT was comparable when it comes to SPE, but significantly lower for LLE. The values were 94 and 97%, and 73 and 89% for SPE and LLE, respectively.

A literature review reveals that SPE is currently used more frequently than LLE in drug analysis. There is, however, no special trend in the selection of extraction columns. Beside C18 adsorbent [6, 13], other types of cartridges – C2 [14,18], C8 [17] and OASIS MCX [4] have also been used. The highest recovery was obtained for extraction of CIT with C2 cartridges, circa 91% from post-mortem blood [18], 98% from serum [14], and 93% in DCIT quantification in postmortem blood [18] and 102% in serum [14]. As regards the remaining cartridges, the extraction efficiency for C8 adsorbent stood between 87 – 93% for CIT, and 74 – 95% for DCIT determined simultaneously in whole blood [17]. The same type of extraction columns, as used in this study, produced slightly lower scores in the range from 78 to 83% for CIT and from 59 to 83% for DCIT quantified in serum [13]. The lowest extraction efficiency was obtained with mixed mode columns – between 49 and 72% [4]. These relatively low values may be due to the simultaneous determination of nine antidepressants and some of their metabolites in saliva. The recovery of CIT and DCIT from plasma following LLE procedure stood at approximately 77 and 53%, respectively [8], while the efficiency of extraction from saliva for citalopram was 94% [19].

The best results of extraction of both analytes were received using Discovery C18 SPE cartridges and 2 mL of redistilled water with the addition of formic acid (pH 3.5) for washing the cartridges. The analytes were eluted with 0.5 mL of methanol. The recovery of both analytes for this procedure was above 90%. Similar results were attained isolating CIT from blood with C2 cartridge [14, 18]. Using C18, the recovery of CIT was lower [13] than obtained in this study. In the literature, there is no information about the results of DCIT extraction from saliva with SPE.

When LLE with dichloromethane was applied, the recovery of CIT was similar to findings in the literature, where ether was used for extraction [19]. It can, therefore, be confirmed that absolute recovery obtained in this study is consistent with literature data.

3.3. Validation of the Method

Validation of the method developed was assessed based on its specificity, linearity, sensitivity, precision and accuracy. The stability of the analytes in saliva was also examined.

3.3.1. Specificity

The specificity was assessed for both SPE and LLE by comparing the chromatograms of extracted blank saliva with those of extracted saliva spiked with various quantities of analytes. As can be seen in Fig. (2), no extraneous peaks in the region of retention time of CIT and DCIT are present in the chromatograms of extracted blank saliva and those spiked before application of SPE and LLE. It should also be noted that in the case of LLE, the peaks of both analytes are not as symmetric as after SPE, which suggests that SPE

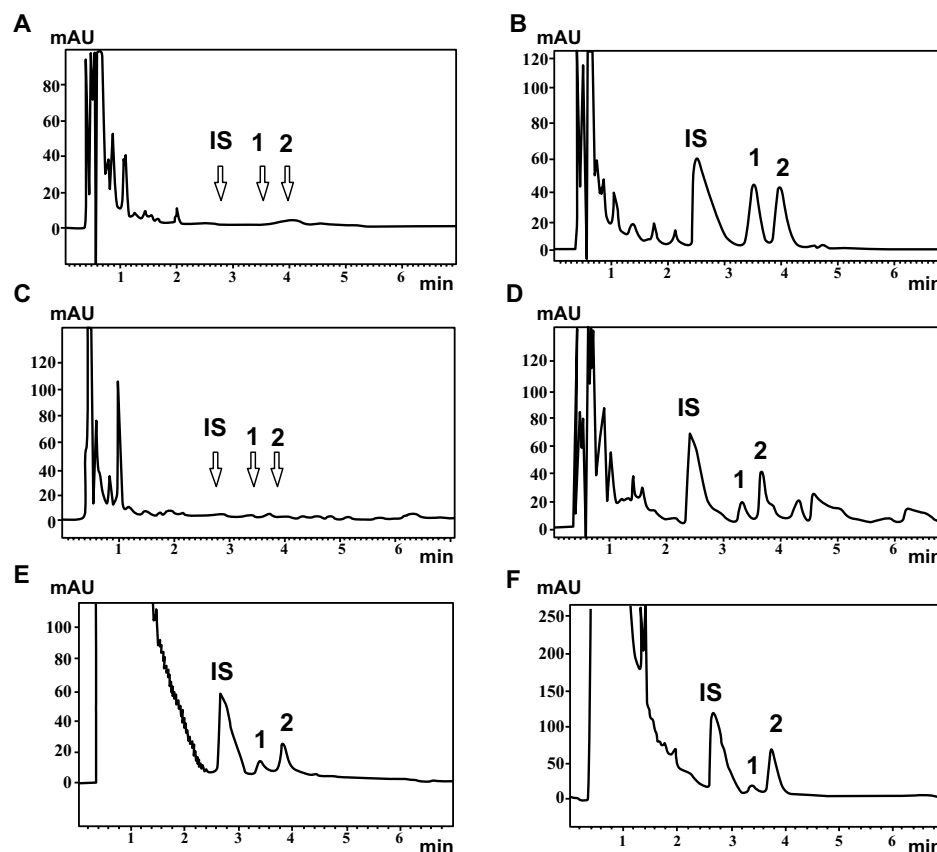


Fig. (2). Chromatograms of human saliva extracts: after SPE – (A) blank saliva and (B) saliva spiked with 200 ng/mL of CIT and DCIT, after LLE – (C) blank saliva and (D) saliva spiked with 200 ng/mL of CIT and DCIT, obtained from depressed patients treated with citalopram after SPE – (E) CIT concentration 11.4 ng/mL, DCIT concentration 4.3 ng/mL, (F) CIT concentration 25.6 ng/mL, DCIT concentration 3.2 ng/mL. (1) DCIT, (2) CIT.

permits more efficient purification of the samples than LLE, as shown in the blank saliva samples in Fig. (2 A and C).

3.3.2. Linearity

Calibration curves for both analytes were constructed based on 6 calibration points in the range from 10 to 1000 ng/mL. As shown in Table 2, both procedures of extraction display good linearity, expressed in high correlation coefficient. For both procedures, the high values of slope (a) confirm the great sensitivity of the method developed. This method is unburdened by statistical error, despite the high value of intercept (b). In both cases, the value of intercept differs statistically from zero.

3.3.3. Sensitivity

The sensitivity of the procedure was characterized by the values of limits of detection and quantification. Limit of detection (LOD) is defined as the smallest measured concentration of an analyte from which it can be detected and limit of quantification (LOQ) is the lowest concentration of analyte from which it can be determined with acceptable precision and accuracy ($\leq 20\%$) [22]. For CIT and DCIT, LOD was established at 2 ng/mL for SPE, and 3 ng/mL for LLE. The LOQ for SPE and LLE was 4 ng/mL and 8 ng/mL for both analytes, respectively.

The literature data shows that the lowest LOQ is obtained using MS detection, which allows determination of both analytes in plasma at a concentration below 1 ng/mL [7]. In this case, the deproteinization was the only stage in sample purification. The same type of detection was used by Lewis *et al.* [17] in the analysis of CIT and DCIT in postmortem fluids and tissues. The LOD determined for whole blood for both analytes was 1.56 ng/mL, while LOQ was 3.33 and 1.56 ng/mL for CIT and DCIT, respectively. In the case of UV detection, the LOD and LOQ produced slightly higher values; only Yu *et al.* [9] obtained LOD at a level of 1 ng/mL. In other studies, LOQ was 16 ng/mL for CIT, 14 ng/mL for DCIT [6], and 20 ng/mL [3] or 25 ng/mL [8] for both analytes.

In the quantification of CIT alone in saliva using UV detection, LOD was found to be between 0.6 ng/mL [19] and 5.48 ng/mL [20], whereas LOQ varied between 1.82 ng/mL [19] and 18.25 ng/mL [20]. Comparable values of LOQ (2 ng/mL) were attained with LC-MS [4].

3.3.4. Precision and Accuracy

The data on the precision and accuracy of the method developed compiled in Table 3 show that the intraday precision expressed by relative standard deviation (RSD) for CIT was between 2.9 and 6.8%, and 3.5 and 12.2% for SPE and LLE, respectively. RSD for DCIT varied in the range of 4.5-9.7%

Table 2. Statistical evaluation of calibration curves for UHPLC quantitation of citalopram and desmethylcitalopram in saliva.

Parameters	Citalopram		Desmethylcitalopram	
	SPE	LLE	SPE	LLE
Linear range (ng/mL)	10-1000			
Slope $a \pm \Delta a$	0.0016 \pm 0.0001	0.0084 \pm 0.0016	0.0015 \pm 0.0001	0.0076 \pm 0.0022
SD of slope	0.0001	0.0089	0.0001	0.0042
Intercept $b \pm \Delta b$	0.0413 \pm 0.05850	0.2612 \pm 0.9040	0.0483 \pm 0.04956	-0.1686 \pm 1.2113
SD of the intercept	0.0162	0.1910	0.0175	0.2866
Correlation coefficient	0.9949	0.9501	0.9958	0.8857
LOD (ng/mL)	2.0	3.0	2.0	3.0
LOQ (ng/mL)	4.0	8.0	4.0	8.0

Table 3. Precision and recovery for UHPLC quantitation of citalopram and desmethylcitalopram in saliva.

Citalopram	SPE			LLE		
Nominal Concentration (ng/mL)	20.0	200.0	750.0	20.0	200.0	750.0
Intraday precision						
Assayed concentration $\bar{x} \pm SD$ (ng/mL)	20.0 \pm 1.37	197.8 \pm 9.7	764.8 \pm 22.3	19.2 \pm 2.3	197.6 \pm 14.3	762.6 \pm 26.6
RSD (%)	6.8	4.9	2.9	12.2	7.2	3.5
Recovery (%)	100.0	98.9	102.0	95.8	98.8	101.7
Interday precision						
Assayed concentration $\bar{x} \pm SD$ (ng/mL)	19.4 \pm 2.2	200.2 \pm 11.0	754.8 \pm 12.0	19.6 \pm 2.0	198.5 \pm 15.6	744.8 \pm 31.7
RSD (%)	11.4	5.5	1.6	10.2	7.8	4.2
Recovery (%)	96.9	100.1	100.6	97.9	99.3	99.3
Desmethylcitalopram						
Nominal Concentration (ng/mL)	20.0	200.0	750.0	20.0	200.0	750.0
Intraday precision						
Assayed concentration $\bar{x} \pm SD$ (ng/mL)	20.3 \pm 2.0	199.9 \pm 11.6	762.8 \pm 34.7	18.3 \pm 1.9	181.6 \pm 2.5	725.1 \pm 18.3
RSD (%)	9.7	5.8	4.5	10.4	1.3	2.5
Recovery (%)	101.7	99.9	101.7	91.6	90.8	96.7
Interday precision						
Assayed concentration $\bar{x} \pm SD$ (ng/mL)	19.4 \pm 2.1	200.8 \pm 12.3	762.0 \pm 22.5	19.6 \pm 2.6	183.9 \pm 18.0	721.2 \pm 21.8
RSD (%)	10.9	6.1	2.9	13.4	9.8	3.0
Recovery (%)	97.2	100.4	101.6	97.8	91.9	96.1

Table 4. Statistical evaluation of solid-phase and liquid-liquid extractions by F-Snedecor and t-Student's tests.

Drug	Citalopram			Desmethylcitalopram		
Nominal concentration [ng/mL]	20.0	200.0	750.0	20.0	200.0	750.0
Intraday precision						
F	0.34	0.46	0.70	1.06	22.11	3.57
F_{α} [$\alpha=0.05$, $f=5$]	0.198			5.05		
T	0.75	0.04	0.15	1.81	3.80	2.35
$t_{\alpha,f}$ [$\alpha=0.05$, $f=10$]	2.228			2.228		
Interday precision						
F	1.21	0.50	0.14	0.66	0.47	1.07
F_{α} [$\alpha=0.05$, $f=5$]	5.05	0.198		0.198	5.05	
T	-0.17	0.22	0.72	-0.08	1.90	3.19
$t_{\alpha,f}$ [$\alpha=0.05$, $f=10$]	2.228			2.228		

for SPE and 1.3-10.4% for LLE. The low values of RSD for both extraction procedures and analytes confirm the precision of the method. However, SPE was found to be more profitable.

F-Snedecor and t-Student's tests were used for statistical evaluation of the precision of both extraction procedures. The results presented in Table 4 demonstrate that the precision of SPE and LLE does not differ statistically, with the exception of some DCIT concentrations, *i.e.* 200 and 750 ng/mL for intraday precision and 750 ng/mL for interday precision (t was higher than T from the test). This confirms the consistency of the results for both the analytes and extraction procedures.

The recovery for both procedures of extraction was also calculated. As shown in Table 3, the recovery of SPE in intraday study was about 100% for both analytes. The values for CIT and DCIT in interday study were slightly lower at a concentration of 20 ng/mL and were about 97%. The recovery of LLE, regardless of the isolated analyte, varied between 91 and 102%. In both cases, the results for DCIT were lower than for CIT. The low score of RSD, which did not exceed 11.4% for SPE and 13.4% for LLE, indicates the precision of the method. Although the results for LLE are slightly higher than SPE, they did not exceed 15% in either case.

3.3.5. Stability

The stability of both analytes was examined during the storage of saliva in a freezer at -20°C or fridge at 4°C . The results show that neither CIT nor DCIT decomposed whatever the storage conditions. The concentration of CIT and DCIT decreased about 2% regardless of the means of storage.

3.4. Clinical Application

Due to the statistically significant differences that were demonstrated by the F-Snedecor and t-Student's tests, the

samples were extracted using only SPE with C18 column. The concentration of CIT in the samples extracted by SPE was between 22.7 and 741.1 ng/mL, and DCIT between 4.6 to 177.3 ng/mL. In one sample the concentration of DCIT extracted with SPE was below LOD. The typical chromatograms of extracted saliva samples obtained from patients treated with citalopram are shown in Fig. (2 E and F).

As claimed in the literature, the concentration of CIT in saliva of patients treated with different doses of this drug varies between 7.6 and 379.1 ng/mL, *i.e.* higher than that established in blood [4]. With the exception of this study, there is no information on the concentration of DCIT in saliva, making the comparison of the results difficult. On the other hand, the concentration of DCIT in blood was lower than CIT [12], in contrast to its concentration in urine, 12 hours after the drug administration [15].

A preliminary study of saliva obtained from patients undergoing treatment with CIT indicates that the concentration of CIT was higher than DCIT. This may be attributed to the time of sampling, *i.e.* shortly after the intake of the drug. Accordingly, the conclusion is that the method developed may prove suitable for the simultaneous determination of CIT and DCIT in saliva.

CONCLUSION

A validated UHPLC-DAD method was developed for the simultaneous determination of citalopram and desmethylcitalopram, its main active metabolite, in saliva. Two procedures of extraction, liquid-liquid and solid-phase, were validated. Both facilitated the separation of analytes from biological material with high extraction recovery, though the recovery of SPE stood higher than 90% for both analytes. The precision of both extraction procedures does not differ statistically, as confirmed by F-Snedecor and t-Student's tests. Moreover, whatever the extraction procedure, the method showed

good linearity, sensitivity, reproducibility and specificity. Additionally, this procedure was defined by low values of LOD and LOQ.

Both SPE and LLE are suitable for the determination of low concentrations of CIT and DCIT in oral fluid. However, SPE enabled determination of DCIT in a very low concentration. In view of the fact that liquid-liquid extraction requires more organic solvent, detrimental to both health and environment, solid-phase extraction should be recommended for routine quantitation of free fraction of CIT and DCIT in human saliva, especially in therapeutic drug monitoring in pediatric and geriatric patients for individualized therapy or polypharmacy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the ethical committee of the Medical University of Gdansk, Poland.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the *Helsinki Declaration* of 1975, as revised in 2008 (<http://www.wma.net/en/20activities/10ethics/10helsinki/>).

CONSENT FOR PUBLICATION

Consent for research involving human has been signed by the participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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