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Rapid determination of letrozole, citalopram and their metabolites by high performance liquid chromatography-fluorescence detection in urine: Method validation and application to real samples

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ABSTRACT

This work reports the validation of a high precision and accuracy method for the simultaneous determination of letrozole, citalopram and their metabolites in urine by high performance liquid chromatography with fluorescence detection. Dilution (urine:mobile phase, 1:2, v/v) was the only sample preparation step. The separation was carried out in a Kromasil C_{18} (150 mm × 4.6 mm) column, and the mobile phase was phosphate buffer 80 mM (pH 3.0) and acetonitrile (65:35, v/v) at a flow rate of 1.0 mL/min. The analytes were detected at 295 nm after excitation at 230 nm. Linearity was observed in the range of 1.0–1000 ng/mL for letrozole and its metabolite and 2.5–1000 ng/mL for citalopram and their metabolites, with limits of detection and quantification between 0.09–1.0 and 0.27–1.65 ng/mL, respectively. The precisions were satisfactory with RSDs between 0.17 and 5.71%. The accuracy was studied by spiking three urines from healthy female volunteers, and the recoveries were from 85 to 103%. The method was applied to urine samples from women under treatment for breast cancer and depression diseases.

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1. Introduction

Patients with cancer are at considerable risk of drug–drug interactions. Typically, such patients receive a large number of drugs during their treatment, including several different cytotoxic agents in multi-drug chemotherapy regimens, hormonal agents, and also supportive care with antiemetics, antidepressants, analgesics, and antiinfective agents, among others. Drug interactions in oncology are of particular importance owing to the narrow therapeutic index and the inherent toxicity of anticancer agents. Interactions with other medications can cause small changes in the pharmacokinetics or pharmacodynamics of a chemotherapy agent that could significantly alter its efficacy or toxicity.

Psychiatric disorders affect about half of the cancer patients [1]. Adjustment disorder is the most frequent diagnosis, followed by major depression, with prevalence going from 4 to 35% and from 3 to 36%, respectively, according to the diagnostic criteria used, the sample studied (outpatient, pre- or postsurgical), and the type and stage of cancer [2–4]. In cancer patients, drug interactions between antidepressants and antineoplastic agents may result in less efficacy of the drug and/or increase of their side effects. Therefore, the choice of antidepressant should be cautious (safe and effective) and well supported.

Aromatase (estrogen synthetase) is an enzyme that catalyses various steps in the conversion of androgens into estrogens. Aromatase inhibitors (AIs) have shown improvements in reducing both mortality and recurrence rates in postmenopausal hormone receptor positive (HR+) early breast cancer patients [5–9]. Letrozole, (4,4′-[1H-1,2,4-triazol-1-yl-methylene]bis-benzonitrile) (LE), is a selective nonsteroidal inhibitor of the aromatase system, that is used for treatment of oesterogen dependent breast cancers [10]. The recommended therapeutic dose for letrozole is 2.5 mg per day. Letrozole is readily and completely absorbed from the gastrointestinal tract. The major route of elimination of letrozole is via metabolism by cytochrome P-450 isozymes (CYP3A4 and CYP2A6) into a pharmacologically inactive carbinol metabolite (ME-LE) [11–14].

The antidepressant citalopram (1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile); (CIT) is a bicyclic phthalate compound approved in 1998 by the US Food and Drug Administration for the treatment of depression. It is also indicated for other central nervous system (CNS) diseases such as anxiety, obsessive–compulsive disorders, various phobias (agoraphobia, social phobia), borderline personality disorders, bipolar disorders as well as as in cases in which inhibition of serotonin reuptake is desired [15]. CIT, is a potent and highly selective serotonin reuptake inhibitor (SSRI). The SSRIs act by

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inhibiting the reuptake of serotonin (or 5-hydroxytryptamine, 5-HT) into the presynaptic nerve terminal, enhancing synaptic concentrations of 5-HT and facilitating serotonergic neurotransmission. Orally administered CIT is well absorbed from the gastrointestinal tract and is cleared from the body primarily by hepatic metabolism, where CIT is stereoselectively metabolized by partial N-demethylation to demethylcitalopram (DCIT) and didemethylcitalopram (DDCIT), as well as by oxidative deamination to a propanoic acid metabolite (CIT-PA) and by N-oxidation to CIT-N-oxide (CIT-NO) [16]. CIT is biotransformed by the specific human hepatic cytochrome P450 enzymes (CYP3A4, CYP2C19, and to a minimal extent CYP2D6) while the inhibition of these enzymes by CIT and DCIT is negligible.

To date, all analytical methods described in the literature for the determination of LE in biological and other matrices involve spectrophotometry [17,18], high performance liquid chromatography (HPLC) [19–24], the microarray approach [25], capillary gas chromatographic method with flame ionization detector [26] or mass spectrometry [27] and by liquid chromatography–tandem mass spectrometry (LC–MS/MS), either with triple quadrupole after electrospray ionization [28] or with a quadrupole-quadrupole linear ion trap instrument with a turbo ion source and with multiple reaction monitoring (MRM) [29].

Ping et al. determined the related substances of LE in its tablet dosage forms using HPLC and thin layer chromatography (TLC) [30] methods.

Only a few methods are reported in the literature for the determination of LE by HPLC coupled to fluorescence detection (FLD). Marfil et al. [31] published a HPLC method with a previous fully automated liquid-solid extraction and fluorescence detection which offers high sensitivity for the quantification of letrozole in plasma and urine, but not of its metabolite in either of those. Zarghi et al. [32] reported a simple, rapid and sensitive HPLC method for the analysis of LE in human plasma. A fluorescence detector was used for the quantitation with excitation and emission wavelengths at 230 and 295 nm, respectively. Sekar et al. [33] described a HPLC method with a fully automated protein precipitation extraction and fluorescence detection offering improved sensitivity for the determination of LE in human plasma.

In contrast, there are a number of analytical methods for the determination of CIT and/or its main metabolites, most of them using HPLC-FLD. Fluorescence detection is more sensitive, specific and selective than ultraviolet-visible detection, which avoids interfering peaks and this is why FLD is preferred among authors. The first analytical HPLC-FLD methods for the determination of CIT and its desmethyl metabolite (DCIT) were carried out by Overo [34] and, a few years later, by Oeyehaug et al. [35], who also coupled liquid chromatography to FLD for the analysis of CIT and its main desmethyl metabolites DCIT and DDCIT.

Matsui et al. [36] developed a new method for the analysis of these compounds in plasma by HPLC-FLD with a successive column-switching technique. Plasma samples were injected directly onto a guard column where the analytes were retained and later eluted employing a six-port valve by the back-flush method.

Kristoffersen et al. [37] proposed a method for the simultaneous determination of the three selective serotonin reuptake inhibitors CIT, fluoxetine (FLX), paroxetine (PRX) and their metabolites in whole blood and plasma. Ohman et al. [38] made a comparison between a new on-line extraction method using an RP-C4-ADS extraction column and an off-line SPE method. Waschgler et al. [39] presented an analytical procedure for the simultaneous quantification of CIT, clozapine (CLZ), fluoxetine (FLX), norfluoxetine (NFLX), maprotiline (MPT), desmethylmaprotiline (DMPT) and trazodone (TRZ) in human serum using HPLC. Enantioselective analysis of CIT and its metabolites DCIT and DDCIT performed in femoral blood from 53 autopsy cases by a chiral HPLC method revealed that the mean (±SD) *S*/*R* ratio for CIT was 0.67 ± 0.25 and that for DCIT 0.68 ± 0.20 [40]. Millan et al. [41] described a procedure for the separation of the enantiomers of DCIT and DDCIT. This assay involved a previous LLE of the analytes from plasma and brain tissue samples followed by a pre-column chiral derivatization.

Unceta et al. [42] presented an analytical application of stir bar sorptive extraction (SBSE) coupled to HPLC-FLD for the quantification of fluoxetine (FLX), citalopram (CIT) and venlafaxine (VEN) and their active metabolites in plasma, urine and brain tissue samples.

Jiang et al. [43] developed a sensitive method for simultaneous determination of citalopram and desmethylcitalopram in plasma samples using LC–MS/MS in multiple reaction monitoring (MRM) mode using a positive electrospray ionization source.

Although there is a growing impact of psychiatric and depressive disorders in cancer patients, literature on the idiosyncrasies of antidepressants used in those conditions and their interactions with antineoplastic agents is scarce. From the analytical point of view, the only approach was carried out by Rodriguez et al. [44], who proposed a method for the determination this mixture of drugs by micellar electrokinetic chromatography (MEKC).

Sharing the same biotransformation pathways enhances the risk of drug interaction, specifically when compounds are inducers, inhibitors or substrates of cytochrome P450 (CYP 450).

In this work, we report the separation optimization and validation of a simple and speed HPLC-fluorescence detection method for the simultaneous determination of LE, CIT and their metabolites in human urine at clinical levels with a sample preparation of the urine that consist only in a dilution step. Besides, the performed pharmacokinetic study would permit us to detect some possible negative interactions between both kinds of drugs that could affect or decrease the efficiency of each one separately.

2. Experimental

2.1. Reagents

LE and ME-LE were supplied by Novartis laboratories (Spain), CIT hydrochloride, DCIT hydrochloride, DDCIT tartrate, CIT-NO were supplied by H. Lundbeck A/S (Copenhagen, Denmark).

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared in deionized water (Milli-Q quality).

Standard stock solutions of LE and ME-LE of 100 mg/L were prepared in ethanol/water 50/50 (v/v). Standard stock solutions of CIT and its metabolites of 100 mg/L were prepared in water. The resulting solutions were stored at 4 °C. Working standard solutions were prepared daily by diluting the standard stock solutions with water.

Na_2HPO_4, NaH_2PO_4, H_3PO_4 and HPLC-grade acetonitrile (Panreac, Barcelona, Spain) were used for the preparation of the mobile phase. The mobile phase was filtered through 0.45 μm filters (HNWP membrane filters, Millipore).

2.2. Apparatus

A Shimadzu model LC-10AD HPLC coupled to a RF-10A_{XL} fluorescence detector was used throughout the work (Shimazdu, Kyoto, Japan). This equipment was fitted with a Rheodyne injection valve with a 20 μL sample loop. The separation was carried out in a Kromasil C18 column (dimensions). The system was controlled by Class-LC 10 software (Shimadzu, Kyoto, Japan), which was used for all measurements and data treatment. Solvent delivery pumps, a model SPD-M10A diode-array detector and model RF-10A_{XL} fluorescence detector and a Rheodyne injection valve with 20-μL sample loop. The system was monitored by means of

a computer equipped with Class-LC 10 software (Shimadzu, Kyoto, Japan), which was used for all measurements and data treatment.

A Crison model pH-Meter GLP 21+ with a combined glass electrode was used for pH measurements.

2.3. Clinical samples

Clinical urine samples were provided by three different female patients. Patient A, 52 years old, was orally treated with 2.5 mg of LE per day for 1 year. Patient B, 50 years old, with 20 mg of CIT per day for 3 months. Patient C, 50 years old, with 2.5 mg of LE and 20 mg of CIT for 1 year.

2.4. Urine sample treatment

Fresh human urine samples were obtained from different healthy female volunteers. Ten microlitres of the standard stock solutions were added to 1 mL of urine. Then, the mixture was vortexed for 1 min, diluted 1:2 (v/v) with mobile phase, vortexed again for 1 min, and finally centrifuged at 5000 × *g* for 2 min. A volume of 20 μL of the supernatant was injected into the HPLC system.

3. Results and discussion

3.1. Optimization of chromatographic conditions

A sample prepared as explained in the sample pretreatment section was used throughout the optimization process. According to their fluorescence spectra, the analytes were monitored at 295 nm after excitation at 230 nm.

The chromatographic parameters optimized for the separation of the analytes were pH, ionic strength, percentage of organic solvent in the mobile phase, and flow rate. Optimization was carried out by modifying the parameters one by one whilst

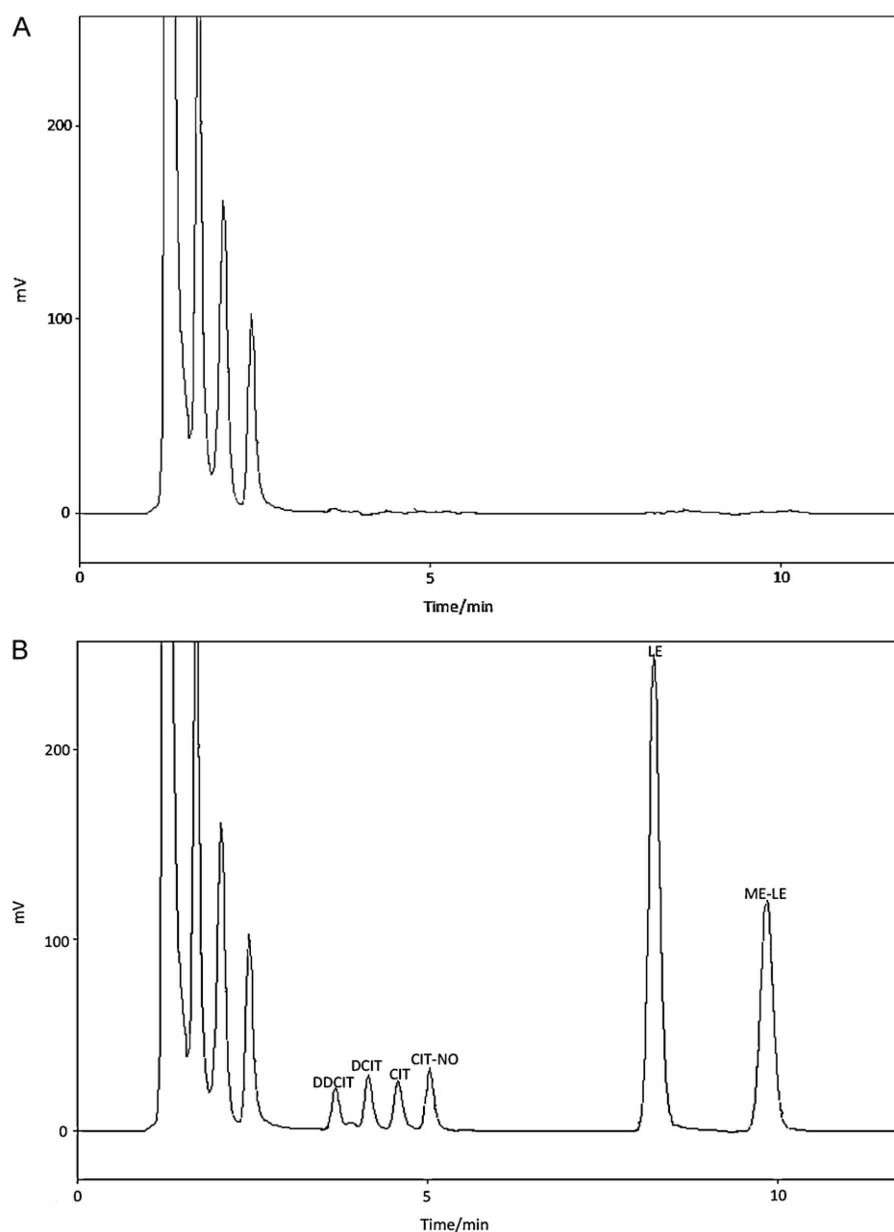

Fig. 1. (A) Chromatogram of a blank urine, (B) chromatogram of a urine sample spiked at 100 ng/mL of the analytes (LE, letrozole; ME-LE, metabolite of letrozole; CIT, citalopram; DCIT, demethylcitalopram; DDCIT, didemethylcitalopram). Operating conditions: mobile phase was phosphate buffer 80 mM (pH 3.0) and acetonitrile (65:35, v/v) at 1.0 mL/min. Excitation wavelength at 230 nm and emission wavelength at 295 nm.

keeping the rest constant, regarding to sensitivity, peak resolution, and run time. Phosphate, acetate, and borate buffers from pH 2 to 10 at concentrations between 20 and 100 mM, combined with either methanol, acetonitrile or mixtures of them as organic modifiers, also at different percentages, were tested as mobile phases. The optimal separation of the analytes was achieved using 80 mM phosphate buffer (pH 3.0) and acetonitrile (65:35, v/v) as mobile phase. Under these conditions, the influence of the flow rate on the separation was studied from 0.5 to 2 mL/min. The best compromise between run time, separation, efficiency, peak width and column backpressure was obtained at 1.0 mL/min. Likewise, the effect of temperature of the chromatographic column on the separation was studied varying this parameter between 18 and 50 °C. A temperature of 40 °C was found selected because it provided the best resolution between all peaks in a short run time. As an example, the chromatograms corresponding to a blank urine and a to a urine spiked with 100 ng/mL of the analytes are shown in Fig. 1. As can be seen, an excellent separation of the analytes was achieved in a run time of 10 min without matrix interferences.

3.2. Validation of the method

Peak area and height at the excitation and emission wavelengths set up previously were the analytical signals monitored throughout the validation process.

The precision of the chromatographic separation was evaluated by injecting a standard solution of all the analytes at 0.1 mg/L under the optimized conditions, nine times a day during two consecutive days. The results for intra-day and inter-day precision, in terms of relative standard deviation (RSD) of retention times and peak areas are shown in Table 1. These values were similar to those described in the literature [22,45,46].

Limits of detection (LOD) and limits of quantification (LOQ) were calculated using the maximal sensitivity allowed by the system and calculating the standard deviation (SD) of this response. LOD and LOQ were estimated by multiplying the SD of blanks by a factor of 3 and 10, respectively. These calculated LODs and LOQs were subsequently validated by the analysis of six standards prepared at their respective concentrations of all the compounds (Table 2).

The analytical curves were constructed using blank urine spiked with standard solutions in the range of 1.0–1000 ng/mL. The corresponding regression equation and other characteristic parameters for the determination of six compounds are shown in Table 2. The analytical curves exhibit excellent linear behavior over the examined concentration range.

The accuracy of the method was evaluated by doing recovery studies of a urine sample spiked with a known amount of the analytes. In different volumetric flasks, urine samples were enriched by adding aliquots of the standard solutions of LE, CIT and their metabolites so that the final concentrations were 50, 75, 200 and 750 ng/mL. These samples were then analyzed by triplicate according to the proposed method. The results are shown in Table 3 and, as can be seen, good recoveries, between 85 and 103%, were obtained.

Ruggedness can be regarded as a measurement of the absence of external influences on the results, whereas robustness measures the lack of internal influences on these ones [47]. In this work, we have tested the influence of variations in both internal (flow rate, temperature, etc.) and external parameters (different days, different patients) at different levels, that is, to study the robustness and ruggedness, respectively. The factors (A–K) and the variations (±) affecting the optimized procedure selected for our model are presented in Table 4. A Plackett–Burman model for the evaluation of both robustness and ruggedness effects (11 factors and 12 experiments) was used and the effects of varying the levels of the factors were investigated on the most critical chromatographic responses of the method. The ranked effects for every selected factor on a

Table 1
Precision.

Compounds	Intra-day precision RSD (%), $n=9$			Inter-day precision RSD (%), $n=2$		
	t_R	Peak area	Height	t_R	Peak area	Height
LE	0.188	1.134	2.071	0.803	1.272	2.125
ME-LE	0.225	1.681	2.162	0.275	1.877	2.312
CIT	0.167	5.019	1.631	1.165	4.721	1.842
DCIT	0.192	5.409	1.541	1.003	5.403	1.736
DDCIT	0.196	5.206	0.985	0.983	5.715	1.469
CIT-NO	0.172	2.596	1.781	1.022	2.732	2.006

Table 2
Statistical parameters of the chromatographic method.

	Calibration equation	R^2	Linearity (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
LE	$Y=(1.3\times10^4\pm110.4)X+(1.8\times10^4\pm2.1\times10^4)$	0.999	1–1000	0.09	0.27
ME-LE	$Y=(7.1\times10^3\pm60.6)X+(3.6\times10^4\pm1.1\times10^4)$	0.999	1–1000	0.14	0.42
CIT	$Y=(893.2\pm7.7)X+(1.1\times10^4\pm1.5\times10^3)$	0.998	2.5–1000	0.47	1.41
DCIT	$Y=(959.4\pm8.7)X+(1.6\times10^3\pm1.6\times10^3)$	0.999	2.5–1000	1.0	3.0
DDCIT	$Y=(651.2\pm13.7)X+(1.3\times10^4\pm2.6\times10^3)$	0.999	2.5–1000	0.29	0.87
CIT-NO	$Y=(1.1\times10^3\pm10.4)X+(9.3\times10^3\pm2\times10^3)$	0.997	2.5–1000	0.55	1.65

LODs, limits of detection; LOQs, limits of quantification.

Table 3
Accuracy of chromatographic method.

Sample compounds	Added (ng/mL)	%R	Added (ng/mL)	%R	Added (ng/mL)	%R	Added (ng/mL)	%R
LE	50	92.9	75	93.8	200	101.2	750	102.2
ME-LE	50	90.8	75	97.3	200	92.3	750	90.1
CIT	50	91.9	75	96.1	200	103.1	750	94.3
DCIT	50	92.9	75	95.9	200	95.4	750	94.9
DDCIT	50	94.5	75	93.2	200	98.9	750	89.9
CIT-NO	50	94.4	75	98.5	200	97.8	750	97.5

Table 4
Variables selected as factors and values chosen as levels.

Factors	External/internal	Optimal	Level (−)	Level (+)
A. Different days	External	–	1	2
B. Different buffers	External	–	1	2
C. Different patients	External	–	1	2
D. [Buffer] (mM)	Internal	80	79	81
E. ACN (%)	Internal	35	34	36
F. pH	Internal	3.0	2.9	3.1
G. Flow rate	Internal	1.0	0.9	1.1
H. Column temperature	Internal	40	39	41
I. λ_{exc} (nm)	Internal	230	229	231
J. λ_{em} (nm)	Internal	295	294	296
K. Column	Internal	–	1	2

Table 5
Values R^2.

	LE	ME-LE	CIT	DCIT	DDCIT	CIT-NO
R^2 (tr)	0.983	0.982	0.984	0.994	0.990	0.987
R^2 (area)	0.977	0.992	0.995	0.979	0.979	0.992
R^2 (height)	0.996	0.993	0.989	0.999	0.995	0.996

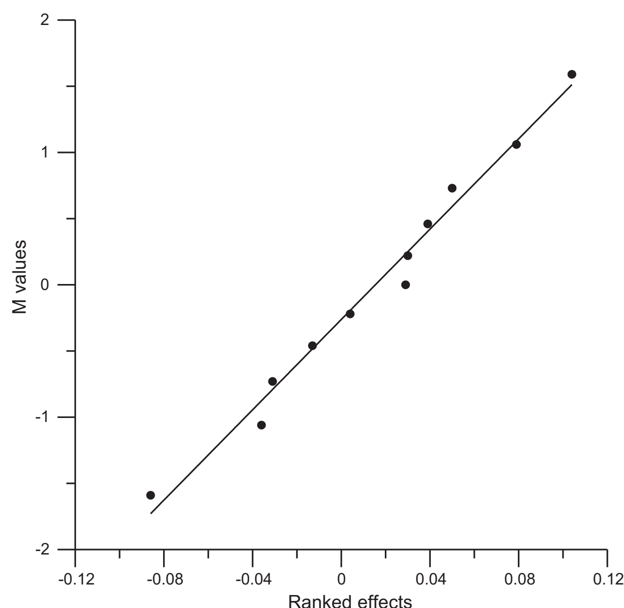

Fig. 2. Values of M for the retention time of CIT versus ranked effects of the 11 selected factors.

specific chromatographic response were calculated by simple addition of its (−) and (+) assay test results, and dividing the total result by half the number of samples. The M values are statistic constants for any given design table with a number of 11 elements, which are the factors in our case [48]. Finally, the obtained ranked effects for the selected 11 factors were plotted (on the x-axis, in increasing order) against the M values (on the y-axis) for each critical chromatographic response. The results from this plot should be close to a straight line. If a value lies outside this straight line, it can be concluded that the method is not rugged/or robust (as classified by its corresponding factor). However, if the results from the plot follow a (nearly) straight line, it can be concluded that the analytical method is rugged and robust over the conditions tested in the run design.

As an example, the plot corresponding to the ranked effect of the 12 selected factors versus M values for the retention time of CIT is shown in Fig. 2. Since similar results were obtained in all cases, it can be stated that the presented procedure is robust and rugged in the terms explained above (Table 5).

3.3. Applications

The presented method was applied to urine samples from patients A, B and C, treated with LE, CIT and both, respectively. Chromatograms of samples from patients A, B and C obtained at 7, 4, and 3.5 h after intake are shown in Fig. 3. The quantification of drugs and metabolites was carried out by both external calibration and standard addition methods.

Urine from patient A was analyzed by duplicate at different times after its administration in order to obtain pharmacokinetic information. Thus, the urinary excretion profile of LE and ME-LE is presented in Fig. 4. As can be seen, LE strongly dominates over its metabolite, showing maximal excretion 14 h after intake.

Urine samples from patients B and C could only be collected 15 and 3 h after oral drug intake, respectively, and they were also analyzed by duplicate. The concentrations of the analytes in these samples are in Table 6, together with the concentrations in patient A, 14 h after intake.

In patient B, the dominant species were the metabolites DDCIT and DCIT, followed by the parent compound, CIT. The least abundant metabolite CIT-NO was only 11% of the main metabolite.

Patient C was the only one that had LE and CIT simultaneously. The concentrations of LE and ME showed an excellent agreement with the pharmacokinetics of LE previously studied in patient A for a time of 3 h (Fig. 4). Concerning to CIT, in this case it is the drug that dominates by 2.5 times over its main metabolites, DDCIT and DCIT, which is the opposite to what happened to patient B. This could be explained because urines were sampled at different times after intake so we have two distant points of the pharmacokinetic curve of CIT. In fact, Rodriguez et al. [44] reported the pharmacokinetic behavior of CIT and its metabolites, and found that the concentration of CIT in urine increased up to 10 h after intake, and then dramatically decreased up to 14 h, when the concentrations of the main metabolites were higher than that of the parent compound.

The results of our study showed that the highest concentration of LE and its metabolite were found 11 h later after the dosing. The presence of the LE in the urine at 24 h is around 25 ng/mL and the

Table 6
Analysis of human urine samples.

	Woman A		Woman B		Woman C	
	Standard addition (ng/mL)	External standard (ng/mL)	Standard addition (ng/mL)	External standard (ng/mL)	Standard addition (ng/mL)	External standard (ng/mL)
LE	59.4	60.1	–	–	33.6	35.2
ME-LE	4.6	4.4	–	–	–	–
CIT	–	–	182.5	183.4	508.1	510.3
DCIT	–	–	200.2	200.8	183.8	184.2
DDCIT	–	–	215.1	213.9	191.9	191.5
CIT-NO	–	–	24.6	25.2	29.7	30.2

Woman A: urine taken after 14 h of letrozole administration; woman B: 15 h; woman C: 3 h.

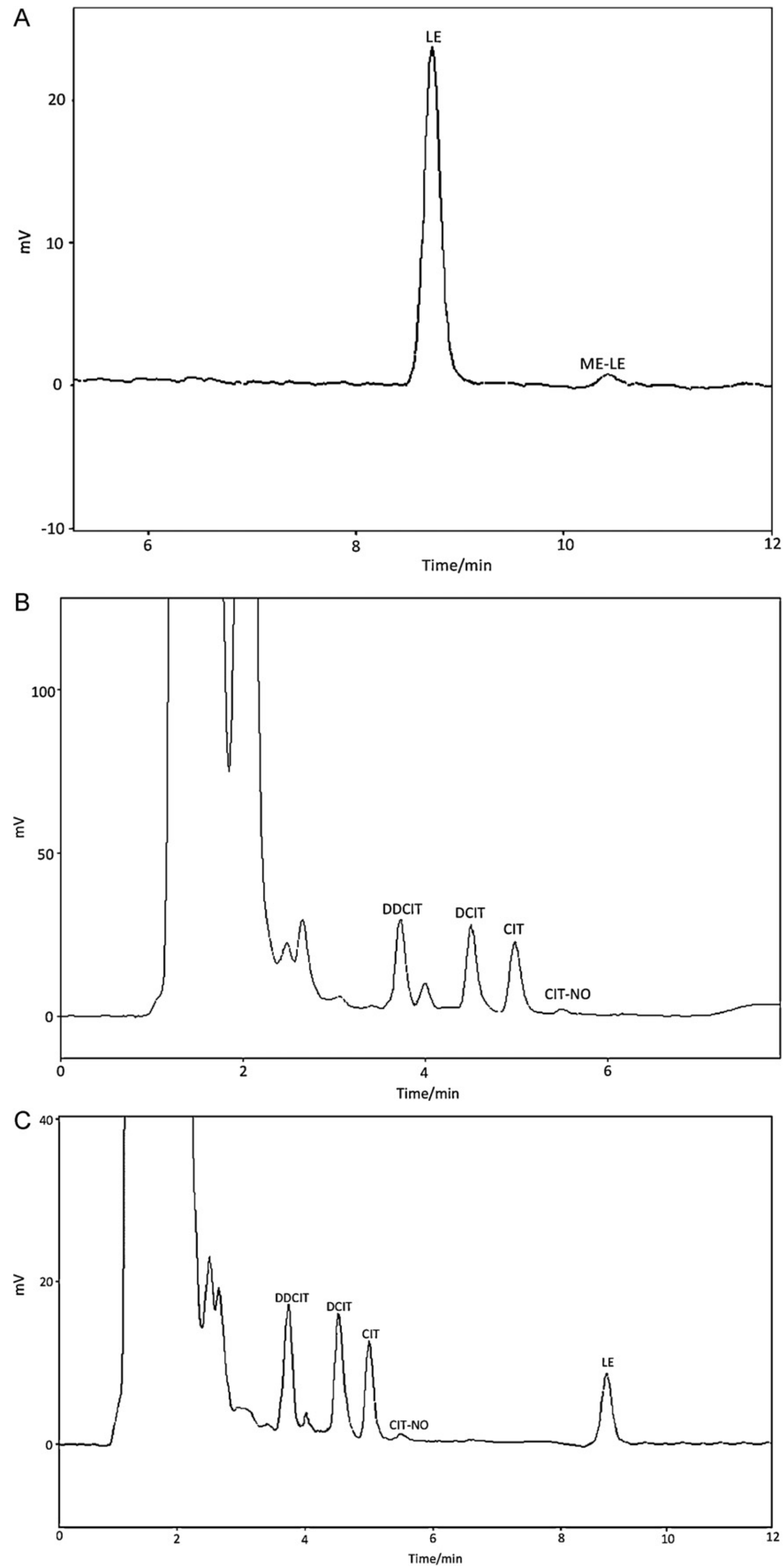

Fig. 3. Chromatograms corresponding to urine samples analyzed after drug intake. (A) Patient A (7 h after LE intake). (B) Patient B (4 h after CIT intake). (C) Patient C (3.5 h LE and CIT intake). Operating conditions: mobile phase was phosphate buffer 80 mM (pH 3.0) and acetonitrile (65:35, v/v) at 1.0 mL/min. Excitation wavelength at 230 nm and emission wavelength at 295 nm.

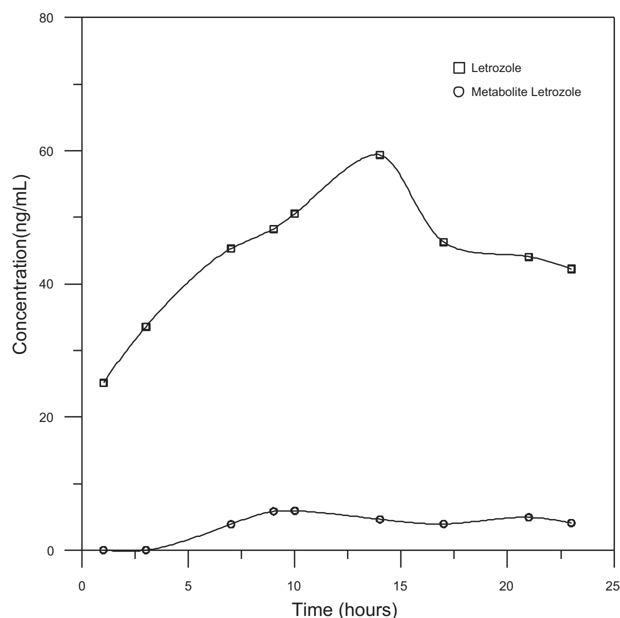

Fig. 4. Urinary excretion profile of LE and ME-LE in a patient treated with 2.5 mg/day of LE. Operating conditions: mobile phase was phosphate buffer 80 mM (pH 3.0) and acetonitrile (65:35, v/v) at 1.0 mL/min. Excitation wavelength at 230 nm and emission wavelength at 295 nm.

absence of ME-LE, shows that the 24 h is time enough to get the next dose of this drug (2.5 mg/day). Letrozole and metabolite are excreted mainly via the kidneys but the urine concentrations of letrozole can show a wide variability among patients.

The pharmacokinetics showed that the presented analytical methodology is useful to obtain relevant and complex clinical information related to bioactivity, $t_{1/2}$, excretion, etc., for this drug and its main metabolite.

4. Conclusions

The developed HPLC-FLD procedure permits the quantification of LE, CIT and their metabolites, minimizing laborious and complicated sample preparation procedures. The selectivity of the fluorescence detector avoids the presence of endogenous and exogenous interfering compounds.

This method is suitable for the analysis of urine samples due to its high sensitivity and selectivity. Also the precision and accuracy obtained in samples confirms its benefits.

This method can also be used as a complementary clinical tool for the evaluation of symptoms produced by interactions between LE and CIT and, consequently, for the establishment of a more efficient and safe dosing.

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