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## Simultaneous quantification of four hormone therapy drugs by LC-MS/MS: Clinical applications in breast cancer patients

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1 **Simultaneous quantification of four hormone therapy**  
2 **drugs by LC-MS/MS: clinical applications in breast cancer**  
3 **patients**

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## 17 **Abstract**

### 18 **Introduction**

19 Aromatase inhibitors such as anastrozole, letrozole, exemestane and selective estrogen down-  
20 regulator (SERD) fulvestrant are used mostly to treat breast cancer estrogen receptor positive  
21 in post-menopausal women. These drugs are given either through the oral route or by  
22 intramuscular injection. They have shown great inter-individual variability with a risk of  
23 cardiometabolic disorders. Hence the importance of their therapeutic drug monitoring not  
24 only for exposure-efficacy but also exposure-toxicity. We describe here a LC-MS/MS method  
25 for the simultaneous quantification of anastrozole, letrozole, exemestane and fulvestrant in  
26 human plasma.

### 27 **Material and methods**

28 Plasma samples were prepared by a single-step protein precipitation. The liquid  
29 chromatography system was paired with a triple quadrupole mass spectrometer.  
30 Quantification were achieved in Multiple Reactions Monitoring mode and the electrospray  
31 ionization was in positive mode.

### 32 **Results**

33 The method demonstrated consistent analytical performance across various parameters,  
34 including linearity, specificity, sensitivity, matrix effect, upper and lower limits of  
35 quantification, extraction recovery, precision, accuracy, hemolysis effect, dilution integrity,  
36 and stability under different storage conditions, in accordance with established guidelines.

37 The analysis time for each run was 4 minutes. Calibration curves exhibited linearity within the  
38 1-100 ng/ml range, with correlation coefficients  $> 0.99$  for the four analytes. Plasma  
39 concentrations from 42 patients were integrated into the selected calibration.

40 Stability assessments indicated that the four drugs remained stable at  $-20^{\circ}\text{C}$  for three months,  
41 15 days under refrigeration, up to 7 days at room temperature, and after three freeze-thaw  
42 cycles

### 43 **Conclusion**

44 We have developed and validated this quantitative method for therapeutic drug monitoring of  
45 those four hormone therapy drugs: anastrozole, letrozole, fulvestrant and exemestane. This  
46 method can be also used for future clinical pharmacokinetics /pharmacodynamics studies.

47 **Keywords:** anastrozole, exemestane, letrozole, fulvestrant, mass spectrometry, Therapeutic  
48 drug monitoring (TDM)

## 49 **1 Introduction**

50 Breast cancer is the most prevalent cancer among women in Europe and is the leading  
51 cause of cancer-related death. The worldwide incidence of breast cancer is estimated to be 2.3  
52 million cases [1]. Approximately 80% of breast cancers are estrogen receptor (ER)–positive:  
53 in such cases, cancer cells proliferate in response to estrogen. Hormone therapy with drugs  
54 such as aromatase inhibitors (AIs) and selective ER downregulators (SERDs) is indicated in  
55 the treatment of this type of cancer in mostly postmenopausal women and to a lesser extent,  
56 premenopausal women when combined with a central blocking. These agents can either  
57 inhibit synthesis of estrogen or cause the degradation of its receptor [2].

58 AIs compete with aromatase, preventing it from converting androgen into estrogen.  
59 Commonly prescribed AIs are anastrozole (ANA), letrozole (LET), and exemestane (EXE)  
60 [3]. Analogous in structure to the natural substrate of aromatase, EXE is a steroidal compound  
61 that inactivates the enzyme by irreversibly binding to it, whereas ANA and LET bind  
62 reversibly to aromatase. Fulvestrant (FUL), a steroidal SERD, is a high-affinity ER  
63 antagonist. When FUL binds to ERs, it induces their degradation [4]. Accordingly, estrogen  
64 levels could serve as biomarkers of treatment efficacy. However, estrogen concentrations  
65 found in patients taking LET or EXE [5] can be subpicomolar, and the quantitative  
66 determination of estrogen in plasma relies on an expensive albeit very sensitive mass  
67 spectrometry method [6].

68 AIs and FUL are administered differently: AIs are taken orally on a daily basis, while  
69 FUL is given intramuscularly once a month. The half-lives of AIs—ANA (48–96 h), LET  
70 (48 h), EXE (24 h)—and FUL (50 d) also differ greatly[7]. These drugs are administered at  
71 fixed doses despite great interpatient variability in concentrations of AIs and FUL: for  
72 example, patients' plasma LET concentrations can vary from 17.0 to 301.3 ng/mL [8].

73 As adjuvant therapy with AIs can last over 5 years, exposure-toxicity evaluation is of  
74 value for therapeutic drug monitoring purposes. The literature offers no definitive exposure-  
75 efficacy studies [3, 9]. However, the review by Nabholz et al. [10] proposed target  
76 concentrations for ANA ( $C_{\min} \geq 34.2$  ng/mL), EXE ( $C_{\max} = 4.1$  ng/mL), and LET ( $C_{\min} \geq 85.6$   
77 ng/mL). The same results was found with a promising TDM recommendation for LET and  
78 exploratory TDM recommendation for ANA and EXE [5]. ANA has a linear relationship  
79 between dose and exposure across doses ranging from 0.5 to 10 mg [11]. Considering the  
80 available data, it may be prudent to contemplate dose escalation of ANA for patients with a  
81  $C_{\min}$  below 34.2 ng/mL [12]. EXE undergoes extensive metabolism, with 17-hydroxy-  
82 exemestane being the sole active metabolite but with a concentration ten times lower than  
83 exemestane concentration and with a less anti-estrogenic effect [5]. Exemestane has not  
84 demonstrated a clear relationship between exposure and toxicity. Generally, it is well-  
85 tolerated, as evidenced by phase I studies where single doses of up to 800 mg and multiple  
86 doses of up to 200 mg were administered, and it has a linear dose-exposure relationship [13].  
87 In the absence of a defined exposure–efficacy target, the median  $C_{\max}$  of 7.7 ng/mL could  
88 serve as a reference for therapeutic drug monitoring (TDM), correlating with a calculated  
89 trough concentration of 4.1 ng/mL [5]. LET has shown a linear dose-exposure relationship  
90 up to the standard dose of 2.5mg once a day while at higher doses the exposure is non-  
91 linear. Until then, the target for LET TDM is  $C_{\min} \geq 85.6$  ng/ml [5]. A pharmacokinetics study  
92 of FUL has proved that  $C_{\max}$  and  $AUC_{(0-28)}$  demonstrate a dose-dependent increase and that  
93 the exposure was proportional to the dose [14].

94 Monitoring of these hormone therapy drugs could offer a better understanding of  
95 pharmacokinetic profiles, such as exposure-response and exposure-toxicity; permit evaluation  
96 of patient compliance; and provide clinicians a tool for personalized breast cancer treatments.  
97 The method presented here aims to simultaneously quantify these commonly prescribed

98 agents after simple pretreatment of plasma samples and has the advantage of rapid run time  
99 for routine laboratory application.

## 100 **2 Materials and methods**

### 101 **2.1 Chemicals and reagents**

102 ANA, LET, EXE, FUL, anastrozole-13C4, fulvestrant-D5, and letrozole-13C2,15N2  
103 were purchased from AlsaChim (Illkirch, France), their purity was higher than 98%.  
104 exemestane-13C,D3, from Toronto Research Chemicals (Toronto, Canada), its purity was  
105 higher than 95%; and methanol, acetonitrile, and DMSO, from Carlo Erba Reagents (Val-de-  
106 Reuil, France), Merck KGaA (Darmstadt, Germany), and PanReac AppliChem (Schiltigheim,  
107 France), respectively. Water used was purified with an ELGA PURELAB system.

108 Drug-free, blank plasma was supplied by the Établissement Français du Sang (French  
109 Blood Establishment) in Paris, France. All reagents used were of the highest available  
110 analytical grades.

### 111 **2.2 Preparation of stock solutions, standards, and quality control samples**

112 Stock solutions were prepared in different solvents: ANA was dissolved in  
113 acetonitrile; EXE in DMSO; and LET and FUL in methanol. Individual stock solutions of  
114 each analyte had concentrations of 1 mg/mL. Quality control samples (QCs) and calibration  
115 standards were obtained by dilution of these stock solutions in methanol:water (50:50, v/v).  
116 QCs were prepared in blank plasma at concentrations of 5, 45 and 90 ng/mL (QC1, QC2 and  
117 QC3, respectively). This quantitative method covers a range of 1 to 100 ng/mL (1, 10, 25, 50,  
118 75, 100 ng/mL) for the four compounds.

119 For samples with a concentration above the linear range, a fourth QC was added  
120 (QC4) at concentrations of 150 ng/mL for ANA, 200 ng/mL for EXE, 400 ng/mL for FUL,  
121 and 1,000 ng/mL for LET, corresponding to the upper limits of quantification (ULOQs)  
122 identified.

123 Internal standard (IS) stock solutions were prepared at a concentration of 1 mg/mL. A  
124 mixture of anastrozole-13C4, exemestane-13C,D3, letrozole-13C2,15N2, and fulvestrant-2H5  
125 was diluted in methanol:acetonitrile (50:50, v/v) to obtain a 75 ng/mL solution.

### 126 **2.3 Analytical conditions**

127 An Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7-μm particle size) (WATERS,  
128 Milford, Massachusetts, US) was used for chromatographic separation. The Nexera® LC40  
129 LC system (Shimadzu Corporation, Kyoto, Japan) was paired with a triple quadrupole mass  
130 spectrometer (LCMS-8060NX, Shimadzu Corporation, Kyoto, Japan) equipped with a 20μL  
131 injection loop. The mobile phase was composed of 0.1% formic acid and acetonitrile and had  
132 a flow rate of 0.4 mL/min. The analysis run time was 4 min. Quantification were achieved in  
133 Multiple Reaction Monitoring (MRM) mode, and electrospray ionization (ESI) was in  
134 positive mode. Transitions (m/z)—294.20 > 225.20 for ANA, 286.10 > 190.10 for LET,  
135 297.20 > 121.10 for EXE, and 607.30 > 589.40 for FUL—were monitored. Data acquisition  
136 and quantification were performed using Labsolution V5.99 SP2 (Shimadzu Corporation,  
137 Kyoto, Japan) and Insight V3.7 SP3 (Shimadzu Corporation, Kyoto, Japan), respectively.

### 138 **2.4 Sample pretreatment**

139 Plasma samples were prepared by one-step protein precipitation: 150 μL of IS solution  
140 was added to 50 μL of patient plasma, a calibrator sample, or a QC. The tube was agitated for  
141 5 min at 2,000 rpm and centrifuged for another 5 min at 13,000 rpm. Next, 100 μL of

142 supernatant was diluted in 100  $\mu\text{L}$  of water and a volume of 10  $\mu\text{L}$  was injected into the LC-  
143 MS/MS system.

## 144 **2.5 Method validation**

145 Validation was conducted in accordance with guidelines of the European Medicines  
146 Agency (EMA) and industry guidance from the US Food and Drug Administration (FDA) for  
147 bioanalytical method validation [15-17]. The parameters verified were selectivity, specificity,  
148 matrix effect, linearity, accuracy and precision, lower limit of quantification (LLOQ) and  
149 upper limits of quantification (ULOQ), carryover, stability, extraction recovery, and dilution  
150 integrity. Hemolysis effect, as described by D’Cunha et al. [18], was also tested.

### 151 **2.5.1 Lower and upper limits of quantification**

152 For each analyte, the LLOQ was matched to the lowest plasma concentration  
153 quantifiable with reliable accuracy and precision, corresponding to a coefficient of variation  
154 (CV) and bias under 20%; and the ULOQ, to the highest quantifiable plasma concentration,  
155 with a CV and bias under 15%.

### 156 **2.5.2 Selectivity and specificity**

157 We tested for drugs potentially associated with these compounds that could interfere  
158 with analyte quantification. Patient plasma samples containing these drugs, but none of the  
159 analytes, were selected. Thus, plasmas from 6 different patients treated with everolimus,  
160 sirolimus, posaconazole, voriconazole, aciclovir, ganciclovir, or ribociclib were used. Spiked  
161 plasma samples were used for tamoxifen and palbociclib. No interference was detected when  
162 the peak area for the plasma containing a cotreatment was <20% of the peak area for the first  
163 calibration standard at the retention time of the analyte. The selectivity and specificity of the  
164 IS was tested through the same procedure but with a limit of 5%.



### 165 **2.5.3 Stability**

166 The stability of the four molecules was measured under different conditions: after  
167 short-term storage for 7 days at room temperature, short-term storage for 15 days when  
168 refrigerated at 4 °C, long-term storage for a total period of 3 months at -20 °C, 3 freeze-thaw  
169 cycles (from -20 °C to room temperature), and leaving in autosampler at 15 °C for 9 days.

170 Stability was assessed by comparing accuracy and precision of QCs at two  
171 concentration levels (QC1 and QC3) before and after exposure to the aforementioned  
172 conditions. Analytes were considered stable when measured concentrations were within ±15%  
173 of the baseline QC3 concentration and ±20% of the baseline QC1 concentration. All QC  
174 levels were measured in triplicate. These tests sought to reproduce all possible storage  
175 conditions to which patient samples could be exposed during routine laboratory operations.

### 176 **2.5.4 Dilution integrity**

177 Dilution integrity was tested to determine whether quantification of a sample, diluted  
178 with blank plasma, whose initial concentration was above the validated calibration range is  
179 precise and accurate. Spiked samples with a concentration above the ULOQ were diluted (1:5  
180 or 1:2) with blank plasma to levels falling within the calibration range. Precision and accuracy  
181 were considered satisfactory when the measured concentration of the diluted sample did not  
182 deviate from the nominal concentration by >15%.

### 183 **2.5.5 Linearity**

184 A weighting factor of 1/X was applied to the linear calibration curve. The calibration  
185 curve was plotted with 6 calibration points (G1 to G6) by determining the ratio of the analyte  
186 peak area to the IS peak area ( $r^2 > 0.99$ ). The validation criteria were: ( $r^2 > 0.99$ ), both bias  
187 and CV of the nominal concentration had to be < 15% for all standards ( G2 to G6) and

188 <20% for the G1. The calibration curve follows a linear regression with a 1/x weighting  
189 factor.

### 190 **2.5.6 Matrix effect, hemolysis effect, and extraction recovery**

191 The matrix effect was evaluated for two QC concentration levels (QC1 and QC3).  
192 Freshly prepared QCs and spiked blank plasma were prepared on the same day. Selected  
193 plasma samples were drawn from patients taking everolimus, sirolimus, posaconazole,  
194 voriconazole, aciclovir, ganciclovir, itraconazole, or ribociclib. The CV of the IS-normalized  
195 matrix factor should be <15%, and the peak area of the spiked plasma should not deviate by  
196 more than 15% of the peak area for QC1 and QC3 levels.

197 In the preanalytical phase, hemolysis could be a source of interference. Hemolysis  
198 effect was tested according to the method described by D’Cunha et al. [18]. The  
199 concentrations of freshly prepared QC1 and QC3 ( $n = 6$ ) were compared with that of 2%-  
200 hemolysis spiked blank plasma extracted in sextuplicate ( $n = 12$ ).

201 Extraction recovery was evaluated for three QC concentration levels (QC1, QC2, and  
202 QC3). No acceptance criteria were established, but CV should be homogenous for all levels  
203 and ideally should not deviate by >20%.

### 204 **2.5.7 Carryover**

205 This parameter was evaluated after an autopurge and tested at the highest (G6) and  
206 lowest (G1) calibration levels. A sequence of three G6 samples followed by three G1 samples  
207 was repeated four times. A G1 sample was injected before this sequence, and its peak area  
208 was used as the reference peak area for the analyte and the IS area. Carryover is not observed  
209 if G1 peak areas are <20% of the reference peak area and <5% of the reference IS area.

### 210 **2.5.8 Precision and accuracy**

211 Intra-day precision and accuracy were measured in sextuplicate ( $n = 6$  for samples at  
212 each QC concentration and G1). Inter-day precision and accuracy were evaluated by  
213 measuring sample at each QC concentration and G1 ( $n = 18$ ) in triplicate over 6 days. CV (%)  
214 and bias (%) should be within 20% for G1 and QC1, and 15% for QC2, QC3, and QC4.

### 215 **2.6 Clinical applications**

216 This validated LC-MS/MS plasma quantification method was applied to a cohort of  
217 15 female breast cancer patients, with a mean age of 63 years. A total of 42 samples of plasma  
218 were analyzed, from patients treated with LET ( $n = 37$ ), EXE ( $n = 5$ ), and FUL ( $n = 4$ ). The  
219 samples of plasma were collected at different time points in the treatment cycle: before  
220 treatment initiation, before achieving a steady state, at steady state, or after the end of  
221 treatment. All LET-treated patients received concurrent ribociclib therapy at a dose of 600  
222 mg, administered once daily 3 weak/month. One patient, who was also undergoing FUL  
223 treatment (administered monthly at a dosage of 500 mg), was additionally treated with EXE at  
224 25mg and ribociclib at 600mg.

## 225 **3 Results**

### 226 **3.1 Optimization of LC-MS/MS conditions**

227 Parameters chosen for each analyte are described in **Table 1**. Phase A was 0.1%  
228 formic acid; phase B, acetonitrile. The chromatograms of LLOQ, blank plasma, and patient  
229 plasma peaks are presented in **Figure 1**.

## 230 **3.2 Method validation**

### 231 **3.2.1 Precision and accuracy**

232 For all QC concentrations and G1, intra-day and inter-day precision and accuracy met  
233 the defined criteria (within 20% for G1 and QC1, and within 15% for QC2, QC3, and QC4)  
234 (Table 2).

### 235 **3.2.2 Stability**

236 The four analytes were stable after short-term storage at room temperature for 7 days,  
237 refrigeration at 4 °C for 15 days, and storage at -20 °C for 3 months. All analytes were stable  
238 after 3 freeze-thaw cycles, following extraction. Extracts were stable for  $\geq 9$  days when kept in  
239 the autosampler at 15 °C. (Table 3).

### 240 **3.2.3 Selectivity and specificity**

241 In all, 9 molecules were tested, including immunosuppressants (everolimus and  
242 sirolimus), antifungals (posaconazole and voriconazole), antivirals (aciclovir and ganciclovir),  
243 tyrosine kinase inhibitors (ribociclib and palbociclib), and tamoxifen. None presented a peak  
244 area >20% of that for G1 or the IS peak.

### 245 **3.2.4 Lower and upper limits of quantification**

246 LLOQs were 0.4 ng/mL for ANA, 0.3 ng/mL for LET, 0.8 ng/mL for EXE, and  
247 1 ng/mL for FUL, each LLOQ corresponding to the lowest concentration with a CV of <20%  
248 and an accuracy of 80%–120%. ULOQs were 150 ng/mL for ANA, 1,000 ng/mL for  
249 LET, 200 ng/mL for EXE and 400 ng/mL for FUL, each ULOQ corresponding to the highest  
250 concentration with a CV of <15% and an accuracy of 95%–115%.

251 **3.2.5 Linearity**

252 Calibration curve equations for the 4 analytes are given in **Table 1**. Coefficients of  
253 correlation ( $r^2$ ) were all  $>0.9900$ . Calibrator CVs were  $\leq 10.54\%$  and accuracy was  $95.28\%$ –  
254  $107.02\%$  (**Table 4**).

255 **3.2.6 Matrix effect, hemolysis effect, and extraction recovery**

256 Plasma of patients treated with four different drug classes were selected: antivirals  
257 (ganciclovir and aciclovir), immunosuppressants (everolimus and sirolimus), antifungals  
258 (posaconazole and itraconazole), and lastly, a tyrosine kinase inhibitor (ribociclib). No  
259 significant matrix effect was observed. Matrix effect ranges were  $82.7\%$ – $108.1\%$  (QC1) and  
260  $92.1\%$ – $112.1\%$  (QC3) for ANA;  $88.4\%$ – $106.5\%$  (QC1) and  $90.1\%$ – $110.1\%$  (QC3) for LET;  
261  $81.3\%$ – $117.5\%$  (QC1) and  $91.6\%$ – $105.2\%$  (QC3) for EXE;  $86.9\%$ – $120.7\%$  (QC1) and  
262  $87.0\%$ – $112.9\%$  (QC3) for FUL. Mean matrix effects for each analyte are given in **Table 5**.  
263 IS-normalized matrix factor CVs were  $2.32\%$  (QC1) and  $2.46\%$  (QC3) for ANA;  $9.21\%$   
264 (QC1) and  $1.72\%$  (QC3) for LET;  $4.84\%$  (QC1) and  $1.99\%$  (QC3) for EXE; and  $6.51\%$   
265 (QC1) and  $1.52\%$  (QC3) for FUL.

266 Concentrations of QC1 and QC3 samples prepared with 2% hemolyzed plasma did not  
267 diverge by  $>15\%$  from the QC1 and QC3 samples prepared with blank plasma. Hence, 2%-  
268 hemolyzed plasma did not appear to interfere with analyte quantification.

269 Extraction recovery data are provided in **Table 5**. CVs varied from  $-6.08\%$  to  $15.43\%$   
270 with all analytes except EXE, for which CVs were homogenous for all 3 concentrations but  
271 varied from  $20.33\%$  to  $23.78\%$ . Considering the linearity, accuracy, and precision of  
272 calibrator standards, no correction factor was applied for EXE quantification.

### 273 **3.2.7 Carryover**

274 No carryover was observed for any of the 4 analytes. Carryover percentages were all  
275 <0.112%. Furthermore, no G1 peak area exceeded 20% of the reference peak area and 5% of  
276 the reference IS peak area.

### 277 **3.2.8 Dilution integrity**

278 Both spiked and diluted samples were within the acceptable range of 15% for the  
279 4 analytes (**Table 6**). Samples with a concentration exceeding the calibration range can thus  
280 be diluted.

### 281 **3.3 Clinical application**

282 All patient plasma samples lay within the validated calibration range for this method:  
283 10.6–283.3 ng/mL for LET, 1.1–2.4 ng/mL for EXE, and 13.9 – 33.0 ng/mL for FUL. Patients  
284 were all treated with combination therapies representative of clinical practice. **Figure 2**  
285 presents analyte concentration ranges, excluding patients with plasma concentrations below  
286 LLOQ. There was no observed interference related to other drugs taken by patients.

## 287 **4 Discussion**

288 In this study, we describe a method for the simultaneous quantification of ANA, LET,  
289 EXE, and FUL in patient plasma samples for therapeutic drug monitoring, and for clinical  
290 research, to assess potential adverse effects of these 4 hormone therapy drugs.

291 Recent combination therapies involve the association of AIs or antiestrogens with  
292 other treatments, such as ribociclib, a TKI [19-22] which may causes cardiometabolic  
293 disorders. Quantification of these drugs may reduce the likelihood of adverse effects.

294 Several LC-MS/MS methods have been proposed in the literature for quantifying one  
295 or more hormone therapy drugs. To the best of our knowledge, however, no other method  
296 simultaneously quantifies ANA, LET, EXE, and FUL.

297 Matrix effect and hemolysis effect were tested, and the results were satisfying  
298 according to the guidelines of the FDA. No carryover was observed, and we are similar to  
299 another study that attempted to use a rinsing solution consisting of 50% methanol to eliminate  
300 the carryover they encountered at the beginning of the method validation for the following  
301 molecules, anastrozole and fulvestrant. Our rinsing solution is composed of 100% MeOH.  
302 The extraction recovery was satisfying and the results was similar to this studie [8].

303 For the quantification of FUL in human plasma, Turkovic et al. [23] successfully  
304 validated a LC-MS/MS method with a range of 5–1,000 ng/mL. Despite the limited number  
305 of samples in our study, the average concentration was found to be 21 ng/mL, with one outlier  
306 at 33 ng/mL. In contrast, Van Nuland et al. [24] reported low sensitivity with their method,  
307 necessitating an additional concentration step for sample extraction when dealing with EXE,  
308 along with a relatively large sample volume of 500  $\mu$ L. Our method permits rapid processing  
309 with short sample preparation and run times (4 min), requires a minimal plasma volume of  
310 50  $\mu$ L, and boasts wider ranges (**Table 7**).

311 For their clinical study, Beer et al. [8] reported concentration ranges of 6.2–102.0  
312 ng/mL (mean: 37.4 ng/mL) for ANA and 17.0–301.3 ng/mL (mean: 107 ng/mL) for LET. The  
313 study revealed significant interindividual variability for these concentrations. Importantly,  
314 these ranges are well within the linear ranges validated for our own method. As such, the  
315 measured plasma concentrations and those reported in the literature did not provide any  
316 compelling reason to further extend our current measurement ranges.

317 In previous research [25, 26], the maximum length of time for assessing short-term  
318 stability of the 4 molecules was 5 days. However, our results suggest that these analytes

319 remain stable for longer periods: up to 7days at room temperature and up to 15days when  
320 refrigerated at 4 °C. The long-term stability of ANA, EXE, and LET was considered by a  
321 previously published method, which confirmed that the molecules remained stable over a  
322 period of 21 weeks. Our study demonstrated their stability over a period of 3 months at  
323 -20 °C.

324 Limitations we faced included certain methodological constraints that hindered our  
325 ability to measure the metabolites of the 4 hormone therapy drugs as well as a lack of plasma  
326 samples from patients being treated with ANA.

## 327 **5 Conclusions**

328 In summary, we have developed and validated a quantitative method for therapeutic  
329 monitoring of ANA, LET, EXE, and FUL. Sample treatment is easy and effective, requiring  
330 only one simple precipitation. We have shown that small sample volumes are suitable for  
331 routine quantification of these analytes. The validated ranges include measured  
332 concentrations in patient plasma samples are aimed to be applied in wider and more diverse  
333 groups of patients and with the intention of describing eventual mechanisms to  
334 interindividual/intraindividual plasma variations. However, we encountered certain  
335 limitations, such as the scarcity of plasma samples from patients treated with those four drugs,  
336 especially anastrozole, and the lack of chemical reagents to expand our measurements and  
337 quantify the metabolites of the four analytes. This method could also apply to  
338 pharmacokinetic/pharmacodynamic studies and clinical trials

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343 validation, visualization, investigation, writing-original draft ,writing-review and editing. All  
344 authors have accepted responsibility for the entire content of this manuscript and approved its  
345 submission.

346 **Declaration of Competing Interest** The authors declare that they have no known competing  
347 financial interests or personal relationships that could have appeared to influence the work  
348 reported in this paper.

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