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► To cite this version:

Omar Ahmed, Yoann Ladner, Junyi Xia, Jérôme Montels, Laurent Philibert, et al.. A fully automated on-line salting-out assisted liquid-liquid extraction capillary electrophoresis methodology: Application to tyrosine kinase inhibitors in human plasma. *Talanta*, 2020, 208, pp.120391. 10.1016/j.talanta.2019.120391 . hal-03488525

HAL Id: hal-03488525

<https://hal.science/hal-03488525>

Submitted on 21 Dec 2021

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1 **A fully automated on-line salting-out assisted liquid-liquid extraction capillary electrophoresis**
2 **methodology: Application to tyrosine kinase inhibitors in human plasma**

3
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16 **Keywords:** automated sample preparation; desalting; in-line CE stacking; protein precipitation, tyrosine
17 kinase inhibitors (TKIs); salting-out assisted liquid-liquid extraction (SALLE).

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25 **Abstract**

26 A fully automated analytical methodology combining salting-out assisted liquid-liquid extraction (SALLE)
27 and capillary electrophoresis (CE) for the analysis of three Tyrosine Kinase Inhibitors (TKIs) in plasma
28 samples is proposed. The automated methodology, called A-SALLE-CE-UV, makes full use of the
29 advantages of both techniques by combining desalting, protein precipitation, automated liquid-liquid
30 extraction, in-line CE stacking and electrophoretic separation of analytes in plasma samples in a fully
31 integrated way. At first, the capillary is used to deliver appropriate micro-volumes of extraction agent
32 solutions (acetonitrile, salt) in the plasma sample. ACN and salting-out agent (NaCl) solutions are added
33 by pressure from outlet vials into the sample vial (inlet) containing human plasma sample spiked with
34 the three tested TKIs. After addition of both ACN and NaCl solutions, mixing is achieved by generating air
35 bubbles leading to a two phases separation and extraction of TKIs in the upper mostly organic phase
36 (ACN). The upper phase containing the TKIs is then injected and analyzed by CE-UV. Due to the presence
37 of ACN, the analytes are stacked in-line and successfully separated in the same capillary. The results
38 obtained in terms of limit of detection (LOD), limit of quantification (LOQ), sensitivity enhancement
39 factor (SEF), repeatability and linearity demonstrate the applicability of the proposed method for
40 possible therapeutic drug monitoring (TDM) of TKIs.

41

42 **1. Introduction**

43 Capillary electrophoresis (CE) is a separation technique extensively used for the analysis of a wide range
44 of drugs in biological matrices such as plasma [1]. However, the presence of interfering compounds (e.g.
45 protein, salts) in the sample can affect the analytical performances of CE methodologies causing peak
46 broadening, baseline distortion and a lack of specificity [2]. Therefore, sample preparation techniques
47 such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are frequently used prior to CE
48 analysis to remove proteins, desalt, concentrate the analytes of interest and obtain efficient
49 electrophoretic peaks [3,4]. However, these methods are based on the use of complex, long procedure
50 and expensive sample preparation protocols (multi-steps) which are not suitable for routine analysis. In
51 the last decade, several improvements of extraction techniques have been reported to address these
52 problems, such as the development of solid phase microextraction (SPME) and liquid phase
53 microextraction (LPME) techniques [5,6]. More recently, new LLE technique based on salting-out
54 assisted liquid–liquid extraction appeared to be well suited for the determination of drugs in plasma
55 samples as it allows desalting, deproteinization and extraction of analytes in the same procedure [7].
56 SALLE is based on the addition of salts (e.g. sodium chloride (NaCl)) into a sample solution composed of
57 a miscible mixture of water and an organic solvent (such as acetonitrile (ACN)), inducing a two phases
58 separation (organic phase from bulk aqueous phase) [8]. SALLE can be easily coupled with CE technique
59 (SALLE-CE-UV) since the high content of ACN in the upper phase allows the in-line CE stacking of the
60 analytes in the capillary even for injected sample volumes exceeding 80% of the capillary volume till the
61 detector window. Excellent peak efficiencies, good resolution and high sensitivity were obtained with
62 SALLE-CE-UV methodology for the analysis of drugs in plasma [7].

63 Since a few years, analytical strategies tend towards automation and on-line combination of sample
64 preparation procedures with separation systems such as liquid chromatography (LC), gas
65 chromatography (GC) and CE [9–11]. Indeed, automation offers many advantages including lab

66 efficiency, reduced human errors and lower operating cost per sample. Many works dealt with the
67 automation of LPME techniques including single drop microextraction [12,13], dispersive liquid-liquid
68 microextraction [9,14–17] and hollow fiber-based microextraction [18–20]. However, only two reports
69 mention the automation of SALLE. The first one is based on the oxidation of diclofenac with potassium
70 ferricyanide in an alkaline medium, was followed by the separation of the ACN phase from the
71 homogeneous sample solution, the simultaneous extraction of the derivative and in-line organic-phase
72 detection by an optical probe [21]. The second work is based on the aspiration of 1-octylamine, urine
73 sample solution containing tetracycline and salting-out agent solution (NaCl) into a mixing chamber of a
74 flow system followed by air bubbles mixing, resulting in isotropic solution formation [11]. After phase
75 separation, the micellar 1-octylamine phase containing analyte is mixed with methanol and analysed by
76 a LC-UV. Both methods require special technical modifications of analytical materials and the use of
77 additional instruments such as external syringe or peristaltic pumps, multi-port selection valves and
78 cone-shaped glass. This include homemade modifications, which are difficult to realize, making them not
79 suitable for routine analysis.

80 Herein, we describe the first demonstration of a fully automated SALLE capillary electrophoresis (CE)-UV
81 methodology, called A-SALLE-CE-UV. The applicability of the proposed A-SALLE-CE-UV methodology was
82 evaluated for the analysis of 3 tyrosine kinase inhibitors (TKIs) namely lapatinib ditosylate, erlotinib
83 hydrochloride and sorafenib in human plasma samples as proof-of-concept analytes. These basic
84 molecules are extensively used for the treatment of malignant tumors such as renal cell carcinoma, lung
85 cancer and breast cancer [22–24]. Different factors including the effects of filling schemes of the sample
86 vial by several solutions (ACN, salt), time/volume of mixing using air bubbles and the type of salting-out
87 agents on the performance of A-SALLE-CE-UV methodology were optimized. Several analytical
88 performances such as the limit of detection (LOD), the limit of quantification (LOQ), sensitivity
89 enhancement factor (SEF), repeatability, and linearity were determined.

90

91 **2. Experimental:**

92 *2.1. Chemicals and reagents:*

93 All reagents were of analytical grade and used as received without further purification. Acetonitrile
94 (ACN, purity \geq 99.9%) was from Honeywell Riedel de Haen (Seelze, Germany). 6-amino caproic acid
95 (purity \geq 99%) was from Acros organics (Geel, Belgium). Ammonium acetate (purity \geq 99%), potassium
96 chloride (KCl, purity \geq 99%), sodium chloride (NaCl, purity \geq 99.5%), sodium hydroxide (NaOH, purity \geq
97 99%), citric acid (purity \geq 99%), dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (Saint-
98 Quentin-Fallavie, France). Sorafenib (purity \geq 99%), erlotinib hydrochloride (purity \geq 99%) and lapatinib
99 ditosylate (purity \geq 99%) were purchased from Molekula (Munich, Germany).

100 *2.2. Apparatus and CE software:*

101 All CE separations were carried out using a Beckman P/ACE-MDQ instrument (Fullerton, USA) equipped
102 with a UV detection system. 600 μ L eppendorf tubes (Thermoscientific, Pittsburgh, USA) used as mixing
103 chamber between plasma sample, ACN and NaCl stock solution. The eppendorf containing the solutions
104 was placed in a plastic 2 ml vial (P/ACE™ MDQ, reference number 970657, France) covered with plastic
105 cap (P/ACE™ MDQ, reference number 144656, grey, France). The cap and the crown of the eppendorf
106 tube was cut to be adjusted in the CE plastic vial (see Figure 1S in supplementary information). 2 ml glass
107 vials (P/ACETM MDQ, reference number 358807, France) covered with vial caps (P/ACETM MDQ,
108 reference number 144648, red, France) were filled with other solutions. Data were acquired by 32 Karat
109 software (Version 8.0, Beckman Coulter, Fullerton, USA). Uncoated fused-silica capillaries (50 μ m ID)
110 were obtained from Polymicro Technologies (Phoenix, USA). The length of the capillary was 31 cm (21
111 cm effective length to the detector).

112 *2.3. Preparation of background electrolyte and stock solutions:*

113 The background electrolyte (BGE) was chosen based on our previous studies [7,25] and was a mixture of
114 citric acid (1800 mM) and 6-amino caproic acid (137 mM) at pH 2.0 and ionic strength (IS) 150 mM. BGE
115 composition with the desired IS and pH was calculated with the software "Peakmaster" 5.3 (Prague,
116 Czech Republic, <https://web.natur.cuni.cz/~gas/>). Stock solutions of erlotinib hydrochloride, sorafenib
117 and lapatinib ditosylate were prepared in DMSO solvent at the concentration of 1.0 g/L due to their low
118 solubility in water. NaCl, ammonium acetate and KCl solutions were prepared at a concentration of 25%
119 (m/v) in water. All stock samples were stored frozen at -20°C. All other solutions were stored in
120 refrigerator at 4°C.

121 *2.4. Samples preparation*

- 122 - Standard sample solution: relevant volumes of distilled water and the three TKIs stock solutions
123 were mixed in an eppendorf tube of 600 µL to obtain a final volume of 80 µL. Then, the
124 eppendorf tube containing the standard solution was placed in the CE plastic vials.
- 125 - Plasma sample solution: relevant volumes of plasma solution and the three TKIs stock solutions
126 were mixed in an eppendorf tube of 600 µL to obtain a final volume of 80 µL. Then, the spiked
127 plasma was mixed during 1 hour at room temperature. Then, the eppendorf tube containing the
128 spiked plasma solution was placed in the CE plastic vials.

129 *2.5. The A-SALLE-CE-UV procedure:*

130 New capillaries were pre-conditioned with 1.0 M NaOH for 3.0 min under 20 psi followed by distilled
131 water for 3.0 min under 20 psi. Then, the capillary was flushed with BGE for 5.0 min under 20 psi. The A-
132 SALLE-CE-UV procedure (Fig. 1) consisted of a commercially available CE apparatus, a silica capillary and
133 a 600 µL tube containing the standard or the plasma sample placed in a plastic vial (mixing chamber). At
134 first, the capillary was used as a tool to deliver 150 µL of ACN and 20 µL of NaCl from outlet vials to the
135 inlet vial holding the 600 µL tube filled with 80 µL of sample containing TKIs (60 psi pressure, reverse
136 mode) were applied (see video 1 in the supplementary information). This was followed by a mixing step

137 using air bubbles at 90 psi during 5.0 minutes (see video 2 in the supplementary information) leading to
138 two phases separation and extraction of TKIs in the upper phase. The capillary was rinsed with water (60
139 psi during 2.0 minutes) between each addition of liquids (sample, NaCl solution and ACN) in the 600 μ L
140 tube and dried with air (30 psi during 1.0 minutes), to start each filling of the sample vial from a cleaned
141 and empty capillary. This rinsing procedure was optimized to ensure that NaCl solution doesn't get in
142 contact with ACN to avoid any precipitation of NaCl inside the capillary. Then, the upper phase
143 containing TKIs was hydrodynamically injected (forward mode) in the capillary at 0.8 psi during 94
144 seconds to obtain an injection volume equivalent to 287 nL (70% of the capillary effective length). All
145 separations were carried out with an applied voltage of 15 kV. The capillary was thermostated at 25°C.
146 The capillary usage life was between 45 to 50 analysis. UV detection was performed at 254 nm. The
147 detailed A-SALLE-CE-UV methodology is mentioned in Table 1S in supplementary information. Corrected
148 peak areas were calculated by dividing each peak area by its corresponding migration time.

149

150 **3. Results and discussion**

151 Automation and on-line combination of sample preparation with analytical separation techniques fit
152 perfectly with the criteria required for analytical methods dedicated to routine analysis such as
153 therapeutic drug monitoring (TDM): simple, fast and cost-effective. TDM of TKIs is required to evaluate
154 the relation between dose and efficacy/toxicity of TKIs. As mentioned before, SALLE applies the salting-
155 out effect to separate water-miscible organic solvent such as ACN from aqueous/biological sample. The
156 separation will result in two phases: upper mostly organic and lower mostly aqueous. SALLE-CE-UV
157 methodology offers a number of features for the analysis of TKIs in human plasma including high
158 separation ability between TKIs, applicability to saline sample matrix, easy operational procedure, low
159 consumption of organic solvent, and environmental benignity.

160 *3.1. Preliminary Trials*

161 According to our previous work [7], SALLE methodology based on a ratio of 60/40 (v/v) ACN/sample in
162 the presence of 2% (m/v) of NaCl gives the best compromise to obtain an efficient plasma protein
163 precipitation, a good separation of the two phases and a moderate dilution of the samples. Moreover, a
164 BGE composed of a mixture of 6-amino caproic acid and citric acid at pH of 2.0 with ionic strength (IS) of
165 150 mM allows an efficient electrophoretic separation of the TKIs. Therefore, these SALLE-CE-UV
166 conditions were used for the automation of the overall procedure of the A-SALLE-CE-UV methodology.
167 The first experimental investigations were realized to check if it was possible to 1/ obtain a two phases
168 separation, 2/ successfully extract the TKIs in the upper (mostly ACN) phase and 3/ separate the TKIs in
169 an automated manner. Three TKIs (Fig. 2) were chosen as proof-of-concept analytes. The length of the
170 capillary was adjusted to be immersed only in the upper phase (mostly organic) of the sample after
171 SALLE extraction. The adjustment of the capillary length passed through several steps. At first, two
172 samples were prepared by SALLE (manually) and by A-SALLE. In order to visualize properly the two
173 phases separation, methyl red indicator was added (Fig. 2S, supplementary results). Estimated heights of

174 the upper phase of both manually and automatically prepared samples was verified to be the same.
175 Accordingly, the length of the capillary inlet was optimized and measured to be immersed only in the
176 upper phase ($L_{\text{tot}} = 31$ cm, $L_{\text{eff}} = 21$ cm). Both ends of the capillary were set at the same level to avoid
177 siphoning effect that would affect the robustness of the proposed methodology. The filling scheme of
178 the sample vial with the different extraction solutions (ACN, salt stock solution) allowing to automate
179 the extraction procedure can be resumed as following: ACN/NaCl/air. ACN and NaCl stock solution were
180 successively added from outlet vials (hydrodynamic injection, reverse mode) to the sample vial
181 containing 160 μL of TKIs at 5 $\mu\text{g}/\text{mL}$ at 80 psi during 8.5 min (300 μL) and at 80 psi during 3.5 min (40
182 μL) respectively. The final volume was fixed at 500 μL for preliminary trials. Mixing of the different
183 solutions was done by generating air bubbles from an empty outlet vial at 80 psi during 5 min. After this
184 step, a two phases separation was observed in the inlet vial confirming the possibility to automate SALLE
185 technique. The upper mostly organic phase was then injected. An electrophoretic separation between
186 the 3 TKIs was obtained. Maximum upper phase injected volume was 10% of the capillary effective
187 length (Fig. 3). This was less than what was obtained by manual preparation (80 % of the capillary
188 effective length for SALLE-CE-UV methodology [7]). This could be attributed to the difference in
189 composition of the obtained upper phases by automated and by manual methodologies in terms of
190 ACN, NaCl and water. Moreover, extraction efficiencies (%) and sensitivity enhancement factors (SEF) of
191 the 3 TKIs obtained by A-SALLE-CE-UV methodology were four and ten times less than those obtained by
192 manual SALLE-CE-UV methodology (98 – 100 % for extraction efficiencies, 144-265 for SEF), respectively.
193 This lack of performance was mainly attributed to an insufficient mixing of ACN, NaCl and sample
194 solutions in the inlet vial affecting both extraction efficiency (%) and sensitivity. However, similar
195 electrophoretic profiles were obtained from A-SALLE-CE-UV and manual methodology in terms of
196 migration times. Good repeatability ($n = 3$) were obtained for the 3 TKIs with RSD of less than 0.7% and
197 of less than 3.6% for migration times and corrected peak areas respectively, confirming the feasibility of

198 combining SALLE preparation procedure with CE separation in an automated manner. Nevertheless,
199 further optimizations were required to improve the efficiency of the A-SALLE-CE-UV methodology.

200 *3.2. Optimization of the mixing efficiency of standard sample solution with ACN and NaCl*

201 The first step in the optimization of the automated extraction procedure was to adjust both filling
202 pressure and time to obtain accurate volumes of both ACN/NaCl solution to the standard sample
203 contained in a 600 μL tube (mixing chamber). Five different pressures ranging from 5 to 80 psi were
204 evaluated with a fixed filling time of 6 min. The mean added volume ($n=3$) of ACN or NaCl (V_x) to the
205 sample was calculated based on mass differences ($V_x \text{ (ml)} = (m_{\text{filled}} - m_{\text{empty}})/\rho$), where m_{filled} is the mass
206 (g) of the 600 μL eppendorf tube filled with ACN solvent or NaCl solution, m_{empty} is the mass (g) of the
207 empty 600 μL eppendorf tube and ρ is the density of the ACN solvent (0.79) or aqueous NaCl solution
208 (1). The mean volumes of both ACN and NaCl increased linearly (correlation coefficient (r^2) higher than
209 0.999) from 4 to 317 μL with good repeatability (RSD < 1.53 %, $n=3$) for the different tested pressures
210 (Fig. 4A and B). Hydrodynamic pressure of 60 psi was chosen as the optimal pressure as it gave the best
211 compromise between low RSD (less than 0.63 %, $n=3$) and rapidity of filling the vials. The same
212 procedure was repeated varying the filling time from 1 to 8 minutes with fixed 60 psi pressure. Mean
213 volumes of both ACN and NaCl increased linearly from 8 to 293 nL ($r^2 > 0.999$) for the different filling
214 times (Fig. 4C and D) with high repeatability (RSD < 0.63 %, $n=3$) whatever the injection time applied.

215 The second step to ameliorate the mixing efficiency was to decrease the sample, ACN and NaCl volumes
216 introduced in the 600 μL eppendorf tube. Indeed, more than 83 % (500 μL) of the 600 μL eppendorf
217 tube was filled with solutions. Consequently, a loss of solutions and a lack of mixing efficiency could
218 occur during the mixing step with air bubbles affecting the repeatability and the performance of the A-
219 SALLE-CE-UV methodology. Moreover, decreasing the solution volumes helps to reduce the time needed
220 to fill the inlet vial. Therefore, the volumes of sample, ACN and NaCl were divided by a factor of 2 and
221 the total volume was fixed at 250 μL . NaCl and ACN were added to the standard samples at 60 psi during

222 2.4 (20 μL) and 4.0 minutes (150 μL), respectively. The pressure applied for the mixing by air bubbles
223 was increased to 90 psi during 5 min without risk of loss of sample. Improvements in the injected sample
224 volume (up to 40%), SEF (up to 125) and extraction efficiencies (up to 45%) for the 3 TKIs were observed
225 confirming the benefit of decreasing ACN, NaCl and sample volumes besides increasing the air pressure
226 used for mixing.

227 The third step in the optimization of the extraction procedure, was to modify the filling scheme of the
228 sample vial by ACN and NaCl. Instead of adding the entire volume of these solutions to the standard
229 sample at once as previously described, NaCl and ACN solutions were added to the sample in 2 steps
230 each to obtain total added volumes of 20 and 150 μL , respectively. So, 75 μL of ACN and 10 μL of NaCl
231 were successively added to the standard sample solution at 60 psi during 2.0 minutes and at 60 psi
232 during 1.2 minutes, respectively. Mixing by air bubbles at 90 psi during 2.0 minutes was performed after
233 the first addition of ACN and salt, and repeated after the next addition of ACN. After the second NaCl
234 solution addition, a final mixing was realized at 90 psi during 5.0 minutes after addition all solutions
235 ($V_{\text{total}} = 250 \mu\text{L}$). The filling scheme of sample vial can be resumed as following:
236 ACN/NaCl/air/ACN/air/NaCl/air. The injected volume of the upper phase could be successfully increased
237 to 287 nl (corresponding to 70% of the capillary effective length) without peak broadening due to in-line
238 acetonitrile CE stacking increasing the sensitivity of the A-SALLE-CE-UV methodology (Fig. 5). Moreover,
239 TKIs were efficiently extracted (60–62% of extraction efficiency) in the upper phase. The injection was
240 repeated three times. RSD ranging from 0.85 to 1.12 % for migration times and from 1.40 to 2.40 % for
241 peak areas were obtained. SEF factors of 94.1, 134.1 and 196.3 were obtained for lapatinib, erlotinib
242 and sorafenib, respectively. All results indicate an improvement of the analytical performances of the
243 proposed A-SALLE-CE-UV methodology due to better mixing efficiency. However, the values obtained in
244 term of extraction efficiency (%) and SEF were about 1.6 times less important than with SALLE-CE-UV (98
245 – 100 % for extraction efficiencies, 144-265 for SEF factors). The mixing generated by a stirrer and a

246 centrifugator in the case of manual SALLE-CE-UV is more efficient than with air bubbles for A-SALLE-CE-
247 UV. Other developments (time and pressure of mixing, volume and shape of the mixing chamber) were
248 realized without technical modifications at the level of the commercially available CE apparatus but
249 didn't give significant improvements. Even though, the performances of the proposed A-SALLE-CE-UV
250 methodology are sufficient to quantify TKIs in human plasma (see 3.4). Consequently, these conditions
251 were defined as being the optimal A-SALLE-CE-UV procedure (see section 2.5; Table 1S in supplementary
252 information) and were applied in the rest of this study.

253 *3.3. Effect of the type of the salting-out agent*

254 Different salts were studied with the aim to choose the optimal salting-out agent. NaCl, potassium
255 chloride (KCl) and ammonium acetate were tested because these salting-out agents gave the best
256 extraction efficiencies in many works [26,27]. It was found that corrected peak area values were 1.3
257 times more important with KCl than for the two other salts (NaCl and ammonium acetate) suggesting
258 better extraction efficiencies (Fig. 6A). However, KCl lead to the reduction of peak efficiencies compared
259 to the other two salting-out agents (Fig. 6B). As KCl is more soluble in a 100% ACN phase (≈ 24 mg/L)
260 than for the two other salts (≈ 3 mg/L), the amount of salt in the upper phase obtained with KCl was
261 probably higher than that of NaCl and ammonium acetate. The presence of salt in the upper phase
262 allows to extract more easily the 3 TKIs due to better solubility. However, the presence of salts in the
263 injected sample, affects the performance of in-line acetonitrile stacking [28]. NaCl and ammonium
264 acetate nearly lead to the same performance in terms of corrected peak areas of TKIs but, NaCl gave the
265 most efficient peaks. Better repeatability ($n=3$) for corrected peaks areas was obtained with NaCl with
266 values of less than 2.5 %. Therefore, NaCl was chosen as the salting out agent for the A-SALLE-CE-UV as
267 it gives the best compromise between extraction efficiency, repeatability and peak efficiency.

268

269

270 3.4. Analysis of human plasma spiked with TKIs:

271 The performance of the proposed A-SALLE-CE-UV methodology was evaluated on plasma sample spiked
272 with the three TKIs. The pH of the BGE was 2.0 and the ionic strength 150 mM. Protein precipitation was
273 observed in the sample vial after the addition of ACN. Figure 7 shows the electropherograms obtained
274 for blank plasma (no TKIs) and TKIs plasma sample (injected volume equal to 70% of the capillary
275 effective length). No interfering peaks of endogenous proteins were observed at the migration time of
276 the TKIs demonstrating the specificity of the proposed methodology. The maximum injected sample
277 volume without peak broadening was similar to that obtained with a standard sample solution (287 nl
278 corresponding to 70% of the capillary effective length). This result indicates that the in-line stacking
279 performances were similar for standard and plasma solutions and confirmed the robustness of the
280 proposed methodology. The analytical performances of the developed methodology were evaluated
281 and are summarized in table 1. LOQ of lapatinib, erlotinib and sorafenib were determined at 1260, 200
282 and 640 ng/ml respectively. These LOQ values were slightly higher than that obtained by manual sample
283 preparation since the type of mixing process was different. However, these values are still lower than
284 mean plasma levels observed for patients treated by TKIs, which are estimated at 1570, 1200 and 1440
285 ng/ml for lapatinib, erlotinib and sorafenib respectively [29–31]. Good repeatability was obtained at the
286 LOQ levels (n = 6) in term of migration time (RSD < 2.88 %) and corrected peak areas (RSD < 2.73%).
287 Good linearity of the calibration curve from 200 to 5000 ng/ml was obtained with r^2 higher than 0.995.
288 Moreover, several advantages are offered by the proposed A-SALLE methodology. The small volume of
289 the sample used (80 μ L compared to 2.0 mL of plasma for manual SALLE methodology) which will be
290 more convenient for the patient's quality of life i.e. less blood sample collected. The reduction in the
291 volume of organic solvent used (about 20x less) compared to manual methodology which will effectively
292 lower the cost and toxicity of analysis per sample. In addition, automation makes analyses easier to
293 realize minimizing manual labor and human error. All the above results confirm the possibility to

294 automate SALLE by CE without the need to make any modification at the level of the commercially
295 available CE machine. Moreover, the applicability of the proposed A-SALLE-CE-UV for the routine
296 analysis of these three TKIs was successfully tested against plasma sample spiked with the three TKIs.

297 **4. Conclusion**

298 A novel automated salting-out assisted liquid-liquid microextraction approach for plasma sample before
299 CE analysis was developed. The capillary was used as a tool to deliver appropriate microvolumes of ACN
300 and NaCl to the inlet vial containing the plasma sample. This was followed by mixing using air bubbles
301 leading to a two phases separation and extraction of the TKIs in the upper phase. NaCl was chosen as
302 the salting out agent as it gives the best compromise between high extraction and peaks efficiency. The
303 A-SALLE-CE-UV methodology has the advantages to eliminate simultaneously biological proteins, salts
304 and to extract the molecules of interest from the sample matrix. This new approach, A-SALLE-CE-UV,
305 was successfully applied for the analysis of 3 TKIs in plasma samples. The extracted solution (upper
306 phase, mostly ACN solvent) could be injected up to 70% of the capillary effective length due to the
307 existence of in-line acetonitrile stacking phenomenon inside the capillary. Thus, it can be considered as a
308 rapid, simple, cheap, convenient and well suited for possible TDM of TKIs and for further applications
309 concerning the use of SALLE. This A-SALLE-CE-UV methodology does not require to make any technical
310 modifications at the level of the commercially available CE apparatus.

311 **Acknowledgments**

312 This work was supported by a PhD grant from Misr University for science and technology (MUST), 6th
313 October City, Egypt. The authors would like to thank Prof. Laila Abdel Fattah, Prof. Joseph Joachim and
314 Prof. Emmanuel Cornillot the coordinators of the collaboration between MUST University and the
315 University of Montpellier. The authors would like also to thank Mr Khaled El-toukhy chancellor,
316 chairman of trustees in MUST university, Pr Mohamed Mostafa, the vice president of MUST university
317 and Dr Yasmin El kashef, the responsible of foreign affairs in MUST university for their support.

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411

412 **Figures captions**

413 - **Figure 1:** A-SALLE-CE-UV procedure. Step (1) Delivering of appropriate micro-volumes of solutions (ACN
414 and NaCl stock solution) from the outlet side to the inlet side (sample vial) by hydrodynamic injection,
415 followed by mixing with air bubbles injected from an empty outlet vial. Step (2) Separation into 2 phases
416 (upper phase: mostly organic phase. Bulk phase: mostly aqueous) after mixing step. Step (3)
417 hydrodynamic injection of the upper phase. Step (4) Separation and detection of the three TKIs by CE-
418 UV.

419 - **Figure 2:** Molecular structures of the studied TKI: (1) lapatinib ditosylate, (2) erlotinib hydrochloride
420 and (3) sorafenib.

421 - **Figure 3:** Analysis of lapatinib, erlotinib and sorafenib after injecting the upper phase (mostly ACN)
422 obtained by A-SALLE-CE-UV methodology. Analysis conditions: silica capillary 50 μm i.d., total length 31
423 cm, effective length 21 cm. BGE: citric acid – ϵ -aminocaproic acid buffer IS 150 mM pH 2.0.
424 Temperature: 25°C. Separation voltage: 15 kV. Sample injection volume: 39 nl (corresponding to 10% of
425 the capillary effective length). Detection: 254 nm.

426 - **Figure 4:** Effect of increasing the hydrodynamic pressure during 6 minutes on the volume obtained of
427 (A) ACN and (B) NaCl. Effect of increasing the time at 60 psi on the volume obtained of (C) ACN and (D)
428 NaCl.

429 - **Figure 5:** Analysis of lapatinib, erlotinib and sorafenib after injecting the upper phase obtained by A-
430 SALLE-CE-UV methodology (3 repetitions). Analysis conditions: silica capillary 50 μm i.d., total length 31
431 cm, effective length 21 cm. BGE: citric acid – ϵ -aminocaproic acid buffer IS 150 mM pH 2.0.
432 Temperature: 25°C. Separation voltage: 15 kV. Sample injection volume: 287 nl (corresponding to 70%
433 of the capillary effective length). Detection: 254 nm.

434 - **Figure 6:** Effect of the type of salting-out agent on (A) corrected peak areas and (B) theoretical plates
435 (N) for the 3 studied TKIs.

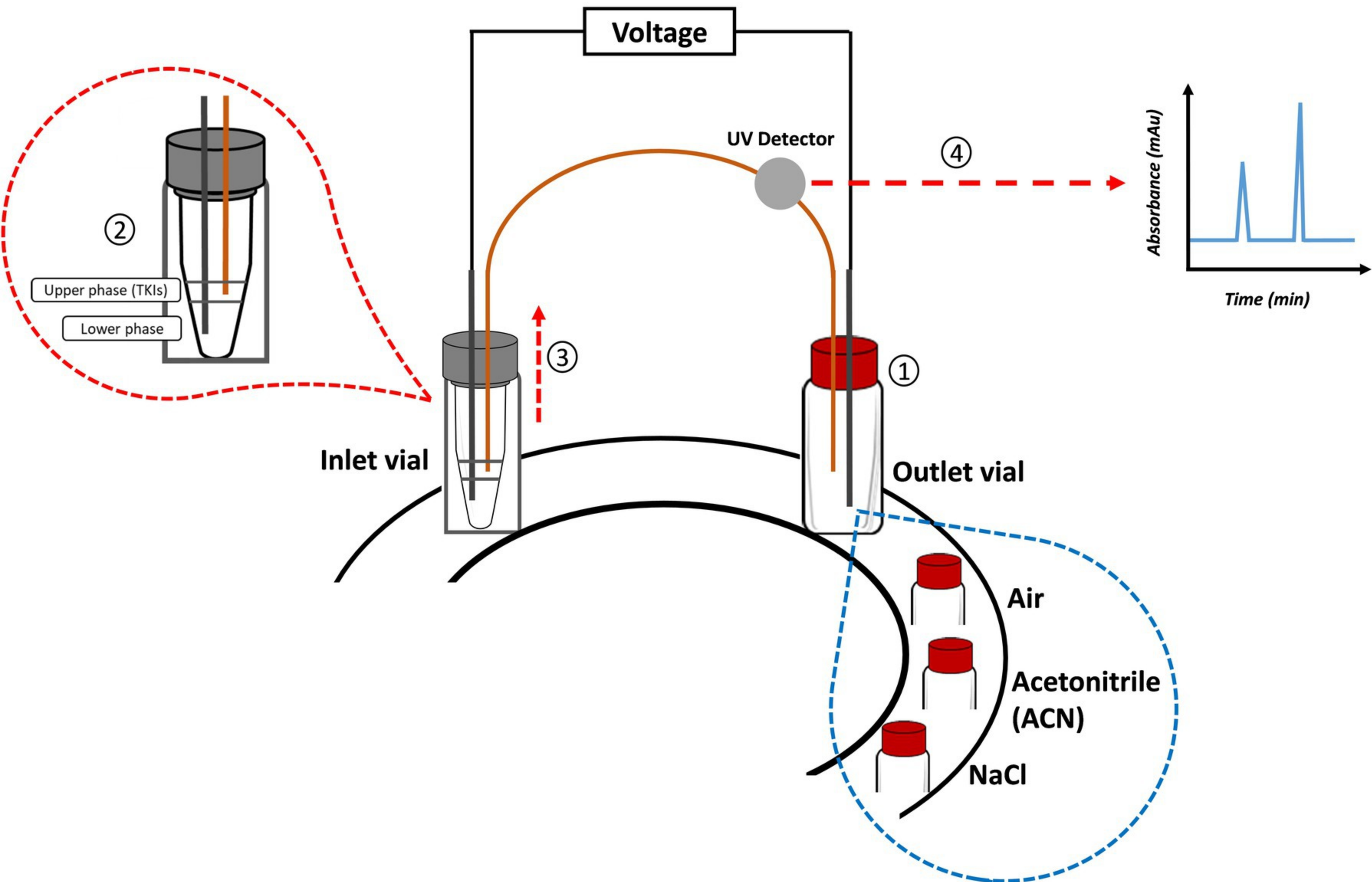
436 - **Figure 7:** Electropherograms obtained for blank human plasma sample and human plasma samples
437 spiked with the three TKIs using the A-SALLE-CE-UV procedure. Analysis conditions: silica capillary 50 μm
438 i.d., total length 31 cm, effective length 21 cm. BGE: citric acid – ϵ -aminocaproic acid buffer IS 150 mM
439 pH 2.0. Temperature: 25°C. Separation voltage: 15 kV. Sample injection volume: 287 nl (corresponding
440 to 70 % of the capillary effective length). Detection: 254 nm.

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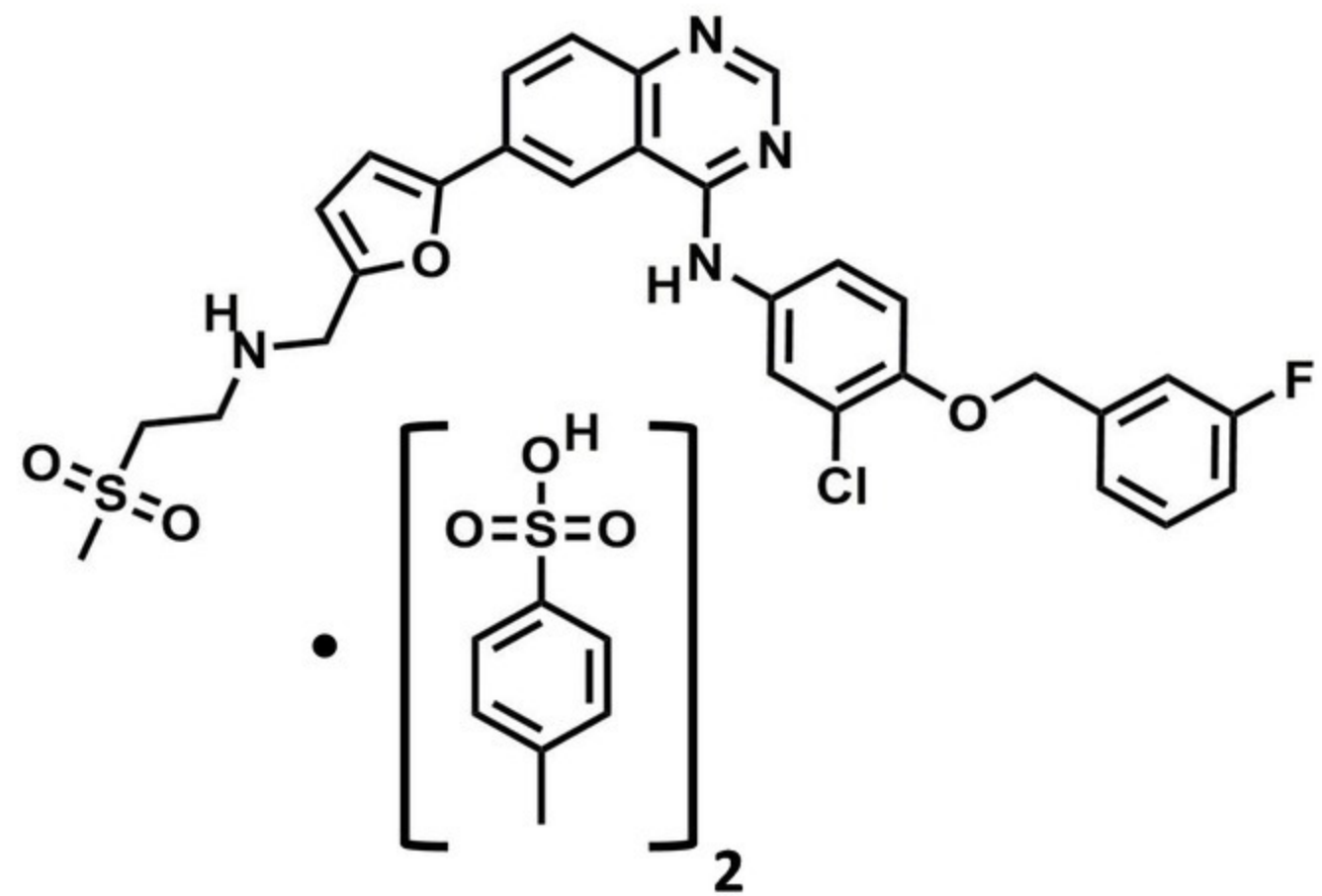
442 **Table caption**

443 - Table 1: Summary of the A-SALLE-CE-UV quantitative results obtained on human plasma samples
444 spiked with lapatinib, erlotinib and sorafenib. Injected sample volume equals 70 % of the capillary
445 effective length.

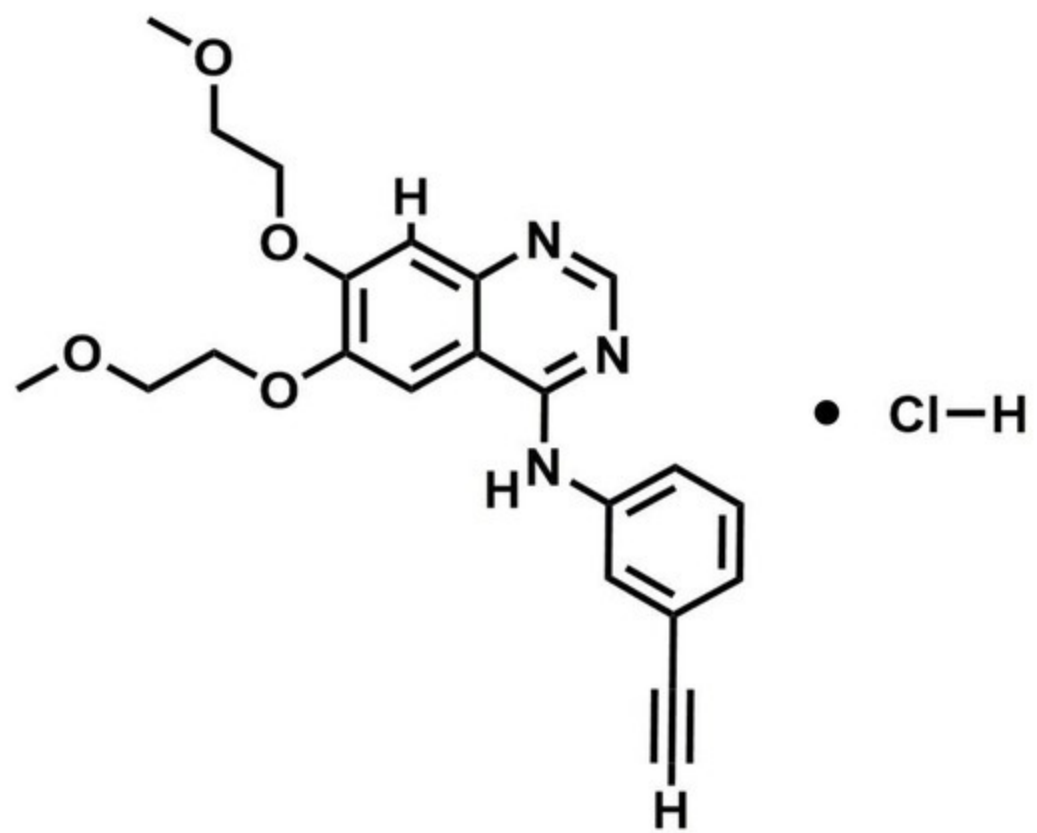
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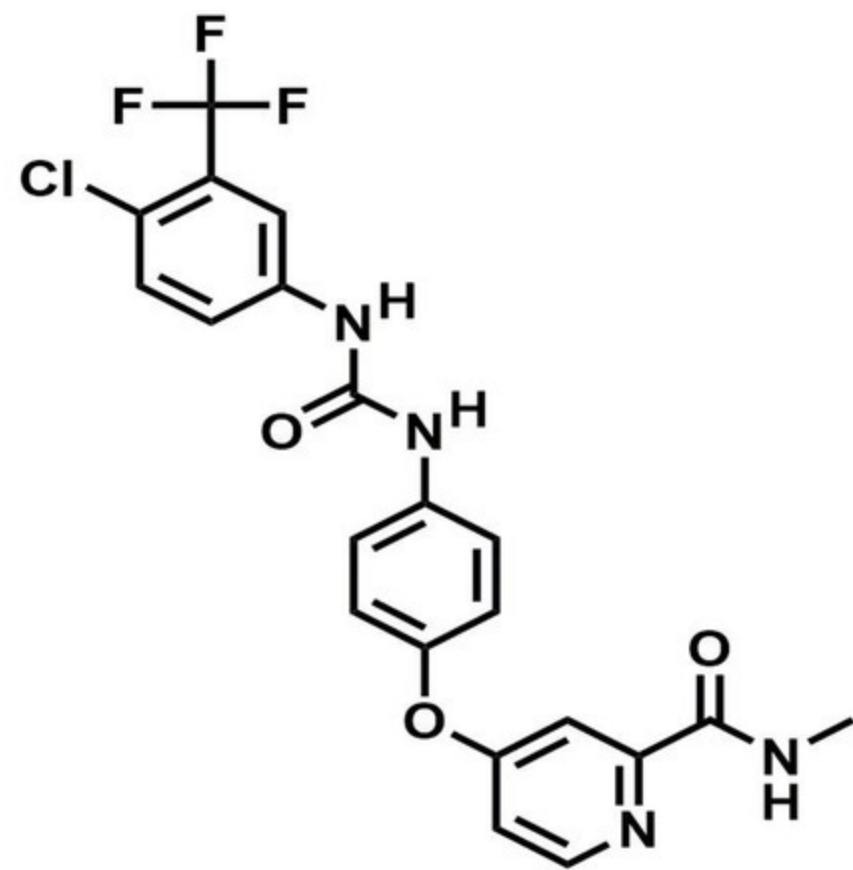
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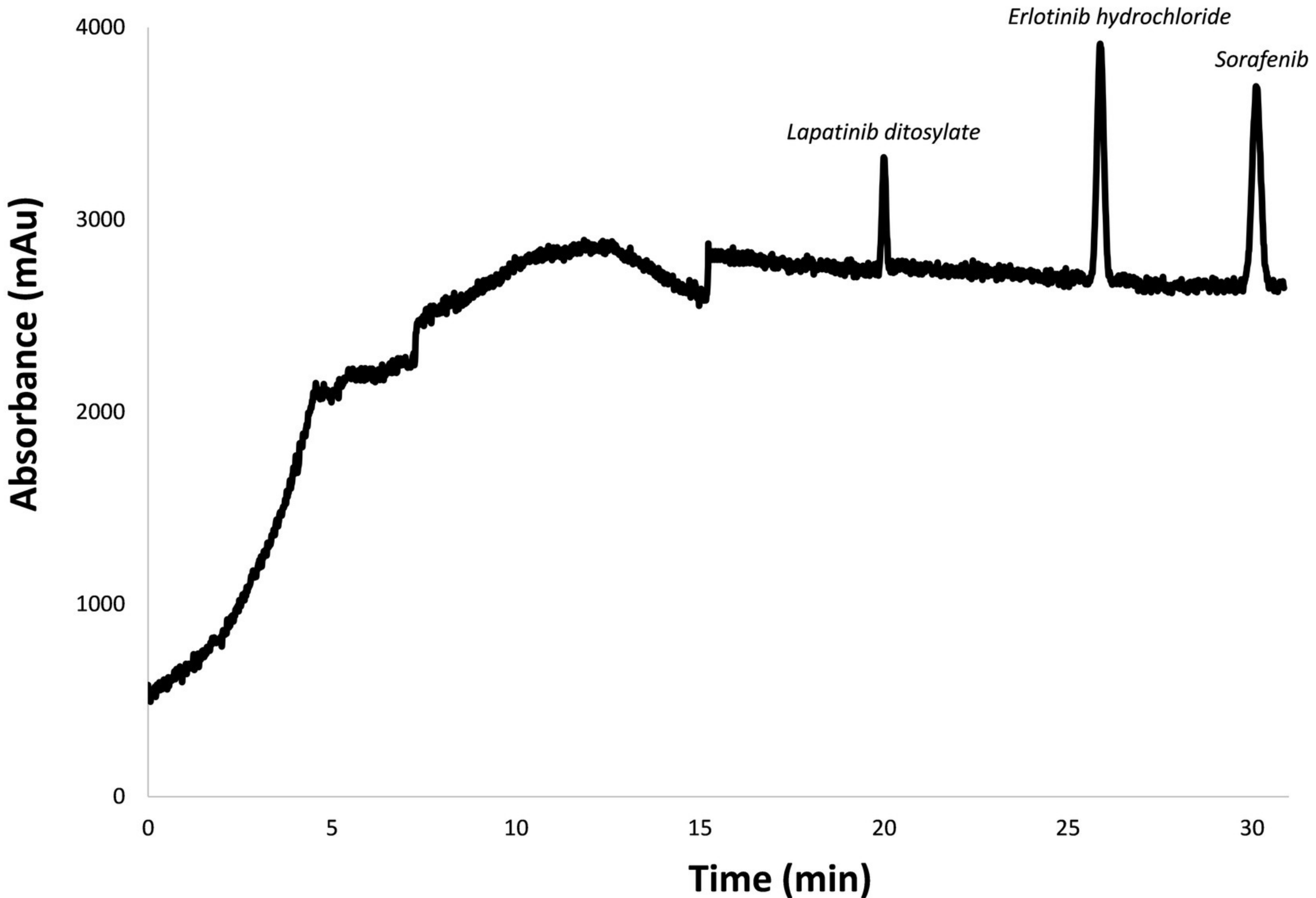


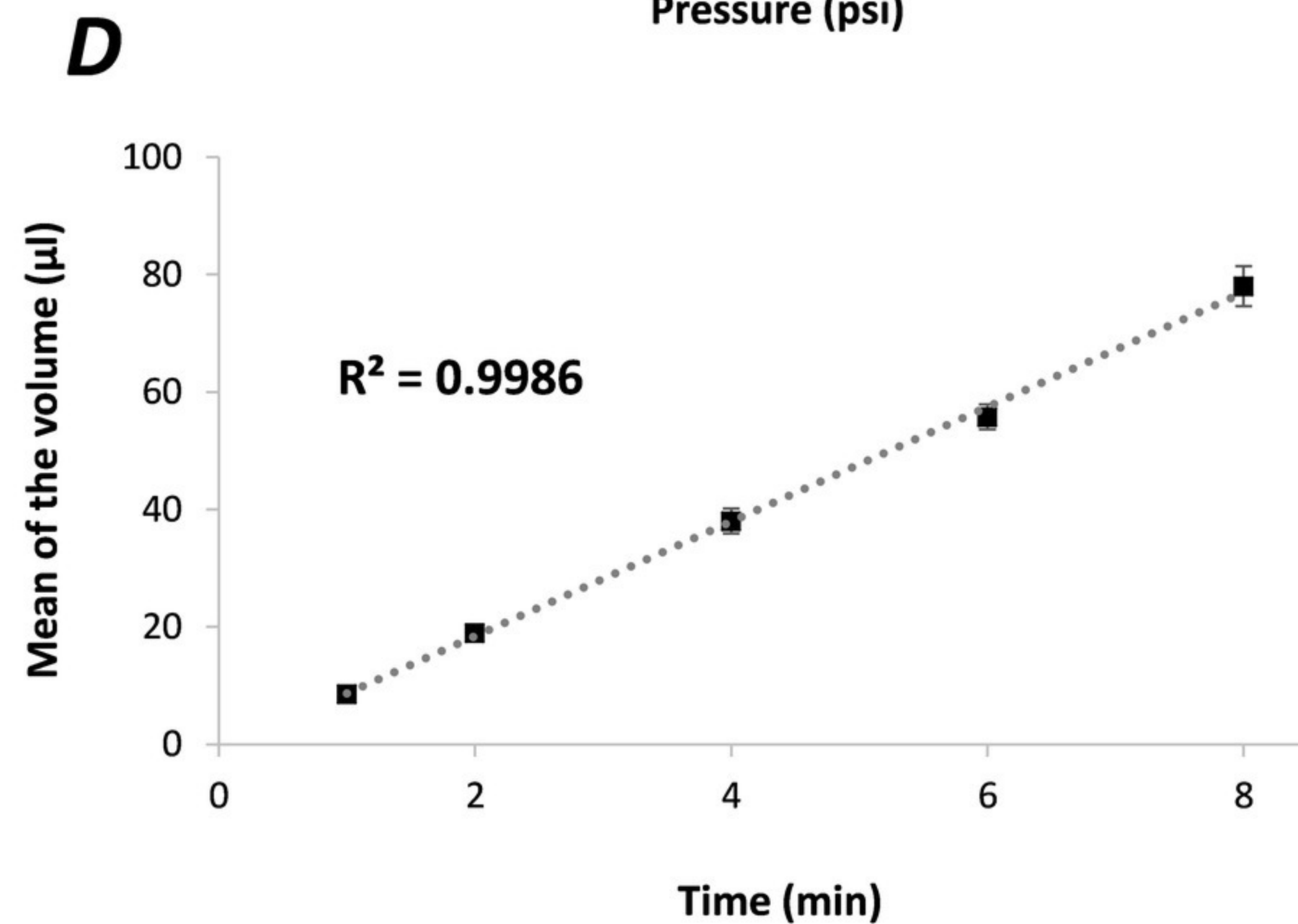
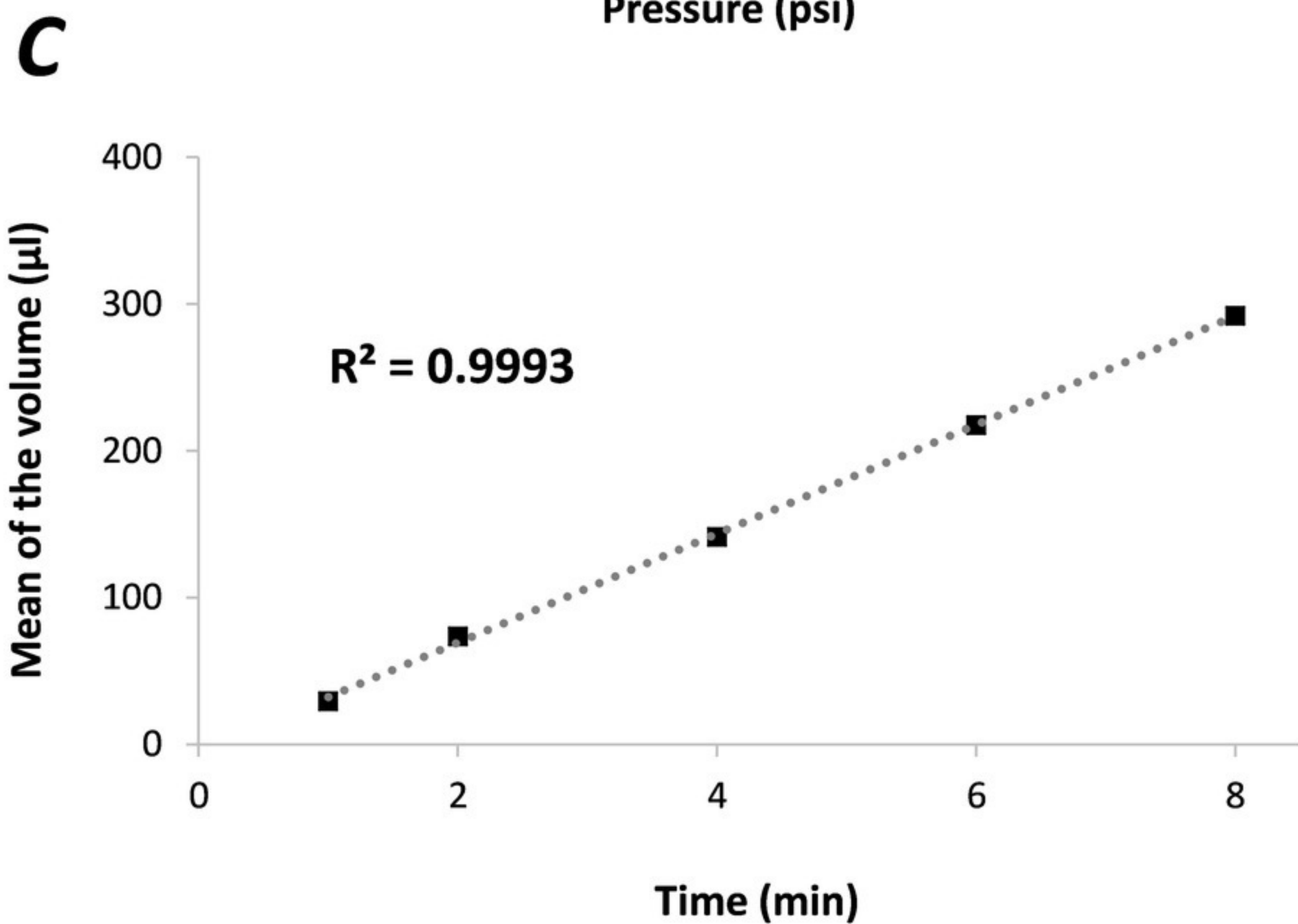
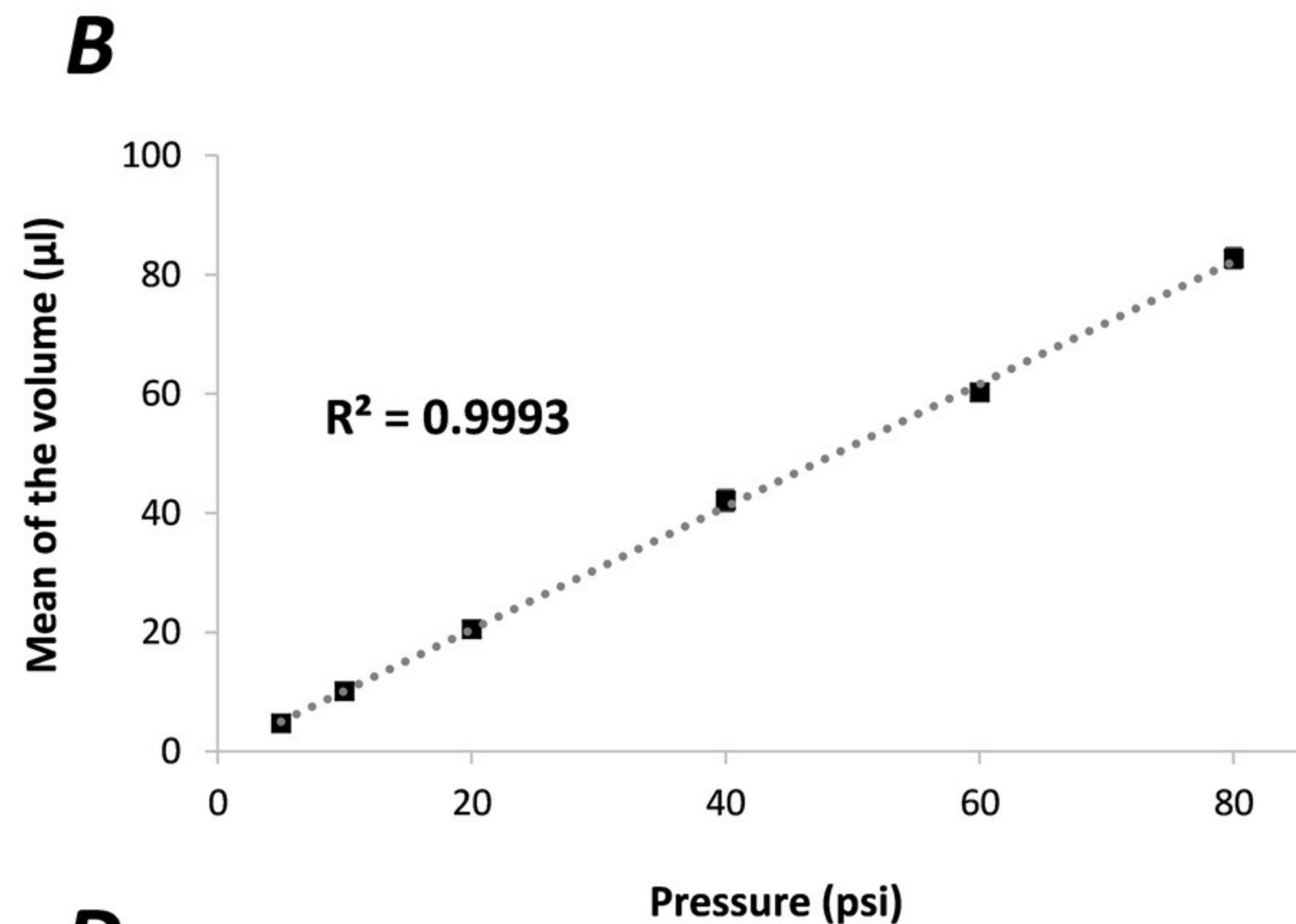
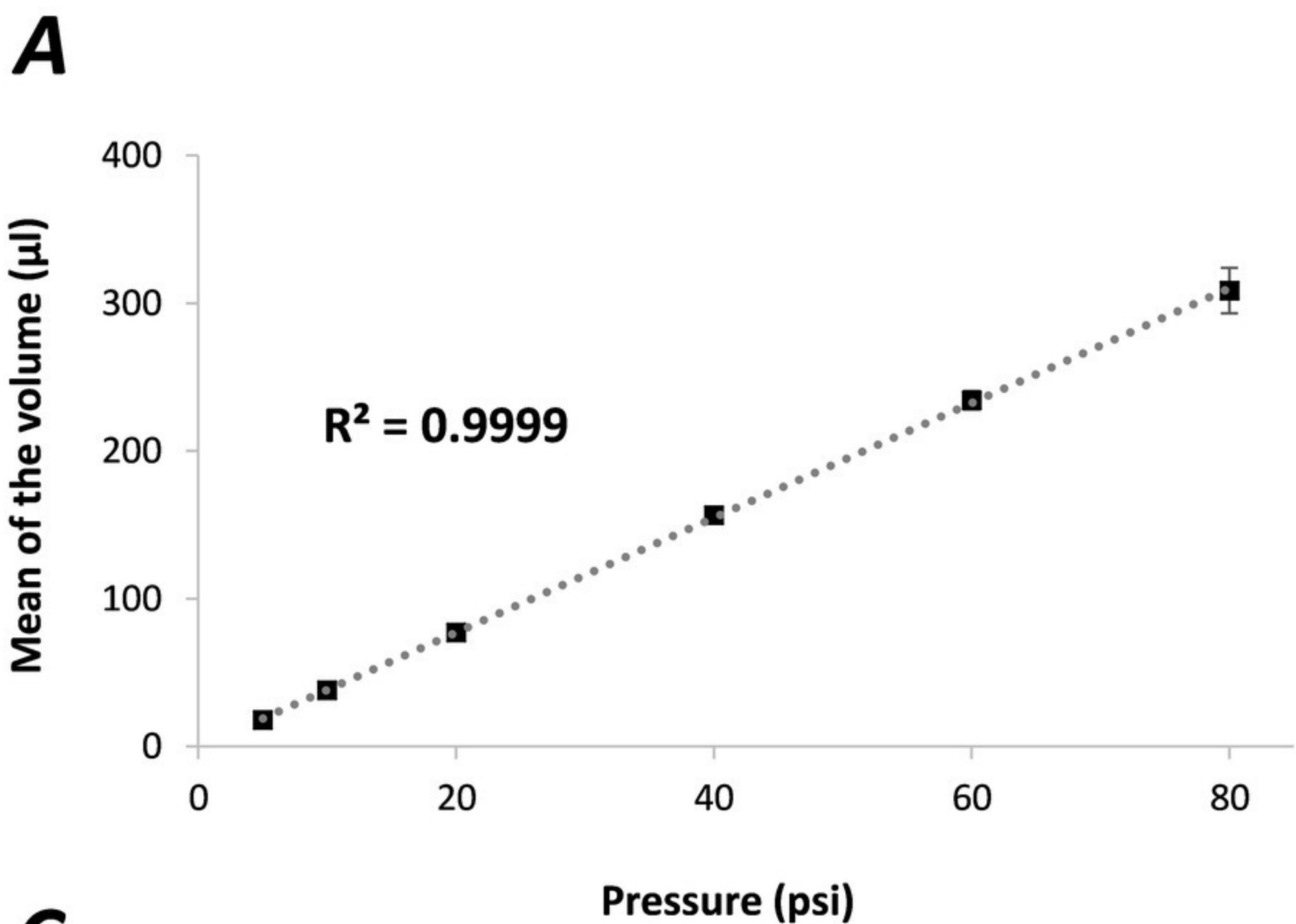
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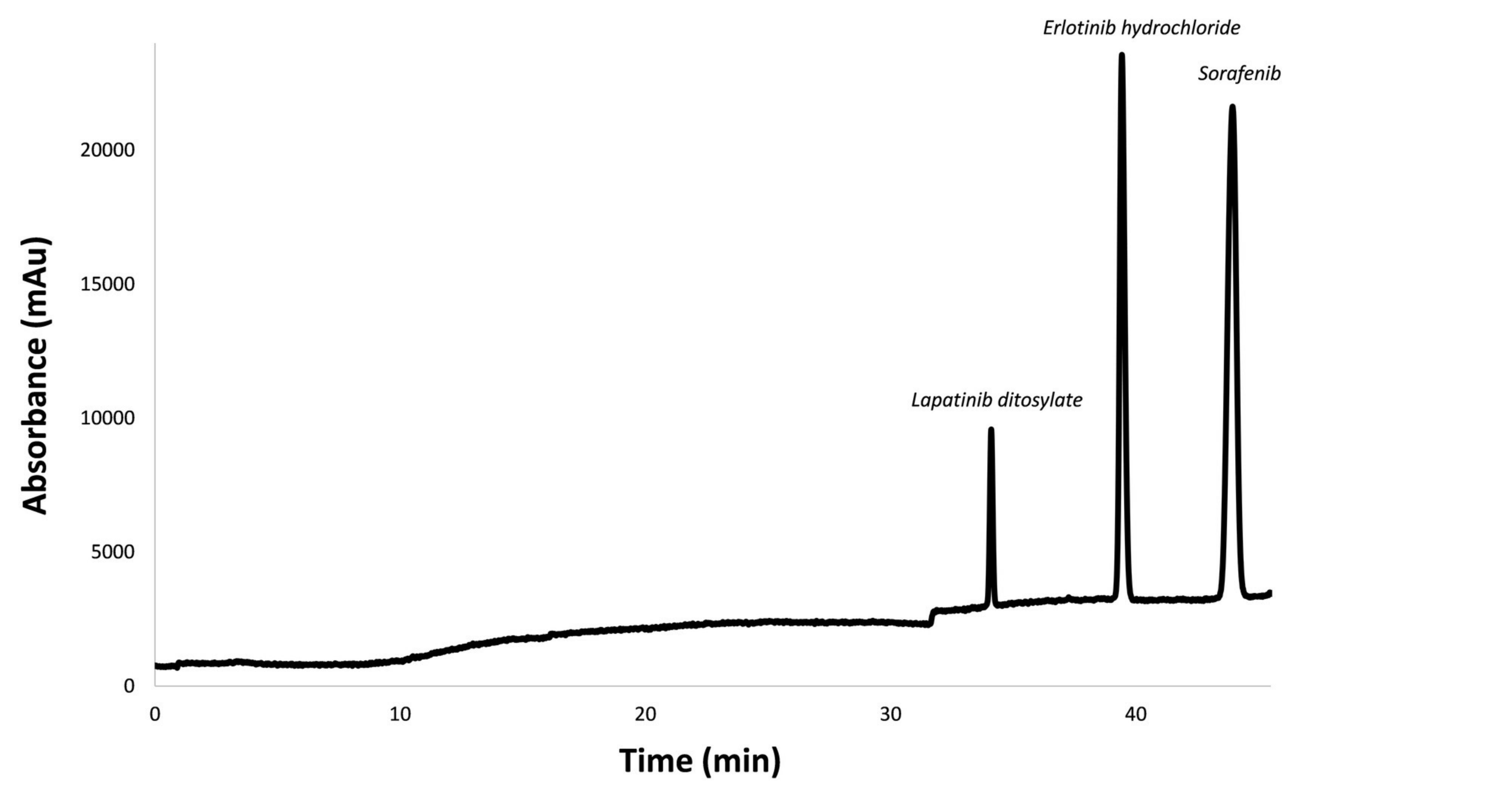


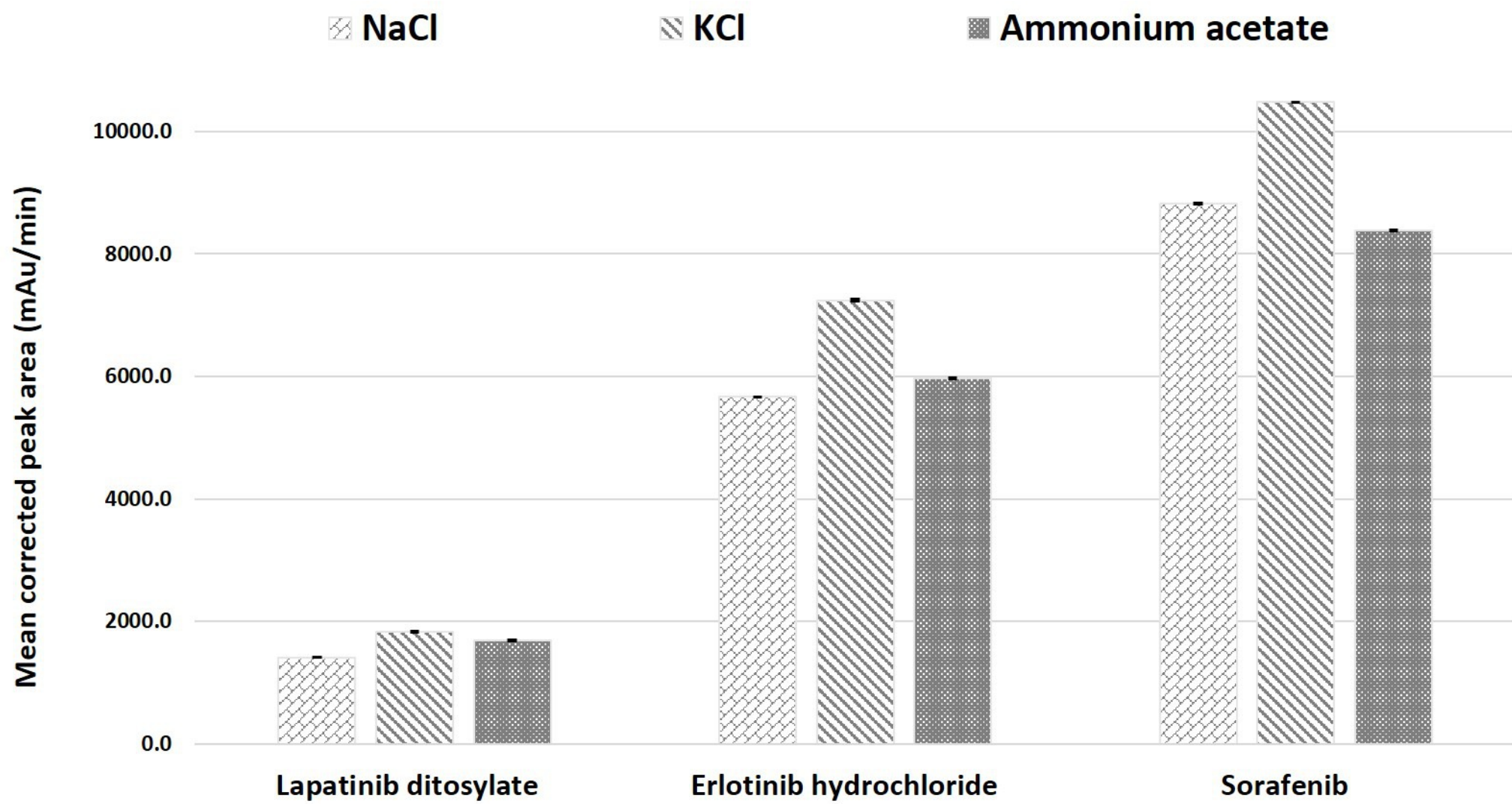
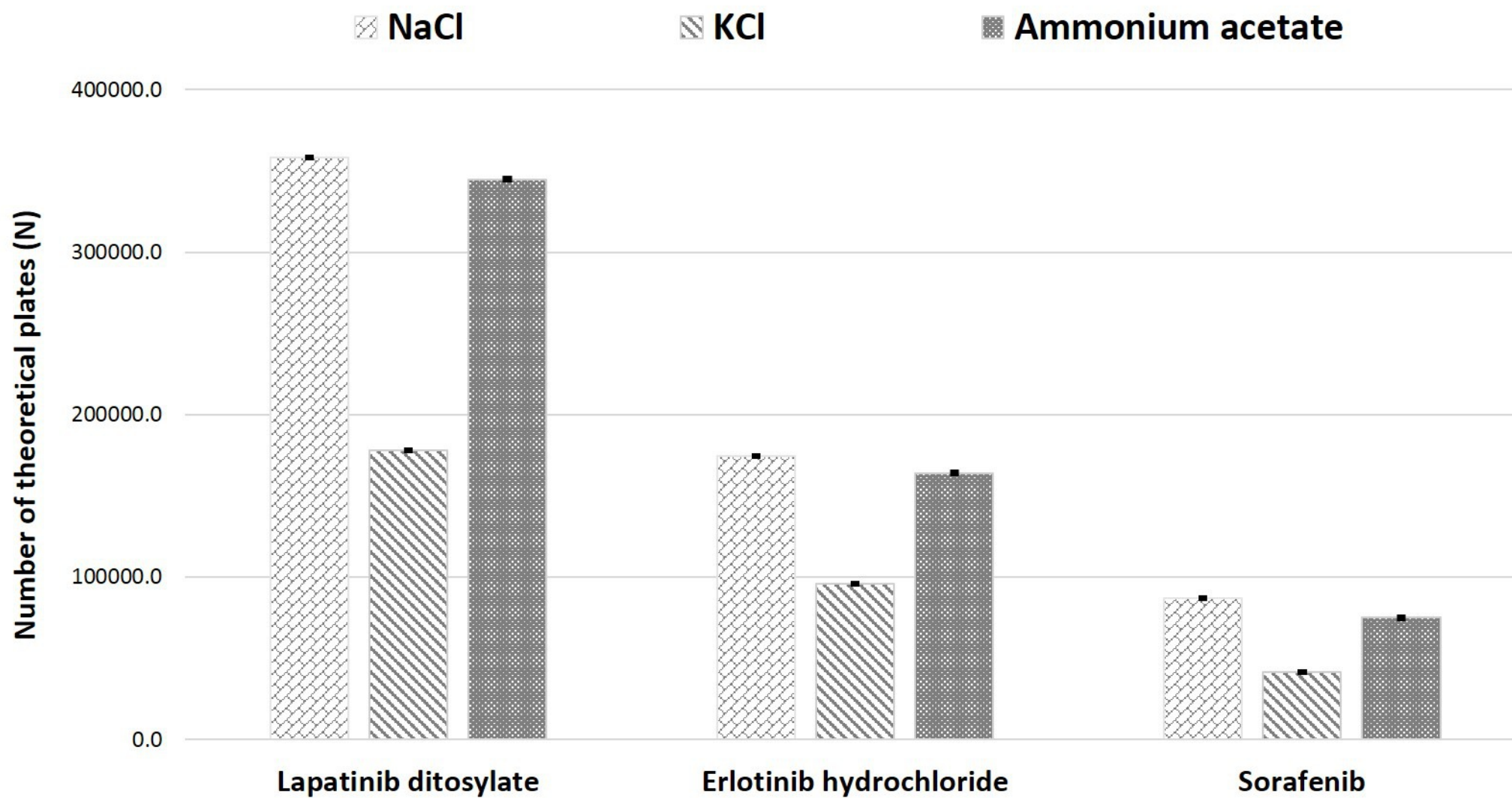
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A**B**

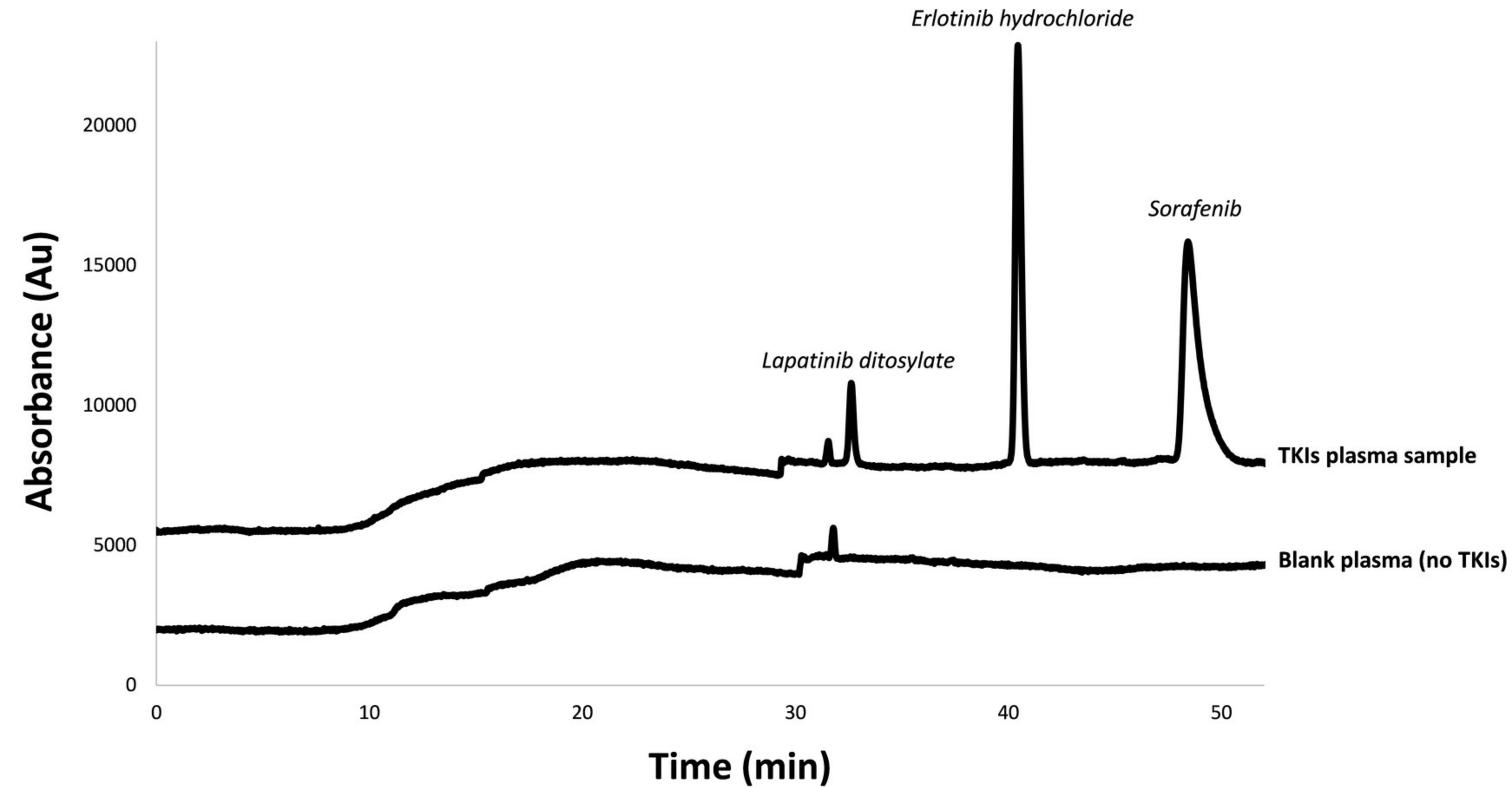


Table 1

| A-SALLE-CE-UV performance parameters | Lapatinib ditosylate | Erlotinib hydrochloride | Sorafenib |
|---|-----------------------------|--------------------------------|------------------|
| Extraction efficiency (%) | 62 | 62 | 60 |
| Specificity | Confirmed | Confirmed | Confirmed |
| Linearity (ng/ml) | 1360-5000 | 200-5000 | 640-5000 |
| Correlation coefficient (r^2) | 0.996 | 0.998 | 0.995 |
| LOD (ng/ml) | 1100 | 80 | 200 |
| LOQ (ng/ml) | 1360 | 200 | 640 |
| % RSD^a (corrected peak area) | 2.19 | 2.73 | 2.67 |
| % RSD^a (migration time) | 2.88 | 1.40 | 2.03 |

^a Calculated for 6 successive runs at the LOQ levels

