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Physical and chemical stability of a generic etoposide formulation as an alternative to etoposide phosphate

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Keywords: Etoposide – Etoposide phosphate – Physico-chemical stability – Supersaturation – Solubility – Degradation products - Computational prediction

Highlights:

- ✓ A method to quantify the decomposition products from etoposide was developed and validated.
- ✓ Degradation kinetics were studied and degradations products structures were elucidated using LC- HR-MSⁿ and computational methods.
- ✓ Physico-chemical stability of etoposide in ready-to-use chloride or glucose solutions was determined
- ✓ The article is a direct consequence of the need to use alternative pharmaceuticals due to a global shortage of etoposide phosphate

Abstract

The generic Mylan[®] etoposide (ETP) has been investigated as an alternative for Etopophos[®], in part due to a global shortage of the latter. The generic alternative is different both in its formulation and in its very limited stability (6 h at 25°C against 4 days for Etopophos[®]) once reconstituted in ready-to-use chloride or glucose solutions. Its intrinsic stability has been

50 thoroughly studied under various conditions. Two degradation products resulting from
51 hydrolysis were characterized by LC-HR-MSⁿ and supported by density functional theory
52 calculations of the frontier molecular orbitals energies, molecular electrostatic potential
53 mapping, and Mulliken charge analysis. Chemical degradation increases with temperature
54 and can be fitted to a zero order kinetic model with a half-life of 119 days and a kinetic
55 constant of 0.0028 mM day⁻¹. Precipitation was only observed in solutions at 5°C and -20°C
56 indicating that at these temperatures the reconstituted solutions are thermodynamically
57 metastable. In conclusion, ETP at concentrations of 0.68 and 1 mM prepared and stored at
58 25°C under good manufacturing practices remained unchanged over a period of 21 days
59 irrespective of the nature of the solvents or the type of container.

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1. Introduction

63

64 Stock-outs of medicinal products of major therapeutic interest have increased lately.
65 According to the ANSM (French National Agency for Medicines and Health Products) up to
66 530 stock-out alerts were reported in 2017, a 30% increase compared to 2016 [1]. The
67 problem is in particular marked in a hospital context, where drug products are frequently
68 subject to additional reconstitution and/or reformulation steps; hence the need to reassess
69 the stability of modified formulations in many cases obtained from new manufacturers. In
70 fact, the time span of drug expiry or “effective stability” is often well beyond that mentioned in
71 the Summary of Manufacturers' Product Characteristics [2, 3]. It is in this context that at the
72 end of 2016, due to a worldwide shortage of the prodrug Etopophos® (ETPO), we sought to
73 replace it by a generic supplied by Mylan® (ETP) formulated with the drug etoposide itself.

74 Etoposide, chemically known as 4'-demethylepipodophyllotoxin 9-(4,6-O-R-Ethylidene-β-D-
75 Glucopyranoside) or (5S,5aR,8aR,9R)-5-[[[(2R,4aR,6R,7R,8R,8aS)-7,8-dihydroxy-2-methyl-
76 4,4a,6,7,8,8a-hexahydropyrano[3,2-d][1,3]dioxin-6-yl]oxy]-9-(4-hydroxy-3,5-
77 dimethoxyphenyl)-5a,6,8a,9-tetrahydro-5H-[2]benzofuro[6,5-f][1,3]benzodioxol-8-one, is a
78 semi-synthetic derivative of podophyllotoxin that shows antitumor activity and is used to treat
79 a broad spectrum of malignancies, among which lung cancer and leukemia [4, 5].

80 There are some notable dissimilarities between the previous and the new formulations. First,
81 Etopophos is a lyophilized powder containing etoposide phosphate, which is a water-soluble
82 (solubility of 150 mM) etoposide prodrug. The compound is rapidly and completely converted
83 to ETP after intravenous injection with similar pharmacological properties and bioavailability
84 compared to ETP. However, etoposide is poorly soluble in water [6] and the formulation of
85 Mylan® contains organic solvents and uses polysorbate 80 to increase its solubility [7].
86 Moreover, the excipients differ from that of etopophos, which contains trisodium citrate and

87 dextran 40, while ETP Mylan[®] contains macrogol 300, anhydrous citric acid, polysorbate 80,
88 benzyl alcohol and ethanol.

89 According to the Summary of manufacturers Product Characteristics (SmPC) the stability of
90 ETP Mylan[®] (6 hours at 25°C) is much less than that of etopophos (24 hours at 25°C and 7
91 days at 4°C) [8, 9]. These stipulated stability ranges are a consequence of the design space
92 and the control strategies [10] of the manufacturers.

93 Nonetheless, pharmaceutical formulations identical to the one of ETP Mylan[®] are
94 accompanied with different and sometimes even contradictory shelf-life recommendations
95 after reconstitution for patient use. For instance, Sandoz[®] identifies the concentration as the
96 key factor impacting the stability of the admixture listing 7 days until expiry for a
97 concentration of 0.2 mg mL⁻¹ and 12 hours for a concentration of 0.4 mg mL⁻¹ irrespective of
98 the storage temperature either around ambient temperature (between 15°C to 30°C) or in a
99 refrigerator (between 2°C to 8°C) [11]. Accord healthcare limited[®] links the stability to the
100 nature of the reconstituted solution at ambient temperature (between 20°C to 25°C) with 96
101 hours in a sodium chloride solution (0.9 % w/v) and 48 hours in a glucose solution (5% w/v)
102 [12]. Mylan Pharmaceuticals[®] ULC-Canada recommends a 24-hour time window for the use
103 of the admixture at ambient temperature (between 15°C to 30°C) irrespective of the
104 concentration between 0.2 and 0.4 mg mL⁻¹ [13].

105 The Parisian Hospitals, depending on the French SmPC for Etoposide Mylan[®] were
106 confronted with the reduced shelf life of the replacing product, which resulted in
107 organizational complications for the hospital production units along with a worsening in
108 patient care, due to the expiration of formulations before administration, lack of available
109 preparations, and the necessity to administer large volumes.

110 Thus, in agreement with Mylan[®] Laboratories, this study was carried out to extend the
111 knowledge space [14] involving the stability behavior of reconstituted ETP improving the
112 identification and the ranges of critical quality parameters defined in the SmPC in terms of
113 effectiveness and safety. Impacts were explored of the concentration (“clinically common” =
114 0.68 mM (0.4 mg mL⁻¹) and “clinically high” = 1 mM (0.6 mg mL⁻¹)), of the nature of the
115 environment (NaCl 0.9% or Glucose 5%), of the interaction between content and container,
116 and of the storage and conditioning temperatures on the drug product behavior. Degradation
117 kinetics have been evaluated and the generated degradation products have been
118 characterized by LC-HR-MSⁿ, followed by an analysis of the chemical and physical
119 processes governing the drug product instability. In addition, density functional theory (DFT)
120 has been used to calculate the HOMO – LUMO energy gap, a molecular electrostatic
121 potential map and the Mulliken charges in a further analysis of the fragility of the drug. *In*
122 *silico* QSAR toxicity studies have been performed to compare the mutagenicity and
123 developmental toxicity of the degradation products with the theoretical data of ETP.

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2. Materials and methods

2.1 Materials and reagents

ETP vials of 34 mM (20 mg mL⁻¹) were kindly provided by Mylan[®] Laboratory (Saint-Priest, France). The ETP standard reference (European Pharmacopoeia, Sigma Aldrich, St Quentin-Fallavier, France) was used to validate the analytical method. Bags filled with 100 mL of sodium chloride (0.9% NaCl) or with glucose 5% (G5%) were obtained from Freeflex[®] (Fresenius Kabi France SA, Sevres, France) and Easyflex[®] (Macopharma, Mouvaux, France), respectively. Chemoprotect[®] Microspike and Take Set[®] (Codan[®], Bischwiller, France), each equipped with a Luer-Lock connection system were stuck respectively into the drug vial and into the bags. Syringes purchased from Becton Dickinson[®] (Rungis, France) were connected to the Microspike for the withdrawal of the drug from the vials, and then injected into the bags. The Take Set[®] was maintained in each bag and served for the sampling of the drug mixture by means of a syringe. Purified water (Fresenius, Sevres, France), formic acid and acetonitrile (VWR, Fontenay-sous-Bois, France) and high grade methanol (Merck, Guyancourt, France) were used for analytical purposes.

2.2 Preparation, storage, and stability study

NHS Pharmaceutical Quality Assurance Committee requirements (Santillo, 2017) were used as the basis for the study design (Table 1). Etoposide solutions in bags (n=128) were produced by diluting ETP Mylan[®] in NaCl 0.9% or G5% to obtain final concentrations of 0.68 and 1 mM, respectively. Easyflex[®] and Freeflex[®] bags, made of polypropylene were used for the preparation. The preparations were carried out under a controlled atmosphere (ISO 5), partly under a laminar airflow containment hood and under aseptic isolators. The bags were stored in a refrigerator at 5 ± 0.1°C or at 25 ± 0.2°C in a drying oven (Thermofisher, Waltham, Massachusetts, USA). The stability of each preparation was tested in triplicate on day 0, 1, 3, 7, 14, 21 and 28. Various parameters were monitored: clearness by visual examination, turbidity by turbidimetry, pH, and characterization of degradation products by LC-HR-MSⁿ. Twelve bags stored at ambient temperature were monitored on a semi-annual basis for 1 year.

Table 1. Study design for robust stability study according to NHS Pharmaceutical Quality Assurance Committee [15].

2.3 Instrumentation and analytical procedures

160 The concentration of etoposide in the different solutions has been monitored as a
161 function of time with an LC system (Dionex UltiMate 3000 HPLC system, Les Ulis, France)
162 equipped with a quaternary pump, a vacuum degasser, an autosampler and a UV detector.
163 The controlling software was Chromeleon® software version 6.80 SR11 (Dionex, Les Ulis,
164 France). The selected analytical column was a Phenomenex C18 (250 nm x 4.6 nm; 5 µm),
165 maintained at 25°C. The flow-rate, the injection volume, and the detection wavelength were
166 set at 1 mL min⁻¹, 100 µL and 220 nm, respectively. In order to prevent any co-elution and
167 include late eluting analytes, the LC parameters were optimized in terms of mobile phase
168 composition, buffer concentration, pH, and temperature as described by Bakshi and Singh
169 enabling the development of stability-indicating assay method (SIAM) capable of
170 discriminating ETP and its degradation products or excipients without interferences with
171 appropriate resolution values ($R_s > 1.5$) and sufficiently sensitive to detect and quantify
172 degradation products [16]. As a result, the optimum composition of the mobile phase
173 consisted of ultrapure water adjusted to pH 3.8 by formic acid (solvent A) and of acetonitrile
174 (solvent B) in gradient mode (0–12 min: 70% A; 12–15 min: 70/50% A; 15–20 min: 50/70%
175 A). [16, 17]. No special protection against ambient light was employed, as ETP is not
176 considered sensitive to light.

177 The identification and structural elucidation of the degradation products have been
178 carried out by LC-HR-MS with the formerly described LC system coupled to an electrospray-
179 LTQ-Orbitrap Velos Pro system consisting of a double linear trap followed by an orbital trap
180 (Thermo Fisher Scientific, CA, USA). Analyses were carried out in positive ion mode using
181 the following conditions: source voltage 3.4 kV, source and capillary temperatures 300°C and
182 350°C, respectively, sheath gas and auxiliary gas nitrogen flows 40 and 20 arbitrary units,
183 respectively S-Lens at 60%, 35% CEL (collision energy level) for high resolution
184 fragmentation studies, mass range 100–800 m/z, a standard solution was used daily for
185 external mass calibration and MS data processing was carried out using Xcalibur® software
186 (version 2.2 SP 1.48).

187 The pH was determined by means of an S40 SevenMulti pH meter (Mettler-Toledo,
188 Greifensee, Switzerland) and limpidity and turbidity of the prepared ETP solutions were
189 assessed at 620 nm using a spectrophotometer DU 640B (Beckman Coulter, Villepinte,
190 France) on storage days 0, 1, 3, 7, 14, 21 and 28.

191 Lastly, interaction between content and container was investigated by exploring the
192 topography of the bag's surfaces (Easyflex® and Freeflex®) in the presence of ETP on
193 storage days 0 and 7, using atomic force microscopy Innova AFM (Bruker, Palaiseau,
194 France). The cantilever resonance frequency was 320 kHz using a silicon probe NCHV
195 (Bruker) with a stiffness of 42 N/m. The scan rate was between 0.5 and 1 Hz, using tapping

196 mode. For each bag, images of three different areas were recorded. The reference images
197 were compared to those of the bags that had been in contact with ETP.

198

199 **2.4 Validation protocol**

200 **ETP standard and drug product**

201 Standard ETP was used as well as the commercial form of ETP provided by Mylan®.
202 Specificity was established on the basis of separations and peak purity analysed using the
203 photodiode array detector. Linearity and accuracy were determined by defining five
204 concentrations of ETP Mylan® (102, 136, 170, 204, 238 µM) on three consecutive days.
205 Validation of linearity was defined by comparing slopes and intercepts at the origin between
206 both products. For reproducibility and repeatability, 6 solutions of 170 µM were injected on
207 three consecutive days. Limits of detection (LOD) and quantitation (LOQ) were determined
208 by graphical method (signal-to-noise ratio) [18]. A solution of ETP 1 mM was prepared and
209 subjected to -20°C and to +40°C to optimize the HPLC method.

210 For the quantification of the etoposide and its degradation products, the solutions were
211 diluted 5 times with water to ensure that they all fell within the range of linearity of the
212 method.

213

214 **Degradation products (DP): Collection and quantification**

215 Degradation products were observed when the product was exposed to 40°C. This meant
216 that etoposide was temperature sensitive and degraded by thermolysis and/or
217 thermocatalysis. As the degradation products were not commercially available, in order to
218 obtain larger quantities of DPs for LC-MS characterization purposes, we have further forced
219 degradation by exposing at 60°C while verifying that only the intensities of the DPs change
220 but that the impurity profiles remain comparable. Subsequently, the DPs were separated
221 using the HPLC and collected after the detector. The sample corresponding to each DP was
222 kept open over a week at 25 °C to concentrate the fraction by solvent evaporation. The
223 concentrations of degradation products were expressed in relative contents, i.e. by
224 normalizing of areas using Chromeleon® software. The chosen method should provide a
225 content estimate very close to the calibration quantification approach since the UV spectra of
226 D1 and D2 have maxima comparable to that of etoposide, suggesting that the different
227 response factors are equivalent under the chosen detection conditions. Therefore, the
228 linearity and the accuracy for the quantification of the degradation products by HPLC were
229 evaluated at the concentration range of 2.1 – 19.2 µM in comparison with ETP.

230

231 **2.5 Degradation kinetics**

232 ETP degradation kinetics were evaluated at 40°C using LC-UV. ETP samples at 0.68 mM
233 were prepared in triplicate and placed in an oven at 40°C (Thermofisher, USA) then removed
234 and analyzed by HPLC at 0, 1, 2, 3, 7, 14, 21, and 28 days. A rate constant (k) and half-life
235 ($t_{1/2}$) were obtained from the experimental data.

236 Simultaneously, the collected degradation products were subjected to the same stress
237 conditions as those of the ETP degradation conditions (pH at 3.8, in saline and glucose
238 solution) to complete the mass spectrometry elucidation of the degradation pathways.

239

240 **2.6 Stability study**

241 For a better understanding of the phenomena around the physical and/or the chemical
242 instability, two sets of stress testing experiments were conducted on ETP reconstituted
243 solutions. First, ETP solutions (n=3) were frozen at -20°C for 2 hours then defrosted at
244 ambient temperature. Second, ETP solutions (n=3) were subjected to 4 cycles of thermal
245 variation, successively for one hour at 5°C and for one hour at 40°C. Possible interactions
246 between the container (Easyflex® and Freeflex®) and the content giving rise to modifications
247 of the container's surface were investigated by AFM.

248

249 **2.7 Computational details**

250 In order to obtain stable molecular structures, the geometrical parameters of ETP were
251 optimized using the hybrid density functional B3LYP method with the 6-31G(d,p) basis set.
252 The calculations were performed with the Gaussian 09 program. The highest occupied
253 molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) analysis
254 provided the most likely charge transfer within the molecule from the ground state (HOMO) to
255 the first excited state (LUMO). The HOMO–LUMO energy gap (ΔE) reflecting the chemical
256 activity of the molecule was also determined. Molecular electrostatic potential (MEP) analysis
257 has been used to highlight the reactive sites of ETP [19]. In addition, Mulliken population
258 analysis was performed to determine the differences in electronegativity of atoms within the
259 molecule.

260

261 **2.8 In silico toxicological assessment**

262 After structural elucidation and intrinsic stability assessment, toxicity of degradation products
263 was evaluated by in silico strategy. Two prediction softwares were used. The first one is
264 Toxtree, aimed to predict the toxicity thresholds based on Cramer classification [20]. The
265 other one, T.E.S.T (Toxicity Estimation Software Tool, version 4.1) was used for the
266 evaluation of potential developmental toxicity and of mutagenic properties as per Ames test.

267 It appeals to a consensual-based approach making an average of the data produced from
268 several in silico strategies [21, 22].

269

270 **3. Results and discussion**

271 ***3.1 HPLC method optimization and validation***

272 The chromatographic peaks of the API and the excipients are well-resolved demonstrating
273 that the method can be used to accurately analyze the drug and its DPs without
274 interferences. Two degradation products generated under stress conditions at 40°C and
275 labeled DP1 and DP2, possess relative retention times of 0.37 and 0.92 compared to the
276 peak of ETP (Figure 1). The HPLC response was linear for both the standard and the
277 commercial form of ETP with respect to the concentration range 102 – 238 µM, yielding
278 correlation coefficients R of 0.9807 and 0.9911 respectively. The method was found to be
279 precise with an RSD of 1.9%. LOD and LOQ were found to be 0.69 µM and 2.10 µM (Table
280 2).

281 As the standards of degradation products were not available, in view of the identical
282 maximum wavelength absorption exhibited by ETP and its DPs (*Figure S1 in the*
283 *Supplementary materials*), the method validation of the degradation products was performed
284 with DP1 assuming a similar molar extinction coefficient to that of the standard solution of
285 ETP. The method was found suitable for the quantification of the degradation products
286 (Table 2).

287

288 **Figure 1.** Chromatograms of ETP and degradation products (DPs) for several time intervals
289 and storage temperatures.

290

291 Table 2; Validation parameters of the HPLC method for ETP standard, ETP Mylan® and the
292 degradation products (DP1).

293

294 ***3.2 Physical-chemical stability of the drug product***

295 ***Degradation kinetics***

296 At ambient temperature (25 °C), DP1 appeared rapidly after just 2 days of storage; however,
297 it remained below the identification threshold for up to 21 days of storage, which is within the
298 recommendations of the International Council for Harmonization on the content and
299 qualification of impurities in new drug products (< 0.2% for a Maximum Daily Dose comprised
300 between 10 mg - 2 g) [23]. A second degradation product (DP2) was observed after 7 and 50

301 days of exposure at 40°C and 25°C, respectively. Neither of the degradation products was
302 detected in the samples stored at 5°C. The formation of the degradation products increased
303 proportionally with increasing temperature (*Figure 1*). The decomposition related to the
304 formation of DP1 was fitted with a zeroth order kinetic model leading to a half-life ($t_{1/2}$) of 119
305 days and a kinetic constant (k) of 0.0028 mM day⁻¹ for ETP in the prepared solutions at 25
306 °C.

307 ***Long term stability of reconstituted ETP***

308 ETP Mylan® solutions, produced under aseptic conditions in polypropylene bags (Freeflex®,
309 n = 64, and Easyflex®, n = 64), did not show any significant degradation or precipitation over
310 a period of 21 days at 25°C irrespective of the tested concentrations, container material, or
311 solutions (based on a 5 % threshold). However, at 5°C, two Easyflex® bags with ETP of 0.68
312 and 1 mM diluted with G5% showed a precipitation at day 15 and 21, respectively,
313 demonstrated by an increase in absorbance at 620 nm. The concentration decreased
314 dramatically down to 80% (day 15) and 65% (day 21) due to precipitation. These bags have
315 been left out of further analysis. For all other bags irrespective of the temperature, clearness
316 results were strongly correlated with turbidimetry measurements demonstrating an absence
317 of precipitation; the concentration variation was found to be <5% over the test period of 28
318 days (*Figure 2 and Table S1 in the Supplementary materials*). The pH value remained stable
319 over time (The standard deviation < ± 2.5%) irrespective of temperature, duration of storage,
320 appearance of degradation products or precipitation (*Figure 2*).

321

322 **Figure 2.** Long term stability of reconstituted etoposide Mylan ® (ETP): a) pH variation; b)
323 Concentration variation of (ETP) for 0.68 and 1 mM in sodium chloride (Easyflex® bags); c)
324 Concentration variation of ETP for 0.68 and 1 mM in G5% (Easyflex® bags)

325

326 ***Exploration of the surface topography (AFM)***

327 As illustrated in [Figure S2](#) (Supplementary materials), on the whole, the surface
328 morphologies of the 2 types of bags (Freeflex® and Easyflex®) were homogenous and
329 reproducible. The roughness values of the surface of the reference bag compared to that in
330 contact with ETP were 20.3 ± 0.7 nm / 17.1 ± 1.5 nm and 17.8 ± 0.8 / 19.2 ± 0.4 for Freeflex
331 and Easyflex bags respectively at 20 x 20 µm² scales. At this stage, no particular
332 modification as a result of the interaction between ETP and the polymers of the bags was
333 observed. It can therefore be concluded that etoposide does not degrade the polypropylene

334 bags. Moreover, etoposide does not appear to be adsorbed to the bags, indicating a
335 complete absence of interactions between the API and its container.

336

337 ***Physical stability***

338 Exposed to a freezing process at -20°C, the bags showed precipitation after defrosting at 20
339 °C (ambient temperature) rendering the solutions not fit for medical use. Those subjected to
340 a successive thermal shock (refrigerator (5°C) and heat (40 °C)) remained clear. As ETP is
341 poorly soluble in water, excipients (ethanol, Macrogol 300 and polysorbate 80) were
342 employed in the formulation to increase water-solubility. Despite the favorable miscibility and
343 solubility of the excipients with water, during the injection of ETP in the bags of sodium
344 chloride or glucose solution, a temporary whitish veil is observed. Rapid stirring quickly
345 disperses the Mylan® formulation and prevents any precipitation, as demonstrated by a
346 dozen of ETP bags stored for over 1 year at ambient temperature that remain clear.

347 The cold increases the supersaturation of ETP, which has a very low water-solubility of about
348 50 µM at 25 °C [24]. This is clearly confirmed by the precipitation of ETP in the bags that
349 were subjected to -20°C. The precipitate has been analyzed by X-ray diffraction and was
350 found to be amorphous in nature (*Figure S3 in the Supplementary materials*). Precipitation
351 was also observed in two solutions stored at 5°C, which indicates that the Mylan® formulation
352 is, from a thermodynamic point of view, not stable at 5°C. Nonetheless, only 2 solutions of 64
353 in total showed precipitation, implying that the formulation provides an active kinetic barrier to
354 precipitation. It is not certain whether the Mylan® formulation is thermodynamically stable at
355 25°C, however no precipitation has been observed in solutions that were properly mixed after
356 introduction of the ETP with its excipients in the bags with NaCl or glucose solutions.

357

358 ***Characterization of degradation products by LC-HR-MS'***

359 The fragmentation pattern of ETP is a key factor to get access to the structure of its
360 degradation products. The origin of each fragment was established through MSⁿ studies and
361 confirmed by high-resolution mass spectrometry analysis. The mass error was calculated to
362 obtain the most probable elemental composition (Table 3). For the sake of simplicity, the
363 nominal m/z has been considered. The ETP mass spectrum represented in Figure 3a shows
364 that the molecular ion of ETP [M+H]⁺ has an m/z of 589 and exhibits eight daughter ions at
365 m/z 435, 401, 383, 363, 299, 247, 229 and 185. Most of the fragments have already been
366 described in the literature [25, 26]. The fragmentation pattern shows two main parallel
367 fragmentation pathways (Figure 3b). First, loss of dimethoxyphenyl has been identified,
368 characterized by the transitions of m/z 589→m/z 435, m/z 401→m/z 247 and m/z 383→m/z

369 229. Then, rearrangement and a neutral loss of the glucopyranosyl moiety by O-dealkylation
370 has been observed, illustrated by the transitions of m/z 589 \rightarrow m/z 401, m/z 589 \rightarrow m/z 383
371 and m/z 435 \rightarrow m/z 229. The fragment of m/z 383, the most intense peak, is generated both
372 by a rearrangement leading to the departure of a 206 Da moiety (589 \rightarrow 383) or by water
373 loss through the ion m/z 401.

374 The two fragmentation paths lead to a common fragment, with an intensive peak at m/z 229
375 (4,6,13-trioxatetracyclohexadeca-1(9),2,7,15-tetraen-12-one) through the loss of two
376 moieties on either side of the four-ring core, namely the glycoside and the dimethoxyphenyl
377 groups. Furthermore, the four-ring core breaks up through different mechanisms, such as the
378 departure of formic acid (H_2CO_2) from the strained lactone ring demonstrated by the
379 transitions m/z 383 \rightarrow m/z 337 and 229 \rightarrow m/z 185, or by rearrangement and neutral losses
380 resulting from the departure of propadiene $C_5H_8O_3$ (m/z 383 \rightarrow m/z 267) and carbon dioxide
381 (m/z 383 \rightarrow m/z 299).

382 The fragments of m/z 383 and 401 were identified to have the same structures and
383 fragmentation patterns as DP1 and DP2 respectively. The accurate mass of the protonated
384 ion of 383.1112 matched the molecular formula of DP1 ($C_{21}H_{19}O_7^+$). The structure was
385 supported by the fragmentation pattern illustrated in figure 3b. Hence, compared to ETP, in
386 DP1 the transition corresponding to the neutral loss of the glucopyranosyl derivative ($C_8H_{12}O_5$
387 or $C_8H_{14}O_6$) was absent implying that this moiety was impacted by degradation. Hence, DP1
388 is postulated to be 10-(4-hydroxy-3,5-dimethoxyphenyl)-4,6,13-trioxatetracyclohexadeca-
389 1(9),2,7,15-tetraen-12-one.

390 DP2 ($C_{21}H_{21}O_8^+$) the late onset degradation product has already been described in the
391 literature as 4'-dimethylepipodophyllotoxin [27].

392

393 **Table 3.** Structures, accurate masses with errors, elemental compositions and MS^n and
394 relevant product ions of Etoposide and its degradation products.

395

396 **Figure 3.** ETP MS spectrum (a) and fragmentation pattern (b).

397

398 ***Postulated degradation pathways of ETP in the drug product***

399 After reconstitution in saline or glucose solution, ETP was shown susceptible to temperature.
400 The appearance and increase of the two DPs are correlated with the increase in temperature
401 and were evidenced by high resolution mass spectrometry. The degradation occurred mainly
402 through a mechanism of acid-catalyzed hydrolysis resulting in the departure of the glycoside
403 chain. Due to its C-O bond, etoposide undergoes O-dealkylation by hydroxylation of α -carbon
404 to form an unstable hemiacetal intermediate, which spontaneously cleaves to D2 and a

405 carbonyl compound, D2' (Figure S4 in the Supplementary materials). Dehydration of D2
406 results in the formation of D1. The chemical degradation mechanism is supported by the
407 theoretical molecular orbital analysis.

408 **Analysis of the frontier molecular orbitals:** As illustrated in Figure 4.B, the HOMO is
409 essentially localized on the phenyl ring (C23, C33, C36, C39) and on the oxygen atoms of
410 the 4-hydroxy-3,5-dimethoxyphenyl moiety. The glycoside moiety does clearly not contribute
411 to the HOMO. The LUMO is mainly located on the 1,3-benzodioxole moiety; the lactone ring
412 (O2, O5 and C21) and the ether oxygen O1 contribute too (Figure 4.B). The calculated
413 values of the ETP HOMO and LUMO energies are -0.2026 eV and -0.0154 eV respectively,
414 leading to a HOMO-LUMO gap of ΔE (ETP) = 0.1872 eV. The small value indicates that only
415 little energy is required for excitation. ETP is therefore expected to be easily polarizable with
416 a relatively high chemical reactivity [28, 29].

417 **ETP electrostatic potential and Mulliken charges.** The calculated electrostatic potential
418 has been provided in Figure 4.C. The potential increases from strongly negative values in red
419 < orange < yellow < green < blue, with blue reflecting the highest positive values. The color
420 code of the map was set between -7.480 au (deepest red) and 7.480 au (deepest blue). As
421 illustrated in Figure 4.C, the most negative electrostatic potential regions (red) were located
422 around oxygen atoms O13, O5, O6 and O8. In contrast, the areas colored in blue, zones with
423 the highest positive potential are comparable to the position of the LUMO. The Mulliken
424 charges distribution obtained from the optimized ETP (Figure 4.A) demonstrated that the C21
425 (lactone), C38 (1,3-dioxole), and C21 have the biggest positive atomic charges of 0.637e,
426 0.567e and 0.522e respectively. This is due to the large negative charges of the O2 (-0.490e)
427 and O5 (-0.516e) of the lactone, the O9 (-0.553e) and O10 (-0.552e) of the 1,3-dioxole and
428 the O1 (-0.546e) of the ether.

429 It can be concluded that the reactive regions are localized around the polarized sites. The
430 formation of DP1 and DP2 are the result of reactions involving the ether O1 directly
431 connected to the electropositive carbon atom C22 of the glycoside. The effect of temperature
432 has clearly been demonstrated in the ETP degradation process.

433 Furthermore, the separate samples of DP1 and DP2 collected by HPLC as described in the
434 experimental section, showed no interconversion between each other while subjected to pH
435 3.8 at 40°C, suggesting independent degradation pathways. It is proposed that an initial
436 protonation of the ether results mainly in DP1 (major product) and to a lesser extent in DP2
437 (minor product). In the literature, ETP was described to undergo a ring-opening reaction of
438 the lactone ring in acidic media (pH<4) and epimerization (pH>6) [30]. Such mechanism was
439 not observed in the present study.

440

441 **Toxicological assessment**

442 The potential toxicity of the identified impurities was evaluated and compared with the ones
443 of the precursor using quantitative structure-activity relationship (QSAR) tools. As described
444 in Table S2 (Supplementary materials), the analysis did not identify any structural alerts
445 related to mutagenicity for ETP or its degradation products based on its most up-to-date
446 database. However, T.E.S.T has associated ETP, DP1 and DP2 to developmental toxicity
447 risks. Based on the Cramer rules, Toxtree indicated that ETP and its degradation products
448 might belong to class III, which includes unknown and unsafe products. Overall, the
449 apparition of degradation products does not seem to lead to additional toxicity.

450

451 **Figure 4.** A) Optimized structure of ETP; B) The frontier molecular orbitals and related
452 energies; C) Molecular electrostatic potential (MEP) surface map for ETP

453

454 **4. Conclusion**

455

456 The present study provides information about the chemical and physical stability of
457 reconstituted etoposide in aqueous solutions containing either NaCl or glucose (0.68 or 1
458 mM). Whereas in solutions kept at 25°C a slow increase in degradation products DP1 and
459 DP2 could be observed, no decomposition was apparent in solutions kept at 5°C for at least
460 28 days. Chemically, the stability depends on hydrolysis followed by the departure of the
461 glycoside moiety as the main degradation pathway of diluted ETP. No effect of the presence
462 of sodium chloride or glucose has been observed.

463 In relation to the physical stability, solutions kept at 25°C did not show any precipitation,
464 whereas at 5°C two solutions contained amorphous precipitates after 14 and 21 days. From
465 this perspective, storage under cold conditions must be avoided as recommended by
466 Sandoz® (SmPC Etoposide Sandoz®, 2015). Although the saturation concentration for the
467 samples at 25°C is not known, the excipients appear to protect the active ingredient ETP
468 against precipitation quite well, even at 5°C.

469 It is clear that the chemical and physical stability behaviors require different storage
470 conditions and limit therefore the total storage time of the reconstituted product. Considering
471 the identification / qualification threshold of the impurities [26] and the physical properties,
472 ETP prepared under good manufacturing practices (i.e. in particular well controlled mixing
473 when introducing the Mylan® formulation in the respective NaCl and glucose solutions)
474 remained unchanged over a period of 21 days at 25 °C irrespective of concentration, nature
475 of the solvents (NaCl or glucose), or type of container. The occurrence of the degradation
476 products above the defined acceptance limits does not appear to be associated with
477 additional toxicity. In the light of these results, by applying a safety margin, the stability period

478 after reconstitution may be set at a period of less than 21 days. In relation to the shelf life of
479 the previously used reconstituted etopophos, i.e. 4 days at 25 °C, the stability data of the
480 present replacement product after reconstitution provide hospitals with an opportunity to
481 improve its production organization, while guaranteeing patient safety. These conclusions
482 should be applicable too to other ETP-based proprietary pharmaceuticals with the same
483 qualitative and quantitative compositions.

484 Since this study has been carried out, more than 20,000 reconstituted solutions have been
485 produced by the Parisian Hospitals and no precipitation or excess degradation products have
486 been observed within the 21 day limit. As a result, the study contributes to diminishing the
487 cost of discarding ready-to-use preparations exceeding the limited official expiry date
488 avoiding the environmental consequences. In addition, it provides the hospitals with a more
489 flexible planning for the reserve of reconstituted solutions leading to less error-prone patient
490 care. Lastly, the study also demonstrates the need of harmonization of the SmPCs on an
491 international level, in particular for formulations that are the same in constituent and
492 concentration even if produced by different manufacturers.

493

494 **Acknowledgements**

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497

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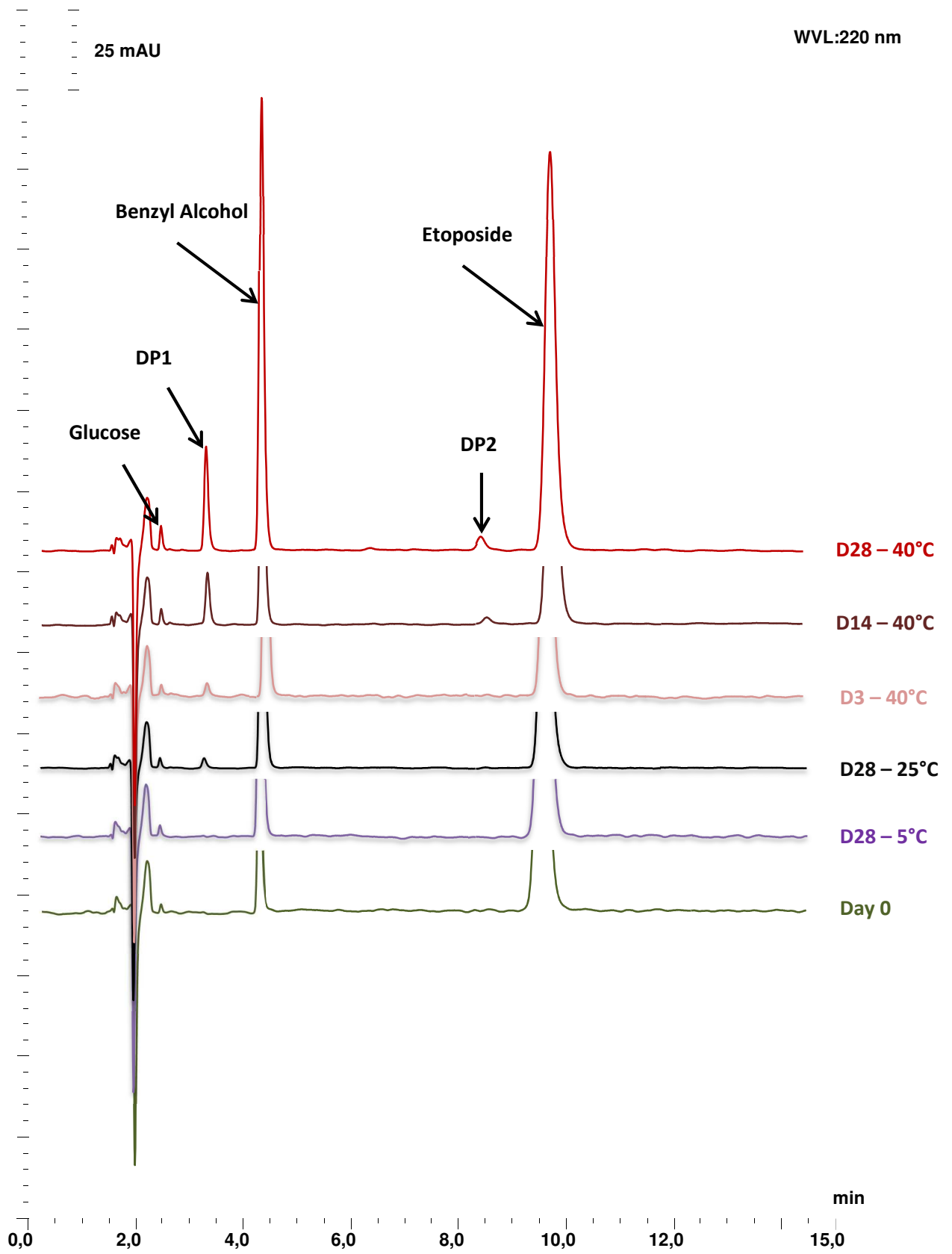
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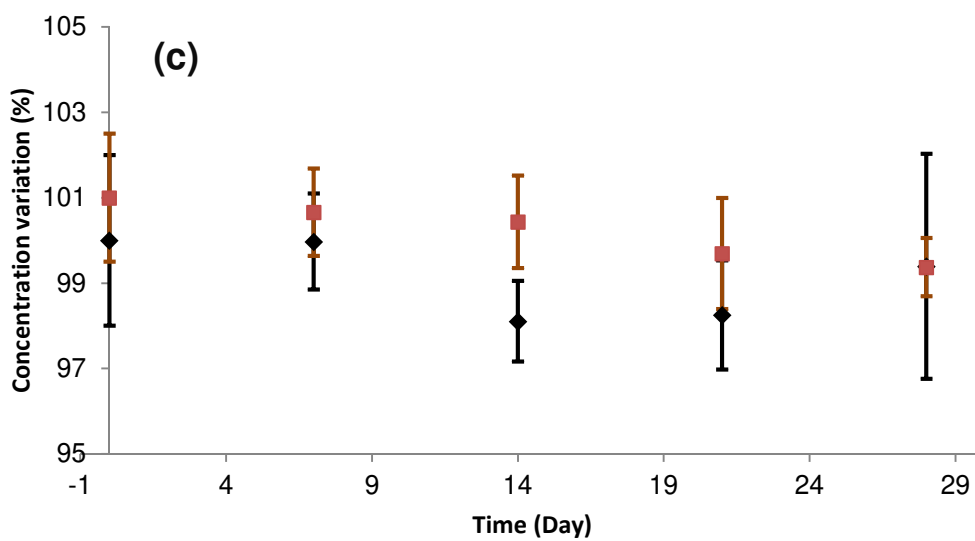
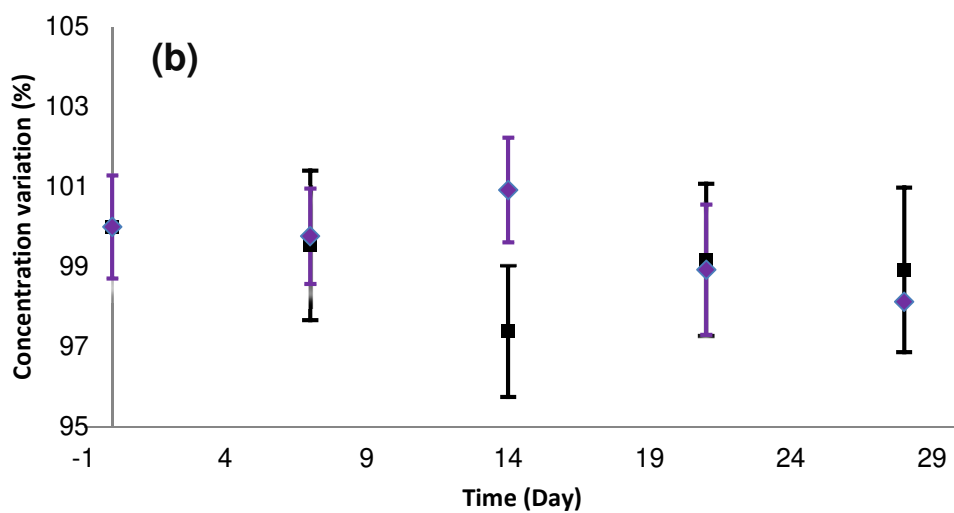
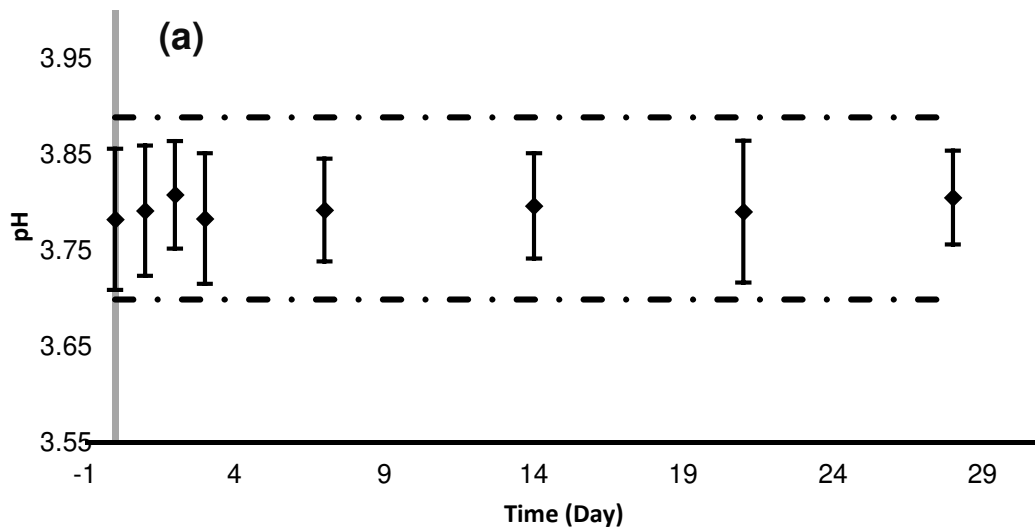
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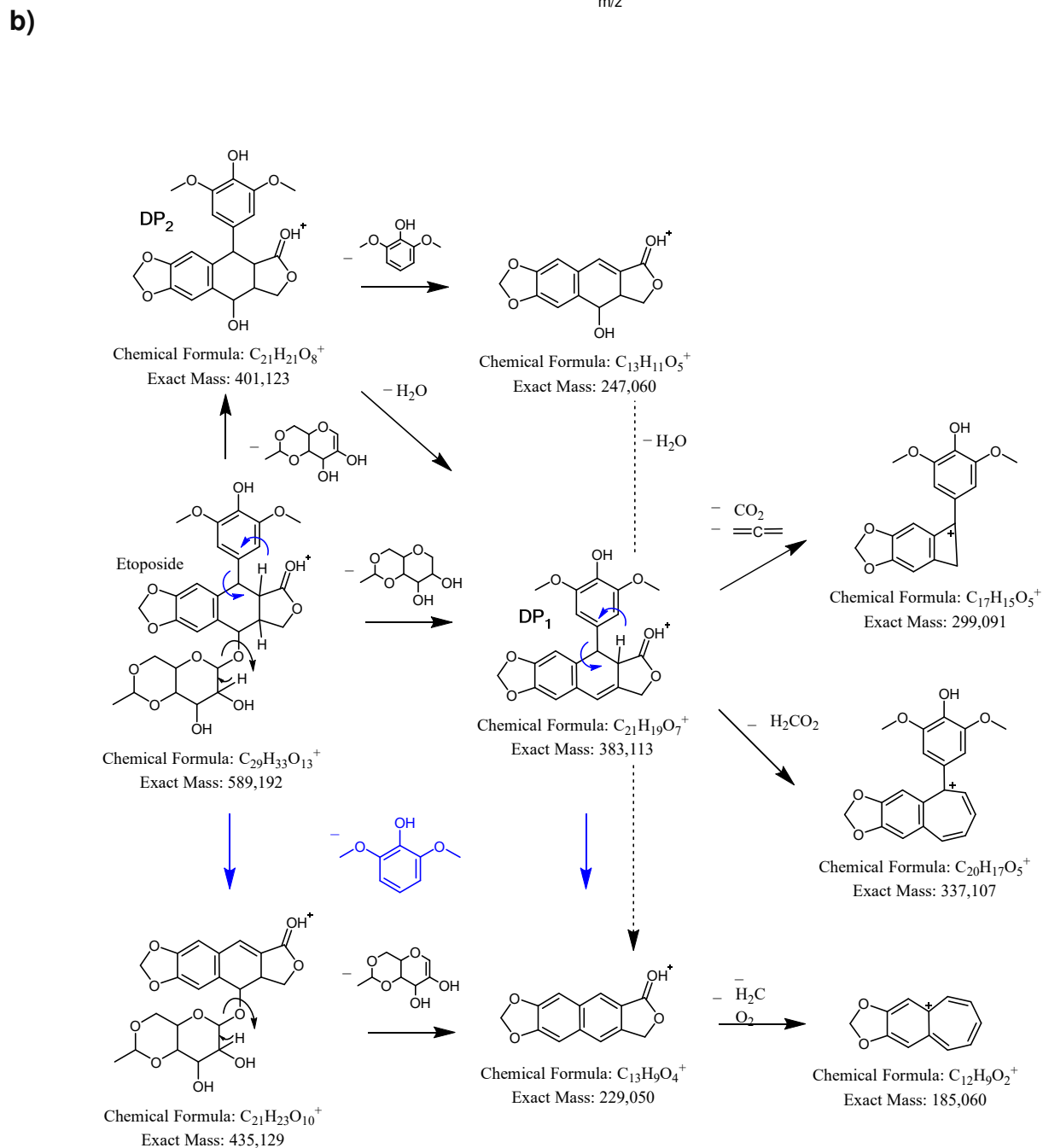
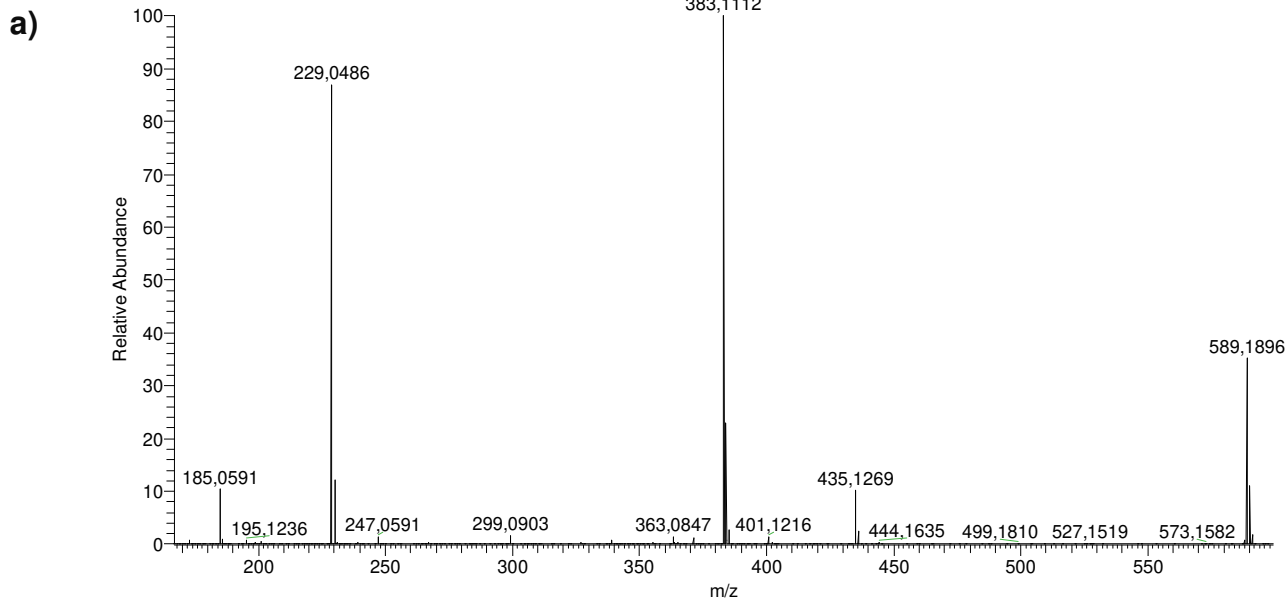
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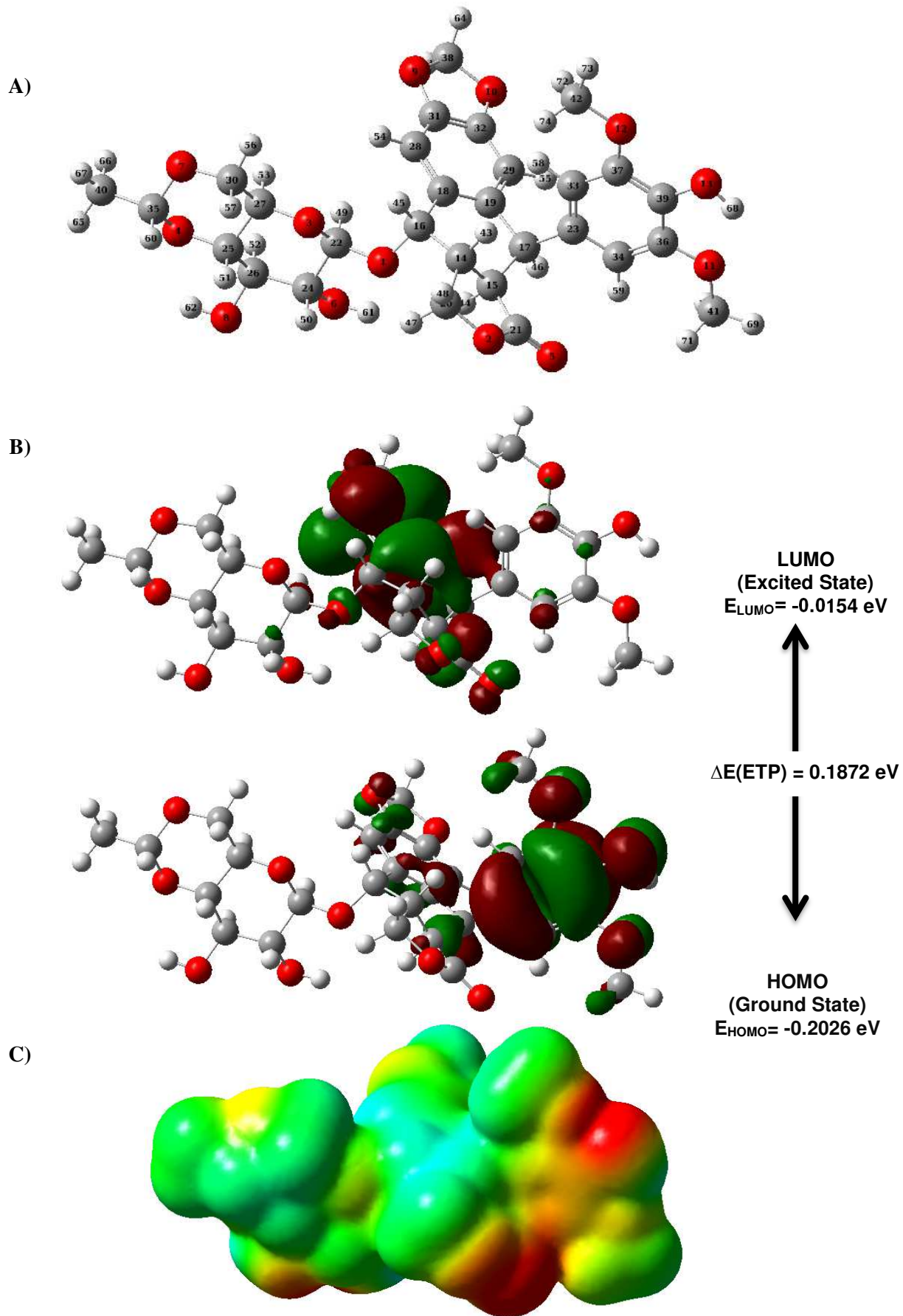


Figure 4: A) Optimized structure of ETP; B) The frontier molecular orbitals and related energies; C) Molecular electrostatic potential (MEP) surface map for ETP

Table 1. Study design for robust stability study according to NHS Pharmaceutical Quality Assurance Committee (Santillo, 2017).

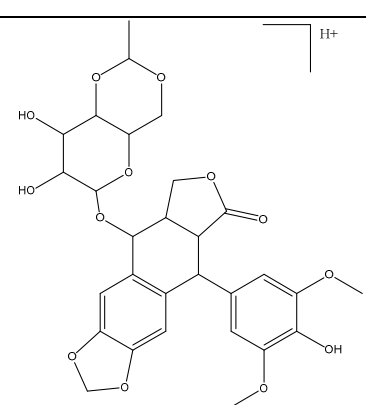
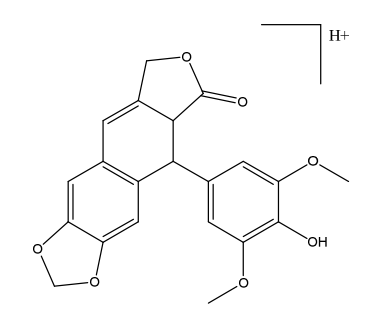
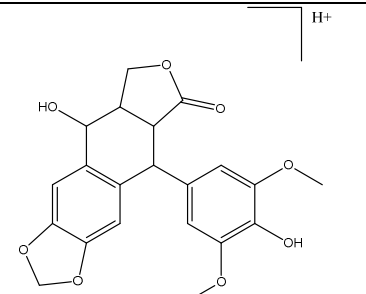
Study parameters	Pharmaceutical Quality Assurance Committee requirements	Element considered in this study
Diluent	As specified in SmPC	0.9% sodium chloride and glucose 5% solution
Containers	Non-PVC	Polyolefin (Freeflex® and Easyflex®)
Storage	Refrigerated (5°C) without exposure to UV light	Refrigerated without protection from light
	Room temperature without exposure to UV light (25°C)	25°C and Room temperature without protection from light
Concentrations	Low & high clinically significant	Two concentrations tested. Clinically common (0.68 mM) and clinically high (1 mM) concentrations
Storage period	Sufficient time to allow critical parameters	28 days storage
Sampling strategy	At least 4 justified time points plus time point zero is the minimum data set required	Sampling performed on each bag at day = 0, 1, 3, 7, 14, 21 and 28 for clinically common and high concentrations
Sample numbers	Three independent batches Three replicates per batch	64 independent batches for each common concentration Three replicates per batch

Table 2. Evaluated parameters of quantitative analytical procedures

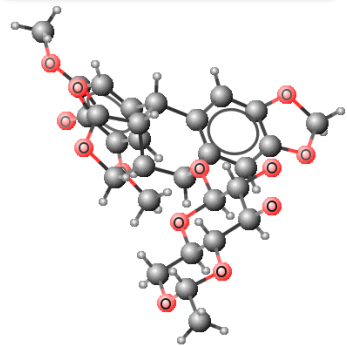
Parameters	Results
Linearity	
Etoposide standard	
Concentrations range (μM)	[102;238]
Slope	0.304
SD* of slope	0.012
Intercept	-6.456
SD* of intercept	2.197
Correlation coefficient (r)	0.978
Etoposide Mylan®	
Concentrations range (μM)	[102;238]
Slope	0.261
SD* of slope	0.0068
Intercept	0.184
SD* of intercept	1.21
Correlation coefficient (r)	0.991
Degradation product (DP1)	
Concentrations range (μM)	[2.1 ; 19.2]
Slope	10.926
SD* of slope	0.470
Intercept	0.059
SD* of intercept	0.244
Correlation coefficient (r)	0.976
LOD (μM)	0.69
LOQ (μM)	2.10
Precision	
Repeatability	0.007%
Reproducibility	1.874%
Accuracy	
Recovery	100.808
SD*	3.378
95% CI	[98.938 ; 102.679]
Specificity	SIAM

* : Standard deviation

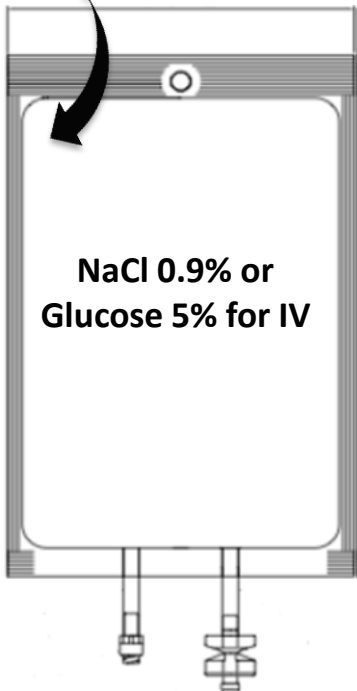
** : Stability Indicating Assay Method

	MS-HR datas	Best possible molecular formula	Theoretical mass m/z (amu)	Error (ppm)	Proposed structures
Etoposide	589.1896	C ₂₉ H ₃₃ O ₁₃ ⁺	589.1876	3.4	
	435.1269	C ₂₁ H ₂₃ O ₁₀ ⁺	435.1286	-3.9	
	401.1216	C ₂₁ H ₂₁ O ₈ ⁺	401.1231	-3.7	
	383.1112	C ₂₁ H ₁₉ O ₇ ⁺	383.1125	-3.4	
	299.0903	C ₁₇ H ₁₅ O ₅ ⁺	299.0914	-3.7	
	229.0486	C ₁₃ H ₉ O ₄ ⁺	229.0495	-3.9	
	185.0591	C ₁₂ H ₉ O ₂ ⁺	185.0597	-3.2	
DP1	383.1112	C ₂₁ H ₁₉ O ₇ ⁺	383.1125	-3.4	
	365.1022	C ₂₁ H ₁₇ O ₆ ⁺	365.102	0.5	
	351.0869	C ₂₀ H ₁₅ O ₆ ⁺	351.0863	1.7	
	337.1074	C ₂₀ H ₁₇ O ₅ ⁺	337.1071	0.9	
	323.0918	C ₁₉ H ₁₅ O ₅ ⁺	323.0914	1.2	
	299.0924	C ₁₇ H ₁₅ O ₅ ⁺	299.0914	3.3	
	229.0505	C ₁₃ H ₉ O ₄ ⁺	229.0495	4.4	
	185.0596	C ₁₂ H ₉ O ₂ ⁺	185.0597	-0.5	
DP2	401,1216	C ₂₁ H ₂₁ O ₈ ⁺	401.1231	-3.7	
	383,1110	C ₂₁ H ₁₉ O ₇ ⁺	383.1125	-3.9	
	299,0907	C ₁₇ H ₁₅ O ₅ ⁺	299.0914	-2.3	
	229,0489	C ₁₃ H ₉ O ₄ ⁺	229.0495	-2.6	
	185,0593	C ₁₂ H ₉ O ₂ ⁺	185.0597	-2.2	

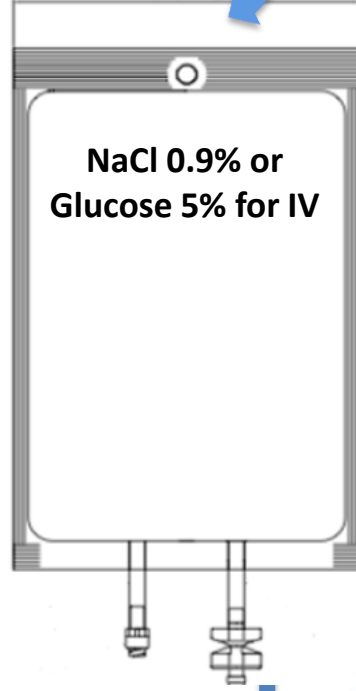
**Branded drug
Etopophos**



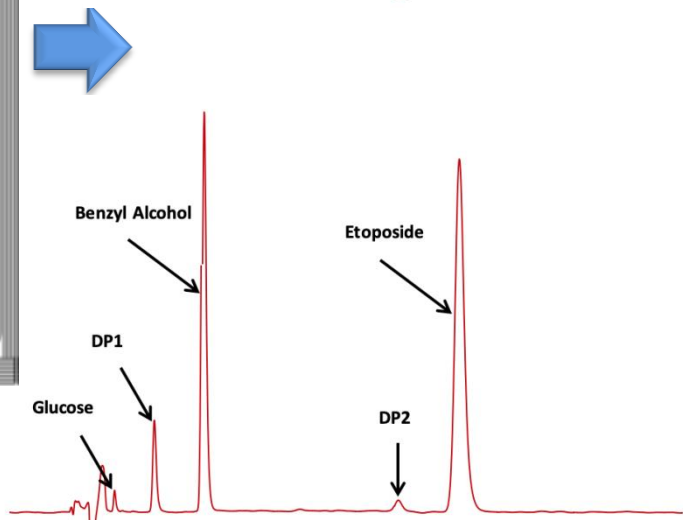
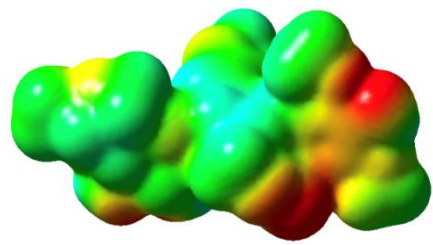
**Generic drug
Etoposide**



4 days



21 days



Expiration at 25°C



Time