IVAX Drug Research Institute Ltd., Budapest, Hungary

Determination of estredox, a compound with sustained estradiol function, and its impurity profile by HPLC

M. Patthy, G. Seres, É. Tóth-Sarudy, I. Hazai, I. Pallagi, A. Simay, N. Bodor

Received October 11, 2007, accepted November 1, 2007

Dr. Antal Simay, 30 Pagony utca, 1124 Budapest, Hungary
antail.simay@yahoo.com

Pharmazie 63: 210–216 (2008) *doi: 10.1691/ph.2008.7321*

The HPLC methods described here for the assay and purity test of Estredox (E_2CDS), a molecule with a redox-based, brain-targeted chemical delivery system for estradiol, allow reliable conclusions to be made on the potency and purity of API and E_2CDS/HPCD complex samples. Extensive work was done to isolate and characterize the major, potential contaminants, and ensure the required stability of solutions of E_2CDS, an inherently labile compound by design. Both the sample solvent and the eluent were thoroughly tested to avoid unwanted changes in sample solutions during analyses. The 12 minute isocratic assay method at 220 or 360 nm is simple, well-founded, highly precise and accurate. Purity profiling of E_2CDS raised several problems in detection, stability and accuracy, owing to the fact that the pattern of the UV spectra and the stability of the compound and those of the potential contaminants often differed greatly. As a result of meticulous analysis of the UV spectra and the factors influencing the behaviour, in solution, of the compounds concerned, the 20 minute gradient method developed for the purity test, at 220 nm, of E_2CDS and E_2CDS/HPCD complex samples has proved to be a reliable means of adequately resolving 15–20 peaks of known and unknown compounds, and establishing the purity of various E_2CDS samples. Sample impurity can be expressed as area % at 220 nm, and/or as approximate w/w % (if needed), since the relative response factors, at 220 nm, of the 6 major, potential contaminants have also been determined.

1. Introduction

Estredox (E_2CDS) is a patented molecule designed to treat peri-menopausal symptoms by delivering estradiol to the central nervous system (CNS) while keeping systemic levels of estradiol within the normal, i.e. pre-menopausal physiological range (Estes et al. 1987, 1994; Rahimy et al. 1990). The lipophilic molecule, which does not survive in the gastro-intestinal tract, can penetrate the CNS from a buccal tablet. Selective targeting is achieved after conversion by two ubiquitous redox enzymes to an inactive, positively charged precursor form which cannot return to the systemic circulation from the brain and is, therefore, "locked in". The quaternary precursor form is thereafter slowly converted by ester hydrolysis to release estradiol in the CNS. The precursor is rapidly cleared from other organs, so the brain becomes the selective site for delivery.

The drug molecule – due to its dihydrotrigonellinate moiety – is susceptible also to non-enzymic oxidation and hydrolysis, which may cause problems with its handling. Besides, E_2CDS and its related potential contaminants absorb UV light at widely different wavelengths, making analytical and stability studies a real challenge.

In order to increase its stability, in general, and solubility in water, E_2CDS is used as an inclusion complex of ca. 3% with 2-hydroxypropyl-β-cyclodextrin (HPCD).

In this report, the elaboration of reliable analytical methods for the assay and purity test of E_2CDS by HPLC has been described.

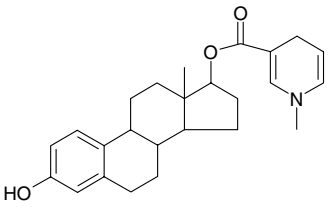
2. Investigations and results

HPLC methods for the assay and purity test of the E_2CDS substance (or active pharmaceutical ingredient = API) and the E_2CDS/HPCD complex have been developed.

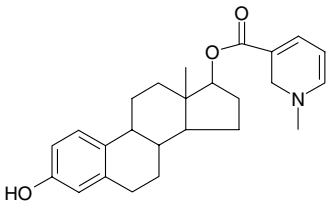
2.1. Assay

The elution solvent elaborated for the assay was a 67 : 33 (v/v) mixture of acetonitrile and aqueous acetate buffer. The retention time of E_2CDS in this isocratic system was 8.5–9.5 min. The sample solvent contained 35 mg of analytical purity Na_2SO_3 in 100 ml of elution solvent to control sample stability in solution.

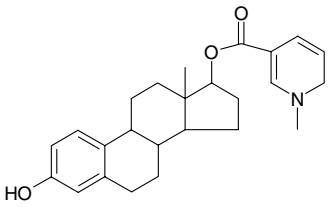
Procedure: Following a "blank run" with 20 µl injected sample solvent (acetonitrile/water = 70/30, v/v), which should not result in peaks with retention times and area counts larger than 2 min and 4000, respectively, 20 µl aliquots of the standard and sample solutions – in parallels of 2 or 3, and prepared immediately or not more than 2 hours with API-s, and 3 hours with E_2CDS/HPCD complexes before injection, each – were injected into the



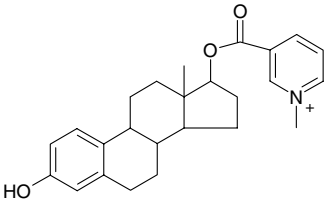
E_2CDS



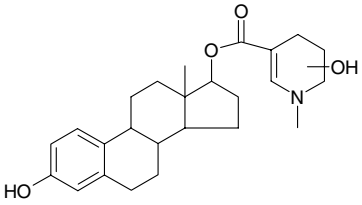
1,2-dihydro isomer
(**G**)



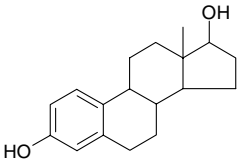
1,6-dihydro isomer
(**E**)



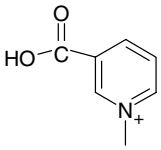
Quaternary product (QUAP)
(**B**)



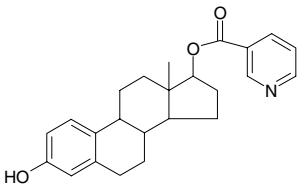
Water addition product (WAP)
(**D**)



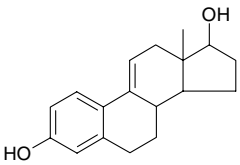
Estradiol
(**C**)



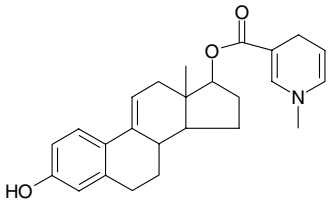
Trigonelline
(**A**)



Estradiol nicotinate
(**F**)



9,11-dehydroestradiol



9,11-dehydro-E_2CDS
(impurity **3**)

chromatographic system. Chromatographic run time was 12 min.

Calibration curve for API-s and E_2CDS/HPCD complexes: Accurately weighed 7–8 mg, 10–11 mg and 14–15 mg portions of the working standard were dissolved in the sample solvent and diluted to volume in three 25.0 ml volumetric flasks. After injecting and running 20 µl aliquots, the area of the main peak was measured and a graph was prepared by plotting the averages of the peak areas (at each concentration point) as a function of working standard concentration. Linearity (R^2) of the graph was better than 0.999 both at 220 nm and 360 nm. (When linearity is that good, the "one-point calibration" method of 2 independent solutions with 2 parallel injections, each, can also be used.)

For the assay of E_2CDS API samples, three accurately weighed 8–15 mg portions of the sample were dissolved in the sample solvent and diluted to volume in three 25.0 ml volumetric flasks. For the assay of E_2CDS/HPCD complex samples, three accurately weighed 120–180 mg portions of the complex were dissolved in the sample solvent and diluted to volume in three 10.0 ml volumetric flasks.

Relative standard deviation (RSD) for the concentration-normalized average of the peak areas in the 6–9 runs did not exceed ± 2.0%.

E_2CDS content of the sample was calculated by means of the calibration graph (or point) and the normalized average of the peak areas in the 6–9 chromatograms of the sample (and taking the potency % of the working standard into account as well, if necessary).

2.2. Purity test

The elution solvents for the gradient purity test runs were as follows: Solvent (a): acetonitrile/sodium acetate buffer = 45/55 (v/v), solvent (b): acetonitrile/sodium acetate buffer = 70/30 (v/v). The gradient steps were: from 100%

(a) to 10% (a) (i.e. 90% (b)) in 6 min; from 90% (b) to 100% (b) in 8 min, hold 100% (b) for 3 min, return to 100% (a) in 3 min. Equilibrate before injection for 10 min.

The retention time of E_2CDS in this gradient system was 15.5–16.5 min.

The retention times (in min) of the known related compounds (referred to by **A**, **B**, **C**, etc. in some figures) and other minor contaminants (referred to as "unknown" or "impurity" **1**, **2** or **3**, whose structural identification has not yet been or has just been completed: Hazai et al., in preparation) were as follows; see also Fig. 1:

A: trigonelline (0.85–1.1, if present; in co-elution with the solvent front), **B**: quaternary product (QUAP) (4.4–5), **C:** estradiol (5.4–5.8), **D**: water addition product (WAP) (8.1–8.4), impurity **1**: unknown (9.1–9.3), impurity **2**: unknown (10.7–11.1), **E**: the 1,6-dihydro E_2CDS isomer (12.7–13.1), **F**: estradiol nicotinate (13.3–13.6), impurity **3**, which is now known to be the 9,11-dehydro E_2CDS analogue (14.7–15.1) and **G**: the 1,2-dihydro isomer (16.1–16.4).

Procedure: Following a "blank run" with 20 µl injected sample solvent (which should not result in peaks with retention times and area counts larger than 2 min and 5000, respectively), 20 µl aliquots of the standard and the sample solutions (prepared immediately or NMT 1 h before injection, each, in parallels of 2) were injected into the chromatographic system. Chromatographic run time was 20 min. The sample solvent contained 35 mg of analytical purity Na_2SO_3 in 100 ml to control sample stability in solution.

For the purity test of E_2CDS substance (API), an accurately weighed 5–6 mg portion of the sample was dissolved in the sample solvent (acetonitrile/water = 70/30, containing the stabilizer, as described above) and diluted to volume in a 10.0 ml volumetric flask. The solution of the working standard was prepared in the same way.

For the purity test of E_2CDS/HPCD *complex*, an accurately weighed 160–180 mg portion of the sample was dissolved in the sample solvent and diluted to volume in a 10.0 ml volumetric flask.

Warning: Sonication of all the solutions to be injected is to be kept to a minimum, to not more than 30 s.

Sample purity: All the peak areas larger than 5000 area counts were recorded. Impurities total (*area %*) was obtained by subtracting the area % values of the main peak, the peaks of the 1,2- and 1,6-dihydro E_2CDS isomers, as well as the solvent peak at the front (if there were no signs of related absorption in the PDA spectrum of this peak at *ca* 1.1 min) from 100. Sample purity (total impurity, *area %*) was calculated by averaging the purity results of the 2 gradient sample runs.

(Trigonelline, whose UV spectrum is shown in Fig. 1, might be present in co-elution with the solvent peak after extensive decomposition of E_2CDS samples, such as oxidation followed by ester hydrolysis. In this case, the micromolar concentration of estradiol in the sample may be indicative of the trigonelline content.)

The approximate w/w % sample purity can be calculated similarly, using the relative response factors (RRF-s) we have determined at 220 nm. Individual w/w % values can be obtained by multiplying the respective area % value with the RRF value shown below. (RRF for E_2CDS = 1.0; for minor contaminants for which response factors were not determined, RRF was arbitrarily taken as 1.0).

RRF for **A** (trigonelline): due to a potential co-elution and unstable baseline at the front, determination of the RRF value was unreliable; **B** (quaternary product, QUAP): 1.07; **C** (estradiol): 1.33; **D** (water addition product, WAP): 1.98*; **E** (1,6-dihydro isomer): 1.39*; **F** (estradiol nicotinate): 0.66; **G** (1,2-dihydro isomer): because of inadequate purity, RRF was not determined; it was arbitra-

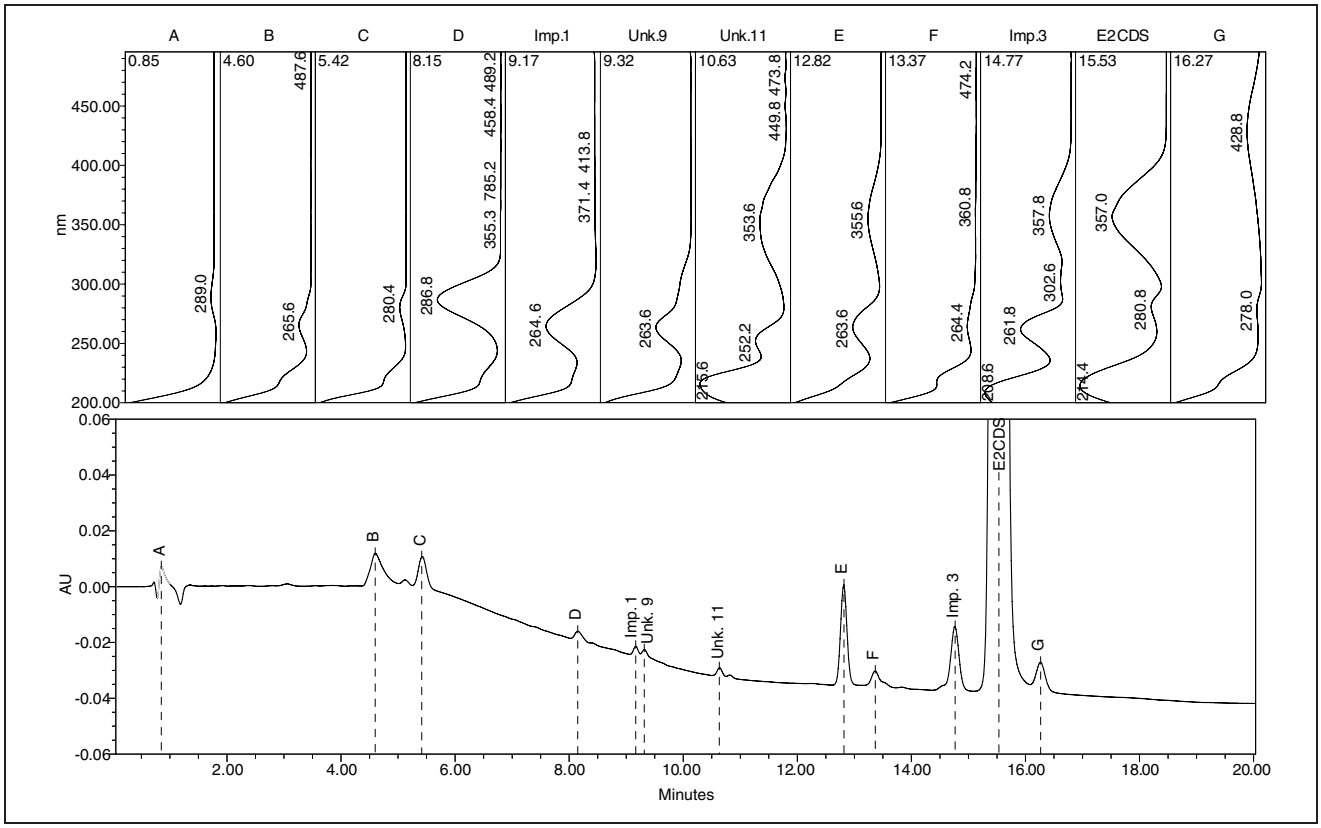


Fig. 1: Representative purity test chromatogram of an E_2CDS/HPCD complex sample (with the relevant UV spectra above)

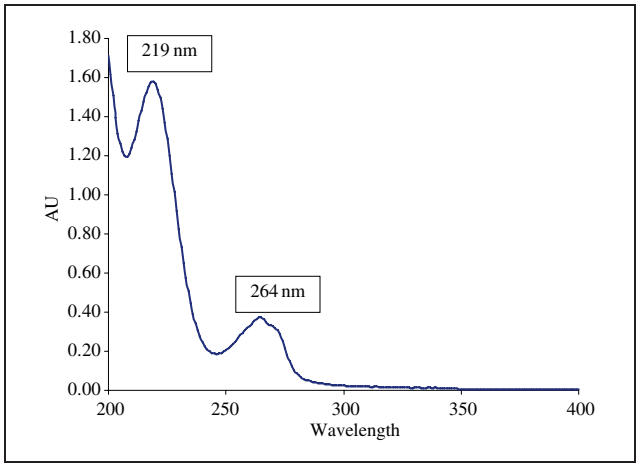


Fig. 2: UV spectrum of trigonelline obtained in the assay eluent with a Varian Cary 3E UV-VIS spectrophotometer

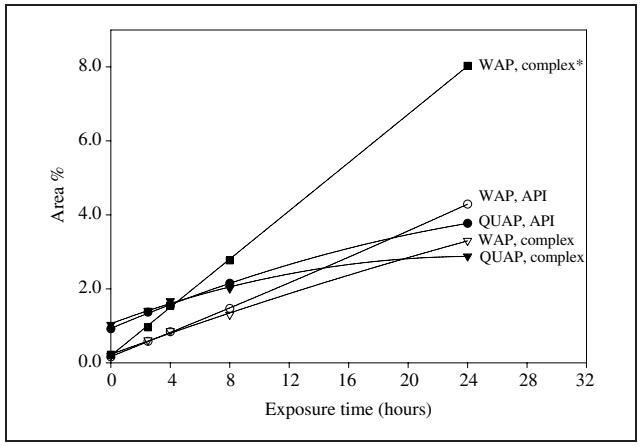


Fig. 3: Changes in the concentrations (area %) of WAP and QUAP as a function of time spent in buffered solutions of API and E_2CDS/HPCD complex samples. The solvent was acetonitrile/aqueous buffer = 65/35, v/v; the buffer was aqueous sodium acetate (as described in 4.2.3) except for one solution (marked with the asterisk) when it was aqueous ammonium acetate prepared with a 10%, w/v, ammonia solution instead of 2N NaOH

rily taken as 1.00; impurity **3** (9,11-dehydro E_2CDS analogue): 0.63*. (*Because the purity of the compound isolated was 80 < 98 area %, RRF value was calculated (corrected) in such a way that, based on its chromatogram, relevant (and known) response data for the main contaminant(s) were also taken into account.)

Limits of detection in the purity test (ng/ml, from a 20 µl aliquot; 3 times the noise as determined by the Waters Millennium 4.0 software) for the major E_2CDS related compounds were as follows: **B**, 79; **C**, 90; **D**, 411; **E**, 84; **F**, 20; impurity **3**, 79.

3. Discussion

Purity profiling of E_2CDS, an inherently labile compound by design, has been a major analytical challenge. Some of the causes are listed below:

(i) The use of UV detection involves difficulty due to the fact that both the intensities and the position of the maxima of the UV absorption bands of the impurities and those of E_2CDS vary greatly (see a representative chromatogram and the UV spectra of the compounds separated in Fig. 1). Thus, area % purity results obtained at a wrongly chosen wavelength may give greatly misleading results. (ii) With the exception of the quaternary product, estradiol and estradiol nicotinate, the known related compounds are not stable or stable enough to be isolated in pure form; consequently, their relative response factors (RRF-s), determined to correct for the differing UV absorptivities at a chosen wavelength, may not be as accurate as they should in converting area % purity results into a more meaningful w/w %. (For some minor, unstable and unknown impurities the determination of RRF-s is almost impossible.) (iii) For the quantitation of impurities in E_2CDS and E_2CDS/HPCD complexes, evaporative light scattering or ELS detection (a universal detection mode compatible with gradient elution, unlike the refractive index detection mode) would, in principle, be adequate. However, data on the comparative evaluation of 4 HPLC detectors (Roda et al. 1993; McCrossen et al. 1998; Toussaint et al. 2000) and our own experience indicate that, when ELS detection is used at concentrations below 20 µg/ml (~1 area %), only log – log peak area *vs* sample concentration curves are linear (making detection less sensitive), and that ELS response is indirectly related to retention time (i.e. higher for poorly retained compounds and lower for more strongly retained ones), yielding discordant results. In addition, the huge peak of the complexing agent at the front of the chromatogram (obtained by ELS detection) of a complex sample may hide polar decomposition products or other related impurities. (iv) Although E_2CDS (and preparations thereof) is/are reasonably stable in solid form (in the refrigerator, under argon gas), the molecule can easily suffer changes in solution – depending on the composition of the solvent and the time spent in it.

In solid form, the main related compounds in E_2CDS substance are the quaternary product (since even a mild oxidative effect can easily cause the API to reconvert to it), the 1,6-dihydropyridine E_2CDS isomer and the 9,11-dehydro E_2CDS analogue (impurity **3**), the latter originating from 9,11-dehydro estradiol, a frequent contaminant of estradiol, the starting material of the synthesis of E_2CDS.

In solution (e.g. in a buffered eluent, at 25 °C, without a stabilizer), especially the concentration of the so called water addition product (WAP) increased rapidly with time, as shown in Fig. 3, with water addition taking place in the 1′,4′-dihydropyridine moiety of E_2CDS. In such solvents, slightly less dramatic increase in the concentration of the quaternary product (QUAP) was also observed.

To address the problems described in points (i), (ii) and (iii), we decided to use UV detection and a detection wavelength at which area % results were the least distorted. Having examined results and UV spectra in the 205–450 nm range, we found that detection at 220 nm offered the best possible compromise for analyzing E_2CDS samples by HPLC, and for presenting purity results as area %. Except for WAP, RRF values at this wavelength, as shown above, fell into the 1 ± 0.5 range, justifying our selection of 220 nm.

A wide range of experiments was carried out in order to improve E_2CDS stability in solution during HPLC analysis (problems described in point iv.). The effects, on E_2CDS stability, of the following factors were studied: previous cooling and/or degassing the sample solvent, the acetonitrile/water ratios (v/v) in non-buffered sample solvents, the nature of the buffer salt in the sample solvent and/or the eluent, the pH of the sample solvent and/or the eluent, the nature and concentration of reducing agents added to the solvent, and sonication time during sample preparation.

In these experiments, the concentration levels of the major contaminants formed during various time periods in solu-

tions, particularly those of QUAP and WAP, served as inverse stability indicators to evaluate the effects measured.

Cooling and/or degassing the sample solvent before sample dissolution resulted in hardly any improvement of sample stability.

Changing the acetonitrile/water volume ratio in the sample solvent, however, had a remarkable effect on the concentration of QUAP in the solvent. As shown in Fig. 4, the concentration (area %) of QUAP showed a strong, almost linear increase with the increase in the volume % of water (or the decrease in the volume % of acetonitrile) in a non-buffered acetonitrile-water mixture, 2.5 h after the dissolution of an API or E_2CDS/HPCD complex sample. At the same time, WAP concentrations in a non-buffered solution of API or a complex sample, showed very little increase. Advantageous as it would be to the stability, HPCD complexes are not soluble enough in solvent mixtures with acetonitrile ratios higher than 70%. Therefore, no data for complexes in such solvent mixtures are presented in Fig. 4.

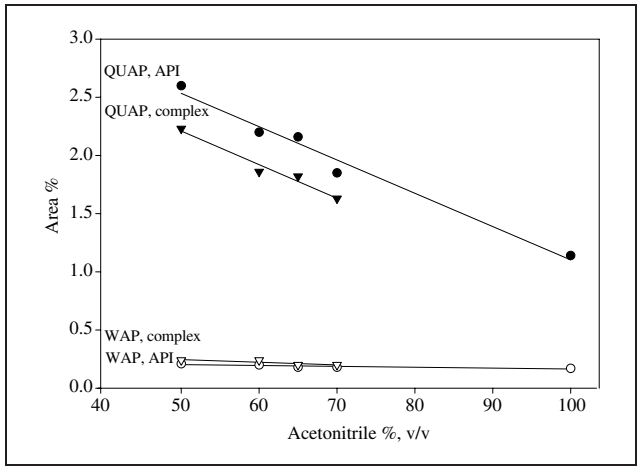


Fig. 4: The concentration (area %) of QUAP and WAP as a function of the volume % of acetonitrile in non-buffered acetonitrile-water mixtures, 2.5 hours after the dissolution of an API and E_2CDS/HPCD complex sample

Buffered sample solvents resulted in a remarkably different stability picture. Figure 3 shows how rapidly the concentration of WAP increases with time in solutions of API and E_2CDS/HPCD complex samples, both solutions buffered with sodium acetate to pH 5.5. When the solution was buffered with ammonium acetate, this increase in WAP concentration was even faster. At the same time, the increase in QUAP concentration was less remarkable.

In general, it appeared from our experiments that amine-containing buffers, both in eluents and sample solvents, were disadvantageous and were to be avoided.

Changing the pH of the sample solvent or the eluent did not result, in itself, in any significant increase in WAP or QUAP concentrations as long as pH was within the range of 3.5–9.5. The retention of QUAP, though, was influenced considerably by pH change in the eluent.

There were, however, significant concentration changes outside this pH range. When the pH of either the sample solvent or the eluent was more acidic, an almost exponential increase was found in WAP concentrations, and when the pH became more basic (or acidic), the concentration of estradiol started to increase as well, owing to the hydrolysis of E_2CDS.

In a strongly acidic milieu, proton catalysis even causes E_2CDS to dimerize on the analogy of water addition, with the 3–OH group taking part in the addition, and resulting in late eluting peaks in a purity chromatogram.

During all the above E_2CDS stability studies, the concentrations of related molecules other than WAP or QUAP, such as estradiol, the 1,6-dihydro E_2CDS isomer, estradiol nicotinate, impurity 3, i.e. the 9,11-dehydro E_2CDS analogue and the 1,2-dihydro E_2CDS isomer, were also monitored. Their concentration changes, generated by the factors examined above, were not significant when E_2CDS/HPCD complex samples were examined. In API sample solutions, however, a slight increase with time was occasionally found in estradiol concentrations.

Reducing agents added to non-buffered sample solvent (acetonitrile/water = 70/30, v/v) in a *ca*. 15-fold molar excess of the reducing molecule on E_2CDS, produced dramatic and often unexpected concentration changes, as shown in the Table.

Two of them, Na_2SO_3 and $NaBH_4$ were found worth further fine-tuning as stabilizing additives. The effective minimum concentration (molar ratio) of these additives was determined and found to be 1.5 and 0.2 for Na_2SO_3 and $NaBH_4$, respectively, to E_2CDS as 1.0. (The concentration of API in the E_2CDS/HPCD sample solutions was mostly 2 μM/ml.) At the effective minimum concentration, Na_2SO_3 did not reduce the QUAP in the sample solution to the 1,6-dihydro E_2CDS isomer, but $NaBH_4$ sometimes did.

Table: Effects of some reducing compounds on E_2CDS stability in solution, as measured by the concentration of related molecules and impurities

Name of the compound added	Area % of peaks when E_2CDS/HPCD sample was injected right after (ra) and 4 h after (4 h) dissolution							
	Peaks at 0–3 min ra/4h	QUAP ra/4h	Estradiol ra/4h	WAP ra/4h	1,6-Isomer ra/4h	Impurity 3 ra/4h	1,2-Isomer ra/4h	sum of Impurities* ra/4h
No addition	0.11/0.07	0.59/1.40	0.49/0.51	0.17/0.21	0.71/0.73	0.64/0.70	0.30/0.21	2.68/3.84
L-ascorbic acid	0.10/0.75	0.86/9.34	0.47/1.09	4.97/69.02	0.78/0.52	0.67/?	0.19/?	7.76/∼90
$Fe(NH_4)_2(SO_4)_2$	0.11/0.23	1.12/7.00	0.61/0.79	0.37/32.08	0.78/0.60	0.72/0.38	0.33/?	3.65/∼43
Hydroquinone	0.43/1.07	0.84/64.96	0.47/7.25	0.18/0.46	0.73/?	0.70/?	0.33/?	3.31/∼100
$NaBH_4$	0.08/0.05	0.00/0.00	0.49/0.80	0.34/0.32	1.29/1.75	0.75/0.79	0.62/0.27	2.45/3.00
$NaHSO_3$	4.18/n.a.	94.82/n.a.	0.52/n.a.	0.03/n.a.	?/n.a.	?/n.a.	?/n.a.	∼100/n.a.
Na_2SO_3	0.11/0.12	0.14/0.16	0.48/0.62	0.20/0.18	1.02/1.43	0.75/0.74	0.60/0.91	2.28/2.68
Na_2S	57.74/59.33	0.0/0.0	0.50/1.88	0.06/0.08	0.30/0.20	0.28/0.25	0.14/0.08	∼59/∼62
$Na_2S_2O_3$	0.18/0.07	0.88/1.79	0.63/0.60	0.19/0.41	0.82/0.79	0.73/0.73	0.36/0.29	3.38/4.43
$Na_2S_2O_4$	0.56/0.10	20.37/92.42	0.38/0.65	0.18/4.48	0.34/0.22	0.54/0.16	0.09/0.17	22.30//∼98
$Na_2S_2O_5$	0.13/n.a.	94.52/n.a.	0.65/n.a.	5.22/n.a.	?/n.a.	?/n.a.	?/n.a.	∼100/n.a.
Quinhydrone	67.24/n.a.	31.0/n.a.	0.52/n.a.	0.05/n.a.	?/n.a.	?/n.a.	?/n.a.	∼100/n.a.

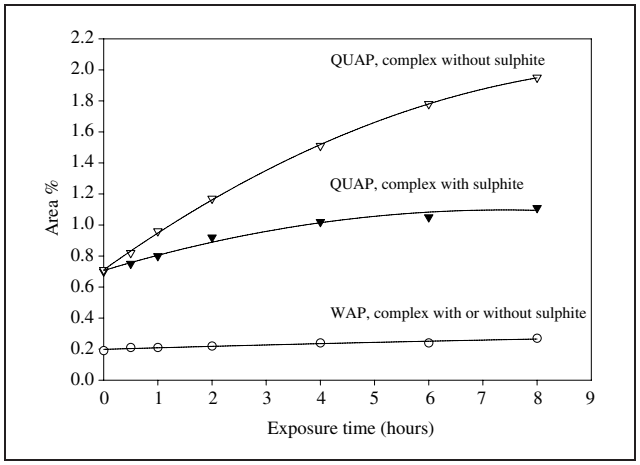


Fig. 5: The stability of E_2CDS in a non-buffered solution (acetonitrile/water = 70/30, v/v) of an E_2CDS/HPCD complex sample, in the presence and absence of the sulphite stabilizer, as measured by the concentration of QUAP and WAP

Based on the results of molar ratio fine-tuning, Na_2SO_3 was chosen as stabilizing additive in the sample solvent, at a 1.5-fold molar excess to the API.

Figure 5 shows the increased stability of E_2CDS, as measured by the concentration of QUAP in a non-buffered solution (acetonitrile/water = 70/30, v/v) of an E_2CDS/HPCD complex sample, in the presence (at the concentration specified above) and absence of Na_2SO_3 as stabilizer. No significant change was found in the concentration level of WAP and the rest of related molecules.

The duration of sonication during sample preparation should preferably be 10–15 s, but not more than 30 s. Sonication of an E_2CDS/HPCD complex sample solution (without a stabilizer) caused QUAP concentration to increase from 0.85 area % to 1.34 and 1.75 area % in 2 and 3 min, respectively. Sonication was more harmful in solvents containing more water. Interestingly, WAP concentration was not affected here, either.

The assay of E_2CDS can be performed without any compromise, both at 220 nm and 360 nm as long as the stability criteria outlined above are met. Even if a 0.3 % increase occured in the QUAP concentration of E_2CDS solutions during the assay (as may be expected on the basis of Fig. 5), the resulting decrease in API concentration would still remain within the limits of an acceptable standard deviation.

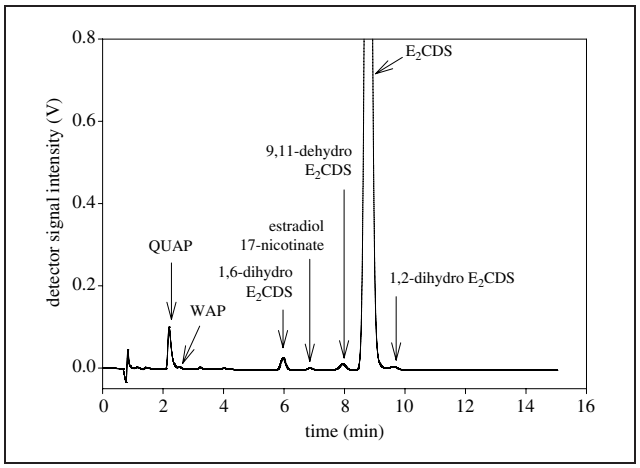


Fig. 6: A representative isocratic chromatogram of an E_2CDS (API) sample obtained under the assay conditions described in 2.1

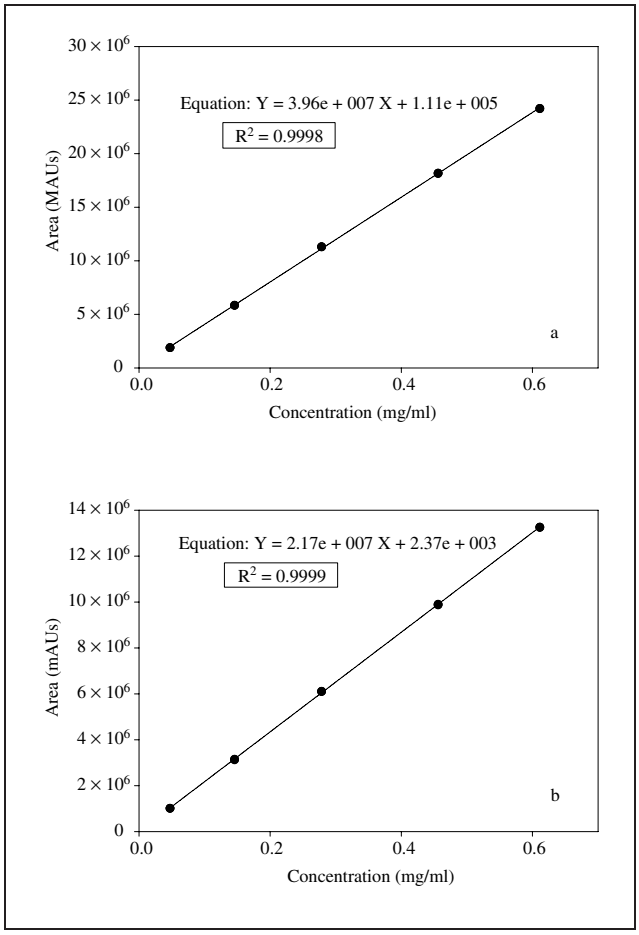


Fig. 7: The linearity of the response of the UV (PDA) detector at 220 nm (a) and 360 nm (b), as measured under the assay conditions described in 2.1

Spectral homogeneity checks on the main peak in Fig. 6 (according to the peak purity test in the Waters Millennium 4.0 software) showed that, in the HPLC system described for the assay, the E_2CDS peak was well resolved, without any signs of co-elution.

As shown in Fig. 7, linearity is slightly better, but detector response is weaker at 360 nm than at 220 nm.

Repeatability was measured from two successive series of 10 independent solutions of E_2CDS (in the concentration range of 0.40–0.44 mg/ml, with 20 µl aliquots) at the above wavelengths.

At 220 nm, the mean of the normalized area (µVs/g) of the E_2CDS peak in the first series was 1,600,482,647 with a % RSD of 0.42; the same data in the second series were 1,600,449,477 and 0.44, respectively. At 360 nm, the mean in the first series was 868,113,107 with a % RSD of 0.47, and the same data in the second series were 868,542,171 and 0.49, respectively.

Accuracy, i.e. the difference between a result (or mean) and the known value (Mehta 1987; Jenke 1996) was determined as follows: Six assays (with triplicate measurements, according to the method protocol) were performed to calculate the mean % recoveries of E_2CDS concentration at 70, 80, 90, 100, 110 and 120 per cent of the expected working concentration (0.4 mg/ml). In order for the recoveries to be considered acceptable, they are to fall between 98 and 102%.

The calculated data in our case were: 98.3, 99.7, 99.2, 100.6, 100.3 and 99.5%, respectively.

4. Experimental

4.1. Materials

All the major eluent components (distilled water included) were of HPLC grade. Acetonitrile, distilled water and sodium dithionite were purchased from Riedel-deHaën (Seelze, Germany), methanol was the product of Merck (Darmstadt, Germany), glacial acetic acid, iodomethane and sodium borohydride were obtained from Sigma-Aldrich (St. Louis, MO, USA), L-ascorbic acid, acetone, chloroform, sodium hydroxide, sodium bicarbonate and sodium sulfite were purchased from Reanal (Budapest, Hungary). Argon gas (99.996 %) was supplied by Messer Hungarogaz (Budapest, Hungary). The Alltima chromatographic column was obtained from Alltech (Deerfield, IL, USA).

Estra-1,3,5(10)-triene-3,17β-diol-17-(1,4-dihydro-1-methyl-3-pyridine) carboxylate (E_2CDS) and some of its intermediates, such as estradiol nicotinate, estra-1,3,5(10)-triene-3,17β-diol-17β-(pyridine-3-carboxylate) and the quaternary precursor (QUAP) estra-1,3,5(10)-triene-3,17β-diol-17β-(3-carboxy-1-methyl) pyridinium cation (as iodide salt) were manufactured by Alchem Laboratories Corp., Alachua, FL (USA).

Trigonelline (1-methylpyridinium-3-carboxylate) and 17β-estradiol were purchased from Sigma, 9,11-dehydro-17β-estradiol was from Maybridge, Tintagel, England. The so-called water addition product (Fig. 1e) and the 9,11-dehydro E_2CDS analogue were prepared in-house from E_2CDS and 9,11-dehydro-17β-estradiol, respectively.

The 1,2-dihydro-1-methyl-pyridine and 1,6-dihydro-1-methyl-pyridine isomers of E_2CDS, which often represent 0.2−0.9 area % in the main product but are not regarded as contaminants because they are also able to deliver estradiol to the CNS, similarly to E_2CDS, were prepared from the iodide salt of the quaternary precursor by reduction with $NaBH_4$ in a suspension with methanol. However, these isomers (especially the 1,2-dihydro isomer) are not stable enough to be isolated and examined in pure, solid form. The HPLC purity of the 1,6-dihydro isomer we have managed to isolate by HPLC was ∼80 area %, at 220 nm.

4.2. Methods

4.2.1. Preparation of the water addition product

E_2CDS (20 mg, 0.05 mM) was dissolved by stirring in 25 ml of a 7/6, v/v mixture of acetonitrile/water containing L-ascorbic acid (284 mg ≈ 1.6 mM in 100 ml). The solution was stirred for 5 h at room temperature, then the organic solvent was partly evaporated, with argon gas bubbling into the system throughout the whole procedure.

The crude residue obtained after freeze-drying was purified by HPLC under similar (but non-buffered) conditions reported below for purity tests. Of the fractions collected those containing the main product were freeze-dried again, providing the water addition product as a white powder, with an HPLC purity of 88 area %. Its structure was confirmed by NMR spectroscopy.

4.2.2. Preparation of the 9,11-dehydro E_2CDS analogue

The first three steps of the synthesis, with 9,11-dehydro-17β-estradiol instead of 17β-estradiol as starting material, were identical with that of E_2CDS (as described in the final report on its manufacturing by Alchem, dated January 7, 2003; see also Estes et al. 1994).

The 9,11-dehydro-estradiol quaternary salt analogue was prepared as follows: To a stirred suspension of 9,11-dehydro-estradiol nicotinate in anhydrous acetone (210 mg or 0.56 mM in 20 ml) 500 μl of iodomethane was added and the mixture was heated at 38–42 °C for 2 days under argon gas, then cooled to 0 °C, and the precipitate was collected by filtration and washed twice with chloroform. The yellow powder was dried under vacuum, at room temperature for 2 h. The yield was 280 mg, with an HPLC purity of 97 area %.

The synthesis of 9,11-dehydro E_2CDS was carried out as follows: Deionized water and acetonitrile (10 ml, each) were placed into a round bottomed flask, then 145 mg (1.72 mM) of sodium bicarbonate and 293 mg (1.68 mM) of sodium dithionite were added while stirring, cooling (4 °C) and bubbling argon into the mixture. After that 140 mg (0.27 mM) of the quaternary salt analogue, as prepared above, was added, stirring at 4–6 °C was continued for 25 min. Then, at 25 min intervals, 6 portions of sodium bicarbonate (25 mg, each) and sodium dithionite (50 mg, each) were added to the reaction mixture, which was then transferred into a separatory funnel, and the layers were separated.

The aqueous layer was discarded and 12 ml of an acetonitrile-water (1 : 1) mixture was added to the separatory funnel, while bubbling argon for 5 minutes.

The solution was concentrated under vacuum (at <1 Hg mm) to a water residue, then the solid product was filtered while covered with argon, and washed 3 times with 2 ml portions of chilled water. The amorphous light yellow powder was dried in vacuum at room temperature, until constant weight, then stored under argon at −20 °C. The yield was 66 mg, with an HPLC purity of 95 area %. ^{1}H NMR (DMSO-d_6) data support the structure expected.

4.2.3. Analytical methods

The HPLC equipment used in the E_2CDS assay and purity tests consisted of a Waters (Milford, MASS, USA) 600E multisolvent delivery system, a Waters 996 photodiode array detector, a Rheodyne (Cotati, CAL, USA) 7125 manual injector with a 20 μl sample loop, a column thermostat (optional) and a Waters Millennium^{32} software for system control and data handling/processing. The reversed-phase HPLC column used was an Alltima C18, 3 μm (100 mm × 4.6 mm, I.D.) column. The elution solvents consisted of an aqueous buffer to which acetonitrile was admixed at varying volume ratios.

Preparation of the aqueous sodium acetate buffer (1 L): To 950 ml distilled water 1.0 ml of glacial acetic acid was added and the pH of the solution was adjusted to 5.5 ± 0.05 using 2N NaOH (dropwise) and a pH meter equipped with a glass electrode. The buffer was then diluted to volume with distilled water. The eluent (aqueous buffer + organic modifier) was filtered and degassed before or during use.

The flow rate and detection wavelength used were 1 ml/min and 220 nm, respectively. (For the assay, detection at 360 nm can also be used.)

Column temperature was 25 ± 3 °C.

This research paper was presented during the 6th Conference on Retrometabolism Based Drug Design and Targeting, June 3–6, 2007, Göd, Hungary.

References

Estes KS, Brewster ME, Bodor N (1994) Evaluation of an estradiol chemical delivery system (CDS) designed to provide enhanced and sustained hormone levels in the brain. Adv Drug Del Rev 14: 167–175.

Estes KS, Brewster ME, Simpkins JW, Bodor N (1987) A novel redox system for CNS-directed delivery of estradiol causes sustained LH suppression in castrate rats. Life Sci 40: 1327–1334.

Jenke DR (1996) Chromatographic method validation: A review of current practices and procedures. 2. Guidelines for primary validation parameters. J Liq Chromatogr Rel Technol 19: 737–757.

McCrossen SD, Bryant DK, Cook BR, Richards JJ (1998) Comparison of LC detection methods in the investigation of non-UV detectable organic impurities in a drug substance. J Pharm Biomed Anal 17: 455–471.

Mehta AC (1987) Drug determination in biological fluids – approaches to method validation. Talanta 34: 609–613.

Rahimy MH, Simpkins JW, Bodor N (1990) Dose and time course evaluation of a redox-based estradiol – chemical delivery system for the brain. 2. Pharmacodynamic responses. Pharm Res 7: 1107–1112.

Roda A, Gatti R, Cavrini V, Cerré C, Simoni P (1993) HPLC study of the impurities present in different ursodesoxcycholic acid preparations – comparative evaluation of 4 detectors. J Pharm Biomed Anal 8: 751–760.

Toussaint B, Duchateau ALL, van der Wal S, Albert A, Hubert P, Crommen J (2000) Comparative evaluation of four detectors in the high-performance liquid chromatographic analysis of chiral nonaromatic alcohols. J Chromatogr Sci 38: 450–457.