Short communication

Investigation of the temperature stability of premarin intravenous using liquid chromatography–mass spectrometry

Hien P. Nguyen[a], Lauren Tedmon[a], Li Li[a], Jane G. Wigginton[b], David Maass[b], Joshua W. Gatson[b],
James W. Simpkins[c], Kevin A. Schug[a,*]

[a] Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, TX, USA
[b] Department of Surgery/Division of Emergency Medicine, The University of Texas Southwestern Medical Center, Dallas, TX, USA
[c] Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Ft. Worth, TX, USA

ARTICLE INFO

ABSTRACT

Stability of Premarin®Intravenous was investigated in dry and reconstituted forms by monitoring major components in samples for a period of six months, using liquid chromatography–mass spectrometry. The components, largely comprising a series of estrogen and steroid hormone sulfates, were considered to be fairly stable (variation $\leq 10\%$) for dry samples stored at room temperature and at $38\,^\circ C$ ($100\,^\circ F$) during the experimental time frame. However, significant variation, especially after 2 months of storage, was observed in reconstituted solutions. This variation was significantly larger for samples stored at elevated vs. room temperature. It was interesting to note that the concentration of equilenin sulfate increased over time, whereas that of other major components were seen to fluctuate and decrease. This phenomenon was partially explained by the conversion of equilin compounds into their corresponding equilenin forms, a phenomenon which was further investigated through a storage study with pure standard solutions and by tandem mass spectrometry.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Neuroprotective effects of estrogens have been investigated in many studies [1–5]. Pre-treatment with estrogens has been demonstrated to reduce ischemic damage in focal cerebral ischemia while post-treatment has also been shown to be beneficial for preserving brain tissue [2–5]. Neuroprotective effects of estradiol were studied when administered after an ischemic insult on rats by Simpkins and coworkers [2,6]. Beneficial effects were observed when the compound was administered within a therapeutic window of three hours after injury [2]. Importantly, the study suggests that estrogens could be useful for treatment of traumatic brain injury (TBI) in humans. The promise of a clinical study to investigate the use of estrogens for treatment of TBI brings with it several points for consideration. Because patients are likely to be unconscious, the drug needs to be administered intravenously. Additionally, because a narrow therapeutic window exists (with diminishing returns over the 0–3 h time span), it is desirable to administer the treatment as early as possible.

Premarin® (Wyeth/Pfizer, PA, USA), in the form of Premarin®Intravenous, was considered for use as an estro-genic therapy for TBI in clinical trials, because it has been approved and widely used since 1942 (www.fda.gov/Drugs/DrugSafety). However, approved protocol requires that the drug be stored in a sterile lyophilized form at $2–8\,^\circ C$, and that it be used immediately after reconstitution with sterile water (www.wyeth.com). Obviously, this requirement does not support the need for immediate treatment if a significant time delay exists between injury and arrival at the hospital. Instead, Premarin®Intravenous should ideally be stored in the ambulance so that the treatment can be given as soon as possible. However, most modern ambulances do not have refrigerators and the stability of Premarin®Intravenous under variable storage temperatures has not been reported. Although synthetically prepared alkali metal estrogen sulfate salts were found to be unstable and hydrolyzed under prolonged exposure to the atmosphere [7], the data for the stability of Premarin®Intravenous are not publicly available. In this work, the stability of solid and reconstituted forms of Premarin®Intravenous was investigated at different temperatures for 6-month periods using LC–MS and tandem MS.

2. Experimental

2.1. Chemicals and reagents

All conjugated estrogen standards were purchased from Organics, L.L.C. (Northbrook, IL, USA), including sodium sulfate conjugates

* Corresponding author at: 700 Planetarium Pl., Box 19065, Arlington, TX 76019-0065, USA. Tel.: +1 817 272 3541; fax: +1 817 272 3808.
E-mail address: kschug@uta.edu (K.A. Schug).

of equilenin (EN-S), 17α- and β-dihydroquilenin (ADHEN-S and BDHEN-S), equilin (E-S), 17α- and β-dihydroequilin (ADHE-S and BDHE-S), estrone (E1-S), 17α- and β-estradiol (AE2-S and BE2-S). Premarin®Intravenous (conjugated estrogens, USP) was obtained from Wyeth/Pfizer (Philadelphia, PA, USA). Amonium acetate was from Sigma–Aldrich (St. Louis, MO, USA). Water and acetonitrile (ACN) were LC–MS grade and obtained from J.T. Baker (Phillipsburg, NJ, USA) and Burdick and Jackson (Muskegon, MI, USA), respectively. Bacteriostatic and sterile water were purchased from Hospira, Inc. (Lake Forest, IL, USA).

2.2. Experimental design

Temperature stability of Premarin®Intravenous was investigated in dry powder and reconstituted forms. Two batches of the drug were used for two consecutive experiments.

In batch 1, the contents of nine Premarin®Intravenous vials from the same lot were removed from their vials and combined. Replicate representative samples were divided and accurately weighed into 8 vials. Samples in six vials were reconstituted in sterile water to the concentration of 47.5 mg/mL. Three of these vials were and stored at room temperature (labeled as R1, R2, and R3), while the other three vials were stored at 38 °C (100 °F) (labeled as H1, H2, and H3). The samples in the remaining two vials were stored at room temperature (RT) (labeled D1) and at 38 °C (100 °F) (labeled D2). In batch 2, eight Premarin®Intravenous vials from the same lot were selected and each of them was reconstituted with 5 mL of sterile water, injected through the rubber cap of the vials in a sterile hood. Samples in four vials were dissolved in sterile water containing 0.9% benzyl alcohol (BnOH), while those in the other four vials were mixed with sterile water without preservative. Two of each were stored at RT and labeled as R4 and R5 for sterile water (no preservative) and as R6 and R7 for the bacteriostatic water. The other four vials were stored at 49 °C (120 °F) and labeled similarly for sterile water (H4 and H5) and bacteriostatic water (H6 and H7), respectively. The experiment temperature was elevated to 49 °C (120 °F) during batch 2 to better mimic temperature variations in ambulances in some areas such as Texas, and to accentuate potential degradation effects observed for batch 1.

2.3. LC–MS conditions

Sampling was performed at monthly time points during a period of 6 or 7 months to determine the variation of sample components. The experiments were carried out on a Shimadzu LCMS-IT-TOF (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). The chromatographic analysis was performed on a Shimadzu (Tokyo, Japan) Shim-pack XR-ODS column (2 mm × 100 mm, 2.2 μm, 120 Å) with an elution gradient of mobile phase A (20 mM ammonium acetate in H_2O) and mobile phase B (ACN) at a flow-rate of 250 μL/min. The run was started at 20%B (held for 5 min), followed by a linear gradient to 40% over 30 min and then to 98% over 20 min (and held for 10 min). The IT-TOF was operated in the negative ionization mode with a spray voltage of −3.5 kV, and a scan range of 100–600 m/z. LCMS Solutions software, version 3.4, was used for data collection and analysis.

3. Results and discussions

3.1. Peak identification

A representative total ion chromatogram of Premarin®Intravenous samples is presented in Fig. 1 with the eight most intense components selected for tracking through the storage experiment.
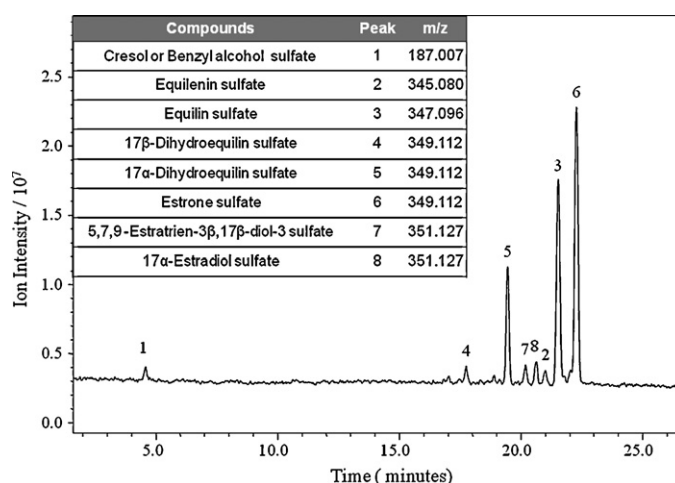

Fig. 1. Reversed phase HPLC–ESI-MS total ion chromatogram of Premarin®Intravenous with the eight most intense components denoted.

The peak investigation was based on matching m/z values and retention times between Premarin®Intravenous samples and pure standards. Six of the eight compounds (peaks 2–6, 8) were positively identified. Assignments for peaks 1 and 7 are tentative. According to Hill et al. [8], peak 1 could be cresol or benzyl alcohol sulfate, but the latter is most likely. Peak 7 (m/z 351) can be 17β-estradiol sulfate or 5,7,9-estratrien-3β,17β-diol-3-sulfate, which are isobaric and have been reported to be present in Premarin by Wyeth-Ayerst [8]. In our analysis, the retention time of peak 7 did not match that of our 17β-estradiol sulfate standard, indicating that peak 7 likely is 5, 7, 9-estratrien-3β,17β-diol-3-sulfate. The standard of this compound was not commercially available to confirm its identification.

3.2. Premarin®Intravenous stability

The stability of Premarin®Intravenous was studied in two consecutive batches. Batch 1 was carried out first. Batch 2 was performed to confirm and clarify results collected from batch 1.

3.2.1. Batch 1

Fig. 2 displays the cumulative results for the dry samples D1 (RT) and D2 (38 °C (100 °F)). The values were anomalously low for the first two time points, and this variation has been attributed to instrumental problems during the sequence analysis. These problems were corrected for the remainder of the analyses. The composition of the two dry samples were stable and did not change significantly after day 93, and for the remainder of the experiment. From day 93–175, the variations of each component were ≤ 10% (most were between 2 and 5% variation), indicating that these non-reconstituted samples were fairly stable during six month time frame when stored at RT or at 38 °C (100 °F).

Samples R1–R3 (presented as an average in Supplementary Fig. 1) show a slight increase for the first month, but more importantly, the relative abundance of the tracked components varied to a greater degree after day 151. Compared to samples R1–R3, samples H1–H3 (stored at 38 °C (100 °F)) showed an even more variable composition after 93 days. In both sample sets, compound 2 subtantially increased while some other compounds, such as compound 4 and 7, decreased. Interestingly, the variation of total signals for steroidal compounds (sum total of relative peak intensities from peak 2–8), at RT and at 38 °C (100 °F), did not vary to a large degree (≤15%). The samples may be considered as being stable for total steroids, although individual components quantitatively varied. An unidentified microbial growth in most of the reconstituted samples
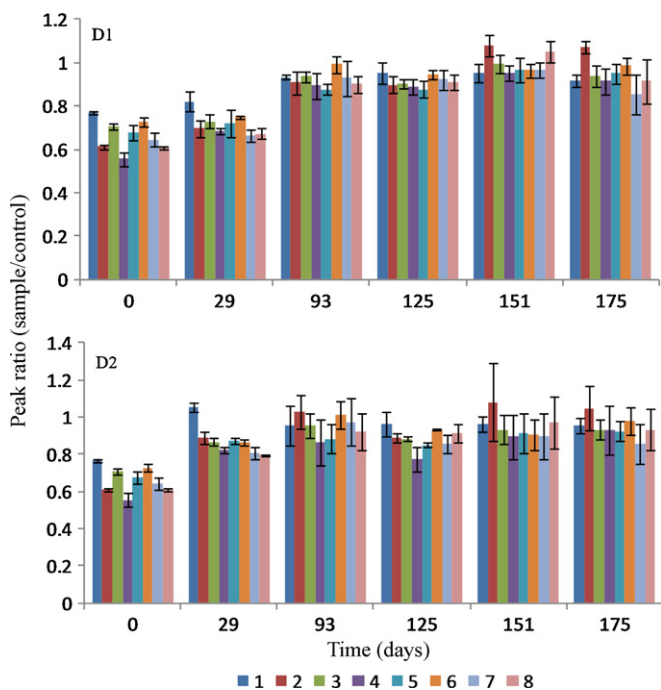
Fig. 2. Variation of dry Premarin®Intravenous within 175 days for D1 at RT and D2 at 38 °C (100 °F). Abnormally low peak ratio values for days 0 and 29 were attributed to instrumentation problems, which were rectified in subsequent sample measurements and time points.

(especially in R2), stored at RT, was noted during sampling on day 175.

3.2.2. Batch 2

Since the microbial growth was observed in the reconstituted samples at RT in batch 1, the experiment with reconstituted solutions was repeated in batch 2 with a better control condition. The samples were reconstituted with sterile water by injection through the rubber caps of the vial to avoid direct contact between the samples and the ambient environment. No precipitation or bacterial growth was observed in reconstituted samples at RT but small rod-shaped particles were detected in those at 49 °C (120 °F) after day 62. The shape of these particles was different from the shape of microbial growth observed in batch 1, which was a furry amorphous shape. The presence of the rod-shaped particles in batch 2 was likely caused by precipitation (possibly, of desulfated steroid hormones) under high temperature condition rather than bacterial contamination.

The variation of steroid hormone components in reconstituted batch 2 samples R4–R5 at RT, as shown in Fig. 3, was not significant for the first 148 days ($\leq$13% for each tracked compound), however some marked variation of some components was observed after this timeframe. The composition of reconstituted samples H4–H5 stored at 49 °C (120 °F) changed to a great degree over 215 days. Compound 2 continuously increased after day 40. The variation of total steroid conjugates was only 12%, although a slight increase with time was noted. At both temperatures, there was no different in composition variation between samples with and without the addition of the BnOH preservative (Supplementary Fig. 2).

In summary, Premarin®Intravenous was considered to be fairly stable in solid form at RT and even at an elevated temperature of 38 °C (100 °F). Variation of steroidal compositions in reconstituted samples at RT was not significant when the experimental conditions were well-controlled and the sample was not exposed to ambient contamination. However, there was a marked change in the abundance of a number of the major components in the
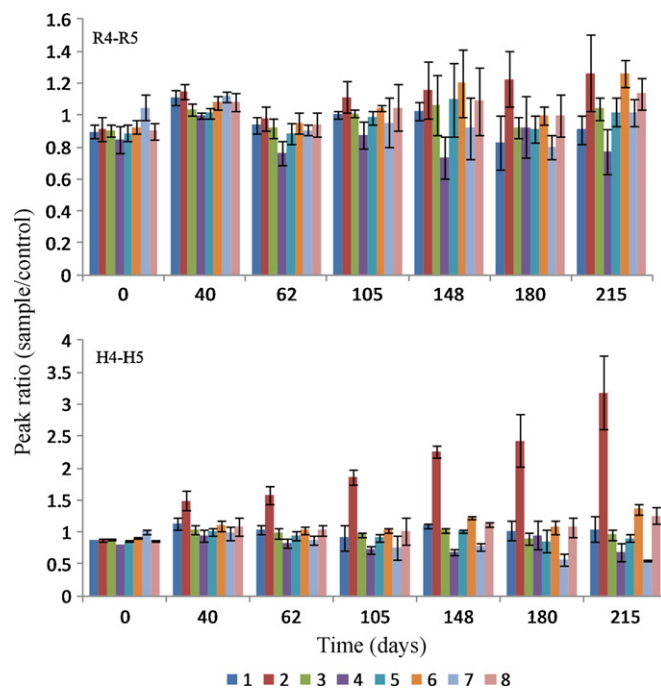

Fig. 3. Variation of reconstituted Premarin®Intravenous samples during 215 days for R4–R5 at RT and for H4–H5 at 49 °C (120 °F) without the addition of BnOH.

drug when high temperature was applied. The increase of compound 2, decrease of some other steroids, and low variation in the sum of total signals led to a hypothesis that interconversion among steroidal components, not degradation of sulfates as might be expected, was responsible for the observed variation in individual components. This hypothesis was investigated by studying the stability of different pure steroid hormone sulfate standards.

3.3. Steroid hormone sulfate conjugate stability in solution

The increase of compound 2 levels and decrease of the other components in reconstituted samples could be caused by conversion between different components into more stable forms. Compound 2, EN-S, contains conjugated A–B aromatic rings; therefore, it is likely more stable than other compounds bearing partial saturation in A–B rings such as compound 3, 4, 5, and 7. Possible conversion from the others into compound 2 could be the reason for its increasing levels over time under elevated temperature condition. This phenomenon was further studied using pure standard solutions. The stability of four standards, E1-S and three equilin sulfates (E-S, ADHE-S, and BDHE-S), were investigated at a concentration of 0.5 µg/mL at 49 °C (120 °F). These compounds were selected because they contain partially saturated A–B rings, which were hypothesized to convert into unsaturated forms under high temperature over a period of time. After being stored at 49 °C (120 °F) for 3 months, additional unknown peaks appeared in the LC–MS chromatograms of the pure standard solutions (Fig. 4). The retention time and m/z values of these peaks matched those of corresponding equilenin sulfate compounds; thus, the conversion of equilin into equilenin was possible. No additional peak was detected with E1-S. Although these unknown peaks were not observed with freshly purchased and prepared standards, they were present at very low levels in freshly made reconstituted samples after storage at −20 °C for 1.5 years.

The unknown peaks were suspected to form from the conversion of equilin species into their corresponding equilenin forms. A supplementary study was implemented to clarify this using tandem mass spectrometry, including MS^2 and MS^3. Three equilenin
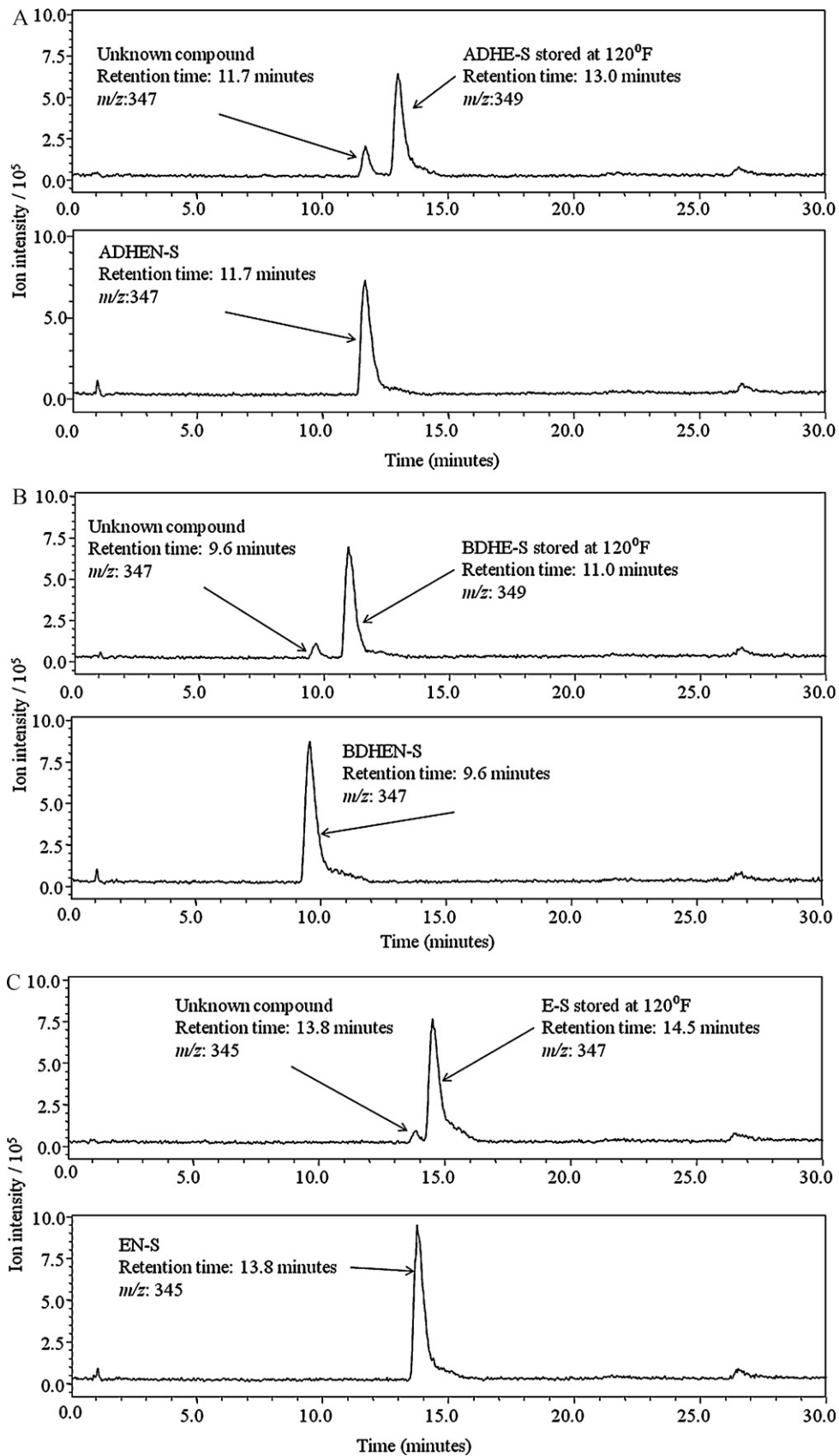
Fig. 4. Chromatograms of (A) ADHE-S stored at 49 °C (120 °F) and ADHEN-S, (B) BDHES stored at 49 °C (120 °F) and BDHEN-S and (C) E-S stored at 49 °C (120 °F) and EN-S.

compounds (ADHEN-S, BDHEN-S, and EN-S) were investigated and compared with the unknown peaks from their corresponding equilin. All compounds lost their sulfate group during MS^2 and the desulfated ions were further fragmented in MS^3. Fragmentation patterns of these unknown peaks were consistent with that of the corresponding equilenin standards.

The conversion from equilin into the corresponding equilenin occurred; thus, the transformation of E-S into EN-S partially explains the increase of EN-S in Premarin®Intravenous solutions. However, further oxidation of the hydroxyl group into a ketone group at position C17 of ADHEN-S and BDHEN-S was not observed. In other words, these compounds were not completely oxidized to EN-S in pure standard solutions. The experiments with the pure standards did not fully explain the dramatic increase of EN-S and decrease of other components in Premarin®Intravenous solutions under high temperature over a period of time. The reason could be that, since this drug is originally from natural sources, there might be other unknown species in the drug possibly initiating or catalyzing the oxidation reaction at C17 of dihydroequilenin compounds and converting them into EN-S.

4. Conclusions

The major components in the dry samples were more stable than those in the reconstituted samples, especially at high temperature. The relative responses in the dry samples D1 and D2 did not change to an appreciable degree after six months; thus, Premarin®Intravenous can be stored in the dry form in the ambulance and reconstituted immediately before use. However, this process is not convenient during the first response treatment. Therefore, if possible, the reconstituted form should be used. The reconstituted samples in batch 2, stored at RT, were considerably stable for the first three months and slightly varied after that. Better control against bacterial interferences was also noted. Although the reconstituted samples stored at high temperature varied significantly for each tracked compound, the variation of total steroids was ≤15%. This is not significantly higher than acceptable varia-

tion ranges of the two main components in Premarin®, estrone and equilin sulfate, recognized by USP [9,10]. The total of these two compounds should comprise 79.5–88.0% of the labeled content of conjugated estrogens [9], a significant allowed variation. The conversion of equilin compounds into their corresponding equilenin forms occurred, partially explaining the increase of EN-S and decrease of other components in Premarin®Intravenous solution. If Premarin®Intravenous is to be stored in a reconstituted form in an ambulance for immediate response and use in TBI, the storage of such samples should ideally be performed at lower temperatures (e.g. in a cooler) and they should be replaced monthly.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.03.003.

References

[1] B.S. McEwen, S.E. Alves, Estrogen actions in the central nervous systems, Endocr. Rev. 20 (1999) 279–307.
[2] J.W. Simpkins, G. Rajakumar, Y.Q. Zhang, C.E. Simpkins, D. Grenwald, C.J. Yu, N. Bodor, L.J. Day, Estrogens may reduce mortality and ischemic damage caused by middle cerebral artery occlusion in the female rat, Neurosurgery 87 (1997) 724–730.
[3] D.B. Dubal, M.L. Kashon, L.C. Pettigrew, J.M. Ran, S.P. Finkelstein, S.W. Rau, P.M. Wise, Estradiol protects against ischemic injury, J. Cereb. Blood Flow Metab. 18 (1998) 1253–1258.
[4] T.J. Toung, R.J. Traystman, P.D. Hurn, Estrogen-mediated neuroprotection after experimental stroke in male rats, Stroke 29 (1998) 1666–1670.
[5] S.-H. Yang, J. Shi, A.L. Day, J.W. Simpkins, Estradiol exerts neuroprotective effects when administered after ischemic insult, Stroke 31 (2000) 745–750.
[6] J.W. Simpkins, O. Rabbani, J. Shi, K.S. Panickar, P.S. Green, A.L. Day, A system for the brain-enhanced delivery of estradiol: an assessment of its potential for the treatment of Alzheimer's disease and stroke, Pharmazie 53 (1998) 505–511.
[7] R.L. Huff, Stabilization of metal steroid alcohol sulfates, U.S. Patent 3608077 (09/21/1971).
[8] E.N. Hill, T.W. Leonard, F.D. Sancillio, K.M. Schlipp, D.G. Shirazi, R.R. Whittle, Pharmaceutical compositions of conjugated estrogens and methods of analyzing mixtures containing estrogenic compounds, U.S. Patent 6855703B1 (02/15/2005).
[9] The United States Pharmacopeia (USP) 34, conjugated estrogens, 2011.
[10] The European Pharmacopeia (USP), seventh ed., conjugated estrogens, 2010.