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STABILITY OF URINARY FEMALE REPRODUCTIVE HORMONES STORED UNDER VARIOUS CONDITIONS

JAMES S. KESNER,* EDWIN A. KNECHT,* and EDWARD F. KRIEG, JR.†
*Experimental Toxicology Branch and †Statistics Activity, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, Cincinnati, Ohio

Abstract — Urinary reproductive hormones afford specific and sensitive evaluation of female reproductive potential in epidemiologic and clinical settings. The goal of this study was to characterize the stability of urinary luteinizing hormone, follicle stimulating hormone, estrone 3-glucuronide, pregnanediol 3-glucuronide, and creatinine during storage as functions of time, temperature, and additives. After 2 weeks with no additives, activity of the four analytes, relative to initial concentrations, ranged from 91.9 to 102.8% at 4 °C, 35.1 to 89.6% at 25 °C, and 7.5 to 66.9% at 37 °C. Antimicrobial additives did not consistently improve stability. Analyte activity for samples stored with no additives for 24 weeks at −80 °C ranged from 69.0 to 101.2%. Glycerol and bovine serum albumin improved analyte stability; activity ranged from 91.1 to 106.3%. Other additives were ineffective. These results reveal conditions for storing reproductive hormone analytes in urine during epidemiologic field studies.

Key Words: luteinizing hormone; follicle stimulating hormone; estrone 3-glucuronide; pregnanediol 3-glucuronide; creatinine; epidemiology; women; urine.

INTRODUCTION

Analysis of reproductive hormones in urine has been shown to afford a convenient, noninvasive means to monitor menstrual function and other aspects of female reproductive well-being. This tool is gaining acceptance for clinical monitoring (1–5) but perhaps holds the greatest potential when applied to population-based epidemiologic evaluations, in which large numbers of women must provide multiple samples over an extended period of time (6–14).

The objective of the current study is to characterize the time-course of stability for urinary luteinizing hormone (LH), follicle stimulating hormone (FSH), estrone 3-glucuronide ($E_1$3G), pregnanediol 3-glucuronide (Pd3G), and creatinine in samples stored at various temperatures. Stability at refrigerated temperatures were compared to warmer temperatures. Storage at continuous freezing temperatures was evaluated because of reports that these conditions cause significant loss of gonadotropin activity (15,16).

Selected additives were also evaluated for efficacy to minimize activity loss. They included antimicrobial compounds; glycerol, which has been shown to reduce gonadotropin degradation in other assay systems (15,16); and bovine serum albumin (BSA), which might reduce gonadotropin degradation by serving as a competitive protein substrate.

MATERIALS AND METHODS

Studies

All studies followed factorial designs. The first experiment determined the time-course of analyte stability at nonfreezing temperatures as a function of various additives (Table 1).

The second experiment consisted of two overlapping parts (Table 1). The first part determined the time-course of analyte stability as a function of antibacterial additives and glycerol at refrigerated or freezing temperatures. The second part evaluated antibacterial additives, glycerol, and BSA at only −80 °C. Data collected under conditions of no BSA and −80 °C were common to both parts of the experiment.

Subjects and samples

Urine samples used to test analyte stability were collected from healthy women 33 to 45 years old.

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Address correspondence to Dr. James S. Kesner, Experimental Toxicology Branch, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, 4676 Columbia Parkway, C-23, Cincinnati, OH 45226-1998. E-mail: JSK4 @ NIOBBS1.em.cdc.gov

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Table 1. Factors tested for effects on urinary analyte stability.

Experiment #1				
Time (in weeks)	Temperature	Additive		
0	4 °C	None		
1	25 °C	0.1% sodium azide		
2	37 °C	0.1% thymol		
		1.0% boric acid		
		0.02% thimerosal		
		0.1% sodium azide and 1.0% BSA		
Experiment #2				
Time (in weeks)	Temperature	Additive	Glycerol	BSA
Part 1				
0	4 °C	None	0	0
2	−80 °C	0.1% sodium azide	7.5%	
7	$N_{2\,Liq} \rightarrow$ −80 °C	1.0% boric acid		
24				
Part 2				
0	−80 °C	None	0	0
2		0.1% sodium azide	7.5%	1.0%
7		1.0% boric acid		
24				

$N_{2\,Liq} \rightarrow$ −80 °C = samples were snap frozen in liquid nitrogen then stored at −80 °C.

Subjects were solicited by announcement, and the first qualified respondents were accepted. The study protocol was reviewed and approved by the National Institute for Occupational Safety and Health Human Subject Review Board. All donating subjects provided signed consent.

Urine samples were collected as first morning voids and immediately refrigerated by the donor until delivered in a chilled thermos to the laboratory. Samples were refrigerated or held on ice in the laboratory until assayed within 6 h. Treatments were added within 3 h of collection.

Five urine samples were tested in the first experiment (n = 4 for Pd3G[1]), and six different urine samples were tested in the second experiment. Each woman donated only one sample per experiment and in most cases donated for both experiments. The follicular, periovulatory, and luteal phases of the menstrual cycle were represented by two samples each for both experiments, except in the first experiment; a single sample represented the follicular phase and a single sample represented the luteal phase for Pd3G. Urine samples received all additives prior to analysis on day 0. Samples were divided into aliquots to accommodate different storage temperatures. Further, samples were divided into aliquots prior to freezing so that samples were thawed only once before assay.

Assays

Urinary LH and FSH were measured using commercial two-site immunofluorometric assays (DELFIA®; Wallac Oy, Turku, Finland; Cat. Nos. 1244-031 and 1244-017), modified and validated for application to urine samples as previously described (17). Urinary $E_1$3G and Pd3G were measured using competitive double-antibody fluoroimmunoassays previously described (18). Urinary creatinine was measured by the Jaffe reaction (19).

Assays calibrators are as follows: FSH = World Health Organization (WHO) Second International Reference Preparation of Human Pituitary FSH and LH for Bioassay; LH = WHO Second International Standard for LH, Pituitary; $E_1$3G = preparation recrystallized by Dr. Delwood Collins, Lexington, KY, USA; Pd3G = Sigma Chemical Co., St. Louis, MO, USA, Cat. No. P-3635; creatinine = Sigma Chemical Co., Cat. Nos. 925-11 and C-4255.

Two urine quality control pools (Lyphochek® Quantitative Urine Controls: Normal, Cat. No. C-390; and Abnormal, Cat. No. C-395; Bio-Rad Labo-

[1]Pd3G concentration in one sample greatly exceeded the calibration curve. Because the urine matrix is integral to the analyte stability, we decided to drop the sample from analysis rather than dilute it with buffer or low-Pd3G urine from another source.

ratories, Anaheim, CA, USA) were duplicated at the beginning, middle, and end of all creatinine assays. Three urine pools, representing the low, middle, and high portions of the standard curves, were run in duplicate (LH and FSH) or triplicate ($E_1$3G and Pd3G) at the beginning and end of each 96-well microtiter assay plate. Intra- and interassay coefficients of variation, respectively, were: LH = 2.9–8.1% and 2.7–6.5%; FSH = 2.3–3.1% and 1.5–2.6%; $E_1$3G = 6.5–7.7% and 5.3–7.9%; Pd3G = 4.5–10.7% and 4.9–11.1%; and creatinine = 3.3–3.6% and 1.1–3.3%.

Statistical analyses

LH and FSH concentrations were calculated using smoothed splines (20). $E_1$3G and Pd3G concentrations were calculated using a 4-parameter logistic model (20). Creatinine concentrations were calculated by linear regression (20).

On the occasions of substantial interassay variation, creatinine and Pd3G values (first study) and creatinine and FSH values (second study) were standardized based on the urine pools. The mean of each pool was calculated for each assay, as was the grand mean of each pool over all assays. A ratio was calculated for each pool and assay by dividing the grand mean of the pool by the assay mean. The ratios for each assay were then averaged over pools. The sample concentrations from each assay were multiplied by their corresponding average ratio to give adjusted values.

Repeated measures analysis of variance was used to analyze the data. The Greenhouse-Geisser estimate of Box's ε (21) was used to adjust the degrees of freedom of all within-subject effects. This estimate adjusts P-values in the event of heteroskedasticity or violation of the assumption of compound symmetry. In the case of significant main effects or interactions, contrasts were performed to determine differences of analyte activity from initial concentrations. Analysis revealed no outliers.

RESULTS

Study #1

The first experiment characterized the effects of nonfreezing temperatures and additives on the activity of the analytes stored for up to 2 weeks (Figure 1). FSH, $E_1$3G, and creatinine decreased more rapidly over the 2-week period at 25 °C and 37 °C than at 4 °C, indicated by the Time × Temperature interactions (FSH, $P = 0.03$; $E_1$3G, $P = 0.05$; creatinine, $P = 0.01$). A similar, nearly significant effect was found for Pd3G ($P = 0.06$). For LH, the main effect of Time ($P = 0.14$) and the interaction of Time × Temperature ($P = 0.29$) were not significant.

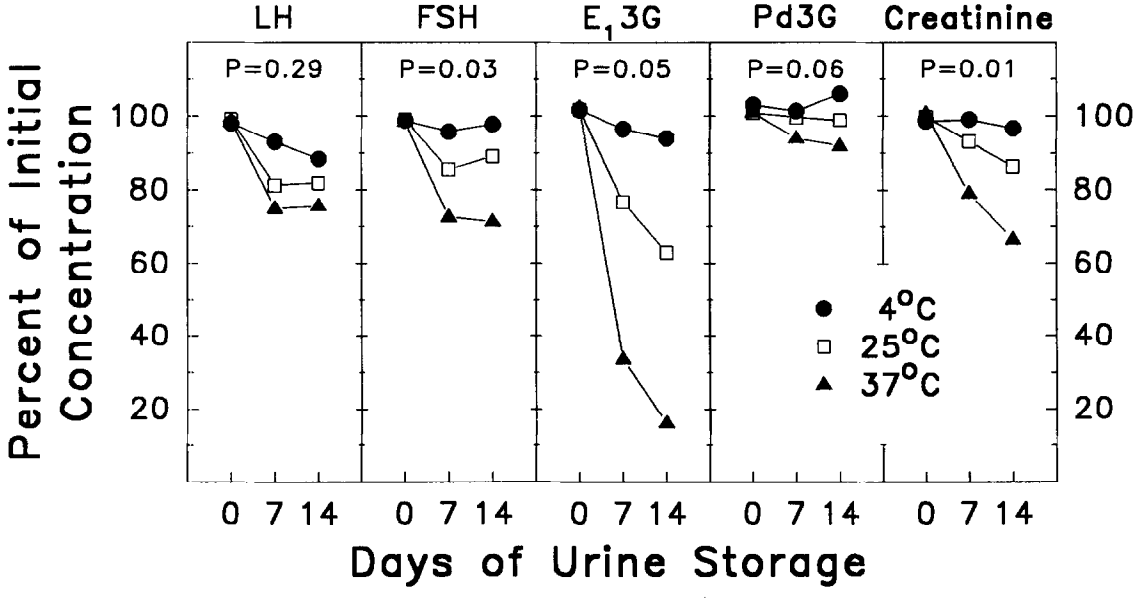

Fig. 1. Relative concentrations of LH, FSH, $E_1$3G, Pd3G, and creatinine in urine samples stored at 4 °C, 25 °C, or 37 °C for 0, 7, or 14 d in the presence of additives (none, sodium azide, thymol, boric acid, thimerosal, or sodium azide plus bovine serum albumin). Five urine samples ($n = 4$ for Pd3G) were tested per factorial cell. P-values at the top of each panel correspond to the Time × Temperature interaction. Neither the main effect nor the interactions involving additives were significant. Thus, values are pooled across additives ($n = 30$; $n = 24$ for Pd3G) and are presented as percent of initial concentrations.

After 2 weeks storage at 4 °C, loss of activity was slight (LH: 9.7%, $P = 0.05$; creatinine: 1.8%, $P = 0.008$) or absent (FSH: 1.0%, $P = 0.54$; $E_1$3G: 7.8%, $P = 0.09$; Pd3G: 2.9% gain, $P = 0.08$). There were no significant effects of additives on analyte activity as indicated by the Treatment × Time × Temperature interaction (FSH, $P = 0.49$; LH, $P = 0.18$; $E_1$3G, $P = 0.22$; Pd3G, $P = 0.24$; creatinine, $P = 0.42$).

Study #2

A second experiment was done to determine the effects of cold storage temperatures, additives (sodium azide and boric acid), and glycerol on analyte activity of samples stored up to 24 weeks. LH activity loss was 11.9% after 2 weeks storage, with no subsequent decrease (Time main effect, $P = 0.009$, Figure 2). The different additives and freezing methods did not alter this effect.

The Glycerol × Temperature × Time interaction for FSH was nearly significant ($P = 0.06$), reflecting about 25% loss of activity after 24 weeks storage without glycerol at −80 °C (with and without snap freezing in liquid nitrogen) compared to less than 1% loss of activity at 4 °C with no glycerol or at all three temperature conditions with glycerol (Figure 2). $E_1$3G activity loss over time was greatest at 4 °C in the absence of glycerol (Glycerol × Temperature × Time interaction, $P = 0.04$).

The Glycerol × Time interaction was significant for Pd3G ($P = 0.0001$), although by 24 weeks the Pd3G activity with and without glycerol was similar (Figure 2). Loss of creatinine was greatest at 4 °C, ranging from 5.8 to 11.2%, depending on the additive (Additives × Temperature × Time interaction, $P = 0.05$). Freezing reduced activity loss.

As part of the second experiment, the effects of BSA and glycerol on samples stored for 24 weeks at −80 °C were determined. No significant effects involving storage time and BSA were found for $E_1$3G, Pd3G, and creatinine.

LH activity loss was greater in samples stored without BSA (6.7%) than with BSA (1.7%) (BSA × Time interaction, $P = 0.009$, Figure 2).

A significant Glycerol × BSA × Time interaction for FSH ($P = 0.009$) revealed that, after 24 weeks, activity loss was different between samples stored without glycerol and BSA (25.7%), samples stored with only BSA (16.3%), and samples stored with glycerol (with or without BSA) (<1%; Figure 2).

Activity loss for urine samples stored for 24 weeks at −80 °C with glycerol and BSA was statistically significant for $E_1$3G (9.9%, $P = 0.005$) but not for the other analytes: FSH (2.5%, $P = 0.14$), LH (0.6%, $P = 0.87$), Pd3G (5.5%, $P = 0.24$), and creatinine (3.3%, $P = 0.09$).

DISCUSSION

Measuring female reproductive hormones in urine affords a convenient, noninvasive means of

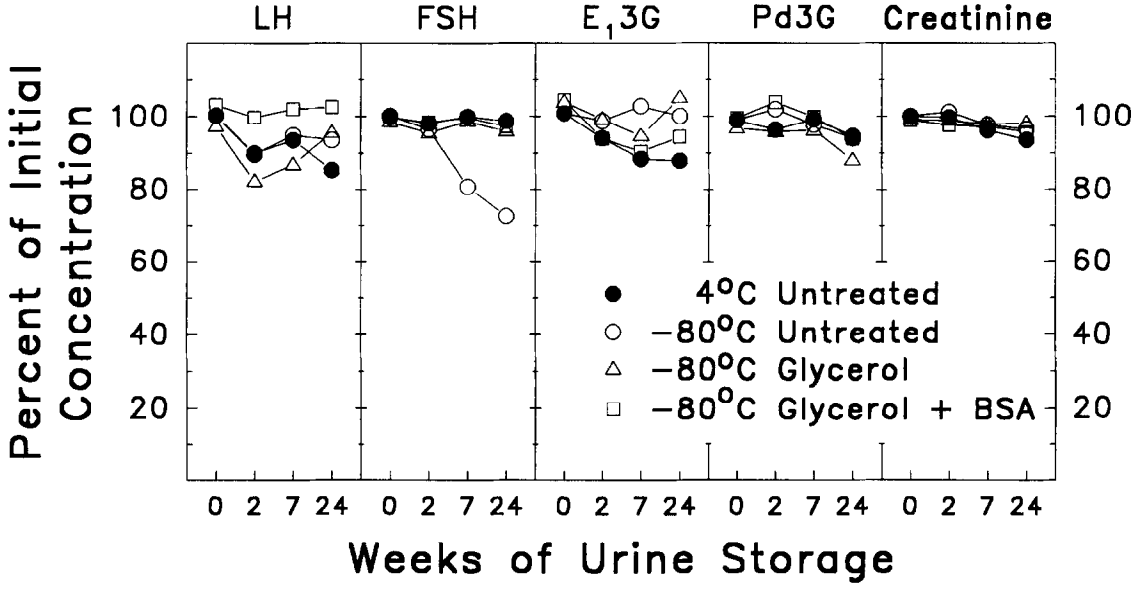

Fig. 2. Relative concentrations of LH, FSH, $E_1$3G, Pd3G, and creatinine in urine samples stored for 0, 2, 7, or 24 weeks at 4 °C, −80 °C with no additives, −80 °C with glycerol, and −80 °C with glycerol and BSA. Values represent percent of initial concentrations ($n = 6$).

acquiring specific and sensitive information about menstrual function (1–14). The present study characterizes stability of these analytes under various conditions, since field studies do not always allow for ideal storage conditions (22). This study also serves to identify the storage conditions for optimal stability of these analytes. While these results generally conform and add to results obtained using other immunoassays (15,16,23), analyte stability is likely to depend on the specific antisera and assay configuration.

Our results indicate that the five analytes studied are stable for samples stored at 4 °C for at least 2 weeks and lose minimal activity after storage for 6 months. $E_1$3G and creatinine stability are significantly compromised at 25 and 37 °C. The loss of LH and FSH activity at these warmer temperatures is consistent with those reported previously (23). Pd3G activity is only minimally affected.

Even though no attempt was made to collect sterile urine samples, additives, including antimicrobial agents, do not abate the loss of activity at the warmer temperatures. Campbell (22) has suggested that the high urea and salt concentrations intrinsic to urine may inactivate lytic factors and retard microbial activity.

Livesey and coworkers previously demonstrated that loss of gonadotropin activity in frozen urine decreases as the temperature is reduced and that no loss is apparent for samples stored at −55 °C for 15 weeks (15). The loss of FSH and LH activity described in the present report for urine samples stored at −80 °C for 24 weeks may reflect a difference in the respective immunoassays to distinguish structural damage.

As reported by others (15,16), LH and FSH activity is stabilized by adding glycerol. BSA further stabilizes LH activity without affecting activity of the other analytes. Beyond the protective effects of glycerol and BSA at freezing temperatures, none of the additives afford preservative effects.

The rationale for testing BSA was to determine if it would serve as a competitive protein substrate, thereby sparing degradation of the gonadotropin glycoproteins. It is not clear if this is how BSA reduces LH activity loss. Finally, boric acid interferes with the $E_1$3G assay, yielding exaggerated levels.

These results indicate that the activities of the basic female reproductive hormones in urine are stable with initial refrigeration followed by storage at −80 °C with 7.5% glycerol and 1% BSA. Efforts to stabilize the analytes at warmer temperatures were not successful. Campbell has described other, novel approaches that can be used where refrigeration and freezing are not options, but these approaches are limited (22). Because of the specific nature of antigen–antibody recognition, the results reported herein may not represent all other immunoassays.

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