

Detection of Xenoestrogens in Serum after Immunoprecipitation of Endogenous Steroidal Estrogens

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In this article we report a simple and efficient method for detecting nonsteroidal estrogens in a biologic sample. This method uses polyclonal antibodies to estradiol (E₂) to immunoprecipitate these major biologically active steroidal estrogens, leaving behind the nonsteroidal estrogens, which are then detected in a cell-based transcriptional activation bioassay for estrogen receptor agonist. The immunoprecipitation method efficiently removed 99% of radiolabeled E₂ and estrone (E₁) from human serum. In experiments in which supraphysiologic concentrations of E₂ and E₁ to human serum, all of the immunoreactive estrogens were still removed by the immunoprecipitation protocol. We carried out an *in vivo* validation study of this method in which we treated female macaques with the xenoestrogen nonylphenol (NP), during the late follicular phase of the menstrual cycle. We used blood samples collected before and after treatment to evaluate and characterize endogenous and exogenous serum estrogens. An immunoassay for E₂ did not detect the NP in treated monkeys. The cell-based bioassay also did not detect the estrogenic activity of NP because of its saturation by the endogenous serum steroidal estrogens. However, when steroidal estrogens were removed by immunoprecipitation, we detected the estrogenic activity of NP in the bioassay. Thus, this approach is appropriate for detecting exogenous, nonsteroidal estrogens in serum samples. **Key words:** cell bioassay, endocrine disruptors, nonylphenol, steroidal estrogens, xenoestrogens. *Environ Health Perspect* 110:791–795 (2002). [Online 17 June 2002] <http://ehpnet1.niehs.nih.gov/docs/2002/110p791-795natarajan/abstract.html>

A great deal of public concern is currently focused on environmental hazards that may have the potential to disrupt endocrine function. Many of these endocrine disruptors have been linked to adverse effects on either embryonic development or reproductive function in wildlife and humans (1–3). Exposures to these chemicals have been shown to stimulate sex hormone signal transduction pathways *in vitro* (4–6) and to cause developmental defects in fish (7) and reptiles (8,9) and egg-shell thinning in birds (10). Nevertheless, the adverse effects of endocrine disruptors on human reproductive health have not been convincingly demonstrated (11). Recently, it has been suggested that *in utero* exposure to environmental estrogens or other endocrine disruptors may be responsible for a global decline in sperm counts of adult men (12–15). Such exposures also have been suggested to cause early puberty in girls (16). Although numerous environmental agents have been shown to have estrogenic activity and thus the potential to exert adverse effects either by acting through the estrogen receptor (ER) and/or by activating estrogen-induced genes (17), documentation of adverse outcomes in humans as a result of exposures to environmental estrogens is lacking.

Previous attempts to link adverse health outcomes with exposures to environmental endocrine disruptors have been hampered by technical limitations in the methodology for

exposure assessment. In many cases, the putative endocrine disruptor may be unknown and/or sensitive assays may not be available to allow accurate measurement of the chemical agent in biologic samples. Even when the chemical agent is known, analytic measurements of the compound in body fluids provide no information as to its ability to produce a biologic effect at the detected concentration. Functional assays, which can measure the biologic activity of a compound or mixture of compounds, have the potential to provide valid and relevant exposure assessments. However, when such assays are applied to biologic samples, they must be able to distinguish the natural, endogenous hormone receptor ligands from the xenoligand mimic(s). In this article, we report the results of experiments that address this problem by a process of immunoprecipitation and removal of all biologically active, endogenous estrogens from serum, followed by measurements of estrogen action with a functional cell-based bioassay for ER-dependent transcriptional activation ligands. Measurements of total ER-mediated cell signals before and after the removal of endogenous steroidal estrogens enable detection of environmental estrogens within a biologic sample as well as assessment of their relative biologic activity in the presence of the endogenous steroidal estrogens.

Materials and Methods

Reagents. We obtained radiolabeled estradiol (¹⁴C-E₂) and estrone (¹⁴C-E₁) from NEN Life Science Inc. (Boston, MA); 17 β -estradiol (17 β -E₂) from Steraloids Inc. (Wilton, NH); nonylphenol (NP) from Aldrich Chemical Co. (Milwaukee, WI); α -minimum essential medium (α MEM), geneticin, fetal bovine serum (FBS), and sodium bicarbonate solution from Gibco BRL (Grand Island, NY); phenol red-free Dulbecco's modified Eagle medium, trypsin, goat anti-rabbit IgG, normal rabbit serum, and human serum from Sigma Chemical Company (St. Louis, MO); charcoal/dextran-treated FBS from Hyclone (Logan, UT); lysis buffer from Promega (Madison, WI); and luciferin from BD PharMingen (San Diego, CA).

We raised polyclonal antibodies in two male rabbits (R614 and R624) to an immunogen that consisted of estradiol-6-carboxy-oxime (Sigma Chemical Company) conjugated to bovine serum albumin (CalBiochem, La Jolla, CA) using a mixed anhydride reaction. Both antisera exhibited 100% cross-reactivity to E₂ and partial cross-reactivity to E₁ when titrated against ¹⁴C-E. The cross-reactivity for E₁ was 81% for R614 and 16% for R624. We pooled retired clinical serum samples from females to obtain human serum pools.

Immunoprecipitation. Radiolabeled estrogens from human serum. We spiked each of three 100- μ L aliquots of human serum (Sigma) with 2 nCi ¹⁴C-E₂ and 2 nCi ¹⁴C-E₁. We treated one of the three aliquots with 100 μ L of a 1:5 dilution of R614 and 100 μ L of a 1:10 dilution of R624. Two control aliquots received the same volume of phosphate buffer (pH 7). After samples were incubated at 4°C for 18 hr, we added 50 μ L of the precipitating antibody (goat anti-rabbit IgG) and 5 μ L normal rabbit serum to the sample that received the antiestrogen

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antibodies and to one of the control samples (secondary antibody control). The second control sample (total count control) received an equal volume of buffer. After a second 18-hr incubation period at 4°C, we centrifuged all samples at $18,000 \times g$ for 30 min at 4°C. We recovered the supernatants and centrifuged them a second time following the same protocol. Finally, 100- μ L aliquots of the supernatants were combined with 10 mL of scintillation fluid and counted for 1 min in a scintillation counter. We repeated the experiment three times.

Estrogens from untreated serum and serum spiked with supraphysiologic levels of E₂ and E₁. We prepared four 100- μ L aliquots of pooled human serum. We spiked two of the four samples with 2,000 pg/mL E₂ and 2,000 pg/mL E₁. The remaining two samples received no additional estrogen. We processed one of the estrogen-spiked serum samples and one of the untreated serum samples by immunoprecipitation with R614 and R624 as described above. The two control samples (one estrogen spiked and one untreated) received phosphate buffer rather than antiestrogen antisera. We incubated and centrifuged the samples as described above in preparation for analysis by immunoassay or bioassay (see below). We repeated the experiment three times with different pools of human serum.

Estrogens from serum spiked with NP or with NP, E₂, and E₁. We spiked each of four 100- μ L aliquots of pooled human serum with 250 pg/mL NP. Two of the four samples also received 2,000 pg/mL E₂ and 2,000 pg/mL E₁. We processed one of the samples treated with NP alone and one of the samples treated with NP and estrogens by immunoprecipitation with R614 and R624 as described above. The two control samples (one treated with NP and one treated with NP and estrogens) received phosphate buffer rather than antiestrogen antisera. We incubated and centrifuged the samples as described above in preparation for analysis by immunoassay or bioassay (see below). We repeated the experiment three times with different pools of human serum.

Treatment of macaques with NP. Three mature female cynomolgus macaques (*Macaca fascicularis*) were individually housed at the California Regional Primate Research Center in compliance with the federal Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (18). All animals experienced regular menstrual cycles as determined by daily observations of the perineum. We administered NP (15 mg/kg body weight/day in corn oil) for 3 successive days by nasogastric intubation during the late follicular phase of the menstrual cycle,

beginning on cycle day 9. We collected blood samples from the saphenous vein from physically restrained, unanesthetized animals. We collected 5 mL of blood 1 day before the first treatment and every day after treatment for 5 consecutive days.

Estrogen immunoassay. We detected estrogens in human serum and monkey serum by immunoassay using the ACS-180 automated chemiluminescence analyzer (Bayer Diagnostics Corporation, Tarrytown, NY). We modified the ACS:180 (E₂-6) immunoassay to enhance sensitivity by altering and lengthening the incubation conditions. The modified immunoassay involves incubation of the sample with a rabbit anti-E₂-6 antibody and subsequent addition of a dimethylacridinium ester-labeled derivative of E₂. Final incubation of sample with label and separation of antibody bound and free all take place after the samples have been placed on the ACS:180. The working assay range is 1.0–250 pg/mL.

ER bioassay. Dose–response curve for the induction of luciferase activity by E₁, E₂, and NP. The bioassay for detecting estrogen-dependent activation of gene expression was originally developed by Rogers and Denison (6). This assay consists of human ovarian carcinoma cells (BG1) that have been stably transfected with a luciferase reporter gene plasmid under the regulation of four estrogen-response elements. These cells are able to detect estrogenic chemicals by ER-mediated transactivation of the reporter gene. We performed the bioassay as previously described (6) with slight modification. Briefly, we grew transfected BG1Luc4E₂ cells in normal cell culture medium (MEM with 10% FBS). When the cells reached approximately 90–95% confluency, they were washed with phosphate-buffered saline, trypsinized for 30 sec, dispersed with cell culture medium, and plated in 150-mm dishes. The next day, after plating, we removed the media and overlaid the cells with estrogen-depleted phenol red-free media (Sigma) supplemented with 10% charcoal-dextran-treated FBS (Hyclone). The cells were maintained in the estrogen-depleted media for 6 days, dispersed with estrogen-depleted media, and then plated in 96-well microtiter plates. After 24-hr incubation, we removed media; overlaid the cells with 250 μ L of estrogen-depleted media containing increasing concentrations of each of E₁, E₂, and NP at 1% final volume; and incubated them for an additional 24 hr. The media were then removed and the cells were lysed using 100 μ L of 1X lysis buffer (Promega). We measured luciferase activity in 45 μ L of lysate in a Dynatech ML2550 microtiter-plate luminometer (Dynatech Laboratories Inc., Chantilly, VA), after the addition of 100 μ L luciferase reagent with a

delay time of 2 sec and integration time of 2 sec. Luciferase activity was expressed as relative light units per milligram of protein. The minimal detection limits were 1 pM for E₂, 10 pM for E₁, and 1 nM for NP.

Detection of estrogenic chemicals. We maintained cells as described above; after 24 hr incubation in 96-well microtiter plates, we removed media and overlaid the cells with 250 μ L of estrogen-depleted media containing E₂ standards and test serum at 1% final volume. We then incubated the cells for an additional 24 hr and determined the luciferase activity as described above.

Calculation of data. We calculated data using an enzyme immunoassay (EIA) program designed by Dennis R. Stewart (Enzyme Immunoassay Program, version 5.2; University of California, Davis, CA).

Calculation of standard curve. The EIA program calculates standard curves using the 2+2 logistic log algorithm (19). Basically, this is a four-parameter logistic log regression, except the parameters are fitted two at a time. The data are fitted according to the equation

$$Y = \frac{A - D}{1 + \left(\frac{X}{C}\right)^B} + D, \quad [1]$$

where Y = the luciferase activity (optical density); X = the concentration of the standard (E₂); A = The zero dose response; B = $-(\text{slope of the logit-log plot})$; C = dose for 50% binding; and D = infinite dose response (maximal luciferase activity measured for a ligand).

The four parameters fitted are A , B , C , and D . These were fitted two at a time. First, we performed a weighted logit-log regression to obtain B and C , which the program used to then calculate A and D , ending the first iteration. The program does five iterations and then determines standard residual variance. The program also calculates the root mean square error, which is a measure of goodness of fit of the line through the points and the standard errors of the fitted parameters.

Table 1. Immunoprecipitation of radiolabeled estrogens from human serum.

Sample	cpm	Percent
Total count ^a	4,177 \pm 24.0	100 \pm 0
Secondary antibody ^b	3,916 \pm 5.5	93.7 \pm 0.24
Immunoprecipitated ^c	51 \pm 9.0	1.2 \pm 0.022

cpm, counts per minute. Values shown are mean \pm SD; n = 3. Values of secondary antibody control and that of immunoprecipitated samples are percentage of total count control. All samples were spiked with 2 nCi ¹⁴C-E₂ and 2 nCi ¹⁴C-E₁.

^aTreated with buffer only. ^bTreated with goat anti-rabbit IgG only. ^cTreated with rabbit antisera raised against E₁ and E₂ followed by goat anti-rabbit.

Calculation of unknowns. Unknowns were calculated as E₂ equivalents. The program calculates the zero dose and the infinite dose for each plate and the percentage binding based on this and relates them back to the standard curve. The information on the number of replicates (which is 3 for this study) is in the format files. The program processes the information and calculates the amount of ligand present (unknowns) based on the standard curve.

We analyzed all samples in triplicate and considered each plate to be an individual assay with standard curve and internal controls included. The interassay coefficients of variation for low and high internal controls ($n = 10$) were 4.5% and 5.2%, respectively. The intraassay coefficients of variation for low and high internal controls ($n = 20$) were 11.5% and 14.3%, respectively. The sensitivity of the assay for E₂, defined as 2 SDs above the average zero dose, was 20 pg/mL.

Statistical methods. We calculated descriptive statistics and comparative analysis (Student's *t* test between controls and experimental) using Microsoft Excel (Microsoft, Redmond, WA).

Results

Immunoprecipitation of estrogens from human serum. Human serum spiked with a mixture of radiolabeled E₂ and E₁ and then treated with polyclonal antibodies raised against the two hormones removed 99% of the added hormones by immunoprecipitation (Table 1). We considered the level of radioactivity after addition of the labeled hormones to be 100%, and after immunoprecipitation, the percentage of radioactivity remaining in the samples was 1.22 ± 0.22 (mean \pm SD, $n = 3$; Table 1). In control samples, treated with

the secondary antibody only, the percentage of radioactivity after the immunoprecipitation procedure was 93.76 ± 0.24 .

Immunoassay of the untreated serum pool revealed that the levels of immunoreactive estrogens were 364.45 ± 65.25 pg/mL (mean \pm SD, $n = 3$; Figure 1A). After immunoprecipitation, no estrogens were detected by immunoassay in the serum pool (Figure 1A). After the pooled serum was spiked with 2,000 pg/mL of E₂ and E₁, we detected the added hormones by immunoassay; immunoprecipitation also reduced these supraphysiologic estrogen levels to undetectable levels (Figure 1A).

When we analyzed the same serum samples using the bioassay, the levels of luciferase induction correlated with the level of estrogens. The immunoprecipitation procedure removed both the endogenous estrogens and the added estrogens from the serum pool (Figure 1B).

Detection of the estrogenic activity of NP in human and macaque serum. When we spiked samples of the pooled human serum with NP and then treated them with the antiestrogen antibodies, we still detected ER-dependent induction of luciferase activity with the bioassay (Figure 2). After we spiked the serum with a mixture of NP, E₂, and E₁, we observed a significant increase in ER-dependent induction of luciferase activity. Most, but not all, of this activity was removed by immunoprecipitation with the antiestrogen antibodies (Figure 2). The level of signal remaining after immunoprecipitation was similar to that of serum spiked with NP alone (Figure 2).

We determined the dose dependence of induction of luciferase activity in BG1Luc4E₂ cells by incubating the cells with an increasing

concentration of E₁, E₂, and NP (10^{-14} to 10^{-6} M) (Figure 3). Induction of luciferase activity was dose dependent, with a minimal detection limit of 1 pM for E₂, 10 pM for E₁, and 1 nM for NP (Figure 3). The estrogenic potency of NP as determined by the bioassay was 1,000 times less than that of E₂ (Figure 3).

Preliminary experiments *in vitro* verified that estrogenic activity of NP could be detected in the macaque serum using the same protocol for immunoprecipitation with antiestrogen antibodies followed by the bioassay for ER-dependent induction of luciferase activity. Serum samples obtained from macaques treated with NP revealed physiologically normal levels of immunoreactive E₂ (90–120 pg/mL) before and after the administration of NP. After immunoprecipitation, however, we did not detect immunoreactive E₂ (< 2 pg/mL) (Figure 4A). Similarly, the bioassay revealed little change in the total bioactive estrogen concentration before and after NP administration. However, after immunoprecipitation, approximately 5–10% of the previous total estrogen bioactivity remained and increased during the 3 days that animals received NP (Figure 4B).

Discussion

Estrogenic endocrine disruptors include natural substances in food (isoflavonoids and lignans), nonsteroidal pharmaceuticals (diethylstilbestrol and ethinyl estradiol), and synthetic chemical compounds that degrade slowly and bioaccumulate in the environment (polychlorinated biphenyls and alkylphenols). After gaining entry into the body, compounds that are ER-ligand mimics are able to bind to the ER on target cells and then act as agonists or antagonists, altering

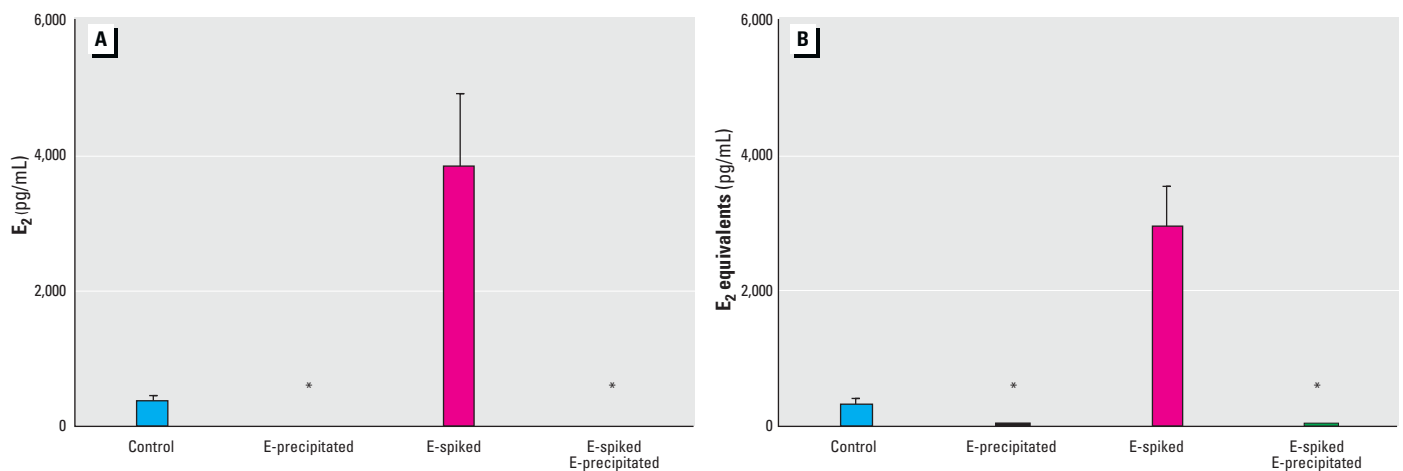


Figure 1. Effect of immunoprecipitation with antiestrogen antibodies on levels of (A) immunoreactive estrogen and (B) bioactive estrogen in human serum. Bars show estrogen levels for untreated, pooled human serum samples (control), pooled serum samples immunoprecipitated with antiestrogen antibodies (E-precipitated), pooled serum samples spiked with E₂ and E₁ (E-spiked), and E-spiked samples after immunoprecipitation (E-spiked E-precipitated). Values shown are \pm SD of triplicate determinations.

*Values significantly different from the unprecipitated samples at $p < 0.05$, as determined by *t*-test.

the ability of endogenous steroids to exert an action by virtue of competitive binding to the ER. The same compound may act as a weak agonist alone but may exert antagonistic effects in the presence of a more potent ER ligand. Because most nonsteroidal estrogens are not tightly bound by circulating binding proteins in human blood, they have an advantage over endogenous estrogens in terms of competing for ER binding because they are unimpeded by carrier proteins. However, because nonsteroidal estrogens can exert a wide range of biologic effects that are determined partly by other compounds in circulation, direct measurements of their concentrations may not be biologically relevant even when the identity of the estrogenic

compound is known. Functional assays can estimate the biologic activity of a wide range of compounds, whether or not the chemical structures are known, but these assays cannot distinguish between exogenous and endogenous estrogenic compounds, and they cannot determine the effects of individual chemicals when a mixture of compounds is present. The present study is a first step in addressing these issues and describes a method that separates steroidal estrogens from xenoestrogens in blood samples and then estimates the biologic activity of the xenoestrogens.

We selected NP as a model environmental estrogen because of its well-defined estrogenic properties (4,20) and widespread use.

In agricultural and industrial communities, alkylphenols are commonly used as wetting agents for pesticides that are delivered to a wide range of crops and, as such, have widespread destination. The potential adverse effects of NP have been investigated *in vitro*, and NP has been shown to stimulate growth in E₂-dependent MCF-7 cells (20), stimulate vitellogen production in cultured trout hepatocytes (4), and bind and activate ligand-dependent gene transcription in ER-transfected mammalian and yeast cells (4–6). *In vivo* studies have demonstrated the ability of NP to increase uterine growth in rodents after treatment with NP (6). The toxicology of NP has not been investigated previously in primate models.

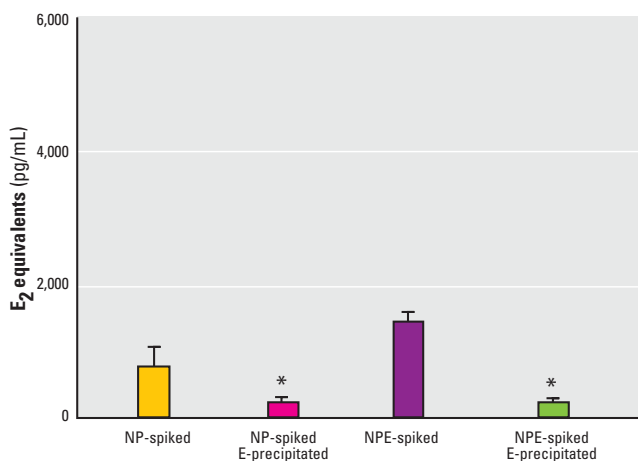


Figure 2. Detection of the estrogenic activity of NP in human serum. Bars show bioactive estrogen levels for pooled human serum samples spiked with NP (NP-spiked); NP-spiked samples after immunoprecipitation with antiestrogen antibodies (NP-spiked E-precipitated); pooled serum samples spiked with NP, E₂, and E₁ (NPE-spiked); and NPE-spiked samples after immunoprecipitation (NPE-spiked E-precipitated). Values shown are mean ± SD of triplicate determinations. *Values significantly different from the unprecipitated samples at $p < 0.05$, as determined by *t*-test.

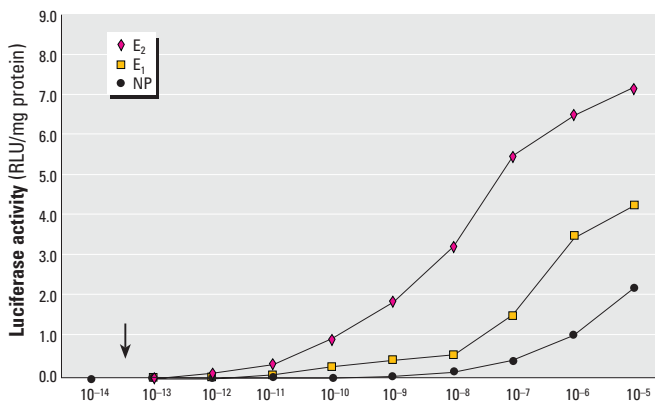


Figure 3. Dose–response curve for the induction of luciferase activity by E₁, E₂, and NP after incubation of the cells with the indicated concentration of E₁, E₂, and NP. RLU, relative light units. Values shown are mean of triplicate determinations. The minimal detection limit were 1 pM for E₂, 10 pM for E₁, and 1 nM for NP. The arrow indicates the sensitivity of the immunoassay for E₂.

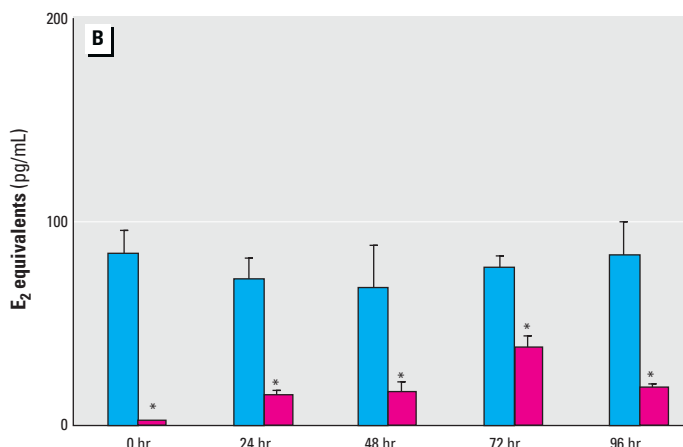
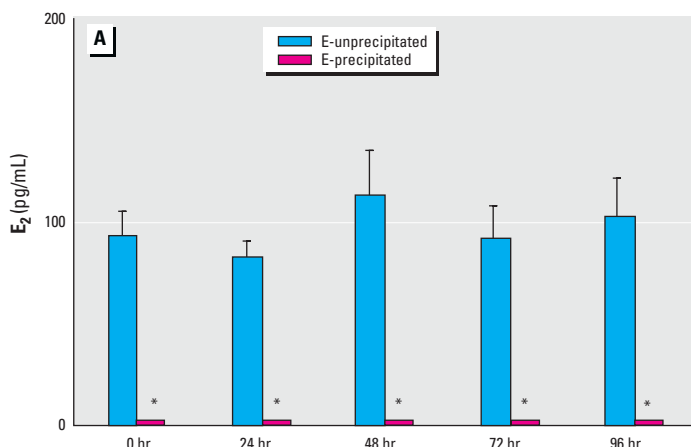


Figure 4. Detection of the estrogenic activity of NP in macaque serum after oral administration of NP for 3 consecutive days. (A) Immunoreactive estrogen levels. (B) Levels of estrogen bioactivity. Bars show estrogen levels on the day before NP administration (0 hr), and on 4 successive days after NP administration (24–96 hr). E-unprecipitated indicates estrogen levels in untreated serum samples, and E-precipitated indicates estrogen levels in the same serum samples after immunoprecipitation with antiestrogen antibodies. Values shown are mean ± SD of three determinations.

*Values significantly different from the unprecipitated samples at $p < 0.05$, as determined by *t*-test.

We used a sensitive immunoassay and a cell-based bioassay in this study to demonstrate that immunoprecipitation effectively separates endogenous steroidal estrogens from exogenous xenoestrogens in human serum samples. Both of these assays confirmed that immunoprecipitation of human serum samples with antiestrogen antibodies completely removed ^{14}C -labeled E_1 and E_2 from the sample. Other experiments demonstrated that the immunoprecipitation method was able to reduce normal concentrations of endogenous ER-binding ligands and supra-physiologic concentrations of E_1 and E_2 to nondetectable levels. Nevertheless, in future studies with other species or types of biologic samples, antibodies to additional steroidal estrogens may be required to remove endogenous estrogens that compete for ER binding.

When we added the xenoestrogen NP to serum samples either alone or in combination with supra-physiologic concentrations of E_2 , NP was not removed by immunoprecipitation, and we subsequently detected it with the bioassay for ER-dependent induction of luciferase activity. The measurement of ER-dependent induction of luciferase activity before and after the removal of steroidal estrogens provides an avenue to measure the relative inducing activity of any xenoestrogens that are present in the sample as well as information on their biologic activity *in vivo*. In our *in vivo* experiments with macaques, we detected the estrogenic activity of NP exposure only after immunoprecipitation of steroidal estrogens.

In summary, we have developed a method for detection of xenoestrogens in biologic samples. This approach can be useful in epidemiologic studies for exposure

assessment and risk assessment. The method can be used for exposure assessment to determine which members of a population have been exposed to potential endocrine disruptors. Traditional analytic chemistry methods would then be needed to identify specific compounds or classes of estrogenic compounds to which the subjects were exposed. In other situations in which xenoestrogens have been identified in the circulation of exposed individuals, our approach of comparing the total ER-dependent inducing activity in the presence and absence of endogenous estrogens can provide important information for assessment of the level of risk to the exposed population. This assay can, for the first time, clearly demonstrate the existence of the biologic activity of non-steroidal estrogens in serum without interference from steroidal estrogens.

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