

Development of a solid-phase extraction—enzyme-linked immunosorbent assay method for the determination of estrone in water

Zhilan Li^a, Shuo Wang^{a,c,*}, N. Alice Lee^a, Robin D. Allan^b, Ivan R. Kennedy^a

^a Faculty of Agriculture, Food and Natural Resources, The University of Sydney, Sydney, NSW 2006, Australia

^b Department of Pharmacology, The University of Sydney, Sydney, NSW 2006, Australia

^c Faculty of Food Science and Bioengineering, Tianjin University of Science and Technology, Tianjin 300222, PR China

Received 14 July 2003; received in revised form 7 October 2003; accepted 10 October 2003

Abstract

Estrone has been identified as a potential endocrine-disrupting chemical (EDC). To facilitate its analysis, a highly selective and sensitive enzyme-linked immunosorbent assay (ELISA) method with a simple solid-phase extraction (SPE) for analysis of estrone in aquatic environments has been developed. The specific polyclonal antibody was produced against a conjugate of estrone-3-hemisuccinate and keyhole limpet hemocyanin (KLH). The obtained ELISA showed specific recognition of estrone, without cross-reactions for three other major estrogenic compounds (17 β -estradiol, estriol, and 17 α -ethynylestradiol) commonly found in water. The ELISA had a limit of detection of 0.14 μ g/l estrone in water. Combining a SPE method to extract and pre-concentrate estrone from water samples and ELISA to specifically quantify estrone content, the SPE-ELISA can detect estrone down to 1.25 ng/l level in water. Good recovery with spiked river water was obtained with this SPE-ELISA method. The developed SPE-ELISA system was applied to analyze the real influent and effluent samples of sewage treatment plant in Penrith (Australia) and the results correlated well with those obtained using GC and HPLC methods. The developed SPE-ELISA method is capable of being applied for the specific detection and routine monitoring of estrone in environmental water samples.

© 2003 Elsevier B.V. All rights reserved.

Keywords: Estrone; ELISA; Solid-phase extraction; Water

1. Introduction

Estrone (Fig. 1) is one of the most potent estrogenic hormones, naturally occurring in the human body. At low levels it can be toxic, and carcinogenic and a strong growth promoter. It has been reported that estrone can have deleterious reproductive effects including inhibition of egg implantation, suppression of spermatogenesis and impotence [1]. Estrone is normally excreted from the human body entering the receiving water through sewage effluent. Although the estrogenicity of estrone is reported to be weaker than that of 17 β -estradiol in the vitellogenin test in rainbow trout [2], it is found to be more abundant in the sewage effluents and river waters [3], thus requiring cautious monitoring for risk assessment and remediation.

Estrogenic hormones in water matrix are usually quantified by instrumental analysis such as gas chromatography–mass spectrometry (GC–MS), GC–MS/MS, high performance liquid chromatography (HPLC), HPLC–MS and HPLC–MS/MS [4–7]. While these methods can be reliable, they have several potential drawbacks, including expensive instrumentation, complex derivatization, extensive clean-up and purification, and require much technical expertise in operation. Such costly and time-consuming instrumental techniques lead to the limitation of their wide utilization and for direct field detection. Therefore easily estimate estrogenic hormones in large numbers of environmental samples, alternative methods based on antibodies such as enzyme-linked immunosorbent assay (ELISA) were developed.

Several immunoassays based on ELISA for determination of estrone in animal or human body fluids were developed in the last few decades [8–10]. Analysis of estrone in air samples using ELISA was reported recently [11]. An application of ELISA kit to estimate estrone in the environmental

* Corresponding author. Tel.: +61-2-9351-2379;

fax: +61-2-9351-5108.

E-mail address: s.wang@agec.usyd.edu.au (S. Wang).

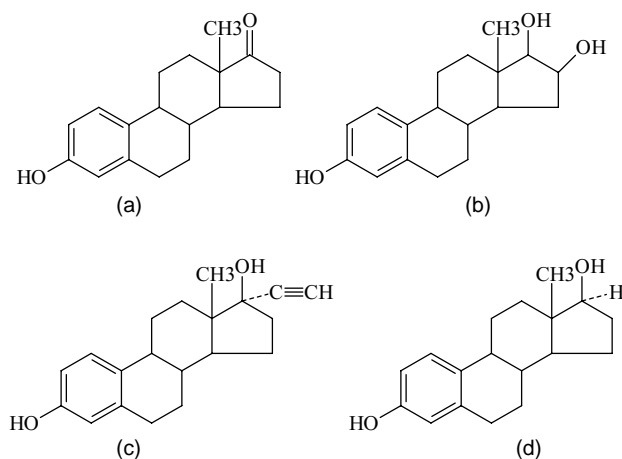


Fig. 1. Chemical structures of estrone (a), estriol (b), 17 α -ethynylestradiol (c) and 17 β -estradiol (d).

samples was also developed [12], but its use was limited due to high commercial price and insufficient sensitivity if no further sample preparation step. Because of low concentrations of estrogenic hormones in aquatic samples, a simple extraction and pre-concentration step was needed prior to measurement. The most commonly used sample preparation methods for aqueous samples are liquid–liquid extraction (LLE) and solid-phase extraction (SPE). The SPE needs less solvent, and it is also possible to achieve trace enrichment and clean-up in one step. For this purpose, SPE was developed for enrichment of estrone in water samples.

The objective of this work has been to develop a cost-effective and fast analytical methodology for the determination of estrone in water at low ng/l concentration levels. Therefore, a SPE followed by ELISA procedure has been developed. This paper reported the development of a sensitive estrone-specific immunoassay for estimation of estrone contents in influent and effluent of Penrith Sewage Treatment Plant of Sydney, Australia.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA) and horseradish peroxidase (HRP) were purchased from Boehringer-Mannheim (Germany). Keyhole limpet hemocyanin (KLH) and ovalbumin (OA), Freund's complete and incomplete adjuvants were purchased from Sigma (St. Louis, MO). Estrone standard (from Sigma) was used without further purification. Thin layer chromatography (TLC) plate was purchased from Merck (Darmstadt, Germany). Maxisorp polystyrene 96-well plates were obtained from Nunc (Roskilde, Denmark). All other organic chemicals of analytical grade used for hapten synthesis were purchased from Aldrich (Milwaukee, WI), Alltech-C₁₈ from Alltech Biotechnology (Victoria, Australia), and Isolute-C₁₈ from Selby Biolab (Victoria,

Australia) were selected for an optimum combination of SPE cartridges.

2.2. Instrumentation

¹H nuclear magnetic resonance (NMR) and ¹³C NMR spectra were obtained using a Varian Gemini-300 spectrometer operating at 300 MHz. Analysis of mass spectra (MS) were carried out at EI mode on a HP 5890 coupled with HP 7391 MSD. Melting points were determined on a Universal V3 OG TA differential scanning calorimeter (TA Instruments, 2920 modulated DSC). *R_f* values refer to TLC under exposure to either UV light or iodine vapor. Evaporation of organic solvent was carried out using a Buchi rotary evaporator with reduced pressure. Immunoassay absorbance were read in dual-wavelength mode (450–650 nm) with a Labsystems microplate reader (Helsinki, Finland). SPE extraction was carried out using Visiprep large volume sampler and Visiprep SPE Vacuum manifold from Sigma–Aldrich (NSW, Australia).

Instrumental analysis of estrone was performed using GC and HPLC. A Hewlett Packard 5890 Series II Gas Chromatograph equipped with an internal ⁶³Ni pulsed electron-capture detector (ECD) or a HP 5971 series mass selective detector (MSD) was used for GC/ECD or GC/MS detection. Two fused silica capillary columns (DB-5, 5%, phenylmethyl silicone, 30 m × 0.25 mm ID, 0.25 μ m film thickness) were conjunct with both detectors and a HP 7673 autoinjector was used for samples injection. The HPLC system consisting of a LC-10AT_{VP} pump and a SCL-10A system controller, together with a SIL-10A_{XL} Shimadzu autosampler. This HPLC system coupled with a Gilson 118 UV/VIS detector (Villiers-le-Bel, France). The separation was performed using a 250 mm × 4.6 mm ID Luna C₁₈ column with particle size of 5 μ m.

2.3. Hapten synthesis

2.3.1. Estrone-3-hemisuccinate

Estrone was succinylated by reaction with succinic anhydride to estrone-3-hemisuccinate (Fig. 2) using the method of Won and Chu [13]. The synthesis scheme is shown in Fig. 2. Estrone (1.00 g, 3.7 mmol) was dissolved in 25 ml of freshly distilled tetrahydrofuran (THF), then succinic anhydride (0.74 g, 7.4 mmol), triethylamine (TEA) (1.02 ml, 7.4 mmol) and 4-(dimethylamino)pyridine (DMAP) (0.45 g, 3.7 mmol) were added and the reaction mixture was stirred overnight at 60 °C. The solvent was removed under reduced pressure and the resulting residue was dissolved in methanol/methylene chloride (0.5:9.5 v/v). The crude product was then purified by column chromatography using methanol/methylene chloride (0.5:9.5 v/v). Appropriate fractions were collected, concentrated, and dried under vacuum: m.p. = 205.1 °C (dec.); TLC, *R_f* = 0.75 (methanol/dichloromethane, 1:9); ¹H NMR (CDCl₃), δ (ppm): 2.61 (s, 4H, CH₂CH₂) and 7.26 (t, 1H, aromatic).

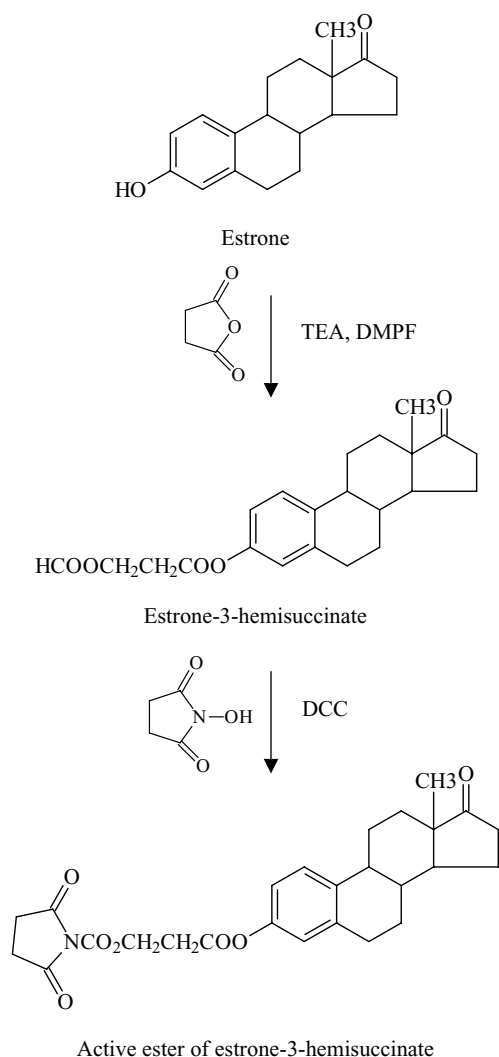


Fig. 2. Reaction scheme for the synthesis of estrone hapten.

2.3.2. Active ester of estrone-3-hemisuccinate

The method for the active ester synthesis was similar to that of benzoylphenylurea-hemisuccinate [14], as illustrated in Fig. 2. Briefly, estrone-3-hemisuccinate (0.432 g, 1.16 mmol) and hydroxysuccinimide (NHS) (0.168 g, 1.45 mmol) were dissolved in 15 ml of freshly distilled THF, then *N,N*-dicyclohexylcarbodiimide (DCC) (0.3 g, 1.45 mmol) was added with stirring. The reaction mixture was stirred at room temperature under N_2 for 3.5 h. The reaction mixture was filtered to remove the dicyclohexylurea precipitate. The combined THF filtrates were evaporated under the reduced pressure and the resulting residue was purified by column chromatography using ethyl acetate/toluene (2:3 v/v). After collecting and drying the appropriate fractions, a white solid of the active ester of estrone-3-hemisuccinate was obtained. The product was then recrystallized from ethylacetate: m.p. = 156.8 °C (dec.); TLC, R_f = 0.68 (ethylacetate/toluene, 2:3); 1H NMR ($CDCl_3$), δ (ppm): 2.99 (t, 4H, succinyl) and 7.26 (d,

1H, aromatic); ^{13}C NMR ($CDCl_3$), δ (ppm): 220.50 (C=O 17-keto); mass spectrum, m/z : 467, 451, 389, 373, 253, 141.

2.4. Preparation of immunogen, coating antigen and enzyme conjugate

For antibody production, the immunogen was prepared by conjugating the hapten to KLH. The coating antigen used in titration studies was prepared by conjugating the hapten to OV. The active ester dissolved in dry dimethylformamide (DMF) was added dropwise to a protein in pre-cooled buffer solution (50 mM K_2HPO_4 , pH 9.1). The reaction solution was gently mixed by hand and let stand at 4 °C overnight. The enzyme (peroxidase) conjugate used in ELISA was prepared using the similar method. The active ester in dry DMF was added to 10 mg of HRP in 2 ml of 50 mM K_2HPO_4 at pH 9.1, and a 13 M excess of hapten was used. The reaction solution was gently mixed by hand, and then left at 4 °C overnight. The conjugated solution was then dialyzed against phosphate buffered saline (pH 7.0) and stored at 4 °C. The concentration of coupled enzyme in solution was determined using an extinction coefficient, $E_{403}^{1\text{cm}} = 2.25$ for 1 mg/ml solution [22].

2.5. Antibody production

Three female New Zealand white rabbits were immunized with estrogen-KLH by intradermal and intramuscular injections. The first immunization was given with 1 mg of immunogen emulsified with 0.5 ml of 0.9% NaCl and 0.5 ml Freund's complete adjuvant. The subsequent booster injections was given with 0.5 mg of immunogen emulsified with 0.5 ml of saline and 0.5 ml Freund's incomplete adjuvant at 2-week intervals. The blood was collected from the marginal ear vein on a monthly basis and the antiserum was isolated by centrifugation, persevered with sodium azide and stored frozen. The antiserum was purified using protein A-sepharose chromatography and stored at 4 °C.

2.6. Preparation of standard solution

For the construction of calibration curve, a standard solution containing estrone in 10% methanol at the concentrations of 0.046–100 $\mu\text{g/l}$ was freshly prepared in glass tubes. Stock solution (10 mg/l in methanol) of estrone was diluted to 1/100, giving a 100 $\mu\text{g/l}$ standard solution (in 10% methanol). Then this 100 $\mu\text{g/l}$ solution was sequentially diluted in 10% methanol to give 33.3, 11.1, 3.7, 1.37 and 0.046 $\mu\text{g/l}$.

2.7. Direct competitive ELISA

The microwell plates were coated with purified anti-estrone IgG at 1 μg per well in 100 μl 50 mM carbonate buffer, pH 9.6, overnight at room temperature. Plates were then washed three times with PBS/T washing solution

(PBS with 0.05% (v/v) Tween 20) and unbound sites were blocked with 150 μ l of 1% BSA/PBS per well for 1 h at room temperature. After the plate was blotted dry, 100 μ l of 10% methanol in water was added to control and blank wells and 100 μ l of standard solution or sample extracts was applied to the allocated wells. 100 μ l of enzyme conjugate was immediately added to each well, except for the blank wells, and the mixture was incubated for 1 h. Following the washing with washing solution five times as before, 150 μ l of substrate solution (1.25 mM 3,3',5,5'-tetramethylbenzidine–1.6 mM hydrogen peroxide in acetate buffer, pH 5.0) was added to each well. The reaction was stopped after 30 min by adding 50 μ l of 1.25 M H₂SO₄, and absorbances were recorded by the microplate reader (Multiskan, Labsystems).

2.8. Solid-phase extraction

The C₁₈ cartridges were consecutively conditioned with 3 \times 2 ml acetonitrile, 3 \times 2 ml methanol and 3 \times 4 ml deionized water. Firstly, the STP influent, effluent and river water samples were filtered through Whatman 47 mm GF/C glass fiber filter with a pore size of 1.2 μ m. The filtrate was then pumped to the SPE cartridges at a flow rate of less than 10 ml/min. The cartridges were washed with 3 \times 5 ml 20% acetonitrile in water and dried for 30 min under the vacuum. The analytes was then eluted from each cartridge with 5 \times 1 ml 90% acetonitrile in water. Finally, the extracts were combined and evaporated to dryness on a heating block with nitrogen. The residues were re-dissolved in 10% methanol for ELISA analysis and in an appropriate solvent for clean-up and derivatization for GC or HPLC analysis.

3. Results and discussions

3.1. Optimization of the SPE extraction process

The SPE of estrone was compared for three C₁₈ cartridges (Alltech, ENVI and Isolute) with spiked tap water at concentrations between 1.25 and 25 ng/l and analyses by HPLC. The recoveries of spiked water using different SPE cartridges are listed in Table 1. The recoveries of the determined compounds varied greatly with different cartridges. Good recovery of 95.8% was obtained with Alltech-C₁₈ SPE

cartridge at higher spiked levels (25 ng/l), but greater than 100% at 5 ng/l spike means an overestimation of estrone content in real sample could occur. With the combination of Alltech-C₁₈ (absorbent material: 0.5 g) and Isolute-C₁₈ (absorbent material: 0.5 g), the satisfied extraction efficiencies were achieved with the recoveries between 94 and 106% at spike levels as low as 1.25 ng/l. This combination of cartridges was selected for the SPE extraction with the following sample analysis.

The maximum capacity of selected SPE cartridge was examined with various volumes of spiked water sample (250, 500, 1000 and 2000 ml) at 5 and 50 ng/l. For samples spiked at 5 ng/l, the capacity of the combined cartridge (Alltech + Isolute) was over 1000 ml, which would allow 1000 ml of water samples at this concentration to be extracted with one combination of the cartridges. Meanwhile, the capacity of SPE cartridge for sample spiked at 50 ng/l was less than 500 ml, but maintained high extraction efficiency. Thus, for different water sample, the volume which can be loaded onto SPE cartridge has to be considered. In this paper, 1000 ml of the sewage effluent or river water samples which are expected to contain very low concentration of estrone were applied in one SPE cartridge, while only 250–500 ml for sewage influent samples which are expected to contain higher concentration of estrone was applied.

After sample loading (spiked tap water at 5 and 50 ng/l), different compositions (10, 20, 25, and 30% acetonitrile in water) of washing solutions were applied to the cartridge. The influence of impurities could not be reduced with 10% composition of washing solution. On the other hand, most of the analytes were lost when the acetonitrile composition exceeded 25%. Finally, 20% solvent composition of washing solution was selected. For selection of the volume used for washing, varying volumes (5.0–25 ml) of washing solutions at the acetonitrile composition of 20% were applied to the selected cartridges. The eluted solution was collected at the volume 5, 10, 15, 20 and 25 ml separately. The wash processing can only cease when the UV absorbance of the eluted washing solution is exactly same as the virgin wash solution. This volume of wash solution was selected for the washing process.

3.2. Hapten selection and synthesis

Estrone contains one reactive phenolic group, ideal for chemical modification to produce a hemisuccinate derivative with a terminal carboxyl group for conjugating to protein and enzyme. Conjugation can then be carried out either by the synthesis of active ester or direct conjugation using water soluble carbodiimide. This approach orientates less common groups of estrone away from the point of conjugation, and as expected, the resulting antibodies were specific to estrone.

Based on the principles of hapten design for ELISA [15], the synthesized hapten should be a near perfect mimic of the

Table 1
Effect of different cartridges on the extraction efficiency

Cartridges with their sorbent	Spike level (ng/l)	Recovery for spiked water (%)
Alltech (1 g)	5	132.3 \pm 7.4
Alltech (1 g)	25	95.8 \pm 4.5
ENVI (1 g) + IST (0.5 g)	25	89.4
IST (0.5 g) + ENVI (1 g)	25	92.8
Alltech (0.5 g) + IST (0.5 g)	5	101.0 \pm 3.4
Alltech (0.5 g) + IST (0.5 g)	2.5	105.8 \pm 4.8
Alltech (0.5 g) + IST (0.5 g)	1.25	93.6 \pm 4.9

target structure in size, shape and electronic properties for antibody production. The hapten handle (the spacer arm for connecting the hapten to a carrier protein) should be of an appropriate length and should not elicit antibody recognition. Generally, the optimal linking group is a alkyl chain of about four to six atoms [16–19] and a spacer arm that is too long may lead to hapten folding [20]. Therefore, a medium size of four-carbon succinyl spacer was selected for the hapten synthesis. The hapten was synthesized by reacting the phenolic group of the estrogen backbone structure with succinic anhydride. The obtained carboxylic acid hapten was converted to the succinimide ester for coupling hapten to carrier protein.

3.3. Assay sensitivity

The optimum conditions for the direct competitive ELISA was determined by titrating the enzyme conjugate against the immobilized antibodies at various concentrations and selecting the concentrations of enzyme conjugate that provide absorbance at 450 nm around one unit. The sensitivities of three anti-estrone antibodies (E1Ab, 99FB and OC31) were studied by running standard curves between 0.046 and 100 $\mu\text{g/l}$ of estrone. The standard curves for three estrone antibodies are shown in Fig. 3. The 99F8 antibody showed the highest sensitivity to estrone with IC_{50} (calculated as the concentration of estrone giving 50% inhibition of color development) of $0.6 \pm 0.046 \mu\text{g/l}$, followed by E1Ab and

OC31 antibodies with the respective IC_{50} of 1.2 ± 0.068 and $4.5 \pm 0.14 \mu\text{g/l}$. The relative standard derivations (RSD) of concentrations in the range from 0.046 to 100 $\mu\text{g/l}$ for this assay were between 17.3 and 2.7%. The limit of detection (LOD), which calculated as the concentration of standard solution causing 15% inhibition of color development, was 0.030, 0.14 and 0.43 $\mu\text{g/l}$ of estrone for 99F8, E1Ab and OC31, respectively.

3.4. Assay specificity

The specificities of these ELISAs were evaluated using three structurally-related natural and synthetic estrogens (17 β -estradiol, estriol, and 17 α -ethynylestradiol, Fig. 1) that may be found in the sewage effluent or in the river water. The IC_{50} value for each of these compounds was above 100 $\mu\text{g/l}$, except for 17 β -estradiol with the 99F8 antibody in which the IC_{50} was 45 $\mu\text{g/l}$. It shows that the assays developed from these three antibodies were very specific to the estrone and the cross-reactions with three other structurally-related compounds commonly found in waste water systems were not high (Table 2). Although the 99F8 antibody exhibited highest sensitivity for estrone, the cross-reaction for 17 β -estradiol, even though it is only 1.3% of the sensitivity for estrone, may cause false-positives if the concentration in water is above the expected low ppb levels. Thus, we chose E1Ab antibody for further validation.

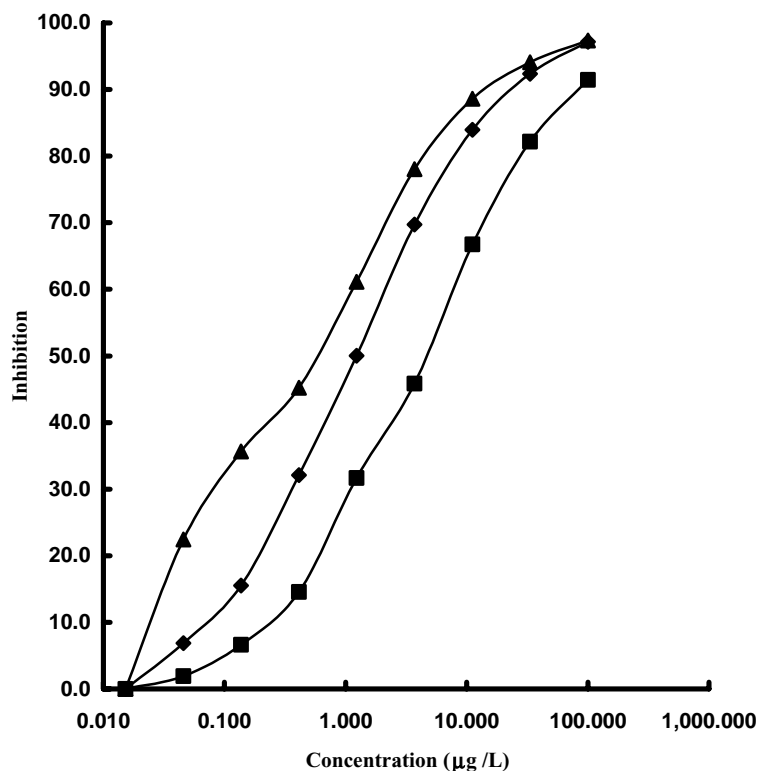


Fig. 3. Estrone standard curves with three antibodies: (■) OC31, (◆) E1Ab, (▲) 99F8.

Table 2

IC₅₀ (μg/l) and cross-reactivity (CR, %) for four estrogenic compounds with three antibodies

Compound	E1AB		99F8		OC31	
	IC ₅₀	CR (%)	IC ₅₀	CR (%)	IC ₅₀	CR (%)
Estrone	1.2	100	0.6	100	4.5	100
17β-Estradiol	>100	<1.2	45	1.3	>100	<4.5
Estriol	>100	<1.2	>100	<0.6	>100	<4.5
17α-Ethinylestradiol	>100	<1.2	>100	<0.6	>100	<4.5

3.5. Assay optimization

Fish skin gelatin (FG) and BSA are commonly used in diluent buffers to reduce nonspecific interactions and to stabilize the enzyme conjugate. In order to investigate their influence on assay performance, four different enzyme-conjugate diluents consisting of BSA and FG in PBS (1% BSA/PBS, 0.1% BSA/PBS, 1% FG/PBS and 0.1% FG/PBS) were tested. It was found these diluents have very little effects on assay performance (Fig. 4). The 1% BSA/PBS was chosen as enzyme-conjugate diluent for routine analysis.

The solvent for preparing standard solutions and samples also can affect the ELISA. Methanol solutions at 5, 10, 20 and 50% in water were tested for their effects by comparing

the standard curves prepared in each of these methanol concentration. Up to 10% of methanol did not alter the sensitivity of the assay and concentration above this shifted the curve to the right, reducing the assay sensitivity (data not shown). Methanol at 10% was chosen for the routine analysis of estrone in water samples.

3.6. Analysis of estrone spiked in water

The matrix effects of the river water were examined by the recovery of river water spiked at 20 and 100 ng/l with three replicates for each concentration. The spiked samples were extracted using the SPE protocol and analyzed by ELISA using E1Ab antibody. The recoveries of spiked river water samples at 20 and 100 μg/l was 90–105 and 99–110%,

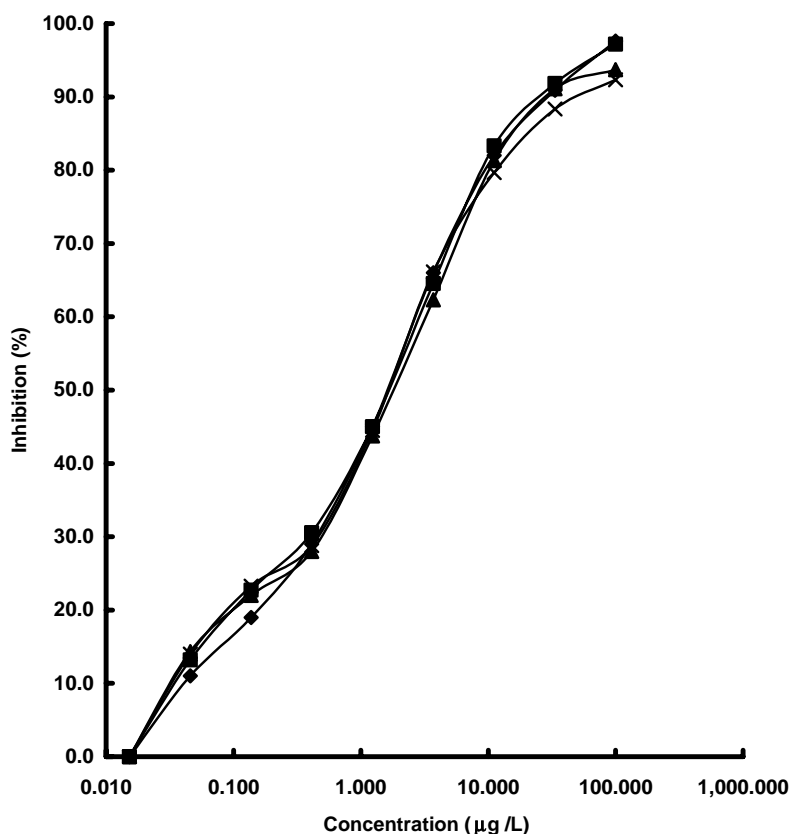


Fig. 4. Effect of enzyme conjugate diluents on estrone ELISA standard curves: (◆) 1% BSA; (■) 0.1% BSA; (▲) 1% FG; (×) 0.1% FG.

Table 3
Comparison of concentrations ($\mu\text{g/l}$) of estrone in influent, effluent, and river water samples using three analytical methods

Samples	GC/ECD ($n = 3$)	HPLC ($n = 3$)	ELISA ($n = 3$)
Influent	0.390 ± 0.0220	0.410 ± 0.0230	0.43 ± 0.0012
Effluent	0.013 ± 0.0010	0.016 ± 0.0014	0.017 ± 0.0008
River water	0.013 ± 0.0011	0.0093 ± 0.0008	0.012 ± 0.0006

respectively, indicating that the river water after SPE extraction did not interfere with the ELISA.

3.7. Accuracy and precision of the assay

The estrone concentration–inhibition standard curve was reproducible, with standard deviation of inhibition values between 2.7 and 17.3% in the range of estrone concentrations of 0.046 and 33.3 $\mu\text{g/l}$. The data were from five experiments conducted in different days. The accuracy of results obtained using ELISA method was investigated by comparison of results obtained with HPLC and GC methods using real sewage water. The results summarized in Table 3 show that this ELISA method gave good correlation with GC and HPLC results, although there was a slight tendency for ELISA values to be slightly higher than GC and HPLC values. It is possible that the clean-up and/or derivatization processes required for GC and HPLC cause some loss of target compounds, resulting in lower estimation, as reported in the previous study with pesticide endosulfan [21].

4. Conclusions

A cost-effective and relatively fast SPE linked with ELISA was developed for quantitation of estrone in river and sewage water. This SPE-ELISA offers an acceptable monitoring technique for routine analysis of estrone in the sewage and river water with a minimum sample preparation. The developed SPE-ELISA exhibited very low cross-reactions with the other estrogenic compounds (17 β -estradiol, estriol, and 17 α -ethynylestradiol) commonly found in the same water sources. The method developed from this study can potentially be applied for the analysis of estrone in other matrices such as biological fluids, soil, and foods.

Acknowledgements

We thank Dr. Nazir Ahmad of Sydney Water, West Ryde, NSW, Australia, for his assistance with sample collections.

References

- [1] R.J. Lewis, Van Nostrand Reinhold, Carcinogenically Active Chemicals, New York, 1991, p. 133.
- [2] E.J. Routledge, D. Shbahani, C. Desbrow, G.C. Brighty, M. Waldock, J.P. Sumpter, Environ. Sci. Technol. 32 (1998) 1559.
- [3] G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Mancini, R. Mastropasqua, M. Nazzari, R. Samperi, Sci. Total Environ. 302 (2003) 199.
- [4] C. Desbrow, E.J. Routledge, G.C. Brighty, J.P. Sumpter, M. Waldock, Environ. Sci. Technol. 32 (1998) 1549.
- [5] M. Torigai, Y. Ishii, S. Yun, T. Hashiba, J. Environ. Chem. 10 (200) 595.
- [6] C.H. Huang, D.L. Sedlak, Environ. Toxicol. Chem. 20 (2001) 133.
- [7] J.K. Fawell, D. Sheahan, H.A. James, M. Hurst, S. Scott, Water Res. 35 (2001) 1240.
- [8] N.M. Czekala, J.E. Gallusser, J.E. Meier, B.L. Lasley, Zoo Biol. 5 (1986) 1.
- [9] J.V. Lee, C.S. Whaling, B.L. Lasley, P. Marler, Zoo Biol. 14 (1995) 97.
- [10] C.J. Munro, L.S. Laughlin, T. Vonschalscha, D.M. Baldwin, B.L. Lasley, Contraception 54 (1996) 43.
- [11] S. Armstrong, Z.F. Miao, F.J. Rowell, Z. Ali, Anal. Chim. Acta 444 (2002) 79.
- [12] Y. Goda, M. Hirobe, A. Kobayashi, S. Fujimoto, M. Ike, M. Fujita, Y. Okayasu, H. Tanaka, Proceedings of the IWA Second World Water Congress on Efficient Water Management, 2001.
- [13] C.Y. Won, C.C. Chu, Carbohydr. Polym. 36 (1998) 327.
- [14] S. Wang, R.D. Allan, J.H. Skerritt, I.R. Kennedy, J. Agric. Food Chem. 46 (1998) 3330.
- [15] F. Szurdoki, H.K.M. Bekheit, M.P. Marco, M.H. Goodrow, B.D. Hammock, in: D.A. Kurtz, J.H. Skerritt, L. Stanker, New Frontiers in Agrochemical Immunoassay, AOAC International, Arlington, VA, 1995, p. 39.
- [16] M. Franek, J. Steroid Biochem. 28 (1987) 95.
- [17] A.S. Hill, D.P. McAdam, S.L. Edward, J.H. Skerritt, J. Agric. Food Chem. 41 (1993) 2011.
- [18] W.H. Newsome, J.M. Yeung, P.G. Collins, J. AOAC Int. 76 (1993) 381.
- [19] J.M. Yeung, W.H. Newsome, in: D.A. Kurtz, J.H. Skerritt, L. Stanker, New Frontiers in Agrochemical Immunoassay, AOAC International, Arlington, VA, 1995, p. 163.
- [20] N.A. Lee, I.R. Kenney, J. AOAC Int. 84 (2001) 1393.
- [21] N.A. Lee, H.L. Beasley, M. Silburn, N. Woods, S. Kimber, J.H. Skerritt, I.R. Kennedy, J. Agric. Food Chem. 45 (1997) 4147.
- [22] D.P. McAdam, A.S. Hill, H.L. Beasley, J.H. Skerritt, J. Agric. Food Chem. 40 (1992) 1466.