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Monitoring of progestins: Development of immunochemical methods for purification and detection of levonorgestrel

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ABSTRACT

A polyclonal antibody (Ab) for the progestin levonorgestrel (LNG) was generated, and immunochemical assays for its detection, clean-up and concentration were developed. A highly specific microplate diagnostic assay for the detection of LNG was developed that used the enzyme linked immunosorbent assay (ELISA) method. The LNG ELISA developed was sensitive and reproducible; it exhibited I_{50} and I_{20} values of $3.3 \pm 1.8\,ng\,mL^{-1}$ and $0.6 \pm 0.4\,ng\,mL^{-1}$, respectively, and the Abs did not cross react with any of the tested steroid hormones. The above Abs were used to develop a sol–gel-based immunoaffinity purification (IAP) method for concentration and clean-up of LNG that is compatible with subsequent immunochemical or instrumental chemical analytical procedures, such as liquid chromatography followed by mass spectrometry (LC–MS/MS). Development of the columns included successful entrapment of Abs within a tetramethoxysilane (TMOS)-based SiO_2 polymer network. The Abs could bind the free analyte from solution, and the bound analyte could be easily eluted from the sol–gel matrix at high recoveries. The Ab selectivity towards the antigen was high, in both ELISA and the sol–gel columns, but the entrapped Abs cross-reacted with two other steroid hormones – ethynylestradiol (EE2) and nortestosterone (NT) – which share similar epitopes with LNG, despite the lack of cross reactivity in the ELISA. The validity of the method was investigated by LC–MS/MS and a good analytical correlation was obtained.

1. Introduction

Studies in the past decade have shown that exposure to environmental chemicals influences human development and reproductive endpoints (for review see [1,2] and references therein). Recent years have seen an accelerating decline in human fecundity, and because many chemicals have been shown to mimic the activities of reproductive hormones, it is potentially possible that environmental exposure to such chemicals influences some human reproductive endpoints [3–5]. Up to now, most of the studies on adverse health effects of environmental contaminants have focused mainly on pesticides of agricultural and/or industrial origin [5], heavy metals [3], and toxic environmental contaminants of industrial origin [5]. One large class of chemicals that has received little attention comprises residues of pharmaceutical products (PPs), which are used in human and veterinary medicine, in quantities comparable to those of the agrochemicals. In the past few years numerous PPs and their metabolites have been shown to contaminate aquatic environments (for review see [1,6–10] and references therein). Despite the vast amount of information that has accumulated in the past few years on the occurrence of PPs in the environment, currently, there is very little information on their

Abbreviation: ACN, acetonitrile; BSA, bovine serum albumin; CMA, (carboxymethoxy)amine hemihydrochloride; CMO, carboxymethyloxime; DCC, N′,N′-dicyclohexylcarbodiimide; DCM, methylene chloride; DDW, double distilled water; DMF, dimethylformamide; EE2, ethynylestradiol; ELISA, enzyme linked immunosorbent assay; FLX, fluoxetine; GC, gas chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; IAP, immunoaffinity purification; IMT, indomethacin; KLH, Keyhole Limpet Hemocyanin; LC, liquid chromatography; LNG, levonorgestrel; LOD, limit of detection; MPA, medroxyprogesterone acetate; MRM, multiple reaction monitor; MS, mass spectrometry; NHS, N-hydroxysuccinimide; NSAIDs, non-steroid anti-inflammatory drugs; NT, nortestosterone; ON, overnight; PBS, phosphate-buffered saline; PBST, sodium phosphate containing Tween; PPs, pharmaceutical products; OVA, ovalbumin; PEG, polyethylene glycol; Py, pyridine; RIA, radioimmunoassay; SPE, solid-phase extraction; SSRIs, selective serotonin reuptake inhibitors; TMB, 3,3′,5,5′-tetramethyl benzidine; TMOS, tetramethylsilane; TMP, thrimethoprim.

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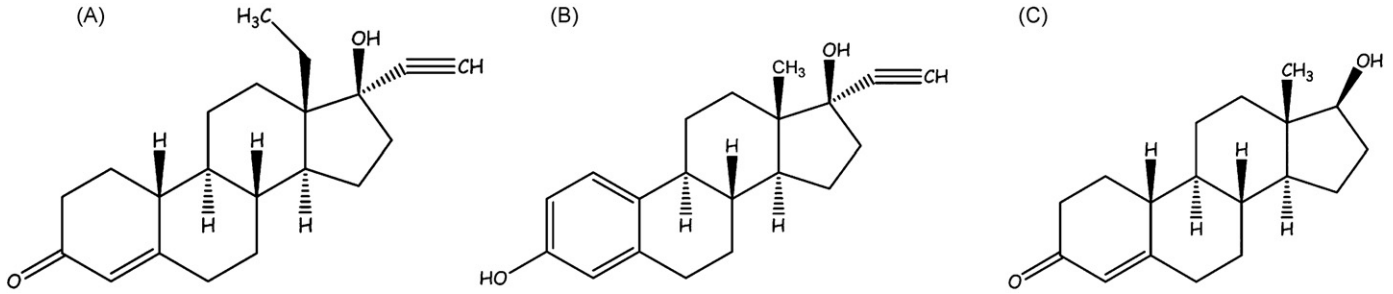

Fig. 1. Structures of the steroid hormones LNG (A), EE2 (B) and NT (C).

environmental fate. Our understanding of the possible transmission of PPs into the food chain is also very limited, and even less is known if and how such contaminants, once ingested, affect human health.

In the past few years we have conducted, within an EU-FP-6 project on Food and Fecundity (F&F), a detailed review of pharmaceuticals with potential to affect human fecundity *via* exposure through the human food chain. Pharmaceuticals were reviewed especially with regard to their mechanism of action, production and consumption volumes, persistence in the environment, and severity of identified adverse health effects in humans. Based on an extensive literature survey, a list of eight potential endocrine-disrupting pharmaceutical products (PPs) had been selected [11]. Among these compounds were a few synthetic steroid hormones, such as: levonorgestrel (LNG, Fig. 1A), ethynylestradiol (EE2, Fig. 1B), nortestosterone (NT, Fig. 1C) and medroxyprogesterone acetate (MPA), which are the main components of contraceptive drugs, and also are used as anabolic steroids; a representative of the non-steroid anti-inflammatory drugs (NSAIDs) – indomethacin (IMT); a representative of selective serotonin reuptake inhibitors (SSRIs) – fluoxetine (FLX); the adrenergic beta blocker atenolol (ATL); and the antibiotic, thrimethoprim (TMP). All of the above compounds exhibit high stability in the environment, are used in large amounts, and, most importantly, were reported to have effects on fecundity. In the present study we focused on LNG, which is a synthetic progestin used mainly as a component of contraceptives (for example see [12,13]). Apart from its obvious effect on women's fertility, LNG was found also to affect men's fertility and exposure to high levels of LNG has been shown to cause azoospermia (no measurable sperm in semen) [14]. So far the available information about the occurrence, fate and bioaccumulation of LNG in the environment or in the food chain is very limited. However, its massive use nowadays, its high environmental stability and long environmental half-life raise the possibility that it has a potential to pose a high risk to human health.

At present the conventional means for monitoring environmental residues are based on chemical analytical methods: namely, advanced mass spectrometry (MS) combined with either gas chromatography (GC–MS) or liquid chromatography (LC–MS) (for review see [8,9,15] and references therein). Unfortunately, although sensitive, precise and reproducible, these methods are not cost effective, as they involve the use of large volumes of toxic solvents for sample extraction and clean-up that are both expensive and require costly storage and disposal arrangements. Furthermore, long and complicated concentration and clean-up procedures must be applied to the tested samples before such analyses can be carried out. In light of these problems, the need for simpler and more economical analytical procedures is of major importance.

Immunochemical methods such as enzyme linked immunosorbent assay (ELISA) can overcome some of the above limitations, basically because they are highly specific, sensitive, simple and inexpensive, and can be implemented in large-scale monitoring in the laboratory and on site. The general objective of our present study focused on development of novel immunochemical methods for clean-up, concentration and monitoring of LNG.

In the present paper, we describe the generation of a polyclonal Ab for the progestin LNG, and employment of this Ab in development of immunochemical assays for its detection, clean-up and concentration. The immunochemical assay focused on development of a microplate ELISA, whereas the clean-up and concentration assay was based on sol–gel-entrapped immunoaffinity purification (IAP), which involves entrapment of the Abs in a ceramic SiO_2 matrix and has been proven by us and others to be a very efficient purification and concentration method, applicable to a wide range of analytes from various matrixes. (For review see [16] and references therein and [17–24].) Using this technology we were able to develop a highly effective and reproducible assay for the affinity purification and concentration of LNG which can be further used to prepare environmental and biological samples for determination of LNG occurrence.

2. Materials and methods

2.1. Immunochemical methods

2.1.1. Antisera

Polyclonal anti-LNG antiserum was generated in rabbits by using a 3′-carboxymethyloxime (CMO) derivate of LNG conjugated to bovine serum albumin (BSA) (Sigma, St. Louis, MI) as an immunogen, as described below (Section 2.1.3). Polyclonal anti-EE2 antiserum was generated in rabbits by using a 6′-CMO derivate of EE2 (Steraloids, Newport, RI, USA) conjugated to Keyhole Limpet Hemocyanin (KLH) (Sigma, St. Louis, MI) (Bronshtein et al., in preparation). Polyclonal anti-NT was generated in rabbits by using a 19-nortestosterone hemisuccinate (Steraloids, Newport, RI, USA)–BSA conjugate (Bardugo et al., in preparation).

2.1.2. Preparation of LNG-3′-CMO

This step involved conjugation of the 3′-ketone group of LNG to (carboxymethoxy)amine hemihydrochloride (CMA) (Aldrich, St. Louis, MI). Briefly, 80 mg of LNG (D(−)-Norgestrel) (Sigma) and 56 mg of CMA were dissolved in 1955 μL of pyridine (Py) (Aldrich). The reaction was allowed to proceed at 60 °C for 90 min, then 2.5 mL of 4N hydrochloric acid (HCl 6N Sequanal Grade ampules) (Pierce, Rockford, IL, USA) were added to the mixture, followed by the addition of 2.5 mL of methylene chloride (DCM, HPLC grade) (J.T. Baker, Phillipsburg, NJ). The mixture was stirred and left for several minutes to allow complete phase separation. The pH of the upper (aqueous) phase was tested for acidity in order to confirm that the base was totally neutralized (the obtained pH was 4.0). Three more separation cycles were executed, using a total volume of 10 mL from each solvent. The organic (lower) phases were collected and evap-

orated under vacuum in a Speed Vac SVC100H (Savant, Wivelsfield Green, UK), and the compound was stored at −20 °C pending further use.

2.1.3. Preparation of LNG-3′-CMO–BSA conjugate for immunization

The antigen for immunization was prepared by conjugation of activated LNG-3′-CMO to bovine serum albumin (BSA) (Sigma). First, LNG-3′-CMO was converted to its ester form by the following procedure: 15 mg of LNG-3′-CMO were mixed with 21.5 mg of N-hydroxysuccinimide (NHS) (Sigma) and 78 mg of N′,N′-dicyclohexylcarbodiimide (DCC) (Sigma), and dissolved in 1.64 mL of dimethylformamide (DMF) (Labscan, Dublin, Ireland). The reaction was allowed to proceed at room temperature for 4 h and the product was then incubated overnight (ON) at 4 °C. The mixture was centrifuged for 15 min at 2500 × *g* at room temperature and 1 mL of the supernatant (10 mg of activated LNG) was added to 10 mg of BSA dissolved in 0.13 M $NaHCO_3$ at pH 9.2. The reaction was allowed to proceed for 1 h at 4 °C, and the unbound hapten and other small-molecular-weight components were separated from the protein hapten conjugate by size exclusion with Centricon 30 (Amicon, Millipore, Billerica, MA). The reaction mixture was spun for 25 min at 2500 × *g* at room temperature, and washed twice with 5 mL of 0.13 M $NaHCO_3$ at pH 9.2. The final volume was adjusted to 5 mL by adding double distilled water (DDW) and the conjugate was kept in 0.5-mL aliquots at −20 °C pending use.

Prior to immunization, 0.5 mL of the conjugate were mixed with Complete Freund's adjuvant (1st injection) or with incomplete Adjuvant (2nd–4th injections). Two rabbits were injected at each time point. Bleeds were collected after each boost and were tested for activity with checkerboard experiments. The 3rd and 4th bleeds were equally active towards the antigen, and both were used for ELISA and sol–gel IAP experiments.

2.1.4. Preparation of LNG-OVA coating antigen

The method was similar to that described above (Section 2.1.3) for preparation of the LNG-3′-CMO–BSA conjugate, except that 2 mg of LNG-3′-CMO were mixed with 2.72 mg of NHS (10 μmol) and with 9.92 mg of DCC, and dissolved in 208 μL of DMF. After incubation and centrifugation as described in Section 2.1.3, 4.65 μL of the conjugate were added to 750 μL of ovalbumin from egg white (OVA), containing 6.67 mg mL^{-1} (Sigma) that was dissolved in 0.13 M $NaHCO_3$, pH 9.2 (molar ratio of 1:1, hapten to carrier protein). The reaction was allowed to proceed for 1 h at 4 °C, and the unbound hapten and other small-molecular-weight components were separated from the protein–hapten conjugate by size exclusion with Centricon 30. The reaction mixture was spun for 25 min at 4000 × *g* at room temperature, and was washed twice with 750 μL of 0.13 M $NaHCO_3$, pH 9.2. The volume was adjusted to 750 μL by adding 0.13 M $NaHCO_3$, pH 9.6, and the conjugate was stored in aliquots at −20 °C pending use.

2.1.5. LNG competitive ELISA

The assay developed was an indirect competitive ELISA, in which tested compounds or LNG standard in solution competed with an antigen–protein conjugate immobilized on a 96-well microtiter plate, for binding to anti-LNG antiserum. The assay served to determine both the cross reactivity of anti-LNG antiserum and the amount of LNG that eluted from the sol–gel IAP columns (see Section 2.2.2). The microtiter plate wells (F96 Maxisorp) (Nunc Immuno Plate, Roskilde, Denmark) were coated with 100 μL of LNG–OVA conjugate, diluted 1:2000 (containing 0.34 μg per 50 μL) in 0.5 M carbonate buffer, pH 9.6. After an ON incubation at 4 °C, the wells were washed three times with phosphate-buffered saline (PBS) that comprised 0.15 M NaCl in 50 mM sodium phosphate, pH 7.2, containing 0.1% (v/v) Tween-20 (PBST) (Sigma), and 50 μL of test (unknown) sample or standard were added to the wells, together with 50 μL of anti-LNG antiserum diluted 1:5000 in PBST containing 10% ethanol. The standard samples comprised 12 serial dilutions of LNG, ranging from 10 to 0.0049 ng per 50 μL. The plates were incubated ON at 4 °C, washed as above with PBST, and 100 μL of secondary Ab conjugated to horseradish peroxidase (HRP) (anti rabbit HRP conjugated, Sigma), diluted 1:30,000 in PBST were added to the plates. The plates were incubated for 2 h at room temperature, rinsed with PBST, and tested for HRP activity by the addition of 100 μL of substrate solution – 3,3′,5,5′-tetramethyl benzidine (TMB substrate chromogen) (Dako, Glostrup, Denmark). The reaction was stopped after 10 min by the addition of 50 μL of 4N sulfuric acid, and the absorbance was measured with a Labsystems Multiscan Multisoft ELISA reader at 450 nm. LNG content in unknown samples was determined from an LNG calibration curve after linearization of the data by transformation to a logit–log plot by means of Microcal Origin software, Version 6.0 (Microcal Software, Northampton, MA, USA). Each sample was tested in duplicate at five dilutions.

Cross reactivity of the Abs with steroid hormones was determined by adding those compounds (instead of LNG) at 12 serial dilutions, ranging from 10 to 0.0049 ng per 50 μL, except in the case of EE2 and NT, for which dilutions ranged from 100 to 0.049 ng per 50 μL, and testing their ability to compete with the LNG–OVA conjugate coating for binding the anti-LNG antiserum. Tolerance of the Abs to various organic solvents was determined similarly except that the primary Ab was added in a buffer containing the tested solvent (10% methanol or ethanol in PBST).

2.1.6. EE2 competitive ELISA

EE2 ELISA was carried out in a Nunc F96 Maxisorp 96-well plate (Nunc Immuno Plate, Roskilde, Denmark) coated with 100 μL of 0.3 μg avidin (Sigma) dissolved in 0.5 M carbonate buffer, pH 9.6. After an ON incubation at 4 °C, the wells were washed three times with PBST and 100 μL of biotin–EE2 conjugate (Bronshtein et al., in preparation) diluted 1:40,000 in PBS were added to the wells. After a 2-h incubation the wells were washed again with PBST, as described above (Section 2.1.5), and 50-μL aliquots of test (unknown) sample or standard were added to the wells, together with 50 μL of anti-EE2 antiserum, diluted 1:4000 in PBST containing 10% ethanol. The standards comprised 12 serial dilutions of EE2, ranging from 10 to 0.0049 ng per 50 μL. The plates were incubated ON at 4 °C and washed as above with PBST. All other steps were carried out as described above for the LNG ELISA (Section 2.1.5). Cross reactivity of the Abs with steroid hormones was determined by adding those compounds (instead of EE2) at 12 serial dilutions ranging from 100 to 0.049 ng per 50 μL, and testing their ability to compete with the immobilized EE2–biotin conjugate for binding the anti-EE2 antiserum.

2.1.7. NT competitive ELISA

NT ELISA was carried out in a similar manner to that described above (Section 2.1.5) for LNG ELISA except that OVA–NT conjugate (Bardugo et al., in preparation), diluted 1:30,000 in PBS was used instead of OVA–LNG conjugate, and anti-NT antisera diluted 1:40,000 was used instead of anti-LNG antisera. The standard curve was generated using 12 serial dilutions of NT ranging from 10 to 0.0049 ng per 50 μL. Cross reactivity of the Abs with steroid hormones was determined by adding those compounds (instead of NT) at 12 serial dilutions ranging from 100 to 0.049 ng per 50 μL, and testing their ability to compete with the immobilized NT–OVA conjugate in binding the anti-NT antiserum.

2.2. Sol–gel IAP

2.2.1. Sol–gel entrapment of anti-LNG antiserum

Entrapment involved a two-step procedure in which hydrolysis was followed by polymerization of tetramethylsilane (TMOS) (Aldrich) as previously described [25]. Briefly, an acidic silica solution was obtained by mixing TMOS with 2.5 mM HCl in DDW at a molar ratio of 1:8, in the presence of 10% polyethylene glycol (PEG-400) (Merck, Darmstadt, Germany), with average molecular weight of 400 g mol^{-1}, corresponding to approximately seven methylene units in the chain. The mixture was stirred for 1 min until a clear solution was obtained, and it was then sonicated for 30 min in a 285-W, 2.75-L Model T-460/H ultra-sonicating bath (ELMA, Singen-Hohentwiel, Germany), in a well-ventilated fume hood. Anti-LNG antiserum, in aliquots of 20–160 μL, were premixed with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, 99.99%) (Sigma) at pH 7.6, to a final volume of 0.5 mL, and added to 0.5 mL of the prehydrolyzed TMOS mixture. Gels in which no antiserum was entrapped (termed 'empty' herein) were prepared by mixing the hydrolyzed TMOS with 0.5 mL HEPES buffer, pH 7.6. The solution was mixed quickly for 5 s, and gelation occurred within 1–2 min. After 30 min, the gels (total volume of 1 mL) were washed with 2 mL of HEPES buffer at pH 7.6 and kept wet under 2 mL of HEPES, at 4 °C pending use. The gels exhibited high stability and could be used for over a month after preparation. In most cases gels were used 24 h post preparation.

2.2.2. Binding and elution of LNG from sol–gel IAP columns

Wet gels were thoroughly crushed and packed in 20-mL, 1.5 cm × 12 cm Chromatography Columns (Bio-Rad, Hercules, CA, USA). The sol–gel columns were washed with 50 mL of 0.15 M NaCl in 10 mM sodium phosphate, pH 7.2 (loading buffer) prior to sample application. To ensure optimal binding, columns were kept under buffer at all times during the experiment. An amount of 100 or 1000 ng of LNG standard was applied, in a volume of 1 mL of loading buffer, to 'empty' sol–gel columns or columns doped with anti-LNG antiserum. Unbound LNG was removed by washing the columns with 10 mL of DDW. Elution was performed with 10 mL of PESTI-S absolute ethanol (Bio-Lab, Jerusalem, Israel) unless otherwise indicated. The eluted fraction underwent vacuum evaporation to remove the eluting solvent, and was further applied on a solid-phase column as described below (Section 2.2.3). Binding experiments were performed with pairs of sol–gel columns that comprised (A) an experimental column containing anti-LNG antiserum (total binding) and (B) an empty control column without antiserum (non-specific binding). Specific binding was defined as the difference between total and non-specific binding. All of the experiments in this study used a standard LNG compound prepared from a stock solution dissolved in ethanol at 1 mg mL^{-1}.

IAP of EE2 and NT was carried out similarly. The amount of entrapped Ab was 80 μL and the amount of analyte loaded on the column was 100 ng. Elution was carried out with 10 mL of ethanol.

2.2.3. Solid-phase extraction (SPE): sample application and elution

Oasis SPE columns (Waters, Milford, MA, USA) were preconditioned by two consecutive washes with 10 mL of PESTI-S absolute ethanol (Bio-Lab, Jerusalem, Israel), followed by 10 mL of 10% ethanol in DDW. Sol–gel-eluted samples were reconstituted in 1 mL of 10% ethanol in DDW. Samples were loaded on the columns, which were then washed with 10 mL of 10% ethanol in DDW. Elution was carried out with 1 mL of absolute ethanol. The samples underwent vacuum evaporation and were then dissolved in 1 mL of PBS. In order to eliminate any residual Abs activity that might have been present in the eluate because of leakage from the sol–gel column, and that might have interfered with the ELISA, samples were pretreated for 10 min at 100 °C prior to analysis. The LNG, EE2 or NT content was determined by competitive ELISA as described above (Sections 2.1.5–2.1.7), over a range of 10–0.0049 ng per 50 μL.

2.3. Chemical analytical methods

2.3.1. LC–MS–MS analysis

A high performance liquid chromatography method with tandem mass spectrometry (LC–MS/MS) was developed in order to measure LNG in sol–gel IAP samples. Sol–gel columns were doped with 80 μL of anti-LNG antiserum and were loaded with 50 ng of LNG. The analyte was eluted with ethanol and passed through an SPE column as described above (Sections 2.2.2 and 2.2.3). These sol–gel/SPE eluates are termed 'experimental eluates'. Sol–gel columns without analyte were processed similarly, and their eluates served either to determine the degree of interference with the LC-MS/MS analysis (termed 'control IAP/SPE eluates') or – following spiking with an LNG standard (termed 'spiked eluates') – to generate a calibration curve. All samples underwent vacuum evaporation. 'Experimental eluates' were reconstituted, prior to analysis, with 150 μL of diluent alone – comprising 30% acetonitrile (ACN) (J.T. Baker, Phillipsburg, NJ), made up in HPLC grade DDW purified with the MilliQ system. 'Control IAP/SPE' eluates were reconstituted with 150 μL of diluent alone and were used to determine the degree of interference of the sol–gel/SPE eluate with the analysis, and 'spiked eluates' were spiked with LNG standard at concentrations ranging from 1 to 100 ng mL^{-1}, made up from a stock solution of LNG in methanol at 100 μg mL^{-1} (J.T. Baker, Phillipsburg, NJ), and were used to generate a calibration curve. Samples were analyzed by LC–MS/MS multiple reaction monitor (MRM) detection in the positive-ion mode, after separation on a reverse-phase C-18 column, attached to a model 2795 HT Waters Alliance HPLC system. The liquid chromatographic separation was carried out on a Phenomenex Gemini C-18 column (50 mm × 2.0 mm, 3 μm particle size, 110 Å pore size); injected volume was 10 μL. Solvent A comprised 10% aqueous ACN containing 0.1% ammonium hydroxide (J.T. Baker, Phillipsburg, NJ); Solvent B comprised 90% aqueous ACN containing 0.1% ammonium hydroxide. For the analysis the solvent initially comprised 65% A and 35% B; after 0.5 min, the solvent was modified over 5 min, according to the Waters linear program, to 100% B; the flow rate was 0.3 mL min^{-1}; the LNG retention time, t_R was 3.3 min. Following the LC analysis, all samples were analyzed with a Micromass Quattro Pt triple-quadrupole mass spectrometer using the electrospray ionization mode. The data were processed with Masslynx v.4.0 and Quantlynx v.4.0 software. The amount of LNG in the samples was determined by comparison with calibration curves based on the spiked samples, and constructed by plotting the concentrations in the spiked samples against the peak areas found in their chromatograms.

2.4. Statistics

Differences between the average values were subjected to Tukey–Kramer one-way ANOVA, at $p < 0.05$.

3. Results

3.1. Development of an LNG ELISA

The first goal of the present study was to develop a highly specific immunochemical assay for monitoring LNG by means of the ELISA method. The development of the LNG ELISA involved two sets of experiments. The first set was intended to determine the optimal concentration of the coating conjugate (LNG–OVA), antiserum and secondary Ab (checkerboard test). The second set

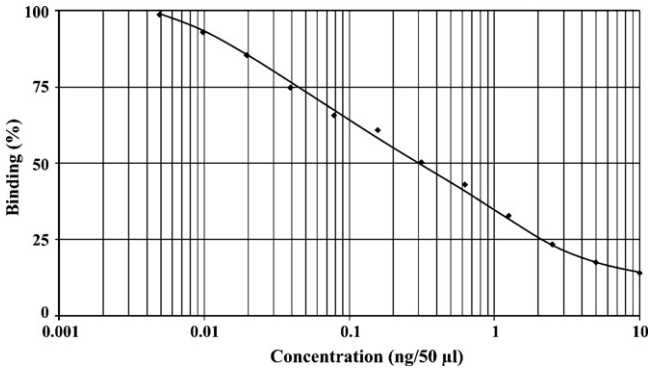

Fig. 2. Representative standard curve of LNG. LNG–OVA conjugate dilution 1:2000; anti-LNG antisera dilution 1:10,000.

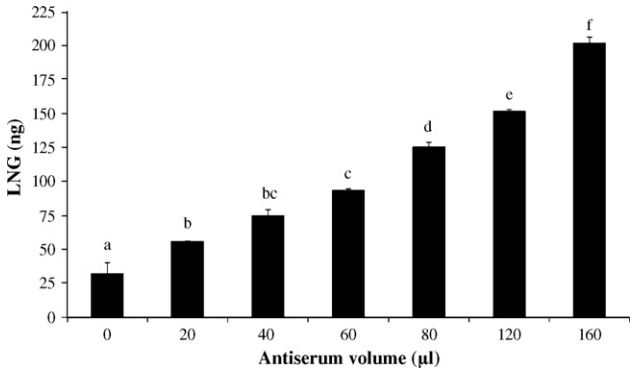

Fig. 3. Comparison of the binding capacity of various amounts of sol–gel-entrapped anti-LNG antiserum. The first column (0 μL) indicates non-specific binding in the absence of entrapped antiserum ('empty' column). Binding was implemented by using the indicated volumes of anti-LNG antiserum and 1000 ng of LNG. Amounts of eluted LNG were determined by ELISA. Each bar represents the mean ± S.E.M. of two experiments. Means with the same letter do not differ significantly at $p < 0.05$.

was intended to determine the I_{50} value and the limit of detection (LOD, I_{20}) of the assay, the tolerance of the Abs to organic solvents, and their cross reactivity with other steroid hormones. The first set of experiments revealed that a 1:2000 dilution of the LNG–OVA conjugate and a 1:10,000 dilution (final) of the anti-LNG antiserum resulted in high binding and a low background (i.e., of non-specific binding). The second set of experiments yielded an I_{50} value of $3.34 \pm 1.8\,ng\,mL^{-1}$ and an I_{20} value of $0.6 \pm 0.4\,ng\,mL^{-1}$ ($n = 20$) (Fig. 2). The experiments also revealed that methanol or ethanol up to final concentrations of 10% were tolerated by the Abs and did not modify the I_{50} and I_{20} values. The values obtained for I_{50} were 1.2, 1.6 and $1.6\,ng\,mL^{-1}$, and those for I_{20} were 0.10, 0.10 and $0.12\,ng\,mL^{-1}$, for PBST, PBST containing 10% methanol, and PBST containing 10% ethanol, respectively. No changes in the maximal absorbance or background signal were observed in the presence of the organic solvent. Analysis of the cross reactivity of the Abs with a variety of steroid hormones revealed no cross reactivity with aldosterone, diethylstilbestrol, estradiol, EE2, hydrocortisone, lynestrol, medroxyprogesterone, MPA, norethisterone, NT, progenolone, progesterone and testosterone. Low cross reactivity (20%) was observed with a racemic mixture of norgestrel (Table 1). Once the ELISA was established and the Abs characterized we set out to develop an IAP method for LNG.

Table 1
Cross reactivity of anti-LNG antiserum with various steroid hormones.

Hormone name	Cross reactivity (%)
Levonorgestrel	100
Aldosterone	0
Diethylstilbestrol	0
Estradiol	0
Ethynylestradiol*	0
Hydrocortisone	0
Lynestrol	0
Medroxyprogesterone	0
Medroxyprogesterone acetate	0
Medroxyprogesterone acetate	0
Norgestrel (+/−)	20
Nortestosterone*	0
Pregnenolone	0
Progesterone	0
Testosterone	0

Cross reactivity represents the ratio (expressed as a percentage) between the concentration of LNG that causes a 50% decrease in the binding of the Ab to the coating antigen absorbed onto the microplate (defined as 100%) and the concentration of the tested compound causing the same inhibition. Cross reactivity of steroids marked with an asterisk (*) was determined over the range, 100–0.049 ng per 50 μL. All other compounds were tested over the range of 10–0.0049 ng per 50 μL. (+/−) represents a racemic mixture of both L and R norgestrel.

3.2. Ab entrapment in sol–gel and LNG binding

A preliminary requirement in the development of an IAP method is determination of optimal conditions for Ab immobilization, including assessment of optimal binding conditions of the analyte. Our previous experience with a variety of sol–gel entrapped Abs [25–27], which included several anti-steroid hormone Abs such as anti-EE2 polyclonal Abs (Bronshtein et al., in preparation), showed that the best sol–gel format for Ab entrapment was a wet gel prepared by the two-step procedure with a TMOS:water ratio of 1:8, containing 10% PEG. In light of these results, we chose the same procedure for entrapment of anti-LNG polyclonal Abs. The first set of experiments in this part of the study was intended to determine the binding of a constant 1000 ng of LNG to various amounts of entrapped anti-LNG Abs. As can be seen in Fig. 3, the entrapment of increasing amounts of antibodies in the sol–gel matrix resulted in increased column capacity: LNG binding capacity ranged from a minimum of 56 ng at 20 μL of Abs to a maximum of 206 ng at 160 μL. The non-specific binding (which may result either from a non-specific adsorption of LNG to the monolith or from its entrapment in the sol–gel pores) was low (32 ng see 'empty' bar, 0 μL of Ab).

Experiments carried out with larger – 2 mL – columns in which 80 μL of Abs were entrapped showed an LNG binding capacity of 115 ± 1 ng, indicating that increasing the column volume without changing the volume of entrapped Ab did not affect the column capacity, because of the higher non-specific binding to the matrix, i.e., 54.6 ± 5.63 and 32 ± 8.29 ng for the 2- and 1-mL columns, respectively (data not shown). Similar results were obtained when 120 and 160 μL of Ab were entrapped in a 2-mL column. In light of the above results, we chose to use 80 μL of antiserum in 1-mL sol–gel columns in all subsequent experiments.

3.3. Conditions for elution of LNG from sol–gel IAP columns

It is well known that the molecular recognition of antigens by Abs is based on strong ionic interactions; therefore, elution of an analyte from an IAP column requires dissociation of these interactions. Organic solvents such as ethanol, methanol or acetonitrile have been shown to have good eluting properties [25–27]. Attempts to elute LNG from anti-LNG Abs-doped columns with ethanol revealed that the solvent was very efficient and that 6–7 mL of absolute ethanol could easily elute 1000 ng of LNG (data not shown). Although ethanol had been shown to be an effective elution agent, other, milder buffers were also evaluated for this function,

Table 2
Cross reactivity of anti-LNG, anti-EE2 and anti-NT antiserums with steroid hormones in solutions and in sol gel.

Antiserum	Antigen	Analyte recovery (ng)	Cross reactivity (%)	
		Sol–gel	Sol–gel	ELISA
Anti-LNG	LNG	93 ± 22	100	100
	EE2	44 ± 8	47	<0.3
Anti-EE2	EE2	114 ± 13	100	100
	LNG	71 ± 20	62	18
	NT	20 ± 1	17	0.3
Anti-NT	NT	72 ± 3	100	100
	EE2	15 ± 1	21	<0.2

Amounts of analyte eluted from the sol–gel columns were determined by the respective ELISAs over the range of 10–0.0049 ng per 50 μL. Cross reactivity values in ELISA represent the ratio (expressed as a percentage) between the concentration of a given steroid hormone (LNG, EE2 or NT) that caused a 50% decrease in the binding of its Ab to the coating antigen absorbed onto the microplate (defined as 100%) and the concentration of any other tested compound that caused the same inhibition. Cross reactivity in ELISA was determined over the range, 100–0.049 ng per 50 μL. Cross reactivity in sol–gel columns was calculated as the ratio (as percentage) between the amount of a heterologous steroid hormone that bound to the entrapped Ab and the amount of the bound homologous hormone (defined as 100%).

in order to minimize the damage caused to the entrapped Ab by absolute ethanol and thereby to enable frequent repeated use of the IAP column. Typically, buffers of extreme high or low pH, as well as reagents of high ionic strength can be used for dissociation. Two different buffers – glycine at pH 2.5 and triethylamine at pH 11.5 – and a high-salt solution (3.5 M $MgCl_2$) were tested to compare their eluting efficiency with that of absolute ethanol. The data indicate that 10 mL of ethanol was much more efficient as an eluant than equivalent volumes of the other tested solutions (data not shown). In light of the above results we were able to develop a highly effective and reproducible assay for IAP and concentration of LNG with a recovery rate of 75–100%, i.e., recovery of 75–100 ng when 100 ng of LNG was loaded.

3.4. Cross reactivity

As indicated in Table 1 under the conditions of the ELISA experiments, the LNG antiserum did not cross react with any of the tested steroid compounds. We have, thus, set to test the cross reactivity of the sol–gel-entrapped Ab with LNG, EE2 and NT by using anti-LNG antiserum and also two other antisera – anti-EE2 and anti-NT – that were generated in our laboratory. Although all the antisera showed their highest reactivity with their homologous analogs, i.e., the hormones toward which they were generated, the entrapped Abs also cross-reacted with the other hormones unlike in the ELISA were cross reactivity was ≤0.3% (Table 2).

3.5. Chemical analytical analysis

Sol–gel/SPE eluates were also tested for their susceptibility to analysis directly by LC–MS/MS. Since the sol–gel columns contain PEG and entrapped antiserum that might leach from the column and interfere with the LC–MS/MS analysis, it was necessary to prove that eluates do not interfere with the chemical instrumental analysis. An eluate ('control eluate') from a sol–gel column doped with 80 μL of anti-LNG antiserum onto which loading buffer without LNG had been applied was subjected to LC–MS/MS, and an identical sample of 'control eluate' was passed through an SPE (designated 'control IAP/SPE eluate') after passing through the sol–gel IAP and was subjected to LC–MS/MS. Analysis of the sol–gel 'control eluate' revealed interference with the method (data not shown), but introduction of an SPE step after the sol–gel IAP purification step ('control IAP/SPE eluate'), gave good results and revealed a small, insignificant peak (Fig. 4A) at the t_R of LNG (3.31 min), indicating that the eluate itself did not interfere with the LC–MS/MS analysis. The small peak, which may have resulted from a bleed over a previous LC–MS–MS run, had an area less than 2% of that of the 'spiked eluate' and was below the limit of detection (Fig. 4B, see below). In view of these results all eluates underwent a combined sol–gel IAP/SPE treatment prior to LC–MS/MS analysis.

The identity of the eluted LNG was also verified by means of the LC–MS/MS method: 50- and 100-ng samples of LNG were subjected to IAP on a sol–gel column doped with 80 μL of anti-LNG antiserum, and then to SPE, and the resulting 'experimental eluates' were tested by LC–MS/MS. Eluates of a control column, spiked with standard LNG, at 1, 5, 10, 50 and 100 ng mL^{-1} (designated 'spiked eluates'), served as a reference for the analysis.

The experimental eluate provided a single peak with a t_R of 3.32 min (Fig. 4C) identical to that of the LNG in the spiked eluate (t_R = 3.30 min; Fig. 4B), confirming the identity of the IAP analytes as LNG, and indicating that sol–gel/SPE samples can be analyzed directly by LC–MS/MS, without any further treatment.

4. Discussion

A polyclonal Ab for the progestin LNG was generated and was used for the development of immunochemical assays for the detection, clean-up and concentration of the steroid hormone LNG. The immunochemical assay focused on development of a microplate ELISA, whereas the clean-up and concentration assay was based on sol–gel-entrapped IAP.

Currently, environmental residues such as steroid hormones in general, and LNG in particular, are monitored mainly by instrumental chemical analysis methods such as GC–MS and LC–MS (see [9,10,28] and references therein). Although the detection limits of these methods are at sub-ppt levels, the need to carry out single or multiple derivation steps (GC–MS) prior to analysis and liquid- or solid-phase extraction and enrichment steps (GC–MS and LC–MS) limited their widespread employment for large-scale monitoring. In addition, chemical analytical methods are not cost effective, as they involve the use of large volumes of toxic solvents that are both expensive and require costly storage and disposal arrangements.

The massive use of synthetic steroid hormones for both human therapeutic and veterinary purposes, and the limitations of the current analytical detection methods have raised the need for simpler and more economical procedures for large-scale monitoring of steroid hormones in the environment and in food. Immunochemical monitoring assays such as the immunoassay described above have recently emerged as preferred approaches to replacement of the conventional instrumental analysis methods, because of their highly specific recognition properties, their adaptability to high-throughput screening both in the laboratory and on site, and their ability to overcome some of the limitations mentioned above. For those reasons these methods have attracted the interest of many researchers, leading to accelerated development of such assays for detection of a variety of steroid hormones.

Recent decades have seen the development of a variety of ELISAs for steroid hormones and other drugs, especially for detection of EE2 (for example [29,30] and references therein), anti-inflammatory compounds in waste water and a variety of humic substances [21,31,32], MPA in human serum [33,34], and NT in athletes' urine samples [35] – to mention just a few.

Several immunoassays have been developed for detection of LNG: two radioimmunoassays (RIAs) and two ELISAs. All Abs were raised against 3′-CMO-derivatives of LNG. Watson and Stewart [36] developed an RIA with a sensitivity level of 47 pg mL^{-1} for LNG, and Li and Nieuweboer [37] reported on the development of a highly sensitive RIA with a sensitivity of 10 pg mL^{-1}. Both of these

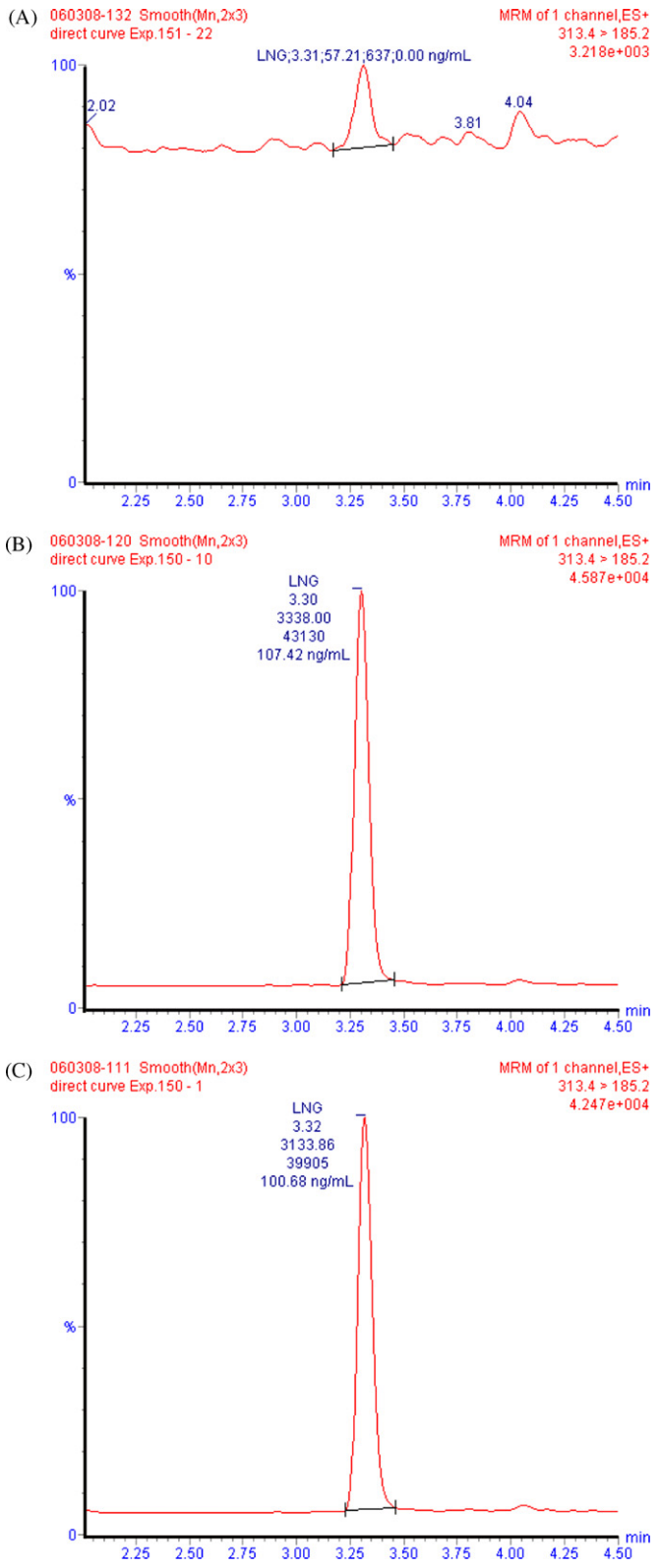

Fig. 4. LC analysis of sol–gel/SPE eluates. Sol–gel columns were doped with 80 μL of anti-LNG antiserum, applied with 50 ng of LNG or with loading buffer, and, after an additional SPE step, the eluates were evaporated, reconstituted in 150 μL of either aqueous 30% acetonitrile (diluent) or with diluent spiked with 100 ng of LNG standard and tested by LC-MS/MS, as described in Section 2.3.1. (A) Chromatogram of a 'control IAP/SPE eluate' with no LNG; (B) chromatogram of a sol–gel/SPE eluate spiked with 100 ng of LNG standard ('spiked eluate'); (C) chromatogram of a sol–gel/SPE eluate containing IAP LNG ('experimental eluate').

Abs exhibited high specificity and showed a high degree of cross reactivity only with LNG metabolites. Both assays were used to determine LNG levels in plasma. In 1996 an ELISA was developed for determination of LNG levels in serum and urine; it used an HRP ELISA format in which the tested analyte competes with LNG-HRP in binding to an Ab adsorbed to the microplate wells [38]. The procedure and performance of this assay were very similar to those applied and achieved in our present study. The detection limit of the assay was very low (5 pg mL^{-1}) and the I_{50} was 0.15–0.19 ng mL^{-1}. Recently, another LNG ELISA was developed in which a 3′-CMO-derivative of LNG with a three-atom spacer arm was used as an immunogen [39]; it was used to monitor LNG in wastewater samples. The assay format was similar to the one described in the present paper, and it resulted in similar I_{50} and I_{20} values (0.9 and 0.07 ng mL^{-1} respectively) to those obtained in our assay when carried out in PBST (1.2 and 0.1 ng mL^{-1}, respectively). Although the sensitivity of our ELISA was not as high as that exhibited by that of Munro et al. [38], we believe that our format and that of Pu et al. [39], in which the coating antigen is adsorbed to the microplate, introduce an advantage especially for the analysis of crude environmental and food samples. In the HRP format the tested sample is added to the reaction mixture together with the HRP–antigen and, therefore, the enzymatic activity might be affected by the presence of interfering elements unrelated to the presence of the analyte, and this might be interpreted as a false positive signal. The assay format used in our present study eliminates this problem, because the sample is washed away prior to the addition of the secondary-HRP conjugated Ab.

Cross reactivity analysis revealed that our Abs were highly specific toward LNG and did not cross react with any of the tested compounds other than with the stereoisomer of LNG (+) (Table 1). The high selectivity of the antisera toward LNG might be due to the hapten:protein conjugate design used for antiserum generation: the carrier protein was conjugated to the LNG 3′ position, thus enabling the Abs to react with its 13′ and 17′ positions, which share a combination of epitopes that differ from those present on the other tested steroid hormones. The Abs described by Munro et al. [38] and by Pu et al. [39] exhibited cross reactivity with two other steroid hormones: norethindrone and EE2. For many years, cross reactivity with endogenous hormones or with synthetic steroid hormones imposed a serious limitation on the application of immunoassays for analysis of synthetic steroid hormones and their metabolites. However, in the case of our Abs, absence of cross reactivity with any of the steroid hormones overcomes this problem and enables the employment of these Abs in immunochemical analysis of LNG.

The need to monitor LNG in environmental and food samples, which in most cases are complex matrixes containing components that might interfere with the monitoring method, raises the need to develop purification methods that can be compatible with both the chemical and the immunochemical analyses. The small amount of such compounds in environmental samples necessitates concentration of the tested samples in order to reach or exceed the detection limits of the above methods. IAP is one of the most powerful techniques for single-step isolation, concentration and purification of individual compounds or of classes of compounds from liquid matrixes (for review see [16] and references therein). In the past few years we have developed an IAP technology, based on the entrapment of Abs in a ceramic SiO_2 matrix termed sol–gel, which facilitates efficient, single-step clean-up and concentration of analytes from large volumes of crude samples. The applicability of this approach to steroid hormones was tested, in this study, with the LNG Abs characterized above. By using the sol–gel technology we were able to develop a highly effective and reproducible assay for affinity purification and concentration of LNG. The entrapped Abs retained their ability to bind the antigen and were very stable

for several weeks at 4 °C. Our experiments indicate that increasing the amounts of Abs entrapped in the sol–gel matrix resulted in increases in column capacity. Increasing the column volume without changing the amount of entrapped Abs did not affect the column capacity, but increased the non-specific binding to the matrix. A binding capacity of 125 ng with low non-specific binding (up to 32 ng) was obtained with 80 μL of antiserum in a 1-mL sol–gel column (Fig. 3) when 1000 ng of LNG were applied to the column, and the most efficient elution solvent was found to be ethanol. The analytes eluted from the sol–gel columns that underwent an additional SPE step could be analyzed directly by means of LC–MS without any further extraction or purification steps.

The use of sol–gel-entrapped Abs for IAP purposes was reported over a decade ago by Altstein and co-workers [40], who entrapped anti-atrazine monoclonal Abs in a TMOS-based sol–gel matrix. That study revealed that the entrapped Abs retained their ability to bind analytes from solution in a dose-dependent manner, within a time frame that did not greatly differ from that in solution. That study was followed by entrapment of several other polyclonal and monoclonal Abs and purification of their respective analytes: dinitrobenzene [27], TNT [25], and pyrethroids [19]. Other laboratories applied the method to other analytes; a good example of Abs activity within the sol–gel matrix was reported by Wang et al. [41], who showed that fluorescent antigens were selectively bound to sol–gel-encapsulated Abs. Those studies were followed by many others in various laboratories, including ours, which explored the ability to encapsulate Abs within sol–gel matrixes. However, only a few compounds have been subjected to sol–gel-based IAP, and even fewer studies have been reported that addressed the use of sol–gel-based IAP for group-selective enrichment and recovery of analytes from real samples. A detailed review of the implementation of sol–gel-entrapped Abs for IAP was published by Altstein and Bronshtein [16]. Additional more recent examples address use of sol–gel-entrapped Abs for IAP of a variety of environmental pollutants such as diclofenac [21], isoproturon [20,23], atrazine, malathion [22], sulfonylurea [17], and diuron [24].

In addition to examining the ability of the Abs to bind LNG the present study also tested the cross reactivity of the sol–gel-entrapped polyclonal Abs. As indicated in Fig. 1 the three steroid hormones used in our study share many similar epitopes, so we expected to see a high cross reactivity with these compounds in both the ELISA and the sol gel. As indicated in Tables 1 and 2, the polyclonal Abs were highly selective toward their homologous antigens in ELISA, and this may have resulted from the design of the hormone:protein conjugate that was used for immunization. Cross reactivity in sol gel, however, was much higher and the Abs did cross react with these compounds in their entrapped format (Table 2).

Other studies also revealed that the cross reactivity of Abs immobilized in a sol–gel affinity column was significantly higher than in solution. Polyclonal Abs targeted against various environmental pollutants such as polycyclic aromatic hydrocarbons have shown marked differences between their cross reactivity pattern in ELISA and in sol gel [42–45]. The reasons for these marked differences are not clear at present. It is possible that the Abs undergo a conformational change during the encapsulation process, and that this might account for the modification in their binding properties, or that the excess of Abs used in the IAP method, in contrast with the practice in ELISA, in which all reagents are used in limiting amounts, might account for such differences. In light of the above results it is clear that ELISA data cannot be used for the quantitative prediction of the cross reactivity of a given Abs immobilized in affinity columns. Improvement of the selectivity of the entrapped Ab might be achieved: by modifications of the sol gel format, possibly through changes in the polymer; by changes in the polymerization conditions; or by addition or omission of additives, e.g., inclusion of detergents, changes in the monomer:water ratio in the course of polymerization, or use of different monomers. Use of monoclonal instead of polyclonal Abs might also lead to a solution in certain cases. Despite the above, it is important to note that high cross reactivity can be an advantage in certain cases, especially when it is desired to purify a group of compounds that share similar epitopes.

In conclusion, a highly specific Ab for LNG and a sensitive ELISA for the detection of LNG has been developed, and also a novel sol–gel-based IAP method for clean-up and concentration of synthetic steroid hormones based on the sol–gel-derived encapsulation process. Development of the IAP method, which is compatible with both instrumental chemical analytical methods and immunoassays (ELISA), adds an important tool to our environmental and clinical monitoring abilities, which so far is only in its infancy. Use of sol–gel techniques for IAP represents a relatively new field of research, and it is envisioned that the next decade will see much faster progress in applications that will lead to the emergence of new and improved devices that will be employed in environmental and clinical monitoring of steroid hormones.

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