DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY AND A BETA-1 ADRENERGIC RECEPTOR–BASED ASSAY FOR MONITORING THE DRUG ATENOLOL

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Abstract—Two approaches for monitoring atenolol (ATL) were applied: an immunochemical assay and a competitive-binding assay, based on the interaction between ATL and its target receptor, β1 adrenergic receptor (*β1AR*). Polyclonal antibodies (Abs) for ATL were generated, and a highly specific microplate immunochemical assay, that is, an enzyme-linked immunosorbent assay (ELISA), for its detection was developed. The ATL ELISA exhibited I50 and limit of detection (I20) values of 0.15 ± 0.048 and 0.032 ± 0.016 ng/ml, respectively, and the Abs did not cross-react with any of the tested beta-blocker drugs. Furthermore, a human *β1AR* (*h-β1AR*) was stably expressed in *Spodoptera frugiperda* cells (*Sf9*). The receptor was employed to develop a competitive-binding assay that monitored binding of ATL in the presence of isoproteranol by quantification of secondary messenger, cyclic adenosine monophosphate (cAMP), levels in the transfected cells. The assay showed that the recombinant *h-β1AR* was functional, could bind the agonistic ligand isoproterenol as well as the antagonist ATL, as indicated by a dose-dependent elevation of cAMP in the presence of isoproteranol, and decrease after ATL addition. The highly efficient and sensitive ELISA and the receptor assay represent two methods suitable for efficient and cost-effective large-scale, high-throughput monitoring of ATL in environmental, agricultural, and biological samples. Environ. Toxicol. Chem. 2013;32:585–593. © 2012 SETAC

Keywords—Atenolol   Beta-blockers   Atenolol   ELISA   Human β1 adrenergic receptor   Pharmaceutical product residues

INTRODUCTION

Studies in the past decade have shown that exposure to environmental chemicals influence human development and reproductive endpoints (for review see Crane et al. [1]). Most studies on adverse health effects of environmental contaminants have focused mainly on agricultural or industrial pesticides [2], heavy metals [3], and toxic industrial wastes and residues [2]. One large class of chemicals that has received little attention in this context 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 [1,4–8]. Despite the vast amount of information that has accumulated in the past few years on the occurrence of PPs in the environment, very little information is available on their environmental fate. Our understanding of the possible transmission of PPs into the food chain is also very limited, and even less is known of whether and how such contaminants, once ingested, affect human health.

In the past few years, within the EU-FP-6 project, Food and Fecundity, we have conducted 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 mechanisms of action, production and consumption volumes, persistence in the environment, and severity of identified adverse health effects in humans. In light of an extensive literature survey, a list of eight PPs with endocrine-disrupting potential has been selected [9], including a few synthetic steroid hormones such as levonorgestrel, ethinylestradiol, nortestosterone and medroxyprogesterone acetate, which are the main components of contraceptive drugs, and are also used as anabolic steroids, a representative nonsteroid anti-inflammatory drug, indomethacin, a representative selective serotonin reuptake inhibitor, fluoxetine, the antibiotic thrimethoprim, and a representative beta adrenergic blocker drug, atenolol (ATL). All of these compounds exhibit high stability in the environment, are used in large amounts, and most importantly, were reported to affect fecundity [9].

Atenolol (Fig. 1A), which is the focus of the present study, is a selective β1 adrenergic receptor (*β1AR*) antagonist, a drug belonging to the group of beta-blockers, a class of drugs used primarily in cardiovascular and blood vessel diseases [10,11]. Introduced in 1976, ATL was developed as a replacement for propranolol in the treatment of hypertension. Currently, more than a quarter of the world's adult population—approximately a billion people—suffer from hypertension [12]. The drug slows down the heart rate and reduces its workload. Unlike propranolol, ATL does not pass through the blood–brain barrier and thus avoids various central nervous system side effects, which makes ATL one of the most widely used beta-blockers worldwide. Unlike most other commonly used beta-blockers, ATL is excreted almost exclusively by the kidneys without undergoing metabolic breakdown and therefore is likely to be found in the environment. The wide use of the drug, its relative stability in the environment, and already proven indications of its influence on organisms in the aquatic environment [13–16] all raise the possibility that the drug poses a potential risk to human health via environmental contamination [11,17–20]. Very little information is available on the fate of ATL in the environment and its adverse effects on human health; therefore, large-scale monitoring of its occurrence in the environment is urgently needed.
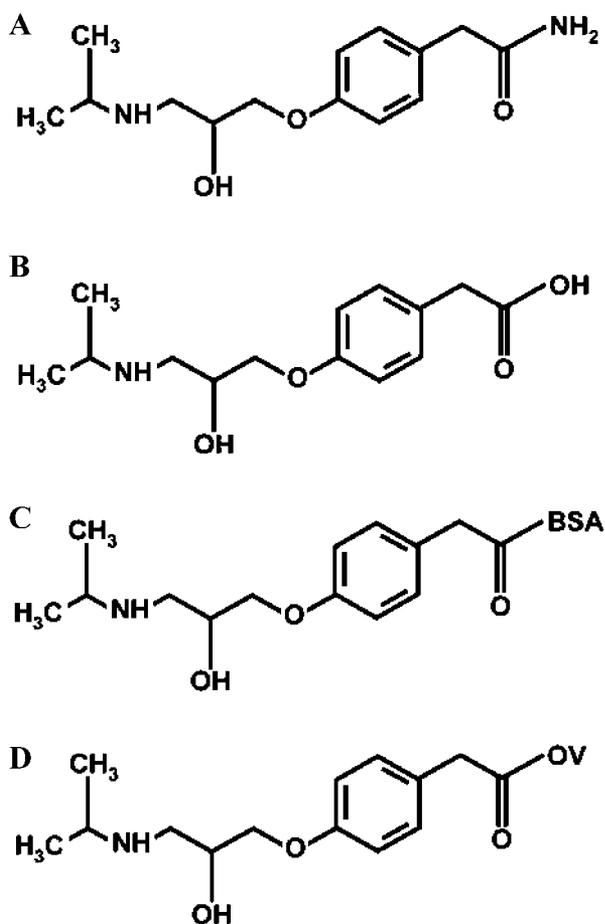
Fig. 1. Structures of atenolol (ATL) (**A**), ATL acid (**B**) and ATL-protein conjugates (**C–D**). ATL-bovine serum albumin (BSA) (**C**): ATL coupled to BSA for immunization; ATL-ovalbumin (OV) (**D**): ATL coupled to OV as a coating antigen.

At present the conventional means for monitoring PP environmental residues are based on chemical analysis by advanced mass spectrometry (MS) combined with either gas chromatography (GC) or liquid chromatography (LC) [6,7,21–23]. Unfortunately, although they are sensitive, precise, and reproducible, these methods are not cost-effective; they involve the use of large volumes of toxic solvents for sample extraction and cleanup, which are expensive in themselves and also require costly storage and disposal arrangements. Furthermore, long and complicated concentration and cleanup procedures must be applied to the tested samples before such analyses can be carried out. In light of the limitations of current analytical detection methods, development of simpler and more economical methods for large-scale monitoring of ATL in environmental and food samples is needed.

Indeed, immunochemical monitoring assays such as the microplate enzyme linked immunosorbent assay (ELISA) have recently emerged as preferred approaches, to replace the conventional instrumental analysis methods. These newer methods are highly specific, can easily be adapted to high-throughput screening both in the laboratory and on-site, are simple and cost-effective, and overcome some of the limitations associated with the chemical analytical methods mentioned. Immunochemical methods have therefore attracted the interest of many researchers in the past two decades, which has led to accelerated development of such assays for detection of a variety of environmental contaminants.

In previous studies, we developed ELISAs and immunoaffinity purification methods for some PPs: steroid hormones, levonorgestrel, ethinylestradiol and nortestosterone [24], medroxyprogesterone acetate [25], and the nonsteroid anti-inflammatory drug indomethacin [26]. In the present study, we used two different approaches for ATL monitoring: an immunochemical approach based on ELISA, and an innovative diagnostic environmental assay based on a receptor-binding assay that can monitor the interaction between ATL and its target receptor $\beta 1AR$. The immunochemical part of the study addressed generation of a polyclonal Ab for the drug ATL, and its employment in development of a sensitive ELISA for detection of ATL. The second part addressed expression of a recombinant human β1-adrenergic receptor (*h-β1AR*) in cells of *Spodoptera frugiperda* (*Sf9*), and its employment in development of a receptor assay for detection of ATL, based on monitoring the level of the secondary messenger cyclic adenosine monophosphate (cAMP). This approach is novel in that although receptor-binding assays are being widely used, to the best of our knowledge, they have not been implemented for residue monitoring. Both assays form a basis for determination of ALT in biological samples to monitor their pharmacokinetic properties; they could be further developed into a high-throughput screening assay and used to study population exposure to ATL, and also to monitor the occurrence of ATL contamination in food, soil, and other environmental samples.

MATERIALS AND METHODS

Immunochemical methods

Antiserum. Polyclonal anti-ATL antiserum was generated in rabbits by using a 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; Sigma) derivate of ATL conjugated to bovine serum albumin (BSA; Sigma) as an immunogen, as described in the following sections.

Preparation of ATL-acid. This step involved generation of a 3′ carboxylic derivative of ATL (Fig. 1B) by acid hydrolysis that replaced the amide group with a carboxylic group. Briefly, 100 mg ATL (Dr. Ehrenstorfer) were dissolved in 5 ml of 10% (v/v) HCl (Sigma), and the reaction was allowed to proceed for 4 h at 100°C. The mixture was then titrated to a final pH of 3 with 30% (v/v) NaOH, and the product and the nonhydrolyzed ATL were analyzed by MS (IMI TAMI Institute for Research and Development). The results showed full conversion of ATL to ATL-acid. The final concentration of the sample was 20 mg/ml. The compound was stored at 4°C pending further use.

Preparation of ATL-BSA conjugates for immunization. The hapten–protein conjugate for immunization was prepared by conjugating ATL-acid (previous subsection) to BSA by using the carboxyl and amine-reactive zero-length cross-linker EDC. EDC first reacts with a carboxyl group to form an amine-reactive ATL-acylisourea intermediate that quickly reacts with an amino group to form an amide bond and release an isourea byproduct. The reaction was carried out as follows: ATL-acid (250 μl, 5 mg/ml) was evaporated in a vacuum evaporator (SVC100H; Savant) to remove the HCl from the solution, after which it was redissolved in 1,250 μl double-distilled water (DDW). Immediately before conjugation, 5 mg EDC was dissolved in 500 μl DDW, and 361 μl of this solution (i.e., 3.61 mg of EDC) was added to the 1,250 μl ATL-acid solution (which contained 5 mg ATL-acid; molar ratios 1:1 ATL-acid:EDC), together with 250 μl 1 M morpholinoethane sulfonic acid (Sigma), pH 6.8. The reaction was allowed to proceed at room temperature for 30 min, after which 250 μl BSA (i.e., 5 mg in

DDW; molar ratio, 334:1 ATL-acid:BSA) was added slowly, and the reaction was allowed to proceed for 2 h at room temperature. The final volume was adjusted to 5 ml by adding DDW, and the conjugate (Fig. 1C), at a concentration of 1 mg/ml, was kept in 0.5-ml aliquots at $-4°C$ pending use.

Before immunization of rabbits, 0.5 ml conjugate was mixed with complete Freund's adjuvant (first injection) or with incomplete adjuvant (second through fourth injections). Two rabbits were injected at each time point. Bleeds were collected after each boost and were tested for activity with checkerboard experiments. The third and fourth bleeds were equally active toward the antigen, and the fourth bleed was used for ELISA.

Preparation of ATL-ovalbumin coating antigen. The method was similar to that described for preparation of the ATL-BSA conjugate. The ATL-acid (50 μl containing 1 mg) was evaporated under vacuum as described, and redissolved in 1,000 μl DDW. Immediately before conjugation, 5 mg EDC dissolved in 1,000 μl DDW was added at a volume of 200 μl (containing 1 mg EDC) to 120 μl (0.12 mg) of the ATL-acid solution (molar ratio of 10:1, ATL-acid:EDC), together with 250 μl 1M MES, pH 3.5, and 200 μl ovalbumin (OV) in DDW (2 mg in DDW; molar ratio of 10:1 ATL-acid:OV), which were added by slow pipetting. The reaction was allowed to proceed for 2 h at room temperature, and the unbound hapten and other small-molecular-weight components were separated from the protein-hapten conjugate (Fig. 1D) by size exclusion with a Centricon 30 centrifugal filter (Amicon, Millipore). The reaction mixture was centrifuged for 25 min at 4,000 g at room temperature, and then washed twice with 750 μl 0.13 M NaHCO$_3$, pH 9.2. The volume was adjusted to 750 μl by addition of DDW, and the conjugate was stored in aliquots at $-80°C$, pending use.

ATL competitive ELISA. The assay that was developed was an indirect competitive ELISA, in which ATL in solution competed with an antigen–protein conjugate (ATL-OV) immobilized on a 96-well microtiter plate (Nunc Immuno Plate, F96; Maxisorb, Roskilde, Denmark), for binding to anti-ATL antiserum. The plate wells were coated with 100 μl ATL-OV conjugate, diluted 1:5,000 in 0.5 M carbonate buffer, pH 9.6. After an overnight (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); 50 μl ATL standard in PBST were added to the wells together with 50 μl anti-ATL antiserum diluted 1:500 in PBST. The standard samples comprised 12 serial dilutions of ATL, ranging from 1 to 0.0005 ng/50 μl. The plates were incubated ON at 4°C, washed as described with PBST, and 100 μl of secondary Ab conjugated to horseradish peroxidase (anti-rabbit horseradish peroxidase conjugated, Sigma), diluted 1:30,000 in PBST was added to the plates. The plates were then incubated for 2 h at room temperature, rinsed three times with PBST, and tested for horseradish peroxidase activity by addition of 100 μl substrate solution: 3,3',5,5' tetramethyl benzidine (substrate chromogen; Dako). The reaction was stopped after 15 min by addition of 50 μl of 4 N sulfuric acid, and the absorbance was measured with a Labsystems Multiskan Multisoft ELISA reader at 450 nm. Each sample was tested in duplicate at five dilutions.

Cross-reactivity and tolerance to organic solvents. Cross-reactivity (CR) of the Abs with other beta-blocker drugs (propranolol, bisoprolol, acebutolol, celiprolol, betaxolol, pindolol) was determined by adding these compounds (instead of ATL) at 12 serial dilutions, ranging from 1 to 0.0005 ng/50 μl, and testing their ability to compete with the ATL-OV coating

conjugate for binding the anti-ATL antiserum. Tolerance of the Abs to various organic solvents was determined as described under ATL competitive ELISA, except that the ATL was added in a buffer containing the tested solvent (20% methanol, or ethanol in PBS) instead of PBST, and the Ab was added in PBS-2xT (PBS containing 0.2% Tween-20).

Molecular methods

Cells. Spodoptera frugiperda pupal ovarian insect cell line (*Sf9*) (2×10^6 cells, unless otherwise indicated) were cultured at 27°C in 4 ml complete growth medium, comprising Grace's medium (Biological Industries, Beit-haEmek, Israel), supplemented with 10% heat-inactivated fetal bovine serum, 1% lactalbumin, 1% yeastolate, 0.5% penicillin G, and 0.5% (v/v) streptomycin (all supplied by Biological Industries), in a 25-cm^2 flask (Corning) for 3 d to achieve approximately 4×10^6 cells. The cells were harvested for treatments or taken for regrowth cycles.

Vector propagation and characterization. The plasmid pIZ/V5His was purchased from Invitrogen. The vector construct pIZ/V5His-*β1AR* (from human placenta) was provided by Dr. S. Gutkind, National Institutes of Dental and Craniofacial Research. The *β1AR* gene was inserted into *EcoRI* and *XbaI* restriction sites of the pIZ/V5His plasmid to generate the pIZ/V5His-*β1AR* vector.

The vector was propagated in competent *Escherichia coli* bacteria (Bio-Lab, Jerusalem, Israel). Briefly, bacteria were thawed on ice, and a mixture of 100 μl DH5α competent *E. coli* bacteria and 1 μl (100 ng) pIZ/V5His-*β1AR* plasmid DNA were incubated in ice for 20 min, and then for 45 s at 42°C. The mixture was transferred to an ice bath for 2 min and added to 950 μl Lysogeny broth (LB) medium that comprised 1% Bacto tryptone, 0.5% Bacto yeast extract (both from Pronadisa), and 0.2% NaCl, pH 7.5; all percentages w/v. The transformed cells were incubated at 37°C for 1 h with agitation at 250 rpm. One hundred microliters transformed bacteria was plated on zeocin (Invitrogen) selective plates (LB medium, 1.1% [w/v] bacterial agar, containing 3 mg zeocin), which were incubated at 37°C for 24 h pending colony growth, and then stored at 4°C. One isolated colony transformed with pIZ/V5His-*β1AR*, and one control empty pIZ/V5His colony were picked and transferred to 3 ml LB medium containing 90 μg zeocin (starter cultures) and incubated at 37°C for 12 h, with agitation at 250 rpm. The cultures were centrifuged at 12,470 g for 1 min, and the plasmids were purified with the commercial DNA-Spin Plasmid DNA purification kit (Intron, Sangdaewon-Dong, Korea) according to the manufacturer's protocol. To prove that the vector contained the required *β1AR* DNA, the *β1AR* DNA was excised from the pIZ/V5His-*β1AR* vector through double digestion with *XbaI* and *EcoRI*. The reaction mixtures (15 μl) consisted of 1× Tango Buffer (Fermentas), 10 U *XbaI* (Fermentas), 10 U *EcoRI* (Fermentas), and approximately 0.4 to 0.5 μg plasmid, and was incubated at 37°C for 1 h with agitation at 250 rpm. The restriction reaction products were characterized by electrophoresis on 1% agarose horizontal slab gel. In addition, the circular construct pIZ/V5His-*β1AR* was sequenced to determine the *β1AR* insert (Hay-Lab). The data revealed 98% sequence homology of the *β1AR* insert with that reported in the literature for human placental *β1AR*.

Stable transfection. The pIZ/V5His-*β1AR* and the control empty vector pIZ/V5His (propagated and purified as described in the previous section) were transfected into the *Sf9* cells with the FuGENE HD transfection reagent (Roche) according to the manufacturer's protocol. Briefly, 0.5×10^6 cells were plated on

1 ml complete growth medium in a 12-well plate (Biofil, Mandhya Pradesh, India) and grown ON at 27°C up to 80% confluency at the time of transfection. The growth medium was replaced with fresh medium, and cells were transfected with 103.2 μl transfection mixture that contained 8 μl DNA (pIZ/V5His-*β1AR* or an empty vector at a concentration of 200 ng/μl made up in DDW) and 92 μl DDW, to which 3.2 μl FuGENE HD transfection reagent was added by slow pipetting. The transfection reagent was vigorously tapped for 5 s and then pre-incubated for 15 min at room temperature before being applied to the cells. The transfection complex was added to the cells (two wells for each vector) below the surface of the medium and swirled to ensure distribution over the entire well surface. Selection of transfected cells was initiated 48 h post-transfection, with zeocin (made up in complete growth medium) at 1,000 μg/ml, and lasted for three weeks, with the growth medium changed every 3 d. Three weeks later, the medium was replaced with regular growth medium, and the cells were grown without zeocin as described under "*Cells*".

Immunocytochemical staining. *S. frugiperda*–transfected pIZ/V5His-*β1AR* cells (at 0.5×10^6 cells/well) were plated on a sterile coverslip (diameter: 18 mm, thickness: 0.13–0.16 mm) (Menzel Glaser, Braunschweig, Germany) placed in a well of a 12-well plate containing 1 ml complete growth medium, and were grown overnight at 27°C. Untransfected *Sf9* cells were plated and grown under the same conditions to serve as a negative control. The growth medium was then removed by aspiration, and the cells on the coverslip were fixed with 0.5 ml 4% (w/v) paraformaldehyde (Sigma) in PBS, pH 7.2, washed twice for 10 min with 0.5 ml PBS, pH 7.2, and incubated with 0.5 ml 0.1 M glycine in PBS, pH 7.2, for 20 min. The cells were washed again with 0.5 ml PBS, pH 7.2, for 5 min and incubated: either with 0.5 ml immunoglobulin G (IgG diluted 1:500 or 1:100 in PBS, pH 7.2, to a final concentration of 0.4 or 2 μg/ml, respectively) generated against the C terminal of *h-β1AR* (Santa Cruz Biotechnology); or with 0.5 ml IgG obtained from normal rabbit serum (Sigma) diluted 1:500 or 1:100 in PBS, pH 7.2, to the same IgG content (0.4 or 2 μg/ml, respectively). Staining with normal rabbit serum IgG provided a control to determine nonspecific binding. Cells were incubated at room temperature for 1 h, washed three times with 0.5 ml PBS, pH 7.2 (5 min each time), incubated for 1 h at room temperature with 0.5 ml blocking solution (5% [w/v] normal goat serum) (Sigma), diluted in PBS and washed again three times with 0.5 ml PBS, pH 7.2, for 20 min each time. From this step onward, the plates were kept under low-light conditions, and the cells were incubated with 0.5 ml Alexa-conjugated goat anti-rabbit secondary Ab (diluted 1:500 in PBS, pH 7.2; Molecular probes; Invitrogen) for 1 h at room temperature. The cells were then washed four times with 0.5 ml PBS, pH 7.2, for 5 min each time, and then incubated for 1 h at room temperature with 1 ml propidium iodide (Bio Chemika) diluted 1:300 in PBS, pH 7.2. After incubation the cells were washed twice for 5 min in 0.5 ml PBS, pH 7.2, the coverslips were removed from the wells, gently dried, and attached to a glass slide with the cells facing the slide, by means of 25 μl Elvanol (comprising 1 g Mowiol 4–88, 4 ml 0.015 M phosphate buffer, and 2 ml glycerol; Sigma). The cells on the glass slides were kept ON in darkness at –20°C and analyzed under an inverted laser scanning confocal microscope (Fluoview 500 IX 81; Olympus) at an excitation wavelength of 488 nm and emission wavelength of 505 to 525 nm (for Alexa); or 543 nm and 610 to 660 nm, excitation and emission, respectively (for propidium iodide). Integrated intensities were analyzed with multi-image analysis software (Cytoview).

Determination of ATL binding by a competitive assay based on a quantitative determination of the secondary messenger cAMP. *Sf9* cells (at 0.5×10^6 cells/well) expressing the *β1AR* were grown ON at 27°C in 12-well plates containing 1 ml complete growth medium. The growth medium was removed by gentle pipetting and the cells were washed for 5 min with PBS, pH 7.4, and then for 5 min with reaction buffer (PBS, pH 7.4 containing 12.5 mM MgCl$_2$, 2 mM ethylenediaminetetraacetic acid, and 0.1% BSA). The reaction buffer was removed by gentle pipetting, and 0.25 ml of the antagonist ATL at a range of concentrations (0, 10, 30, 100, and 300 μM made up in reaction buffer) was added to the wells. The cells were incubated for 10 min at 27°C, followed by addition of 0.25 ml (−)Isoproterenol (Fluka) at 0.1 or 0.3 μM. Control wells contained 0.25 ml reaction buffer instead of (−)isoproterenol, and 55 μl 3-isobutyl-1-methylxanthine (IBMX) made up in DDW was added, to a final concentration of 0.5 mM. The cells were incubated for 10 min at 27°C, washed carefully with 0.5 ml PBS, pH 7.4, and lyzed with 110 μL 0.1 M HCl containing 0.5 mM IBMX. The plates were incubated for 20 min with shaking, the cells were scraped with a Drigalski spatula (Corning), the cell lysate was transferred to Eppendorf test tubes, and the lysates were centrifuged at 1,000 g for 10 min at room temperature. The supernatants were collected and used for quantitation of cAMP by means of a cAMP enzyme immunoassay kit (Cayman Chemicals) that monitored cAMP levels. The reaction was carried out according to the manufacturer's protocol. Briefly, the supernatant samples were acetylated in accordance with the manufacturer's protocol, diluted 1:100 with the ELISA reaction buffer supplied with the kit, and tested for cAMP content. Protein contents of each sample were determined with the Bradford reagent (Sigma) according to the manufacturer's protocol. Most samples contained protein at 0.4 to 0.5 mg/ml.

Statistics. Differences between the average values were subjected to Tukey-Kramer one-way analysis of variance, at $p < 0.05$.

RESULTS

Development of an ATL ELISA

The first goal of the present study was to develop a highly specific immunochemical assay for monitoring ATL by means of an ELISA method. The development of the ATL ELISA involved two sets of experiments. The first set was intended to determine the optimal concentrations of the coating conjugate (ATL-OV), antiserum, and secondary Ab (checkerboard test). The second set was intended to determine the I50 value and the limit of detection (I20) of the assay, the tolerance of the Abs to organic solvents, and their CR with other beta-blocker drugs. The first set of experiments revealed that a 1:5,000 dilution of the ATL-OV conjugate and a 1:1,000 dilution (final) of the anti-ATL antiserum resulted in high binding and a low background, namely, nonspecific binding. The second set of experiments revealed that the Abs could indeed recognize ATL. This set of experiments yielded an I50 value of 0.15 ± 0.048 ng/ml and a limit of detection (I20 value) of 0.032 ± 0.016 ng/ml ($n = 15$; Fig. 2). The experiments also revealed that methanol or ethanol, up to final concentrations of 10%, were tolerated by the Abs and did not seriously modify the I50 and I20 values. The I50 and I20 values obtained in the presence of 10% methanol or ethanol in PBST were 0.4 and 0.08 ng/ml, respectively—similar to those obtained in the presence of PBS (0.6 and 0.06 ng/ml, respectively). Analysis of the CR of the Abs with a variety of
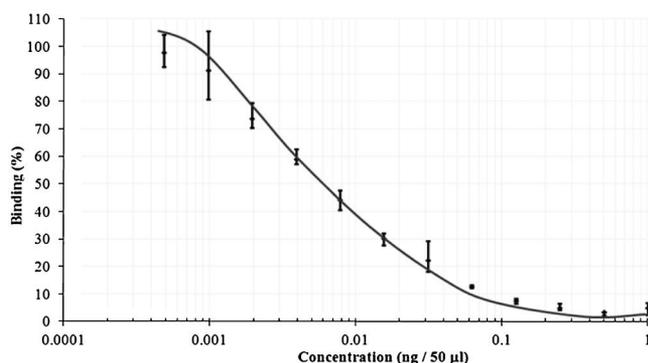
Fig. 2. Representative standard curve of atenolol (ATL). Atenolol-ovalbumin (OV) conjugate dilution 1:5,000, anti-ATL antisera dilution 1:1,000. Each point on the curve represents the mean $\pm$ SEM (n = 3).

beta-blockers revealed no cross-reactivity with acebutolol, propranolol, or celiprolol, and low cross-reactivity with betaxolol and bisoprolol, at 10 and 17%, respectively (Table 1).

Expression of the h-β1AR in Sf9 cells

A preliminary requirement in the development of an assay for detection of ATL through its interaction with the *h-β1AR* was establishment of a stable cell line that could express the receptor in large amounts. *Sf9* cells were transfected with FuGENE HD transfection reagents, and a fluorescent immuno-cytochemical staining method that employed anti–*h-β1AR* IgGs was used to visualize the receptor expressed on the transfected cell membrane. High expression of the *h-β1AR* was obtained in the *Sf9* cells transfected with pIZ/V5-His *β1AR* that were stained with the anti–*h-β1AR* IgG (Fig. 3A), and no staining was obtained when the cells were stained with IgG of normal rabbit serum (Fig. 3B). Control cells (transfected with the pIz/V5-His vector that did not contain the *β1AR*) that underwent the same treatments did not show any staining (Fig. 3C, D).

Employment of a multi-image analysis software enabled quantitation of the laser confocal microscope images into numerical values. Quantitative analysis of the *h-β1AR* expression generated high signal intensity per unit area when IgG anti–*h-β1AR* IgG was used at 2 μg/ml; the intensity decreased when a lower concentration (0.4 μg/ml) of the IgG was used, and no signal was obtained after treatment with IgG obtained from normal rabbit serum (Fig. 4).

Table 1. Cross-reactivity[a] of anti-ATL antiserum with various beta-blocker drugs

Compound	Cross-reactivity (%)
Atenolol	100.0
Propranolol	0.0
Bisoprolol	17.0
Acebutolol	0.4
Celiprolol	0.0
Betaxolol	10.0
Pindolol	5.0

[a] Cross-reactivity represents the ratio (expressed as a percentage) between the concentration of atenolol (ATL) that caused a 50% decrease in the binding of the Ab to the coating antigen adsorbed onto the microplate (defined as 100%) and the concentration of the tested compound that caused the same inhibition.
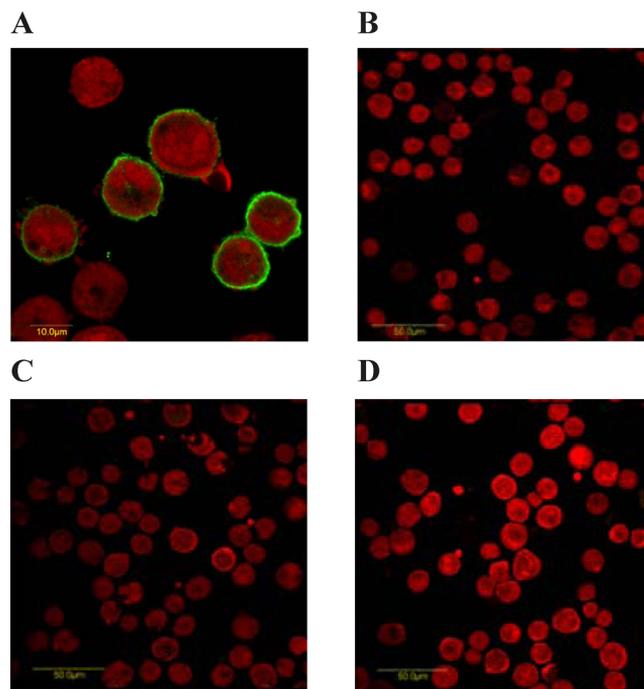
Fig. 3. Immunofluorescent confocal microscope images of *Sf9* cells expressing the *h-β1AR* stained with rabbit anti-*h-β1AR* IgG (2 μg/mL) (**A**), or with an equivalent amount of NRS IgG (**B**), *Sf9* cells with an empty pIz/V5-His (without the receptor) stained with rabbit anti-*h-β1AR* IgG (2 μg/ml) (**C**), or with an equivalent amount of NRS IgG (**D**). Goat anti-rabbit secondary Ab coupled to an Alexa fluorophore (green color) was used to visualize IgG binding. Cell nuclei were stained with propidium iodide (red color). Bar A represent 10 μm. Bars B–D represent 50 μm. Control treatments indicate no background staining or non-specific binding of the Alexa fluorophore (B–D). Transmitted light images were obtained using Nomarski differential interference contrast (DIC) microscopy.

Determination of ATL binding by a competitive assay based on a quantitative determination of the secondary messenger cAMP

The ability of the cloned receptor to monitor ATL was determined with a functional competitive binding assay in which the antagonist ATL competed with a *β1AR* agonist for binding to the receptor, and the degree of agonist binding was monitored by measuring the elevation of the level of the
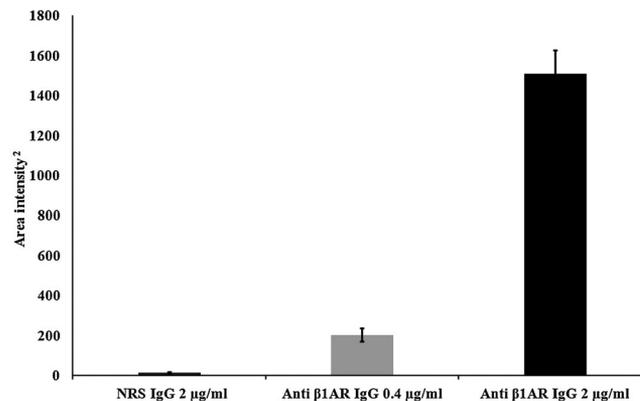
Fig. 4. Multi-image analysis of *Sf9* cells expressing the *h-β1AR* stained with rabbit anti-*h-β1AR* IgG at 0.4 or 2 μg/ml, or with NRS IgG at 2 μg/ml. Goat anti-rabbit secondary Ab coupled to an Alexa fluorophore was used to visualize IgG binding. The relative intensity of the fluorescence signal was estimated by calculating average pixel intensity from each image, with MICA software (Multi-Image Analysis, Version 1.0; CytoView, Israel). Results were based on 10 scanned areas in each treatment. Data represent means $\pm$ SD (n = 10).
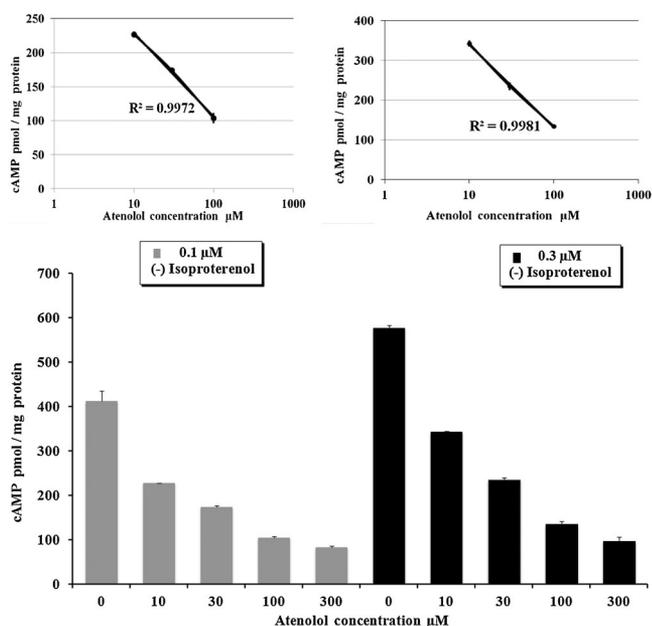
Fig. 5. Competitive functional assay showing a concentration-dependent inhibitory response of the antagonist atenolol (ATL) on the intracellular cAMP level in *Sf9* cells expressing *h-β1AR* stimulated by the agonist isoproterenol at 0.1 and 0.3 μM. Levels of cAMP/mg protein were detected with the commercial Cayman Chemical cAMP EIA kit under competitive conditions, as indicated in the *Materials and Methods* section. Data represent means ± SEM (n = 2).

secondary messenger cAMP. The agonist that was chosen for the study was isoproteranol, and cAMP was monitored, in the presence and absence of ATL, by means of a commercial cAMP enzyme immunoassay kit that exhibited a detection limit of 0.1 pmol/ml (at 80% B/B_0) for acetylated cAMP and a median inhibitory concentration value of 0.5 pmol/ml. The ability of ATL to compete with the isoproteranol for binding to the *β1AR* was tested at agonist concentrations of 0.1 and 0.3 μM and ATL concentrations of 10, 30, 100, and 300 μM. The data in Figure 5 clearly reveal a positive response of the isoproteranol agonist, which resulted in the generation of cAMP at 412 and 576 pmol/mg protein, respectively. Addition of the ATL resulted in a dose-dependent decrease of cAMP, indicating a functional binding of the antagonist to the receptor. For both agonist concentrations, the decrease in cAMP binding was linear (with $R = 0.9972$ and $R = 0.9981$) within the ATL range of 10 to 100 μM (Fig. 5, upper two panels).

DISCUSSION

Currently, environmental residues, including pharmaceutical products in general, and ATL in particular, in sediments, sludge, and surface, waste, and effluent water are monitored mainly by instrumental chemical analysis methods such as GC-MS and LC-MS [7,21,22,27,28]. These methods can detect ATL in environmental residues at the low ng/ml range, (0.017 to 0.360 ng/ml).

The chemical analytical methods used for monitoring ATL present some disadvantages, especially in large-scale monitoring, because they require highly purified samples that, in some cases, need to undergo single or multiple derivatization steps before analysis (GC-MS). Furthermore, chemical analytical methods are not cost- or time-effective: they involve the use of large volumes of toxic solvents that are both expensive and require costly storage and disposal arrangements; sample

preparation is time consuming; the analysis cannot be performed on site; and the equipment and personnel required for the operation are usually very costly. Immunochemical methods have therefore emerged in the past two decades as an efficient, simple and cost-effective means for large-scale and on-site monitoring of environment-contaminating residues.

The present study presents the development of an immunochemical assay in the format of a microplate ELISA for detection of ATL. The ATL ELISA was found to be highly specific and sensitive, with an I50 value of 0.15 ± 0.048 ng/ml and a detection limit (I20) of 0.032 ± 0.016 ng/ml (Fig. 2), which is lower than that of most chemical analytical methods currently used to monitor ATL [29–31]. The assay tolerated the presence of methanol and ethanol up to a final concentration of 10%, CR analysis revealed that the Abs that were generated in the course of the study were highly specific toward ATL and did not cross-react with any of the tested beta-blocker compounds. To the best of our knowledge, this is the first ELISA developed in a competitive indirect immunoassay format. The only other available immunoassay for ATL monitoring is a commercial ELISA kit (Abnova) based on a direct-competitive format in which standard or sample ATL in the sample competes with ATL-alkaline phosphatase conjugate, for binding to rabbit anti-ATL adsorbed onto microplate wells. Comparison of our ELISA with the commercial ELISA kit revealed that the assay developed in the present study was an order of magnitude more sensitive than the ELISA kit (with its estimated I20 of 0.2 ng/ml), and an I50 value of 8 ng/ml, which is over 50 times higher than that of our ELISA. No information is available on the CR of the ATL kit Abs with other beta-blockers or related compounds, or on their tolerance to organic solvents. The tolerance of the present assay to ethanol and methanol is an important finding, in light of the fact that in many cases environmental and even food extractions are carried out in the presence of organic solvents to increase recovery yields, and consequently, the immunochemical assays used to monitor it must tolerate the presence of such solvents. The ELISA described in the present study does fulfill this requirement.

Furthermore, the indirect competitive ELISA format described in the present paper, in which the coating antigen is adsorbed onto the microplate, offers an advantage, especially for the analysis of crude environmental and food extracts. In the direct format, the tested sample is added to the reaction mixture together with the enzyme–antigen conjugate, and therefore, the enzymatic activity might be affected by the presence of interfering elements unrelated to the presence of the analyte, and thus might be interpreted as a false-positive signal. The indirect competitive assay format developed in our present study eliminates this problem, because the sample is washed away before the addition of the secondary enzyme-conjugated Ab. Taken together, the indirect format, the low detection limit together with the low CR with related compounds, ability to tolerate organic solvents, and cost-effectiveness definitely represent advantages for the large-scale application of the present ELISA method for detection of ATL in environmental, agricultural, and biological samples. Recently another ELISA kit, which is not specific to ATL and which cross-reacts with a large variety of beta-blockers (Neogen, Cat. No. 1003319), was used to monitor ATL in spiked urine, and to compare the ELISA method with chemical instrumental analytical methods, LC-MS or postderivatization GC-MS [32]. The findings revealed a limit of detection of 49.6 ng/ml which was more than three orders of magnitude higher than that obtained in our ELISA. The method was much less sensitive than LC-MS analysis, which had a limit

of detection of 0.49 ng/ml, also higher than that obtained with our ELISA.

The Abs developed in the present study could be employed to develop immunoaffinity purification (IAP) methods for cleanup and concentration of the sample before its analysis by chemical instrumental analytical methods or immunoassays. IAP methods emerged in recent decades as one of the most powerful techniques for single-step isolation, concentration, and purification of individual compounds or of classes of compounds from liquid matrixes [33]. In the past few years, we have developed highly effective and reproducible IAP technology, based on the entrapment of Abs in a ceramic SiO_2 matrix termed sol-gel, which facilitates efficient, single-step cleanup and concentration of analyzates from large volumes of crude samples. The applicability of this approach was tested with Abs raised in our laboratory against a variety of PPs such as the steroid hormones levonorgestrel, ethinylestradiol, and nortestosterone [24], medroxyprogesterone acetate [25], the nonsteroid anti-inflammatory drug indomethacin [26], several other pesticides (pyrethroids and atrazine) [34,35], forensic compounds (TNT) [36], and environmental contaminants [37,38]. Implementation of the Abs for the development of a sol-gel based ATL IAP method is under development.

In parallel to the development of the immunochemical assay, the present study focused also on an innovative experiment to develop a diagnostic environmental assay based on a receptor-binding assay; like the ELISA, it could be further converted into a high-throughput screening assay. We have developed such an assay by stable expressing the *h-β1AR* in *Sf9* cells using pIZ/V5His vector, which is based on a stable expression system. Unlike transient expression systems, the stable transfection enables functional assays to be performed with intact cells, in response to their stimulation with various agonists and antagonists, and to follow ligand-mediated intracellular events without needing to transfect the cell with the G-protein components (on top of the ability to extract membrane proteins and carry out studies on the purified molecules).

Transfection with the pIZ/V5His vector was carried out by using the cationic liposomal transfection reagent FuGENE HD, which resulted in a high and stable transfection efficiency. By use of a cationic lipid mixed with a neutral lipid, unilamellar liposome vesicles are formed that carry a net positive charge to the highly positive amine groups on the cationic molecules. The DNA nucleic acids, which serve as the vector, adsorb to these vesicles and gain access to the inside of cells, most likely by fusion of the liposome with the plasma membrane to form an endocytic vesicle. Under carefully optimized conditions (data not shown), this liposome-mediated transfection method was found to be highly efficient and much easier to use than earlier methods that used the baculovirus expression vector pVL941 system for gene expression in *Sf9* cells.

Sf9 cells have been used by many research groups to express mammalian *β1AR* and *β2AR* and, interestingly, the first G-protein coupled receptors to be expressed in *Sf9* cells were these two receptors [39]. However, most of these studies used the pVL941 vector and a transient expression system [40]. To the best of our knowledge, the present study was the first attempt to express the *h-β1AR* in *Sf9* cells by using the liposome-mediated transfection method with this vector, and this study is one of only a few in which the *h-β1AR* was expressed stably in *Sf9* cells. This system was used in our present study to determine the ability of ATL to antagonize the *β* agonist isoproterenol. The receptor's expression in the stably transfected cells was proved

by means of immunocytochemical staining, using a commercial IgG generated against the C terminal of *h-β1AR*, and the extent of primary antibody binding was monitored with a secondary antibody labeled with a fluorophore (Alexa). The experiment showed high expression of the recombinant receptor in the cells.

Classically, the *h-β1AR* signaling pathway is considered to be a three-component system that involves a seven-transmembrane-domain receptor, a trimeric G-protein complex (G_α, G_β, G_γ), and an effector. When activated, the receptor associates with the G-protein complex and this causes the exchange of guanosine diphosphate (GDP) bound to G_α for guanosine-5'-trephosphate (GTP), followed by the dissociation of G_α-GTP from the complex. The activated subunit G_α can couple to downstream effectors to stimulate the intracellular messenger adenylyl cyclase, which, in turn, catalyzes the conversion of cytoplasmic adenosine triphosphate to cAMP, leading to an increase in intracellular cAMP. The concentration of cAMP in a cell is a function of the ratio between its rate of synthesis from adenosine triphosphate by adenylate cyclase and its rate of breakdown to adenosine monophosphate (AMP) by specific phosphodiesterases. Consequently, after activation of the *β1AR* expressed on the cells by known concentrations of the agonists, such as (−)isoproterenol, we can expect a concentration-dependent elevation of the intracellular level of cAMP, a level that is decreased when an antagonist such as ATL is added to the system.

Receptors, particularly those belonging to the G-protein coupled receptors group, offer many advantages: they exhibit high affinity toward their ligands, usually in the nanomolar range; they can be used to develop quantitative assays; and their proteins are relatively easily cloned, which enables high-level expression in transfection systems. Furthermore, the system enables insertion of mutations and generation of receptors with binding properties different from those of the native receptor. Such mutated receptors are capable of selectively binding a drug such as ATL, without identifying native adrenergic agonists of this family. Indeed, several studies were carried out in a variety of stably expressed cell lines (with the exception of *Sf9*) in which cloned and mutated *β1A*- and *β2ARs* were characterized and the interactions with their ligands and G-proteins were determined [41]. However, despite the many advantages presented by cloned and expressed receptors, so far only a few G-protein coupled receptor–based assays have been developed for environmental or food diagnostics.

In the present study, a competitive binding assay was developed with the recombinant cloned receptor, which quantified the secondary messenger cAMP (by means of a commercial immunochemical test kit) in response to activation by isoproteranol in the presence and absence of ATL. The assay showed that the recombinant cloned receptor was indeed functional, that it bound the agonistic ligand isoproterenol as well as the antagonist ATL, as indicated by a dose-dependent elevation of cAMP in the presence of isoproteranol at varied concentrations, and its decrease once ATL was added to the reaction mixture. The detection of ATL was found to be linear in the range of 10 to 100 μM. The assay can thus be used for monitoring ATL in real-world samples, and for further studies of its pharmacological properties.

In conclusion, a highly specific Ab for ATL and a sensitive ELISA for ATL detection have been developed, along with a novel receptor-based assay for monitoring the drug. Each method introduces a specific advantage: the ELISA is highly sensitive and can detect ATL at sub-parts per billion levels (comparable to the detection limit of analytical chemical

methods); the receptor-binding assay, although less sensitive, enables differentiation between adrenergic agonists and antagonists, and forms a basis for future selective monitoring of $\beta 1AR$ ligands (by means of mutated receptors, specific for the various compounds) that cannot be differentiated by polyclonal Abs employed in the ELISA. Thus, the two approaches are complementary and provide a basis for analysis of ATL and other adrenergic compounds in a wide variety of samples. The combination of both analysis methods could provide information on the pharmacokinetic properties of ATL and related drugs. This would facilitate the study of population exposure to them and enable monitoring of their occurrence in contaminated food, soil, and other environmental samples.

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