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ORIGINAL PAPER

Monitoring of the non-steroid anti-inflammatory drug indomethacin: development of immunochemical methods for its purification and detection

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Abstract The present research focused on the development of an immunoassay and an immunochemical sol–gel-based immunoaffinity purification (IAP) method for purification and detection of the non-steroid anti-inflammatory drug (NSAID) indomethacin (IMT). A polyclonal antibody (Ab) for IMT was generated, and two sensitive microplate assays for the detection of IMT were developed (termed OV and HRP formats), based on the enzyme-linked immunosorbent assay (ELISA) method. The limits of detection of the assays were 15±1.25 ng mL^{-1} (n=50) and 12± 0.17 ng mL^{-1} (n=4) for the OVA and HRP formats, respectively. The Abs exhibited slight cross-reactivity with other NSAIDs. The Abs were also used to develop a sol–gel-based IAP method for clean-up and concentration of IMT. Several sol–gel formats with various amounts of antibodies were examined; the best and most reproducible format was at a TMOS:HCl molar ratio of 1:6 in which 120 μL of IMT Abs was entrapped. The binding capacity under these conditions was ca. 100 to 250 ng of IMT with very low non-specific binding (less than 5% of the applied amount). The sol–gel IAP method, combined with solid-phase extraction, successfully eliminated serum interference to a degree that enabled analysis of spiked serum samples by ELISA. The method was also found to be fully compatible with subsequent chemical analytical methods, such as liquid chromatography followed by mass spectrometry. The approaches developed in this study form a basis for analysis of IMT in biological samples in order to monitor their pharmacokinetic properties, and may be further used to study population exposure to IMT, and to monitor the occurrence of IMT contamination in water samples.

Keywords Indomethacin · ELISA · Immunoaffinity chromatography · Sol–gel · Pharmaceutical residues · Residue monitoring · Serum monitoring

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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). Up to now, most of the studies on adverse health effects of environmental contaminants have focused mainly on agricultural pesticides, heavy metals, and toxic industrial contaminants [3, 4]. One large class of chemicals that has received little attention for many years comprises residues of pharmaceutical products (PPs) that are used in human and veterinary medicine in quantities comparable to those of agrochemicals.

In recent years, however, the presence of PP residues in food and the environment, and the inadvertent exposure of populations to those drugs via contaminated food have roused public and scientific awareness which together with the extensive food safety legislation controlling drug residue levels in food initiated studies that focus on occurrence of PPs in food and the environment, and on the possible correlation between food and environmental contamination, on the one hand, and adverse health effects, on the other hand. These studies revealed that numerous PPs and their metabolites do indeed contaminate aquatic environments (for review, see [1, 5–9] and references therein), but currently, there is very little information on their environmental fate, and our understanding of the possible transmission of PPs into the food chain and of human exposure is also very limited. Even less is known regarding whether and how such contaminants, once ingested, affect human health.

In order to evaluate the extent of the problem, it is necessary to carry out large-scale monitoring programs that will enable to determine small quantities of PP residues in environmental, food, and biological samples. 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 that have the potential to affect human fecundity by exposure via 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. Based on an extensive literature survey, eight potential endocrine-disrupting PPs were selected [10]. These compounds comprised four steroid hormones, i.e., levonorgestrel (LNG), ethynylestradiol (EE2), nortestosterone (NT), and medroxyprogesterone acetate (MPA), which are the main components of contraceptive drugs, and are also used as anabolic steroids; a representative of non-steroid anti-inflammatory drugs (NSAIDs)—indomethacin (IMT, Fig. 1a); a representative of selective serotonin re-uptake inhibitors (SSRIs)—fluoxetine (FLX); the antibiotic, trimethoprim (TMP); and the beta-blocker atenolol (ATL). All of these compounds exhibit high stability in the environment, are used in large amounts, and most importantly, have been reported to affect fecundity [10].

In a previous study, we developed an enzyme-linked immunosorbent assay (ELISA) and an immuno-affinity purification method for the steroid hormone LNG [11]. In the present study, we focused on the NSAID IMT. So far, the available information about the occurrence, fate, and bioaccumulation of IMT in the environment or in the food chain is very limited. However, its massive current use, high environmental stability, and consequently, long environmental half-life raise the possibility that it has the potential to pose a high risk to human health.

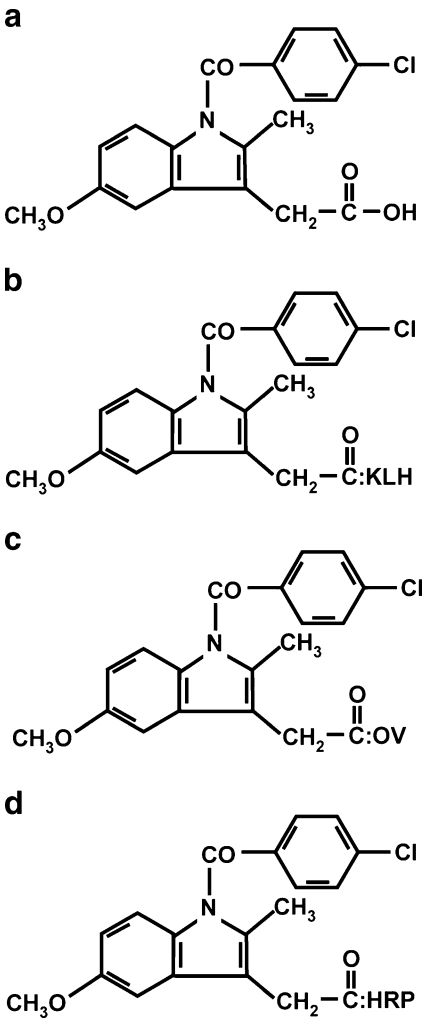

Fig. 1 Structure of IMT (**a**) and IMT-protein conjugates (**b**–**d**). IMT-KLH (**b**): IMT coupled to keyhole limpet hemocyanin; IMT-OVA (**c**): coupled to ovalbumin; and IMT-HRP (**d**): coupled to horseradish peroxidase

IMT is used to reduce fever, aches, and inflammation; it is similar in its mode of action to Ibuprofen and Naproxen [12–15], i.e., it involves downregulation of prostaglandin synthesis by inhibiting the enzymes cyclooxygenase 1 and 2 ($COX_{1,2}$). Similarly to any other drug, IMT has side effects [16–20], and studies have shown that the effective serum concentration of IMT when used as an anti-inflammatory drug is a little less than 1 μg/mL, and the concentration considered toxic in humans is slightly more than 5 μg/mL [21]. The side effects of the drug and the extremely narrow range between the effective and toxic doses indicate the need to maintain accurate identification and monitoring of small amounts of IMT in environmental, water, and food samples, in order to prevent exposure of "non-target" populations to the drug.

Today, there are several effective methods—e.g., capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), high-performance liquid chromatography

(HPLC), and colorimetric assays—to monitor IMT in biological and environmental samples (for example, see [21–25] and references therein). Immunochemical methods have also been developed, including fluorescence polarization immunoassay (FPIA) [26] and radioimmunoassay [27]. Also, a single ELISA to assay IMT in water samples has been reported [28].

In order to be able to perform large-scale monitoring and to be able to detect small amounts of analytes, it is necessary to develop simple, fast, and cost-effective diagnostic methods and also sample-preparation protocols with high recovery. This is especially true for environmental and food samples, which contain, in most cases, components that may interfere with the analysis, whether chemical or immunochemical. Although immunochemical methods tolerate matrix interference to a greater extent than instrumental chemical methods, it is still necessary to purify the analyte from the sample before it can be tested by ELISA. For many years, immunoaffinity purification (IAP) has been considered to be one of the leading technologies for purification, concentration, and isolation of chemical and biological compounds. In the past two decades, our laboratory has developed an IAP method that uses antibodies (Abs) entrapped in a SiO_2 ceramic matrix. This method has been successfully applied by many laboratories, including ours, for purification of a large number of compounds in the analysis of a variety of environmental, food, and biological samples (for review, see [29–32] and references therein and [33–40]).

In the present study, we developed an ELISA and a sol–gel-based IAP for IMT. A polyclonal Ab was generated, and two sensitive and highly specific ELISAs were developed. The antiserum was also used to develop a sol–gel-based IAP method for purification and concentration of IMT. Several sol–gel formats, containing various amounts of antiserum, were examined under diverse experimental conditions. The sol–gel IAP method was tested for its capability to eliminate serum interference with ELISA, and its compatibility with chemical analytical procedures was tested by means of LC-tandem mass spectrometry (MS/MS). The approaches developed in this study form a basis for analysis of IMT in biological samples in order to monitor their pharmacokinetic properties, and they could be further used to study population exposure to IMT and also to monitor the occurrence of IMT contamination in food, soil, and other environmental samples.

Materials and methods

Immunochemical methods

Antiserum

Polyclonal anti-IMT antiserum was generated in rabbits by using IMT conjugated to Keyhole Limpet Hemocyanin (KLH; Sigma, St. Louis, MI, USA) as an immunogen (Fig. 1b), as described below. Each injection was carried out with 0.8 mg/0.5 mL of the IMT-KLH conjugate.

Preparation of IMT-KLH conjugate for immunization

IMT was first converted to its active ester form as follows: 16 mg of IMT was mixed with 26.6 mg of *N*-hydroxysuccinimide (NHS; Sigma) and 92.5 mg of *N*′,*N*′-dicyclohexylcarbodiimide (DCC; Sigma), and dissolved in 1,944 μL of dimethylformamide (DMF; Labscan, Dublin, Ireland). The reaction was allowed to proceed at room temperature for 4 h and was then further incubated at 4 °C for 12 h. The mixture was centrifuged for 15 min at 4,000×*g* at room temperature (25 °C), and 1,215 μL of the supernatant, containing 10 mg of activated IMT, was added, drop-wise, to 10 mg of KLH dissolved in 1 mL of 0.13 M $NaHCO_3$ at pH 8.5. The reaction was allowed to proceed for 1 h at room temperature, and the solution was then dialyzed against 4 L of 0.13 M $NaHCO_3$ at pH 8.5 for 3 days at room temperature. The solution was changed three times daily. The hapten IMT-KLH conjugate was stored as aliquots at −20 °C pending injection into rabbits.

Prior to immunization, 0.5 mL of the conjugate was mixed with Complete Freund's adjuvant (first injection) or with incomplete Adjuvant (second to 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 almost equally active towards the antigen, but only the fourth bleed was used for ELISA and sol–gel IAP experiments.

Preparation of IMT-OVA coating antigen

The method was similar to that described above for preparation of the IMT-KLH conjugate, except that 1.95 μL of the supernatant, containing 16.1 μg of activated IMT, was added, drop-wise, to 2 mg of ovalbumin from egg white (OVA, Sigma), dissolved in 750 μL of 0.13 M $NaHCO_3$ at pH 8.5 (molar ratio of 1:1, hapten:carrier protein; Fig. 1c). The conjugate was stored in aliquots at −20 °C pending use.

Preparation of IMT-HRP conjugate

The method was similar to those described in the previous two subsections, except that 9.9 μL of the supernatant, containing 81 μg of activated IMT, were added, drop-wise, to 1 mg of horseradish peroxidase (HRP) dissolved in 1,500 μL of 0.13 M $NaHCO_3$ at pH 8.5 (molar ratio of 1:10, carrier protein: hapten; Fig. 1d). The reaction was allowed to proceed at room temperature for 1 h, and the unbound hapten and other

small molecular weight components were separated from the protein/hapten conjugate by size exclusion with a Centricon 30 (Amicon, Millipore, Billerica, MA, USA). The reaction mixture was spun for 25 min at 4,000×*g* at room temperature and washed twice with 1 mL of 0.13 M $NaHCO_3$ at pH 8.5. The final volume was adjusted to 1,500 μL by adding 0.13 M $NaHCO_3$ at pH 8.5, and 1,500 μL of ethylene glycol (Sigma) were added to the conjugate solution, which was then kept at −20 °C pending use.

IMT competitive ELISA

Two indirect competitive chemiluminescent ELISAs were developed, designated as OVA format and HRP format, respectively.

For the OVA format, ELISA microtiter plate wells (96 F Maxisorp White Microwell, Nunc, Roskilde, Denmark) were coated with 100 μL of IMT-OVA conjugate, diluted 1:5,000 (containing 0.96 μg/mL) in 0.5 M carbonate buffer, pH 9.6. Six wells (designated as background, Bg, wells) were coated with an equivalent amount of OVA and served as controls to determine non-specific binding. After an overnight (ON) incubation at 4 °C, the wells were washed three times with 0.05 M phosphate buffer containing 0.15 M NaCl and 0.1% Tween-20 (PBST), and 50 μL of IMT (12 serial dilutions ranging from 0.049 to 100 ng per 50 μL in (PBS+20% methanol)—PBS-M) or any other tested sample were added to the wells in duplicate, together with 50 μL of anti-IMT antiserum diluted 1:2,000 (for a final concentration of 1:4,000) in PBS–2xT (PBS containing 0.2% Tween 20, pH 7.2). The Bg wells and six additional wells received only PBS without IMT and served to determine maximal binding (designated as 100%) in the absence of competing analyte. The plates were incubated overnight at 4 °C, washed as above with PBST, and 100 μL of secondary Ab conjugated to HRP (goat anti-rabbit HRP conjugated; Sigma) diluted 1:40,000 in PBST was 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 chemiluminescent 3,3′,5,5′ tetramethyl benzidine (TMB) substrate (SuperSignal ELISA Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Rockford, IL, USA). The reaction was measured after 5 min with a chemiluminescent ELISA reader (Lucy 2, Microplate Reader; Anthos, Eugendorf, Austria).

The tolerance of the assay to various concentrations of organic solvents was tested similarly, except that the IMT standard was made up in either (PBS+40% methanol), (PBS+20% ethanol), or (PBS+40% ethanol), and the Bg and maximal-binding wells were tested in the presence of each of these solvents instead of PBS-M.

The effect of normal human serum on the ability to generate an IMT calibration curve was tested similarly, except that the calibration standard curve was made up in 4% serum diluted in PBS-M, and the Bg and maximal binding wells received 4% serum diluted in PBS-M instead of just PBS-M.

For the HRP format ELISA, microtiter plate wells (transparent F96 Maxisorp or 96 F maxisorp white microwell; Nunc Immuno Plate, Roskilde, Denmark) were coated with 100 μL of protein A (Sigma) at 1 μg/100 μL made up in 0.5 M carbonate buffer, pH 9.6, in duplicate, and were incubated overnight at 4 °C. The plates were washed three times with PBST, and 100 μL of anti-IMT antiserum (diluted 1:4,000 in 0.5 M carbonate buffer, pH 9.6) was added to the plate and incubated overnight at 4 °C. The plates were washed three times with PBST, and 12 serial dilutions of IMT standard or any other tested compound, diluted in PBS-M at concentrations ranging from 0.049 to 100 ng/50 μL, were added to the plates together with 50 μL of IMT-HRP conjugate diluted 1:25 (i.e., a final dilution of 1:50) in PBS–2xT, in duplicate. Six wells to which PBS without the primary Abs was added served as a reaction Bg control. Another six wells with no competing IMT, to which 50 μL of PBS-M were added, served to determine maximal binding (designated as 100%). The reaction was incubated for 2 h at room temperature, the plates were washed three times with PBST, and 100 μL of either a chemiluminescent or a colorimetric TMB substrate (TMB Substrate Chromagen; Dako, Glostrup, Denmark) was added. The chemiluminescent reaction was monitored as described above. The color reaction was stopped after 10 min by the addition of 50 μL of 4 M H_2SO_4, and the absorbance was measured with an ELISA reader at 450 nm.

Cross reactivity (CR) of the Abs with a variety of NSAIDs was determined by adding the IMT or the tested compounds at 12 serial dilutions all ranging from 0.049 to 100 ng/50 μL, in duplicate, and testing their ability to compete with the IMT antiserum adsorbed to the microplate, for binding the IMT-HRP conjugate.

Sol–gel IAP

Sol–gel entrapment of anti-IMT antiserum

Entrapment involved a two-step procedure in which hydrolysis was followed by polymerization of tetramethylsilane (TMOS; Aldrich) as previously described [41]. Briefly, an acidic silica sol solution was obtained by mixing TMOS with 2.5 mM HCl in double-distilled water (DDW) at molar ratios of 1:6 (unless otherwise indicated). The mixture was stirred for 1 min until a clear solution was obtained, and it was then sonicated for 30 min in an ultrasonicator bath (model T-460/H, 285 W, 2.75-L; ELMA, Singen-Hohentwiel, Germany). The reaction was performed in a well-ventilated fume hood. Anti-IMT antiserum (120 μL equivalent to

6.72 mg protein, unless otherwise indicated) was premixed with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic 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") were prepared by mixing 0.5 mL of hydrolyzed TMOS with 0.5 mL of 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, each of total volume of 1 mL, were washed with 2 mL of HEPES buffer at pH 7.6 and stored 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; however, in most cases, they were used within 24 h.

Binding and elution of IMT from sol–gel IAP columns

Wet gels were thoroughly crushed and packed into 20-mL, 1.5×12-cm, Econo-Pac disposable chromatography columns (Bio Rad, Philadelphia, PA, USA). These sol–gel columns were washed with 50 mL of PBS, pH 7.2 (loading buffer) prior to sample application. To ensure optimal binding, the columns were kept under buffer at all times during the experiment. Aliquots of 1,000 ng of IMT standard in 1 mL of loading buffer or 1 mL of normal human serum spiked with 1,000 ng of IMT that had been kept for 1 h at room temperature were applied to "empty" sol–gel columns or to columns doped with 120 μL of anti-IMT antiserum (unless otherwise indicated). The eluate was collected and applied to the column once again to ensure better binding results. Unbound IMT was removed by washing the columns with 20 mL of DDW. Elution was performed with 10 mL of acetonitrile (PESTI-S; Bio-Lab, Jerusalem, Israel). The eluted fraction was evaporated under nitrogen at 50 °C to remove the eluting solvent, and the eluate was reconstituted in 500 μL of PBS-M for ELISA analysis. In cases where sol–gel eluates underwent an additional concentration step on solid-phase columns (see "Solid-phase extraction (SPE): sample application and elution," below), samples were reconstituted in 1 mL of DDW before being applied to the solid-phase columns. All IAP experiments were performed with pairs of sol–gel columns that comprised an experimental column containing anti-IMT antiserum (total binding) paired with an empty control column without antiserum (non-specific binding), and the specific binding was determined from the difference between the eluates of the two columns. All the experiments in this study were performed with a standard IMT compound prepared from stock solution dissolved in PESTI-S absolute methanol (Bio-Lab, Jerusalem, Israel).

The IMT content in sol–gel IAP eluates was determined by means of the OVA chemiluminescent ELISA format. The reconstituted samples were heated for 10 min at 100 °C prior to the ELISA analysis, to inactivate residual antibodies that may have leached from the IAP columns. All samples were tested in duplicate at five successive two-fold dilutions (undiluted to 1:16) that were within the range of the standard curve. Aliquots of 50 μL of the tested sample were added to the wells, together with 50 μL of anti-IMT antiserum in PBS–2xT (as described above, for the OVA format). The IMT content in tested samples was determined from an IMT calibration curve after linearization of the data by transformation to a logit-log plot by means of the Origin software, Version 6.0 (Microcal Software, Northampton, MA, USA). Slopes of the curves obtained for all the samples were tested for parallelism with the standard curve by testing for homogeneity of regression slopes, according to Sokal and Rohlf [42].

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 methanol (Bio-Lab, Jerusalem, Israel), followed by 10 mL of DDW. Sol–gel-eluted samples were reconstituted in 1 mL of DDW (as described above, under "Binding and elution of IMT from sol–gel IAP columns" section). Samples were loaded onto the columns, which were then washed with 10 mL of DDW. Elution was carried out with 1 mL of absolute methanol. For ELISA analysis, the samples were evaporated under nitrogen at 50 °C, reconstituted in 500 μL of PBS-M, and heat-inactivated as described above (under "Binding and elution of IMT from sol–gel IAP columns" section). For LC-MS/MS analysis, samples were dissolved in 1 mL of a 2:8 acetonitrile: (water+ 0.1% NH_4OH) mixture. The IMT content was determined either by analysis with the competitive OVA chemiluminescent ELISA format as described above, over a concentration range of 0.049 to 100 ng/50 μL, or by LC-MS/MS analysis (see "LC-MS/MS analysis," below).

Chemical analytical methods

LC-MS/MS analysis

A LC-MS/MS method was developed, to assess the compatibility of sol–gel IAP with chemical analyses. This was done by determining the degree of precision with which IMT content in spiked sol–gel/SPE eluates could be determined. Empty sol–gel columns (1:6 format) and columns doped with 120 μL of anti-IMT antiserum were loaded with 200 mL of DDW instead of IMT, and the "samples" were eluted with 10 mL of acetonitrile, evaporated under nitrogen at 50 °C, and then dissolved in 1 mL of DDW and passed through an SPE column as described above. All samples were again vacuum evaporated and

were reconstituted, prior to LC-MS/MS analysis, with 1 mL of a 2:8 acetonitrile (ACN):(water+0.1% NH_4OH) mixture (LC running buffer). Eluates from doped and empty columns were then spiked with IMT at 10 ng mL^{-1} and subjected to LC-MS/MS analysis. Eluates from doped columns were also spiked with IMT standard made up from a stock solution of IMT in methanol at a series of concentrations ranging from 1 to 200 ng mL^{-1}. These samples were used to generate a calibration curve.

Samples were analyzed by LC-MS/MS multiple-reaction monitor (MRM) detection in the negative-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 (50×2.0 mm, 3 μm particle size, 110 Å pore size); injected volume was 5 μL. Solvent A comprised 10% aqueous ACN containing 0.1% ammonium hydroxide (J. T. Baker, Phillipsburg, NJ, USA); solvent B comprised 90% aqueous ACN containing 0.1% ammonium hydroxide. For the analysis, the solvent initially comprised 80% A and 20% B; after 1.5 min, the solvent was modified to 100% B over 4 min, according to the Waters linear program; the flow rate was 0.3 mL min^{-1}, and the IMT retention time, t_R, was 3.35 min. Following the LC analysis, all samples were analyzed with a Micromass Quattro Pt triple-quadrupole mass spectrometer operating in the electrospray ionization mode. The data were processed with Masslynx v. 4.0 and Quantlynx v. 4.0 software. The amount of IMT in the samples was determined by comparison with calibration curves constructed by plotting the concentrations in the spiked samples against the peak areas found in their chromatograms.

Statistics

Differences between the average values were subjected to Tukey–Kramer one-way ANOVA at $p<0.05$ (95%).

Results

Development of an IMT ELISA

Two immunochemical assays for monitoring the NSAID IMT were developed that used two ELISA formats: OVA and HRP. In the OVA format, microplates were coated with a hapten-carrier protein (OVA) conjugate, and the analyte (standard or unknown sample) competed with the hapten conjugate adsorbed to the microplate for binding to the Ab. In the HRP format, Abs were adsorbed onto the microplate via Protein A, and the analyte competed with a hapten-enzyme conjugate (HRP) for binding to the adsorbed Ab. The development of the IMT ELISA involved two sets of experiments: the first set was intended to determine the optimal concentrations of the coating conjugate IMT-OVA (OVA format), the IMT-HRP conjugate (HRP format), the antiserum, and the secondary Ab (checkerboard tests); the second set was intended to generate a standard curve, 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 NSAIDs.

The first set of experiments revealed that for the OVA chemiluminescent format, dilutions of 1:5,000 for the IMT-OVA conjugate and 1:4,000 (final) of the anti-IMT antiserum resulted in high binding and a low background, i.e., of non-specific binding. In the HRP format, dilutions of 1:4,000 for the coating Ab and 1:50 for the HRP-hapten conjugate resulted in a good signal-to-background ratio (data not shown). The second set of experiments determined the working range of the assay for both formats (0.1 to 30 ng/50 μL; Fig. 2) and the I_{50} and I_{20} values for both assay formats. Basically, there were no marked differences in the working ranges of the respective formats, and the I_{50} and I_{20} values were 132±9.3 and 15±1.25 ng mL^{-1} (n=50), respectively, in the OVA ELISA and 91±2.59 and 12±0.17 ng mL^{-1} (n=4), respectively, for the HRP format. The inter- and intra-assay accuracies were 92% and 85%, respectively. Because of the very low solubility of IMT in neutral aqueous buffers, both assays were carried out in the presence of 10% methanol. The presence of a higher percentage (20%) of ethanol or methanol caused marked decreases in both the sensitivity and affinity of the Ab: I_{50} and I_{20} of 1,800 and 170 ng mL^{-1}, respectively, for methanol, and 640 and 80 ng mL^{-1}, respectively, for ethanol. In the light of these results, 10% methanol in PBST was chosen as the reaction buffer for all subsequent experiments.

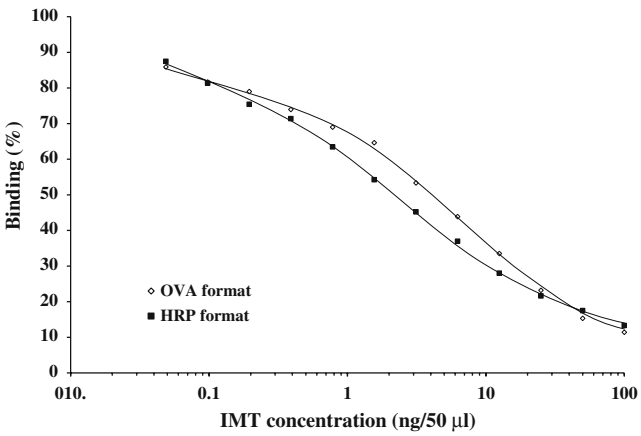

Fig. 2 Representative standard curves of IMT OVA and HRP chemiluminescent ELISA formats. Chemiluminescent ELISA was carried out in PBST containing 10% methanol

Cross reactivity

Once the assay had been optimized and the sensitivity determined, the ELISA was used to characterize the antiserum for specificity and CR with other closely related compounds (Table 1). The CR with all of the listed compounds was determined by means of an HRP ELISA format. As indicated in Table 1, under the tested conditions, the IMT antiserum cross-reacted to a small extent (<9%) with Tolmetin and Diclofenac. The CR with Suulindac, Ketorolac, and Benzydamine was <3% (at concentrations up to 2 ppm 100 ng/50 μL). Thus, the results demonstrated high affinity towards IMT with slight CR with other NSAIDs.

IMT sol–gel-based IAP

A sol–gel-based IAP method was developed for purification and concentration of IMT. The ability of the sol–gel-entrapped Abs to bind the analyte was tested with several different sol–gel formats and various amounts of entrapped antiserum. Recovery of the bound analyte was tested with various eluting solvents. The amounts of IMT recovered from the sol–gel columns were analyzed with the OVA chemiluminescent ELISA format.

The effects of several different sol–gel formats on the activity of the entrapped Abs were examined at four different TMOS:HCl ratios. Figure 3 shows the differences between performances of the various formats: the best results were obtained at a TMOS:HCl ratio of 1:6, which resulted in a binding capacity of 111 ng (out of 1,000 ng applied on the column) when 120 μL of Abs was entrapped in the sol–gel matrix. Lower binding capacities—of 87 and 60 ng—although the differences were not significant, were obtained with 1:4 and 1:12 formats, respectively. A significantly lower binding capacity—of 33 ng—was obtained with a 1:8 format and the same amount of entrapped Abs. Non-specific binding of IMT to the sol–gel matrix in all of the tested formats was negligible, i.e., less than 1% of the amount applied on the IAP column.

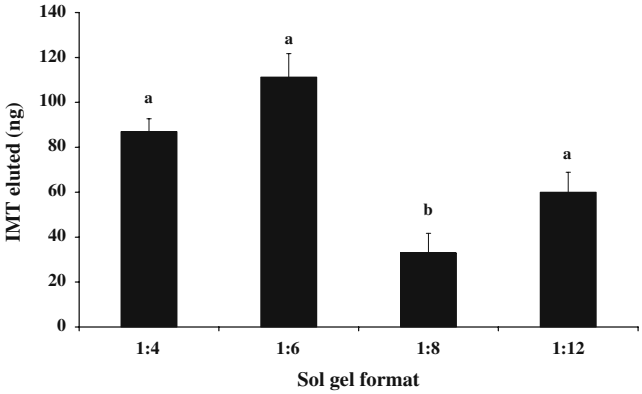

Fig. 3 Effects of various sol–gel formats (based on four TMOS:HCl ratios—1:4, 1:6, 1:8, and 1:12) on the activity of entrapped anti-IMT antiserum. IMT (1,000 ng) was loaded on sol–gel columns prepared with the above TMOS:HCl ratios and doped with 120 μL of anti-IMT antiserum. Elution was carried out with 10 mL of acetonitrile. Amounts of eluted IMT were determined with the OVA chemiluminescent ELISA format. Values were obtained after logit-log transformation of the data by means of Microcal Origin software. Each *bar* represents the mean±SEM of five to nine measurements (1:4, $n=9$; 1:6, $n=7$; 1:8, $n=8$; 1:12, $n=5$). *Means with the same letter* do not differ significantly at $p<0.05$

Next, the column binding capacity was examined with various amounts of entrapped Abs, ranging from 30 to 240 μL of antiserum (equivalent to 1.68 to 13.44 mg protein), in a 1:6 sol–gel format. The best results were obtained with 120 and 240 μL of entrapped antiserum, which resulted in binding of 111 and 130 ng of IMT, respectively (Fig. 4). The amounts of IMT recovered with 30 and 60 μL of antiserum revealed very low binding: 10 ng with each amount of antiserum. The non-specific binding of IMT to the sol–gel matrix, in the absence of entrapped antiserum, was 10 ng. Since no significant differences were noticed between the binding capacity of columns that contained 120 and 240 μL of entrapped antiserum, we decided to use 120 μL of anti-serum in 1:6 format sol–gel columns in all subsequent experiments.

The method was also optimized with respect to the eluting solvent. Four different solvents were tested for their ability to dissociate IMT from the sol–gel IAP column: absolute ethanol, methanol, acetone, and acetonitrile. The results clearly indicated that acetonitrile was the only solvent that enabled IMT elution; the other three were ineffective as eluting solvents and resulted in a recovery of less than 10% of the applied analyte (data not shown).

Although sol–gel-entrapped Abs are strongly retained within the monolith, our preliminary experiments revealed

Table 1 Cross reactivity of the anti-IMT antiserum with various NSAIDs

Compound	Cross reactivity (%)
Indomethacin	100
Sulindac	<3
Ketorolac	<3
Benzydamine	<3
Tolmetin	<9
Diclofenac	<9

Cross reactivity represents the ratio (as a percentage) between the concentration of free IMT that caused a decrease of 50% in the binding of IMT-HRP conjugate (final dilution 1:50) to an anti-IMT antiserum (diluted 1:4,000) adsorbed to the microplate (HRP format) and that of any of the tested compounds that caused the same inhibition. Analysis was by a colorimetric ELISA. All compounds (including IMT) were tested at a range of 0.049 to 100 ng/50 μL

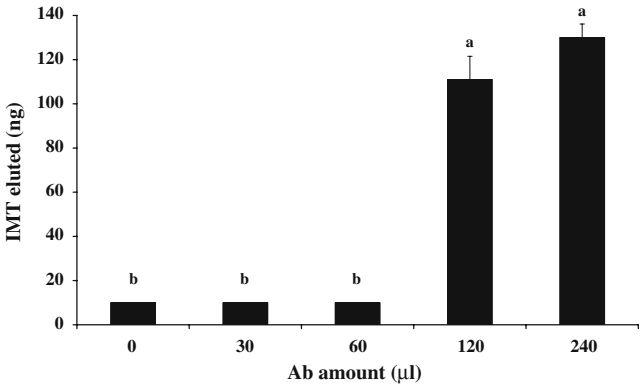

Fig. 4 Comparison of the binding capacity of various amounts of sol–gel-entrapped anti-IMT antiserum, ranging from 30 to 240 μL (equivalent to 1.68 −13.44 mg protein). The sol–gel format used was 1:6. Binding in the absence of entrapped antiserum (0 μL, "empty" column) indicates non-specific binding. Binding was tested with 1,000 ng of IMT. Elution was with 10 mL of acetonitrile. Amounts of eluted IMT were determined with the OVA chemiluminescent ELISA format. Values were obtained after logit-log transformation of the data by means of Microcal Origin software. Each *bar* represents the mean±SEM of three to seven measurements (for 0, 30, and 60 μL, $n=5$; for 120 μL, $n=7$; for 240 μL, $n=3$). *Means with the same letter* do not differ significantly at $p<0.05$

that very small amounts of antiserum—1 to 2 μL—did leach from the column during analyte elution and interfered with the ELISA that was used to monitor IMT recovery from the IAP columns. Therefore, there was a need to add a heat-inactivation step to the IAP method prior to analysis of the eluates. In order to prove that the eluate did not contain any interfering factors that might impair the accurate determination of the analyte eluted from the sol–gel IAP columns, empty and doped columns were subjected to the entire process in the absence of an analyte; the eluates were then further concentrated in an SPE column, heat inactivated, and then spiked with 1,000 ng of IMT and tested with the OVA chemiluminescent ELISA. Un-spiked eluates from empty and doped columns served as controls to evaluate interference with the ELISA by the eluate itself, in the absence of an analyte. The results (Table 2) confirmed high accuracy in the determination of IMT content in the spiked eluates from both the empty and the doped sol–gel columns (94 and 120%, respectively), and no IMT could be detected in un-spiked samples, indicating that the samples did not contain interfering factors and could be analyzed directly with the immunochemical assay according to this protocol.

Analysis of spiked and un-spiked serum samples

In order to test the ability of the ELISA to monitor IMT in biological samples, two sets of experiments were carried out: in the first, normal human serum was spiked with IMT (1,000 ng), and the degree of interference of various percentages of serum (0%, 0.5%, 2%, 4%, 8%, 16%, and 50%) with the assay was monitored; in the second set, a calibration curve was generated in 4% serum (diluted in PBS-M) and compared with a curve made up in PBS-M alone. The first experiments revealed that serum at 8% and above heavily interfered with the IMT ELISA: the presence of the serum affected both the Bg of the reaction, (i.e., the non-specific binding of the primary Ab and/or the secondary Ab-HRP conjugate to the OVA coated on the microplate well), and the binding of the primary IMT Ab to the coating antigen (Table 3). Lower concentrations of serum had negligible effects and, as can be seen in Fig. 5, the IMT standard curve generated in the presence of 4% serum was almost identical with that generated in the normal reaction buffer (PBS-M), with only a slight increase in the I_{50} values: 32 and 50 ng mL^{-1} in buffer and 4% serum, respectively.

Sol–gel IAP of IMT from spiked human serum samples

The interference of human serum with the IMT ELISA indicated the need to purify samples prior to the immuno-

Table 2 Determination of IMT content in spiked eluates after sol–gel IAP and solid-phase concentration

Eluate origin	IMT spiked (ng)	Recovery (ng)	Recovery (%)
Sol–gel empty column and SPE	0	0	0
Sol–gel empty column and SPE	1,000	938	94
Sol–gel doped column and SPE	0	0	0
Sol–gel doped column and SPE	1,000	1,199	120

Sol–gel columns (1:6 format) were doped with 120 μL of anti-IMT antiserum. The columns were washed with buffer (with no IMT) and elution solvent (10 mL of acetonitrile), and the eluate was applied on an SPE column and further eluted with 1 mL of methanol. The SPE eluates were evaporated under nitrogen, reconstituted in 1 mL of PBS-M, and spiked with 1,000 ng of IMT. The IMT content in the eluates was monitored with the OVA chemiluminescent ELISA format. Sol–gel columns with no entrapped antiserum (empty columns) and un-spiked eluates from doped and empty columns served as controls. Percentage recovery represents the ratio between the amount (in nanograms) in the eluate, as determined by ELISA, and the amount of spiked analyte

Table 3 Degree of interference of various percentages of normal human serum with IMT ELISA

Human serum (%)	Background (%)	Activity (%)	Interference (%)
0	100	100	0
0.5	135	95	5
2	204	90	10
4	339	82	18
8	552	57	43
16	878	12	88
50	1,117	0	100

Background values represent non-specific binding of primary IMT and/or secondary Abs to OVA adsorbed to the microplate wells. Activity values represent the specific binding of primary IMT Abs to the IMT-OVA coating antigen adsorbed to the microplate wells. All numbers are expressed as the ratios (expressed as percentages) between the OD values obtained in the presence and absence, respectively, of the various amounts of serum, with the OD value in the absence of serum being designated as 100%. Interference is represented by 100 minus the percentage of activity. The experiment was performed with the OVA chemiluminescent format

chemical analysis because high dilution of the serum, i.e., 4% or less, might minimize the matrix interference in the assay, but might also impair the detection of low levels of IMT residues in "real world" samples. Therefore, un-spiked human serum samples and samples spiked with 1,000 ng of IMT were applied on sol–gel IAP columns that had or had not been doped with 120 μL of Abs, and the IMT content of each eluate was monitored by ELISA. Spiked serum samples that had not undergone sol–gel IAP served as controls to monitor the efficiency of the IAP process. The results presented in Table 4 reveal the high recovery rate (82%) of IMT from spiked serum samples and clearly demonstrate the high efficiency of the sol–gel IAP method for removing interfering components from undiluted human serum samples, in a manner that enabled quantitative determination of IMT. Negligible amounts of IMT were detected in un-spiked serum samples that had undergone IAP, and in spiked and un-spiked samples that had passed through "empty" sol–gel columns. Untreated samples (i.e., "before IAP") interfered with the assay, and the yields that were obtained from those spiked serum samples were above 3,000 ng, which clearly indicated a false positive result.

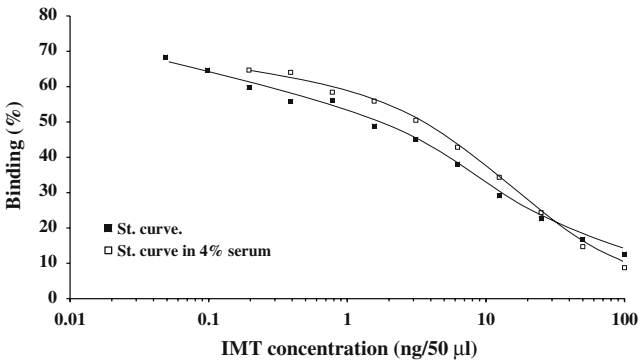

Fig. 5 Comparison of calibration curves of IMT made up in PBS-M or in 4% normal human serum (diluted in the same buffer). ELISA: OVA chemiluminescent format

Compatibility of sol–gel IAP with chemical analytical analysis

Sol–gel eluates were also tested for their compatibility with LC-MS/MS analysis, by examining the extent to which sol–gel eluates that underwent further concentration by SPE interfered with the analysis. For that purpose, 200-mL water samples were applied on empty and doped sol–gel columns; the eluates were subjected to SPE concentration, then spiked with IMT at 10 ng mL^{-1}, and analyzed by LC-MS/MS. The results summarized in Table 5 revealed high accuracy in the determination of IMT content in the spiked eluates from both the empty and the doped sol–gel columns, with a small and insignificant detection deviation of 1.4% to 3.3%. The results in Fig. 6 show that the IMT in the spiked eluates was identical with an IMT standard and generated only a single peak with no impurities. The results indicate that the eluates that originated from 200 mL of tap water that had been concentrated did not interfere with the LC-MS/MS analysis, and indicate that sol–gel IAP/SPE samples could be analyzed directly by LC-MS/MS according to the above protocol, without any further treatment.

Discussion

The present study focused on the development of an immunochemical and a sol–gel-based IAP method for purification and detection of the NSAID IMT. A polyclonal Ab for IMT was generated, and immunochemical assays for its detection, purification, and concentration were developed. The immunochemical detection assays were based on a microplate ELISA; the purification and concentration

Table 4 Recovery of IMT from spiked and un-spiked normal human serum samples before and after sol–gel IAP

Sample	IMT recovery	
	ng	%
Before IAP		
IMT standard	1,000	100
Spiked serum	3,056	306
Un-spiked serum	230	–
After IAP		
IMT standard	250	100
Spiked serum	205	82
Un-spiked serum	76	–
After IAP (empty columns)		
IMT standard	0	–
Spiked serum	11	–
Un-spiked serum	0	–

Normal human serum samples were spiked with 1,000 ng of IMT and applied on sol–gel columns (1:6 format) that contained 120 µL of entrapped anti-IMT antiserum. Sol–gel columns without antiserum (empty columns) served as controls. The IMT content was determined with the OVA chemiluminescent ELISA format. All serum samples were tested at five twofold dilutions in PBS-M: those "before IAP" were diluted 1:2 to 1:32 (equivalent to 50% to 3.125% serum content); those "after IAP" were diluted 1:0 (undiluted) and 1:2 to 1:16 (equivalent to 100% to 6.25% serum). Recovery was calculated as the ratio (expressed as a percentage) between the eluted amount and the amount applied on the column

assay was based on sol–gel IAP. Our previous experience in development of ELISAs revealed that different ELISA formats resulted in differing sensitivities and cross-reactivity patterns, and that different formats reacted differently to the presence of interfering factors in "real world" samples. Therefore, it is essential to develop more than one assay format and to choose the best one for each specific purpose. This is especially important in cases where biological, food, and environmental samples are analyzed because such samples are not homogeneous; they may contain pigmentation and other factors that might interfere heavily and in differing ways with different assay formats. In the light of the above considerations, we

Table 5 Interference of spiked water samples with LC-MS/MS analysis of IMT, following sol–gel IAP and solid-phase concentration

Eluate origin	IMT recovery ± SD (ng mL^{-1})
Sol–gel doped column and SPE	11.81±0.41 (n=9)
Sol–gel empty column and SPE	11.98±0.17 (n=3)

Sol–gel/SPE eluates (concentrated from 200 mL of tap water) were spiked with IMT at 10 ng mL^{-1}. IMT recovery was calculated from a standard curve that ranged from 1 to 200 ng mL^{-1}. Samples were tested by LC-MS/MS, as described in "Materials and methods" section. Limit of detection of the method, 1 ng mL^{-1}

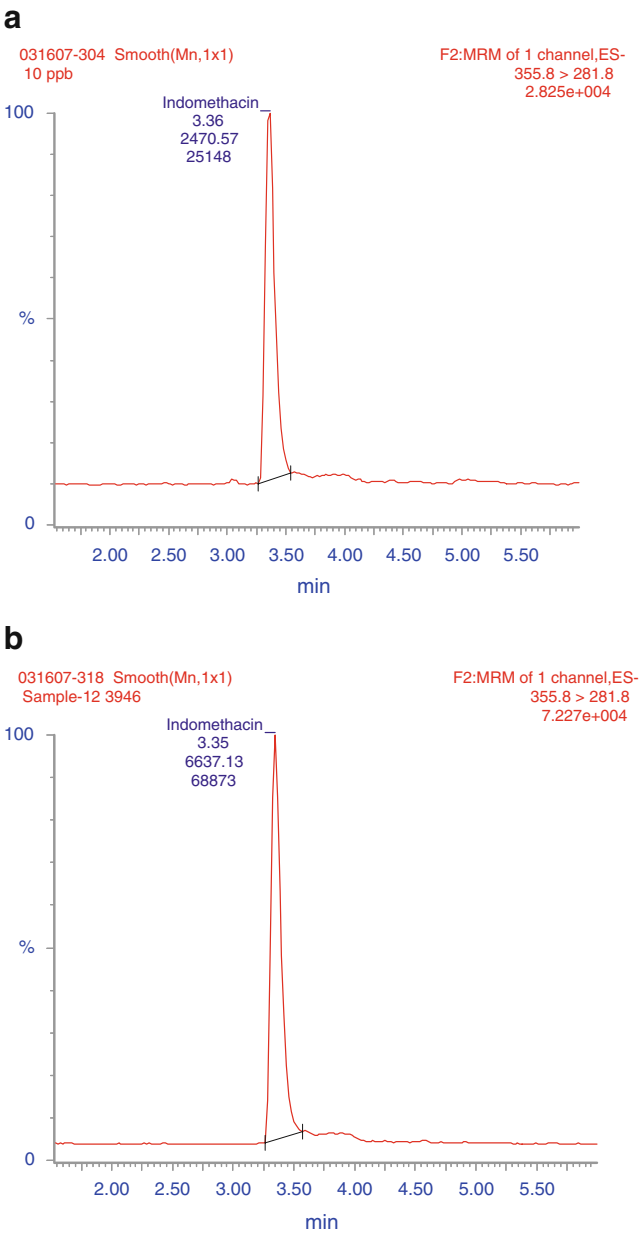

Fig. 6 LC-MS/MS analysis of IMT standard in LC running buffer (**a**) and sol–gel/SPE eluates (concentrated from 200 mL of tap water) spiked with IMT at 10 ng mL^{-1} (**b**). Sample reconstitution and analysis were carried out as described in "Materials and methods" section

developed two different assay formats, designated as OVA and HRP. Both formats exhibited high affinities and low detection limits, and similar I_{50} and I_{20} values: 132±9.3 and 15±1.25 ng mL^{-1} (n=50), respectively, for the OVA and 91±2.59 and 12±0.17 ng mL^{-1} (n=4), respectively, for the HRP format. The assays exhibited tolerance toward 10% ethanol or methanol, and under the test conditions, the IMT antiserum showed high affinity towards IMT and cross reacted to a small extent (<3–9%) with the tested NSAIDs at concentrations up to 2 ppm. The tolerance of the assay to

both of these organic solvents is an important finding in light of the fact that IMT is soluble in organic solvents, i.e., ethanol or methanol in the present study, and is sparingly soluble in neutral aqueous buffers. The low solubility of IMT in aqueous buffers necessitates its extraction and analysis in the presence of organic solvents, and consequently, the immunochemical assays used to monitor it must tolerate the presence of such solvents. The ELISAs described in the present study do fulfill this requirement. Only two immunochemical assays—one ELISA and one RIA—have been described previously [27, 28], and only one has been successfully implemented for monitoring IMT in environmental samples, i.e., water from various sources [28]. A wide variety of chemical methods are currently used to monitor IMT (see [25] and references therein), and most of them exhibit significantly higher limits of detection than the immunochemical assays, e.g., 900 ng mL^{-1} [25], 100 ng mL^{-1} [24], and 50 ng mL^{-1} [22]). The low detection limits introduced by the ELISAs, together with their simplicity and cost effectiveness in implementation, definitely represent advantages for the large-scale application of this method.

The Abs that were developed in the present study were also used in development of a sol–gel-based IAP method for purification and concentration of IMT. For many years, IAP has been considered to be one of the leading technologies for purification, concentration, and isolation of chemical and biological compounds. Recently, IAP was revealed to be a very useful and reliable method for the purification and concentration of agricultural and environmental residue samples [43–48]. The simplicity of using the IAP method and the capability of the method to reduce the levels of organic solvents that are often used to extract the tested analyte makes IAP suitable for agricultural and environmental applications.

The current expansion of applications of the IAP method has emphasized the general need to improve and simplify the method and, simultaneously, to decrease the costs of its use. Application of IAP to agricultural and environmental samples (food, soil extracts, effluent water, etc.), in contrast to medical applications, requires, in addition to other features, the ability to protect the Abs against factors, e.g., denaturing factors or organic solvents, that are present in extracts of the tested samples and that might impair the activity of the Abs. One of the main goals of this research has been to develop a method that fulfills these requirements and is also highly effective in purification of the analyte obtained from biological, environmental, and food samples. In the present study we have, therefore, focused on the development of an effective sol–gel-based IAP system for purification and concentration of IMT. To this end, IMT antiserum was entrapped in a SiO_2 sol–gel polymer, and the binding properties of the column—capacity, antiserum leaching, non-specific binding, and reproducibility—were characterized under various polymerization conditions and with diverse amounts of entrapped antiserum. Recovery of the bound analyte was tested with several different eluting solvents. The best and most reproducible format was 1:6 to 120 μL of IMT Abs were entrapped, and the only solvent that eluted IMT was acetonitrile. The binding capacity under these conditions was about 100 to 250 ng of IMT; binding was dose dependent, although it did not increase in a linear manner with concentration, and exhibited very low non-specific binding, i.e., less than 1% of the applied amount. It is interesting to note that the binding capacity did not increase gradually with the increase in the amount of entrapped antiserum being low (10 ng IMT) at amounts of up to 60 μl (equivalent to 3.36 mg protein) and increasing by a factor of 10 (to 111 ng) at 120 μl. The fact that entrapped molecules do not always obey kinetic properties similar to those obtained in solution may result either from interactions between the entrapped biomolecules and the sol–gel monolith or from spatial interferences in Ab-antigen interactions which necessitates to pass a certain "threshold amount" of entrapped antiserum in order to obtain binding.

The sol–gel composition and preparation conditions are known to greatly influence the structure of the polymer. Previous studies have shown that the properties of the biocomposite, i.e., a sol–gel in which a biomolecule is entrapped, can be drastically affected by changes in the TMOS:HCl ratio, and by the involvement in the sol–gel process of additives such as hydrophobic moieties; polymers, e.g., polyethylene glycol (PEG), glycerol, and polyvinylimidazole; surfactants; liposomes; organic solvents, e.g., cyclohexane; polysaccharides, e.g., dextran, cellulose, or chitosan; cofactors, e.g., redox modifiers; or even biological or synthetic materials. Such additives may alter the physical properties of the gel, e.g., its rigidity, mechanical stability, pore size, and optical or electrochemical clarity; they may affect the interactions of the gel with the entrapped biomolecules and enhance its overall activity and stability [49–51]. In all of our previous studies, PEG significantly improved the binding capacity of a variety of analytes and reduced their non-specific binding [52–54]. Interestingly, in the present study, addition of PEG did not improve the binding capacity of IMT (data not shown).

Once the method had been optimized, it was tested for its efficiency in purifying IMT from spiked serum samples. The sol–gel IAP method successfully eliminated interference to a degree that enabled analysis of the purified serum samples by ELISA. Interestingly, the binding capacity of the sol–gel IAP column in the presence of the serum was very similar to that of the IMT standard applied on the column in buffer only—205 and 250 ng, respectively—indicating that the presence of the serum did not signifi-

cantly affect the binding capacity of the entrapped Abs for the analyte; untreated samples interfered strongly with the IMT ELISA. These findings reveal once again an important characteristic of the sol–gel method: the high stability of the entrapped bio-molecule. This results from the protective characteristics of the polymer, which reduce the potential mobility of the entrapped molecule and thereby lower its susceptibility to being denatured or losing activity because of matrix interference. The high stability of sol–gel-entrapped biomolecules has been reported in many other studies ([29] and references therein).

A wide variety of Abs (monoclonal, polyclonal, and purified immunoglobulins—IgGs) have been entrapped in sol–gel polymers, and their applications in IAP of serum and other samples has been reported by many laboratories, including ours [29–32]. The overall advantages of the sol–gel technique, and the successful entrapment of a wide variety of Abs, which enables development of efficient IAP protocols, indicate the generic nature of the method and the practicability of implementing it for purification of a wide range of analytes.

The IMT content in serum or plasma samples is currently monitored by a variety of chemical analytical methods [21, 22, 24]. Not only are those methods less sensitive than immunochemical methods, they also require extensive extraction, heating, and/or hydrolysis or derivatization prior to analysis (to eliminate matrix interference), all of which result in analyte loss, affect accuracy, and limit the widespread employment of these methods for large-scale monitoring. Sample dilution of crude extracts, which is often used to reduce interferences, is not always a valid option, especially in cases where low analyte levels need to be detected. In addition, chemical analytical methods are not cost effective because they involve the use of large volumes of toxic solvents that are both expensive and necessitate costly storage and disposal arrangements. To the best of our knowledge, ELISA has not so far been used for monitoring IMT in serum samples; the only samples that have been monitored by ELISA were water samples from various sources [28], and although ELISA is known to tolerate matrix interference, even some water samples had to be diluted in order to eliminate such interference. Human serum, which is a pigmented matrix, exhibits a much higher potential to interfere with the assay, either by elevating the background "signal" of the reaction or by affecting analyte–Ab interactions, thereby causing false-negative or false-positive results. Indeed, the present study indicated that the assay could tolerate the presence of not more than 4% of the matrix; higher percentages strongly interfered with the assay. Pretreatment of the samples by IAP significantly reduced matrix interference, eliminated the need to adjust pH values for compatibility with the assay, and provided concentrated, ready-to-use samples which enabled analysis of as much as 50 μL of an undiluted serum sample.

The sol–gel IAP was also found to be fully compatible with LC-MS/MS, after the sample had been further concentrated by SPE. A spiked sol–gel/SPE eluate, which consisted of a concentrate of 200 mL of tap water, did not interfere with the analysis, and the qualitative and quantitative analysis results were identical with those yielded by an IMT standard applied in LC running buffer. The fact that purified immuno-affinity samples can be analyzed by LC-MS/MS without further extraction or purification introduces a major advantage to the chemical analytical method because such analyses can be carried out only with highly pure samples, whose preparation necessitates tedious, long, and expensive multi-step processes, which may result in low yields of the tested analyte and production of large volumes of toxic waste. The sol–gel/IAP method described and discussed above eliminates the need for all of these steps, and enables rapid, simple, easy, and inexpensive preparation of high-purity samples that are ready for chemical analysis. It may very well be that further improvement in the sol–gel IAP method will eliminate the use of the SPE step, as in the case of consecutive ELISA analysis. To the best of our knowledge, LC-MS/MS has not yet been implemented for IMT analysis, although the method offers many advantages over the chemical and instrumental methods currently used to monitor IMT. The results of the present study may provide a good basis for further analysis by a combination of a sol–gel IAP method followed by LC-MS/MS.

Summary and conclusions

At present, immunochemical assays such as ELISA can be developed for almost any compound—artificial or natural—and they offer many advantages for quick, low-cost, and efficient analysis of large numbers of samples. However, development of a reliable ELISA requires optimization with respect to the assay format, reagent quantities, signal-to-noise ratio, detection limits, reproducibility and precision, and especially, with the way the samples are prepared for analysis, since this preparation must eliminate interference of the tested samples with the assay. The ELISAs described in the present paper provide sensitive and reproducible assays for monitoring IMT (with I_{50} and I_{20} values of 132±9.3 and 15±1.25 ng mL^{-1} (n=50), respectively, for the OVA format and 91±2.59 and 12±0.17 ng mL^{-1} (n=4), respectively, for the HRP format). The sol–gel technology developed in this study overcomes most of the difficulties introduced by residue analysis in biological samples, e.g., serum, and offers many advantages over the commonly used IAP methods based on Abs that are entrapped or adsorbed by, or covalently bound to, various matrixes. The sol–gel method fulfills the IAP requirements for purification of analytes from crude samples, without the need for any

preliminary treatment prior to the IAP step; it simplifies examination of the samples and decreases the analysis time and cost by enabling sample analysis—by either immunochemical or instrumental chemical analytical methods—after only a single purification step. A combination of both approaches could provide a basis for analysis of IMT in biological samples in order to monitor their pharmacokinetic properties, and their implementation could be extended to studying population exposure to IMT and also to monitoring occurrence of IMT contamination in food, soil, and other environmental samples.

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