Rapid, Direct Enzyme Immunoassay of 11-Keto-Thromboxane B$_2$ in Urine, Validated by Immunoaffinity/Gas Chromatography–Mass Spectrometry

René Djurup,* Chiara Chiabrando, Achim Jörres, Roberto Fanelli, Marie Foegh, Hanne U. Soerensen, and Peer N. Joergensen

We have developed a direct enzyme immunoassay (EIA) for quantifying immunoreactive 11-keto-thromboxane B$_2$ (iKTXB) in unprocessed human urine. Cross-reactivity with other thromboxane metabolites and prostanooids was negligible. Analytical recovery of 11-keto-TXB$_2$ in urine specimens was 97.4% to 99.8%. Total imprecision for two clinical specimens was 8.5% and 12.2%. Intake of acetysalicylic acid decreased the measured concentration of iKTXB. Cardiopulmonary bypass, a procedure known to activate platelets, increased the mean excretion rate of iKTXB 10-fold. Simultaneous gas chromatography–mass spectrometry analysis of 11-keto-TXB$_2$ and 11-keto-2,3-dinor-TXB$_2$ in urine specimens (n = 17) from healthy subjects indicated that urinary iKTXB concentrations measured by EIA represented a sum of the two 11-keto metabolites. We conclude that the direct EIA is sufficiently sensitive, rapid, simple, and specific to allow screening for alterations in thromboxane biosynthesis in patients.

Indexing Terms: platelets • coagulation • thrombosis

Thromboxane A$_2$ (TXA$_2$) is formed from arachidonic acid in several cell types. Under physiological conditions, platelets are the quantitatively most important source of TXA$_2$, with small amounts also being formed in the kidneys and monocytes. TXA$_2$ stimulates secondary platelet aggregation and secretion and takes part in the renal autoregulation. The major breakdown products of TXA$_2$ are thromboxane B$_2$ (TXB$_2$), 2,3-dinor-TXB$_2$, 11-keto-TXB$_2$, and 11-keto-2,3-dinor-TXB$_2$, all of which are found in human urine (1). Measurement of urinary concentrations of TXA$_2$ metabolites may be of interest in the diagnosis of conditions with increased platelet activation or turnover, such as thrombosis in various parts of the body, e.g., coronary artery thrombosis and deep venous thrombosis (2, 3). Clinical use of such assays is hindered by the fact that available assays take several hours to days to complete and may require specialized equipment or preprocessing of the urine (4–10). Furthermore, so far, no direct immunoassay has been convincingly shown to give valid results in urine specimens obtained from human subjects under relevant clinical conditions, i.e., inhibition or stimulation of TXA$_2$ turnover.

We describe here the development and validation of a rapid, direct, competitive enzyme immunoassay (EIA) for measuring the sum of two main metabolites of TXA$_2$, namely, 11-keto-TXB$_2$ and 11-keto-2,3-dinor-TXB$_2$.

Materials and Methods
Preparation of 11-Keto-TXB$_2$ Immune-Stimulating Complexes

Immune-stimulating complexes (ISC) containing 11-keto-TXB$_2$, N-acetylmuramyl-l-alanyl-d-isoglutamine (muramyl dipeptide (MDP)), and keyhole limpet hemocyanin (KLH) were prepared by a modification of the method of Mozes et al. (11):

1. To transform all molecules to the δ-lactone form, we incubated 1.0 mg (2.71 μmol) of 11-keto-TXB$_2$ with 100 µL of glacial acetic acid for 1 h at ambient temperature, then evaporated the acetic acid under a stream of N$_2$. The residue was washed two times with 1.0 mL of methanol, and the methanol was also evaporated under N$_2$.

2. The 11-keto-TXB$_2$ residue was dissolved in 50 µL of dimethylformamide, and 1.55 µmol of MDP was added in a volume of 50 µL. To form amide bonds between the carboxy groups in 11-keto-TXB$_2$ and MDP, and amino groups in KLH (see below), we added 4.7 µmol of N-hydroxybenzotriazole (HOBr) in 94 µL of dimethylformamide, and 4.7 µmol of N-ethyl-N'-(dimethylamino propyl)carbodiimide hydrochloride (EDCI), also in 94 µL of dimethylformamide, to the 11-keto-TXB$_2$/MDP solution. The 11-keto-TXB$_2$/MDP/EDCI/KLH solution was incubated for 1 h at ambient temperature.

3. We then added this solution (in 10 aliquots of 30 µL) to a dark glass vial containing 2.0 mg of KLH in 1.2 mL of 0.1 mol/L NaHCO$_3$ buffer, pH 8.5 (± 0.05), with continuous vigorous mixing. The mixture was incubated for 3 h at ambient temperature. Finally, 1.5 mL of phosphate-buffered saline (PBS; 0.1 mol/L sodium phosphate buffer, pH 7.5, 0.15 mol/L sodium chloride) was added to the vial containing the 11-keto-TXB$_2$/MDP/EDCI/KLH.
4. The ISC samples containing 11-keto-TXB$_2$ were stored in 100-$\mu$L aliquots at $-20^\circ$C.

Immunization of Rabbits

To immunize each of eight rabbits, we diluted 100 $\mu$L of ISC with 900 $\mu$L of PBS. The ISC/PBS solution was mixed with 1.0 mL of incomplete Freund’s adjuvant. The mixture was injected subcutaneously at four different sites, $-0.5$ mL at each site. The immunization was repeated at weeks 3 and 6 and every 4 weeks thereafter. After the third immunization, the rabbits were bled 1 week after each booster immunization. Blood was collected in tubes containing Na$_2$EDTA and indomethacin (approximate final concentrations 28 mmol/L and 15 $\mu$mol/L, respectively). The anticoagulated blood was centrifuged at 2000 $\times$ g at 4 $^\circ$C immediately after bleeding. The antibody-containing plasma was stored in aliquots at $-20^\circ$C.

Purification of IgG from Antibody-Containing Plasma

The antibody-containing plasma was thawed in an ice/water mixture. After thawing, the antiplasma was centrifuged at 7250 $\times$ g at 4 $^\circ$C to remove any remaining platelets. The platelet-poor plasma was then heated to 37 $^\circ$C. To prevent any fibrin precipitation during subsequent IgG purification, we added CaCl$_2$ and thrombin to the plasma to final concentrations 65 mmol/L and 5000 U/L, respectively. After incubation for 10 min at 37 $^\circ$C, and then at ambient temperature for 1 h, the treated plasma was centrifuged at 7250 $\times$ g for 30 min at 4 $^\circ$C. The supernate obtained after centrifugation (henceforth called “antisera”) was used for further purification.

The IgG fraction of the antisera was purified by Protein G Fast Flow (Pharmacia, Uppsala, Sweden), with use of a potassium phosphate buffer, 0.02 mol/L, pH 7.0, for equilibrating, loading, and washing the column, and a citrate/phosphate buffer, pH 2.7 (0.083 mol/L citrate, 0.035 mol/L phosphate), for elution. The fractions were collected in tubes containing one fraction volume of sodium carbonate/hydrogen carbonate buffer, 0.5 mol/L, pH 9.2. After pooling the IgG-containing fractions, we dialyzed the pool against a potassium phosphate buffer (0.1 mol/L potassium phosphate, pH 7.5, 0.15 mol/L sodium chloride) and stored the dialysate in aliquots at $-20^\circ$C.

Preparation of Enzyme–Antibody Conjugate

An enzyme–antibody conjugate (detector conjugate) was prepared by conjugation of an aliquot of the IgG fraction with horseradish peroxidase (POD; EC 1.11.1.7). The preparation was done with minor modifications of the method described by Iabikawa et al. (12):

**Thiolation of IgG.** We added 5170 nmol S-acetylmecaptopcursincin anhydride in 15 $\mu$L of dimethylformamide to $-87$ nmol IgG in 1.1 mL of potassium phosphate buffer, 0.1 mol/L, pH 6.5. One of the two reactive carboxyl groups in each molecule of the anhydride forms amide bonds with amino groups in IgG at a high rate. After incubating the mixture for 30 min at ambient temperature, we added the following reagents in the sequence stated (to deacetylate the acetylthioacetylated IgG: 40 $\mu$L of Na$_3$EDTA, 0.1 mol/L; 200 $\mu$L of Tris-HCl, 0.1 mol/L, pH 7.0; and 200 $\mu$L of hydroxylamine-HCl, 1.0 mol/L, pH 7.0. After further incubation at 30 $^\circ$C for 10 min, the conjugate mixture was transferred to 0.1 mol/L potassium phosphate buffer, pH 6.0, containing 0.05 mol/L Na$_4$ EDTA, by means of gel filtration. The volume of the effluent, containing thiolated IgG (IgG-SH), was 2.2 mL.

** Incorporation of maleimide groups into POD.** Maleimide groups were incorporated into POD by use of the reagent sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). After dissolving $1.5$ mmol (40 mg) of POD in 4.0 mL of potassium phosphate buffer, 0.1 mol/L, pH 7.0, we added 13.1 mg of sulfo-SMCC (30 $\mu$L) dry powder to the solution. We incubated this for 30 min at 37 $^\circ$C, then separated the sulfo-SMCC/POD conjugate (which may contain a small amount of free POD) from free sulfo-SMCC by gel filtration on a Sephadex G25 Fine column (Pharmacia). Fractions of 1.0 mL were collected. The two fractions giving the highest absorbance at 403 nm were pooled and used for conjugate preparation (see below). These two fractions contained about 1000 nmol of maleimide-activated POD (sulfo-SMCC/POD), which can react with thiolated IgG (see below).

**Formation and purification of enzyme–antibody conjugate.** The 2.2 mL of IgG-SH (50 nmol of IgG-SH) was incubated with the 2.0 mL of sulfo-SMCC/POD overnight at 4 $^\circ$C. The reaction was stopped by adding 55.0 $\mu$L of N-ethylmaleimide in 0.5 mL of potassium phosphate buffer, 0.1 mol/L, pH 6.0. After incubation for 20 min at 37 $^\circ$C, the enzyme–antibody conjugate was purified on a 1.6 cm x 70 cm column containing Superose 12 prep (Pharmacia) by using automated Fast Protein Liquid Chromatography (Pharmacia) equipment. Potassium phosphate buffer, 0.1 mol/L, pH 7.0, was used for equilibration of the column and elution of the enzyme–antibody conjugate. The fractions containing molecular masses in the range 235 to 470 kDa were pooled. The POD/IgG ratio in the pool was 2.7.

Preparation of 11-Keto-TXB$_2$–Bovine Serum Albumin

The “capture conjugate” was prepared as follows:

1. The methyl acetate of 1.0 mg of 11-keto-TXB$_2$ in 10 g/L methyl acetate was evaporated with Na$_2$. The 11-keto-TXB$_2$ residue was washed three times with 0.5 mL of methanol. Methanol was evaporated with Na$_2$.

2. The 11-keto-TXB$_2$ residue was incubated with 100 $\mu$L of acetic acid (100%) for 1 h at ambient temperature. Acetic acid was evaporated with Na$_2$. The residue was washed two times with 0.5 mL of methanol.

3. The 11-keto-TXB$_2$ (27 $\mu$L) was dissolved in 100 $\mu$L of dimethylformamide, and 2.7 $\mu$L of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in 50 $\mu$L of dimethylformamide was added. The mixture was incubated for 1 h at ambient temperature. The dihydroquinoline forms a mixed anhydride with 11-keto-TXB$_2$, and the mixed anhydride then reacts with amino groups in bo-
vine serum albumin (BSA) (see below), forming amide bonds.

4. This solution was then added (in 10-μL aliquots) to 0.27 μmol (19 mg) of BSA in 1.5 mL of distilled water and incubated for 3 h at ambient temperature, with mild stirring. The conjugate was isolated by means of gel filtration on a small disposable column (PD-10; Pharmacia) equilibrated with PBS. The volume of the eluate, which contained the capture conjugate, was adjusted to 3.5 mL with PBS and stored in aliquots at -20 °C.

Coating of Plates with 11-Keto-TXB₂-BSA

The 11-keto-TXB₂-BSA capture conjugate was diluted to 20 mg/L in sodium carbonate/hydrogen carbonate buffer, pH 9.2 (± 1). Each well in the microtiter strip (Nunc, Roskilde, Denmark; code no. 473709) was incubated with 200 μL of diluted capture conjugate overnight at ambient temperature. Nonspecific binding sites in each well were then blocked by overnight incubation with 300 μL of blocking buffer (9 g/L NaCl, 60 g/L sorbitol, 5 g/L BSA, and 0.5 g/L sodium azide, pH 7.7 ± 0.1). After aspirating the blocking buffer from the wells, we added 25 μL of blocking buffer, covered the plate with an adhesive plastic foil, and stored the plate in a bag containing a 10 × 10 cm cloth saturated with a 1 g/L solution of the preservative Germall II.

Preparation of 11-Keto-TXB₂ Calibrators

All calibrators were prepared gravimetrically: An approximate volume was pipetted in a preweighed vial, and the exact volume dispensed was calculated by dividing the weight of the solution in question by its density. The volume of the diluent needed to give the desired concentration was calculated and expressed as a mass by multiplication by the density of the diluent. The needed mass was then added by weighing. First, 50 μL of 11-keto-TXB₂ in 10 g/L methyl acetate (Cayman Chemical, Ann Arbor, MI; cat. no. 19500) was diluted into glycine buffer (0.1 mol/L, pH 9.0) to give a stock solution containing 100 000 ng/mL. The stock solution was kept at ambient temperature overnight to ensure the opening of the lactone ring in 11-keto-TXB₂. The stock solution was further diluted in several steps in calibrator buffer (0.2 mol/L glycine buffer, pH 9.5, containing 1 g/L BSA, 50 g/L mannitol, and 50 mg/L thimerosal) to give calibrators with concentrations of 4.00, 2.00, 1.00, 0.500, 0.250, 0.125, and 0.00 μg/L. The calibrators were freeze-dried and stored at 4 °C. Before use in the assay, the calibrators were reconstituted with distilled water.

Standard Assay for Immunoreactive 11-Keto-TXB₂ (iKTXB₂)

Preincubation. Add 100 μL of buffer A (0.2 mol/L glycine, pH 9.5, containing 1 g/L BSA and 50 mg/L thimerosal) to a Minisorp test tube (Nunc), then add 100 μL of unprocessed urine and incubate for 1 h at ambient temperature.

Incubation. Wash the polystyrene strips coated with 11-keto-TXB₂-BSA four times with washing buffer (0.1 mol/L glycine buffer, pH 9.5, containing 0.5 mL/L Tween 20, and 50 mg/L thimerosal), then pipette 20 μL of the preincubated urine specimens or control or 20 μL of undiluted calibrator into the wells of the washed strip. To each well add 200 μL of anti-11-keto-TXB₂-POD detector conjugate (diluted to ~1 nmol/L (measured as POD) in buffer B). Incubate the mixture on a plate shaker for 1 h at ambient temperature, then wash the strips four times with washing buffer and add 200 μL of substrate buffer (containing 0.8 g/L o-phenylenediamine and 0.66 g/L sodium perborate in 0.1 mol/L citrate buffer, pH 5.2). After incubation for 8 min on a plate shaker at ambient temperature, stop the reaction by adding 100 μL of 2 mol/L sulfuric acid. Read the plate absorbance at 490 nm (we used an SLT ELISA reader), using 650 nm as reference.

Calculation of results. For curve fitting, we used a weighted four-parameter logistic plot, calculated with Multicalc software from Pharmacia.

Fast assay. The fast version of the assay for iKTXB₂ was carried out the same as the standard assay but with the following modifications: (a) preincubation was reduced from 60 to 5 min; (b) incubation was reduced from 60 to 15 min; (c) only two strips (corresponding to 24 wells) were handled in one setup (to reduce the risk of drift).

Imunoaffinity Extraction

Endogenous cross-reactants. For qualitative studies aimed at identifying endogenous cross-reacting species interfering in direct EIA of urinary 11-keto-TXB₂, urine was extracted by immunoaffinity chromatography. We prepared an immuno sorbent, using the same antibody as in the EIA described above. The IgG fraction (prepared as described above) was immobilized onto CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer’s instructions (10 mg of protein per gram of Sepharose). Immunoaffinity columns were prepared with 0.25 mL of settled gel. Urine extraction and derivatization were carried out as described below (4, 13) without addition of internal standards. Samples were analyzed by gas chromatography–negative ion chemical ionization mass spectrometry (see below) in the full scan mode (mass range 200–800 amu).

11-Keto-TXB₂ and 11-keto-2,3-dinor-TXB₂. For quantitative measurement of 11-dehydro-TXB₂ and 11-keto-2,3-dinor-TXB₂ (identified as a major cross-reacting species in the EIA system; see below), we used an immunosorbent prepared with an antibody raised against 2,3-dinor-TXB₂ (13% and 17% cross-reaction with δ-lactone forms of 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂, respectively). The columns linearly extracted 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂ (0–5 ng each) in the presence of a constant amount of their deuterated analogs, as described for other thromboxanes (4, 13). In brief, urine samples (1–5 mL) were diluted to 20 mL with phosphate buffer (pH 7.4, 50 mmol/L) containing deuterated internal standards, [6H₅]11-keto-TXB₂ and [6H₅]11-keto-2,3-dinor-TXB₂. [6H₅]11-Keto-TXB₂ was purchased from Cayman Chemicals, and [6H₅]11-keto-2,3-dinor-TXB₂ was synthesized as described below. Samples were acidified to pH 2 with 1 mol/L HCl and left to stand overnight to ensure lactonization of 11-keto metabolites. Sample pH was
adjusted to 7.4 just before extraction. After sample percolation at a reduced flow-rate, columns were washed with water and eluted with a small volume of acetone/water (95/5, by vol). Samples were then dried and derivatized as follows: (a) Add 30 µL of pentafluorobenzyl bromide/acetonitrile (1/20, by vol) and 5 µL of diisopropylethylamine to the samples and incubate at 40 °C for 5 min, then dry; (b) add 20 µL of methoxylamine hydrochloride and 30 µL of acetonitrile and incubate at 60 °C for 1 h, then dry; (c) add 50 µL of N,O-bis(trimethylsilyl)trifluoroacetamide and incubate at 60 °C for 15 min.

Preparation of 11-Keto-2,3-Dinor-TXB₂

11-Keto-2,3-dinor-TXB₂ was prepared from 2,3-dinor-TXB₂, as described by Barrow et al. (14) for preparing 11-keto-TXB₂ from TXB₂. In brief, 100 µg of 2,3-dinor-TXB₂ dissolved in chloroform/methanol (1/1, by vol) was reacted for 30 min with 2 mg of silver oxide under N₂. After elimination of the silver oxide, the reaction mixture was purified by thin-layer chromatography (silica gel plate developed with ethyl acetate/isooctane/acetic acid (100/15/10, by vol). A small aliquot of putative 11-keto-2,3-dinor-TXB₂ (eluted at Rₜ = 0.39) was derivatized and analyzed by gas chromatography–mass spectrometry (GC-MS). The compound showed the expected GC behavior and mass spectrum, in comparison with its C₁₂₀ homolog, 11-keto-TXB₂. The mass spectrum of 11-keto-2,3-dinor-TXB₂ was similar to that of 11-keto-TXB₂, both being characterized by intense [M–pentafluorobenzyl]⁻ ions at m/z 483 and 511, respectively. This indicated the expected difference of 28 amu (two methylene units) between the C₁₂₀ and C₁₂₀ homologs. 11-Keto-2,3-dinor-TXB₂ was standardized against 11-keto-TXB₂ by using [²H₄]11-keto-TXB₂ as internal standard and by using selected ion recording of their carboxylate anions (m/z 483, 511, and 515, respectively). [²H₄]11-Keto-2,3-dinor-TXB₂ was prepared from [²H₄]2,3-dinor-TXB₂, as described above for the unlabeled compound.

Measurement of Urinary 11-Keto-TXB₂ and 11-Keto-2,3-dinor-TXB₂ by Negative Ion–Chemical Ionization GC-MS

Thromboxane metabolites were analyzed as previously described (4) by negative ion–chemical ionization GC-MS with a Finnigan 4000 mass spectrometer (Finnigan MAT, San Jose, CA), directly interfaced with a 6500 DANI gas chromatograph (DANI, Monza, Italy) (GC capillary column: CP-Sil 5 CB 25 m x 0.32 mm (i.d.), 0.12 µm film thickness), and equipped with a VECTOr ONE (Teknivent, St. Louis, MO) data system. For quantification we used the stable isotope dilution assay, monitoring the [M–pentafluorobenzyl]⁻ ions of endogenous metabolites and their corresponding deuterated analogs (respectively, m/z 511 and 515 for 11-keto-TXB₂ and [²H₄]11-keto-TXB₂, m/z 483 and 487 for 11-keto-2,3-dinor-TXB₂ and [²H₄]11-keto-2,3-dinor-TXB₂).

Results

Assay Performance

Calibration. Curve fitting was performed by use of the MultiCalc software (Pharmacia), with a weighted four-parameter logistic plot. Figure 1 shows the calibration curves for the standard assay (2 h) and the fast assay (30 min) of iKTXB.

Sensitivity. The sensitivity of the assays was estimated as the minimal detectable concentration (MDC), determined with use of the statistical routine supplied with Multicalc software. In this program, MDC is defined as: MDC = concentration at (y₀ – 3σ) where y₀ is the fitted raw response at the concentration 0.00 μg/L. dY is the maximum numerical value of the estimated error from the response–error relationship and the estimated standard deviation on the 0.00 μg/L calibrator. MDC for the standard iKTXB assay was measured in 52 consecutive assays, each comprising 96 single measurements; the results were MDC = 0.037 ± 0.025 (SD) μg/L.

MDC for the fast iKTXB assay was measured in nine consecutive assays, each comprising 24 single measurements. The results were: MDC = 0.076 ± 0.058 μg/L (± SD). All subsequent data reported were obtained with the standard assay.

Cross-reactivity. Cross-reactivity was first evaluated with available compounds structurally related to 11-keto-TXB₂, i.e., TXB₂, its metabolite 2,3-dinor-TXB₂, and other prostanoids. Increasing amounts (10–10 000 μg/L) of the substances in question were added to the assay. The analyte 11-keto-TXB₂ itself was added in concentrations from 0 to 128 μg/L. For these substances the cross-reactivity was calculated as 100 × IC₅₀(11-keto-TXB₂)/IC₅₀(eicosanoid) where IC₅₀ is the concentration at which one-half of the prostanoid binding is inhibited. In the actual experiment the IC₅₀ value for 11-keto-TXB₂ was 0.32 μg/L.

We found the following cross-reactivities: prostaglandin A₂ = 0.016%, prostaglandin B₂ <0.003%, prostaglandin D₂ = 0.024%, prostaglandin E₂ = 0.003%, 6-keto-

![Fig. 1. Calibration curve for the enzyme immunoassay of immunoreactive 11-keto-TXB₂: +, standard assay; ■, fast assay](image-url)
prostaglandin \( F_{1\alpha} < 0.003\% \), \( \text{TXB}_2 = 0.005\% \), and 2,3-dinor-TXB\(_2\) \(< 0.003\%\). After identifying 11-keto-2,3-dinor-TXB\(_2\) as a putative endogenous cross-reactant (see below), we tested the compound in the EIA system. The reactivity for 11-keto-2,3-dinor-TXB\(_2\) in the assay was indeed increased. Cross-reactivity, measured by adding increasing amounts (0–12.8 \( \mu \)g/L) of 11-keto-2,3-dinor-TXB\(_2\), ranged from 113\% at 0.4 \( \mu \)g/L to 58\% at 12.8 g/L.

Recovery. To urine specimens from 11 apparently healthy subjects we added appropriately diluted solutions of 11-keto-TXB\(_2\), either 0.3 or 3.0 \( \mu \)g/L. The concentration of iKTXB in the specimens without added 11-keto-TXB\(_2\) ranged from 0.789 to 4.43 \( \mu \)g/L.

As control, all specimens were supplemented with the dilution buffer. With 0.3 \( \mu \)g/L added 11-keto-TXB\(_2\), the recovery was 99.8\% \( \pm \) 9.9\% \( (x \pm SD); range 80.3–110\%\). With 3.0 \( \mu \)g/L added, recovery was 97.4\% \( \pm \) 4.9\% \( (range 87.5–105\%\).

Parallelism. Parallelism was examined as suggested \( (15) \) by serial dilution (1:2 to 1:16) of urine specimens obtained from normal subjects and from patients with increased concentrations of iKTXB. The dilution was performed at the preincubation step. Values lower than lowest calibrator \( (0.125 \mu \)g/L) after predilution were rejected. The results are presented in Figure 2. Visual inspection of the curves shows no systematic deviation from linearity for specimens diluted up to eightfold.

Imprecision. Within-assay imprecision was estimated by running three clinical specimens, in 2 replicates, each on two different plates. One obvious outlier was excluded. The results (Table 1) show a CV (calculated on the basis of single determinations) in the range 4.9–7.4\%. Total imprecision was estimated for two clinical specimens (mean concentrations 0.702 and 1.30 \( \mu \)g/L) that were assayed 33 and 27 times, respectively, within 18 months. The total CVs of the assay (based on double determinations) were 8.5\% and 12.2\%, respectively.

![Fig. 2. Test for parallelism: urine specimens were diluted twofold serially and the concentration measured was multiplied by the dilution factor.](Image)

<table>
<thead>
<tr>
<th>Specimen/plate*</th>
<th>Concentration, ( \mu )g/L</th>
<th>CV, %*</th>
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<td>3/2</td>
<td>4.33</td>
<td>4.9</td>
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* Three clinical specimens, each measured in 20 replicates on two plates.

**Interference.** To test pH interference, we adjusted the pH of a urine pool to either 4.0 with 1 mol/L HCl or to pH 8.5 with 1 mol/L NaOH. The same urine pool with an identical volume of distilled water added was used as control. No significant interference was observed.

To test interference from NaCl and urea, we added to a urine pool enough 3 mol/L NaCl solution to give final added NaCl concentrations of 0.3 or 0.6 mol/L. Aliquots of the same urine pool, but with added identical volumes of distilled water instead of saline, were used as controls. No significant interference was observed. Adding a 6 mol/L urea solution to the urine pool to final added concentrations of 0.6 or 1.2 mol/L urea showed no significant interference.

Protein interference was tested by supplementing a urine pool with 50 \( g/L \) solutions of BSA or bovine gamma globulin solution to give final added concentrations of 5 or 10 \( g/L \). Bovine gamma globulin at 10 \( g/L \) increased the measured value by 23\%; otherwise, no significant interference was observed.

**Normal Values**

No proper reference interval was established. However, we collected urine specimens for 6.5 to 17 h from 17 apparently healthy women (ages 24 to 48). The range of urine volume collected was 0.170–1.00 L. The means \( (\pm SD) \) of measured concentration, excretion rate, and ratio of iKTXB \( (\mu g/L) \) to creatinine \( (g) \): 1.83 \( \pm \) 1.43 \( \mu \)g/L, 1.15 \( \pm \) 0.75 \( ng/min \), and 1.47 \( \pm \) 0.92, respectively \( (n = 16) \). Data from one subject was excluded because her urine concentration of iKTXB was 7.9 SD above the mean for the remaining 16 subjects.

**Biological Validation**

**Effect of a single dose of 500 mg of acetylsalicylic acid (ASA) in normal subjects.** Nine adult subjects volunteered to take a tablet containing 500 mg of ASA after voiding. On the following morning a spot urine specimen was obtained from each subject. In all subjects a decrease in iKTXB was observed. The values obtained \(-24 \) h after the intake of ASA were 0–77\% of their respective basal values. Five subjects displayed a decrease of >80\% in urinary concentration of iKTXB; in the other four subjects, the decrease ranged from 23\% to 48.7%.

**Effect of multiple doses of 30 mg of ASA in normal subjects.** Intake of 30 mg of ASA per day for several days has been shown to selectively inhibit platelet cyclooxygenase and therefore platelet TXA\(_2\) production \( (16) \).
Urinary excretion of metabolites of platelet-derived TXA2 metabolites is therefore progressively inhibited by repeated intake of 30 mg of ASA per day. We studied four healthy subjects (ages 25.5 ± 1.7 years, weight 74.5 ± 9.9 kg) before, during, and after oral administration of 30 mg of ASA per day for 8 days (16). ASA was taken at 1100, immediately after voiding. Urine was collected from 1100 to 1700 for three consecutive days before aspirin intake (B1 to B3), on days 5 (A5) and 8 (A8) during ASA intake, and on days 1 (P1), 4 (P4), and 8 (P8) after the drug was discontinued. The urine specimens were examined for iKTXB, 11-keto-TXB2, and 11-keto-2,3-dinor-TXB2 (see also below). The urinary excretion of iKTXB decreased markedly in all four subjects during low-dose administration of ASA (Figure 3).

Effect of in vivo platelet activation. We evaluated 16 patients before and after cardiopulmonary bypass. Urinary excretion rates of iKTXB (mean ± 1 SEM) increased from 2.13 ± 0.34 to 23.4 ± 3.6 ng/min (17).

Method Comparison

In the first phase of the study, iKTXB2 values obtained by EIA were compared with 11-keto-TXB2 values obtained by GC-MS in 17 specimens obtained from patients undergoing cardiopulmonary bypass surgery. Urinary excretion rates (ng/min) of iKTXB (y) were well correlated to those of 11-keto-TXB2 (x); y = 2.55 (SE 0.20) x + 1.96 (SE 1.16) \( r = 0.956 \) (root-mean-squared error (RMSE) 3.40), \( P <0.00001 \). Because the intercept (1.96) was not significantly different from zero (1.69, \( P = 0.111 \)), the regression analysis was repeated with the intercept set at zero. The regression equation then became: y = 2.79 (SE 0.15) x [\( r = 0.977 \) (RMSE 3.60), \( P <0.00001 \). The results of the regression analysis are shown in Figure 4.

In a later phase of the study, when we had discovered that EIA also measures 11-keto-2,3-dinor-TXB2, we compared values of iKTXB2 with 11-keto-TXB2 or with the sum of 11-keto-TXB2 and 11-keto-2,3-dinor-TXB2 in 17 urine specimens obtained from the four healthy subjects (see above) before, during, and after repeated doses of ASA (30 mg/day). Figure 5 shows the correlation obtained between concentrations of iKTXB (y) and 11-keto-TXB2 plus 11-keto-2,3-dinor-TXB2 (x). The regression equation was y = 0.98 (SE 0.10) x + 0.29 (SE 0.11) [\( r = 0.93 \) (RMSE 0.22), \( P <0.00001 \). If we considered the correlation between concentrations of iKTXB (y) and 11-keto-TXB2 alone (x), the resulting regression equation was y = 1.46 (SE 0.14) x + 0.248 (SE 0.11) [\( r = 0.94 \) (RMSE 0.21), \( P <0.00001 \). In both cases, the intercept values were significantly different from zero.

The concentrations of iKTXB, 11-keto-TXB2, 11-keto-2,3-dinor-TXB2, and 11-keto-TXB2 plus 11-keto-2,3-dinor-TXB2 in relation to repeated administration of ASA (30 mg/day) in the four subjects examined were shown in Figure 3.

Major Cross-Reacting Urinary Metabolite

GC-MS analysis of a human urine pool extracted by immunoabsorption with the antibody used in the EIA revealed the presence of a major component, in addition to 11-keto-TXB2 itself. This compound was first tentatively identified as 11-keto-2,3-dinor-TXB2 by GC-MS, by
comparison with its homolog 11-keto-TXB₂ and by the other structurally related homologous pair TXB₂/2,3-dinor-TXB₂. 11-Keto-2,3-dinor-TXB₂ was thereafter synthesized and purified (13). Endogenous 11-keto-2,3-dinor-TXB₂ was identified by showing its identical GC-MS behavior with the pure compound (coincident retention time on a 25-m capillary column and the presence of the base peak at m/z 483 with identical isotopic cluster ions) (13). The cross-reactivity of 11-keto-2,3-dinor-TXB₂ in the EIA was thereafter demonstrated as described above.

Discussion

We have developed a sensitive direct enzyme immunoassay for measuring immunoreactive 11-keto-TXB₂ in human urine. In the first phase of the study, we undertook a complete classical validation of the assay. Recovery, linearity, and imprecision were found to be fully satisfactory for use in clinical measurement of iKTXB₂ in human urine specimens. We also studied the potential influence of widely different variations in the concentrations of sodium chloride, urea, and proteins, which can be found in human urine specimens under unusual circumstances. Except for an increased value after the addition of a high concentration of bovine gamma globulin, we found no significant interference (defined as deviations exceeding the long-term imprecision of the assay).

A major analytical effort was then devoted to defining the specificity of the assay. Cross-reactivity was first evaluated by means of classical procedures, studying available compounds structurally related to the analyte, i.e., TXB₂, 2,3-dinor-TXB₂, and other prostanoids. Despite the negligible cross-reactivity found when testing these compounds, we found that urinary concentrations of 11-keto-TXB₂ measured by EIA were higher than those measured by GC-MS in patients undergoing cardiopulmonary bypass (17), although the values were strongly correlated. Because additional metabolites structurally related to 11-keto-TXB₂ were not available to further test cross-reactivity, we decided to look for major endogenous cross-reactants directly in urine. Our approach to identify unknown endogenous cross-reactants with a given antibody was based on the use of the immobilized antibody to extract reactive compounds from the biological matrix of interest for subsequent GC-MS identification (13). In this case, the conclusive evidence that 11-keto-2,3-dinor-TXB₂ was indeed a major source of EIA immunoactivity in human urine was obtained after several steps. First, GC-MS analysis of antibody-bound compounds isolated from a human urine pool revealed the presence of a major component, in addition to 11-keto-TXB₂, tentatively identified as 11-keto-2,3-dinor-TXB₂. Second, 11-keto-2,3-dinor-TXB₂ was synthesized and purified to obtain final GC-MS identification of the endogenous metabolite. Thereafter, testing the cross-reactivity of pure 11-keto-2,3-dinor-TXB₂ in the EIA system showed that this metabolite displayed noticeable reactivity in the assay.

Finally, parallel measurements by EIA and GC-MS of urine specimens from normal subjects during basal or ASA-inhibited TXA₂ turnover suggested that, under these conditions, iKTXB values represent the sum of 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂. Comparing these results with those obtained earlier in the group of patients undergoing cardiopulmonary bypass (where 11-keto-2,3-dinor-TXB₂ had not been measured), we noted that the correlation coefficient between iKTXB₂ (EIA) and 11-keto-TXB₂ (GC-MS) was higher in this latter group (see Method Comparison). At present, we cannot establish whether the higher correlation coefficient in the cardiopulmonary bypass series may be due to a different proportion of the two 11-keto metabolites in these subjects or to the EIA measurement of some other immunoreactive compound(s). This discrepancy might also be due to the different method used for converting urinary 11-keto thromboxanes into the δ-lactone form for GC-MS analysis in the two phases of the study. In fact, for the cardiopulmonary bypass series (17), we used a shorter incubation based on the current methods (pH 2 for 1 h), whereas for the ASA study, samples were incubated overnight at pH 2. This modification was based on the observation that, because of pH variation of individual urine specimens, incubation at pH 2 for 1 h resulted in incomplete conversion of 11-keto-TXB₂ to the δ-lactone for some samples, with consequent underestimation of the endogenous levels (Chibrando et al., unpublished results).

We therefore postulate that the EIA measures the sum of 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂ under normal and decreased (ASA-inhibited) TXA₂ turnover, and possibly also during increased TXA₂ turnover. More importantly, we found a very good correlation between results obtained with the EIA and the GC-MS method during inhibited, normal, and stimulated TXA₂ turn-
over. To the best of our knowledge, these findings make the present assay the most extensively characterized and best validated direct EIA of 11-keto-TXB₂ metabolites in human urine.

Although the assay will most probably give a very reliable estimate of the sum of 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂ under a wide variety of physiological and pathological conditions, we emphasize that the assay may also measure other substances closely related to 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂. Roberts et al. (1) identified 20 urinary metabolites of TXB₂, of which 16 belonged to the series of compounds formed by dehydrogenation at the 11-position. GC-MS measures individual compounds separately, but an EIA will almost certainly have a broader reactivity—as also found here. The deviation between GC-MS results and EIA results on day P1 in subject 4 (Figure 3) may be caused by such differences in specificity. The most probable clinical use of this assay is to screen urine samples from patients suspected of having ongoing thrombosis, e.g., coronary artery thrombosis or deep venous thrombosis (2, 18, 19). However, the assay could also be useful in monitoring transplanted recipients (20, 21). Earlier findings (2, 17) indicate that an increase in iKTXB may be expected in patients with thrombosis, before significant organ damage has occurred. If this hypothesis is correct, the availability of a rapid assay for detecting iKTXB could be of interest to identify patients who might benefit from intervention with antithrombotic therapy. This hypothesis gained support in a recently completed study (Foegh M, et al., unpublished results) involving assay of urine specimens from 369 patients presenting in the emergency department with acute chest pain. In this group of patients, a positive result for iKTXB in urine, i.e., >0.7 μg/L as measured by the present assay, had a predictive value of 0.67 for myocardial infarction.

We have furthermore shown that it is possible, with the available reagents and format (solid-phase EIA), to obtain reliable results within 30 min. Use of a more appropriate assay format, with faster reaction kinetics (e.g., an immunofiltration assay), should give fully reliable results within 15 min. The antibody we used in the EIA was developed by an immunization technique favoring production of high-affinity antibodies. The antibody was especially selected for its fast reaction kinetics, narrow specificity for 11-keto-TXB₂-related compounds, and lack of undesired cross-reactivities. Therefore, the development of the present assay may represent a significant step towards a clinically useful assay for the measurement of thromboxane metabolites in human urine.

We conclude that this direct EIA for urinary iKTXB results in very reliable estimates of the formation of 11-keto-TXB₂ and 11-keto-2,3-dinor-TXB₂ in the human body. The assay is rapid, simple, and sufficiently specific to allow the detection of alterations in thromboxane biosynthesis in human subjects.

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References