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Development of an enzyme immunoassay for linoleic acid diols in urine

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Abstract

An enzyme-linked immunosorbent assay for the diol derivatives of linoleic acid, *cis*-9,10-dihydroxyoctadec-12(Z)-enoic acid (leukotoxindiol, LTXD) and *cis*-12,13-dihydroxyoctadec-9-(Z)-enoic acid (*iso*-leukotoxindiol, *iso*-LTXD), was developed. Polyclonal antibodies were generated in rabbits using an isomeric LTXD and *iso*-LTXD mixture conjugated with keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA). Coating antigens were synthesized by conjugation of LTXD/*iso*-LTXD, dihydroxystearic acid, ricinoleic acid (OLE), ricelaidic acid (ELA) or 12-hydroxystearic acid to BSA or ovalbumin (OVA). Various linoleic acid derivatives did not cross react significantly. Using the ovalbumin conjugate of ricinoleic acid as a coating antigen, the assay yielded an IC_{50} value of 8 μg/l LTXD/*iso*-LTXD and was applied to the analysis of urine samples. Urine samples were treated with glucuronidase to release LTXD/*iso*-LTXD from its glucuronic acid conjugate. An increase of the LTXD/*iso*-LTXD signal was clearly observed after glucuronidase incubation. Recent evidence suggests that these diols may be involved in diseases such as acute respiratory distress syndrome and cardiovascular diseases, thus this assay will be important in assessing the significance of these compounds as biomarkers for these disease states.
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Keywords: Leukotoxin; Leukotoxin diol; Immunoassay; Biomarker

1. Introduction

The two diol regioisomers of linoleic acid, *cis*-9,10-dihydroxyoctadec-12(Z)-enoic acid (leukotoxindiol, LTXD) and *cis*-12,13-dihydroxyoctadec-9-(Z)-enoic acid (*iso*-leukotoxindiol, *iso*-LTXD), have been identified as the most toxic metabolites of the cytochrome P450 monooxygenase and epoxide hydrolase mediated pathway of linoleic acid [1,2]. The diols as well as the epoxides of polyunsaturated fatty acids, especially linoleic acid and arachidonic acid, are involved in severe diseases [3], e.g. the acute respiratory disease syndrome [4], peroxisomal disorders [5] and cardiovascular diseases [3].

The synthesis starting from linoleic acid via its epoxides, *cis*-9,10-epoxyoctadec-12(Z)-enoic acid (leukotoxin, LTX) and *cis*-12,13-epoxyoctadec-9(Z)-enoic acid (*iso*-leukotoxin, *iso*-LTX), is depicted in Fig. 1 [1,2,4–6]. LTXD and *iso*-LTXD are themselves substrates for human UDP-glucuronyltransferases [6] with UGT2B7 being the major isoform [7]. The conjugation to glucuronic acid [8] represents an effective detoxification of LTXD/*iso*-LTXD. Street et al. [5] report high levels of the glucuronic acid conjugate of LTXD in the urine of patients with generalized peroxisomal disorders compared to healthy humans. The glucuronide of LTXD/*iso*-LTXD was identified by FAB/MS and GC/MS. Urinary background levels

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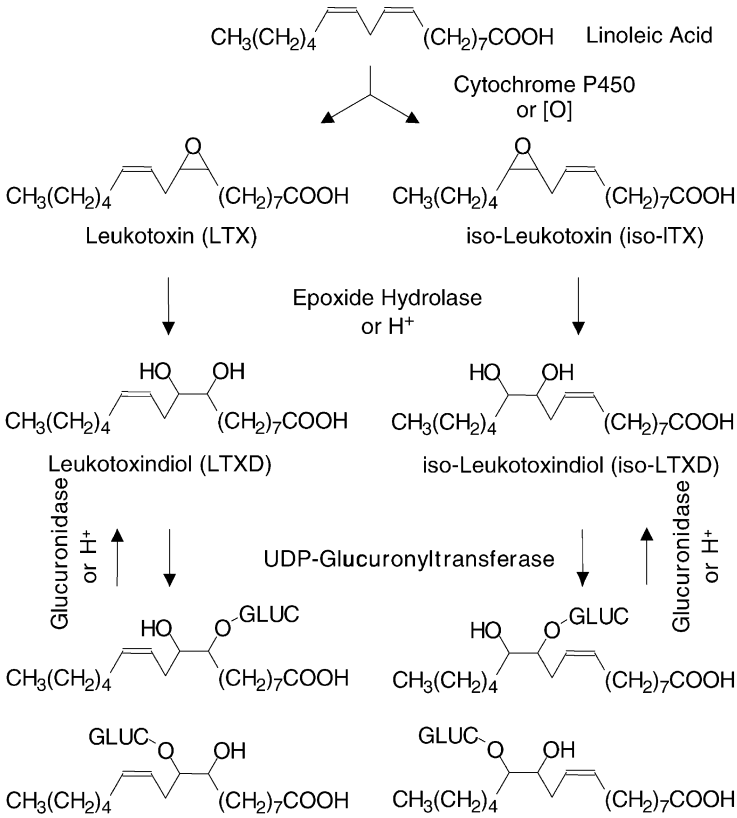

Fig. 1. Metabolic pathway leading to the formation and glucuronidation of LTXD/*iso*-LTXD.

of LTXD/*iso*-LTXD in humans have not yet been investigated. Knowledge of the background levels of endogenous or exogenous toxicants/xenobiotics is crucial for physiological data interpretation. This type of study requires a simple and fast screening assay to monitor large numbers of samples. Immunoassays [9,10] have proven to be valuable tools for this purpose in many clinical investigations [11] and environmental applications [12].

Nagao et al. [13] report an immunoassay for LTX, the epoxide precursor of LTXD, for immunohistochemical applications in rat lung tissue. However, LTX is not suitable as a biomarker for the cytochrome P450/epoxide hydrolase mediated pathway in urine due to the high reactivity and metabolic instability of the epoxide.

The target of this work was to establish an ELISA for LTXD/*iso*-LTXD in urine samples. The major focus was on developing a simple screening method to analyze samples from human control subjects. This ELISA can serve as tool for a first investigation of the significance of a linoleic acid cascade comparable to the well-established arachidonic acid cascade in humans.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals and reagents

Fatty acid epoxides and diols were synthesized in isomeric mixtures according to [2]. Ricinoleic acid (OLE), ricelaidic acid (ELA) and 12-hydroxystearic acid were purchased from Nu-Chek Prep (Elysian, MN). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) and ovalbumin (OVA) in lyophilized form, glucuronidase from *Helix pomatia*

and goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP) were obtained from Sigma (St. Louis, MO). Linoleic acid, Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB) and other chemicals for the preparation of buffers, the synthesis of immunoconjugates and organic solvents, including HPLC grade, were purchased from Aldrich (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA).

2.1.2. ELISA buffers and reagents

All buffers were prepared in water from a Sybron/Barnsted Nanopure II water system (Newton, MS). Coating buffer consisted of 0.05 M sodium carbonate–bicarbonate (pH 9.6). Blocking buffer contained 0.5% BSA in phosphate buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4). Wash buffer contained PBS diluted 1:10 with water (pH 7.4) including 0.0025% Tween-20. Assay buffer was comprised of PBS including 0.0025% Tween-20. TMB substrate solution consisted of 100 mmol/l sodium citrate–acetate buffer (pH 5.5, 10 ml), 1% H_2O_2 (40 μl) and TMB (0.6% w/v in DMSO; 160 μl) [14].

2.1.3. Instrumentation

Absorbance measurements were performed with a UVmax microtiter plate reader from Molecular Devices (Sunnyvale, CA) equipped with data evaluation software (Softmax Pro 1.2). Maxisorb Immunoplates (Nunc, Roskilde, Denmark, part no. 442044) were used for ELISAs. HPLC/MS/MS measurements were carried out with a Micromass Ultima Triple Quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with electrospray ionization (ESI). The chromatographic system consisted of the following components: Waters 2790 HPLC system with 2487 Waters UV–VIS detector 2487 (Waters, Milford, MA), Supelco Discovery HSC18 column 150 mm in length, 2.1 mm i.d., 3 μm particles (Bellefonte, PA).

2.1.4. Synthesis of the immunogen

An isomeric mixture of LTXD/*iso*-LTXD was conjugated to KLH and BSA using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) as a coupling reagent. LTXD/*iso*-LTXD (13.8 mg; 44 μmol) were dissolved in 500 μl DMSO, added drop wise to EDAC (16.9 mg; 88 μmol) in 500 μl phosphate buffer (10 mM, pH 5.05) and stirred for 20 min. The mixture was combined with BSA (25 mg) dissolved in 2 ml borate buffer (100 mmol/l, pH 8.55) and stirred for 1 h at room temperature. The BSA–LTXD/*iso*-LTXD conjugate was dialyzed for 12 h against PBS and for another 8 h against water. The product was lyophilized and stored at −20 °C [15,16].

A KLH–LTXD/*iso*-LTXD conjugate was prepared in the same way from LTXD/*iso*-LTXD (11.3 mg; 36 μmol), EDAC (13.8 mg; 72 μmol) and KLH (20 mg) in the same amount of solvents and buffers.

2.1.5. Immunization

Five female New Zealand white rabbits were immunized. Three with LTXD/*iso*-LTXD–KLH and two with the BSA conjugate. The initial immunization of each animal consisted of 100 μg (measured as protein) of antigen dissolved in sterile PBS and mixed (1:1) with Freund's complete adjuvant to a total volume of 0.5 ml. The immunogen was delivered subcutaneously to multiple sites on the hindquarters. After 30 days, a boost of 100 μg of immunogen was given in Freund's incomplete adjuvant. Fourteen days later, a test bleed was taken. Another boost was given 14 days after the test bleed. This boost and bleed schedule was continued for a total of seven boosts. Fourteen days following the seventh boost, the animal was exsanguinated. All blood samples were allowed to clot. The clot was removed and the serum centrifuged to remove any extraneous cells. The clear serum was frozen at −20 °C until use. Antisera were raised in rabbits 13457, 13498 against the BSA conjugate of LTXD/*iso*-LTXD and in rabbits 13461, 13462, 13465 against KLH conjugate of LTXD/*iso*-LTXD.

2.1.6. Screening of antisera

All antisera were screened for specific binding using a two-dimensional titration, non-competitive ELISA experiment [14]. The antisera 13457 and 13498 were assayed with the KLH–LTXD/*iso*-LTXD conjugate as coating antigen, antisera 13461, 13462 and 13465 with the BSA–LTXD/*iso*-LTXD conjugate, respectively.

2.1.7. Synthesis of coating antigens

LTXD/*iso*-LTXD, (OLE), (ELA), dihydroxystearic acid and 12-hydroxystearic acids were conjugated separately to BSA and OVA. The haptens (13.3 μmol) were activated with EDAC (16.7 μmol) and sulfo-NHS

(20 μmol) in DMF by stirring under N_2 overnight. Twenty-five milligram of protein were dissolved in 6 ml PBS (pH 7.4) at 4 °C for each individual conjugate. The activated hapten solution was added drop wise to the protein solution and the mixture was stirred overnight. The conjugates were dialyzed against PBS for 36 h with buffer changes every 12 h. Conjugates were subsequently lyophilized and stored frozen [16].

Solutions of 1000 μg/ml lyophilized conjugate in water were prepared for further ELISA experiments and stored at 4 °C.

2.1.8. Screening of coating antigens

All synthesized coating antigens were subjected to a two-dimensional titration with antisera 13465. Comparison of the results was achieved by normalizing the data against the highest absolute absorbance value for a coating antigen concentration of 2.5 μg/ml and an antisera dilution of 1/4000. All coating antigens were subsequently screened with antisera 13465 in a competitive inhibition ELISA.

2.1.9. Competitive enzyme immunoassay

All assays were performed using the antigen-coated competitive format [9,14] with at least three replicates per well. Microtiter plates were incubated overnight at 4 °C with 2.5 μg/ml of coating antigen OVA–OLE in coating buffer (100 μl per well). The next day, the plates were washed (three times) with wash solution and incubated for 30 min with 200 μl per well of blocking solution. The plates were washed again (four times). A stock solution of 1000 ppm (m/v) LTXD/*iso*-LTXD in acetonitrile was used to prepare the standard dilution series. Standards were diluted in assay buffer. For the inhibition step, 50 μl per well analyte in assay buffer and 50 μl per well antisera 13465 diluted (1:4000) in assay buffer were added and incubated for 60 min at room temperature. After another washing step (four times), the plates were treated with 100 μl per well goat anti-rabbit immunoglobulin HRP conjugate in assay buffer (1:3000) for 60 min at room temperature. After washing (four times), 100 μl per well of substrate solution was added for an incubation time of 20 min. The enzymatic reaction was stopped by addition of 50 μl per well 2 mol/l of sulfuric acid. The absorbance was read at 450 nm.

For the generation of standard curves, absorbances were plotted against the logarithm of analyte concentration. The curves were fitted to a four parameter logistic equation:

$$y = \frac{A - D}{[1 + (x/C)^B]} + D$$

where A is the maximum absorbance at zero concentration, B the slope at the inflection point, C the analyte concentration (x) giving 50% inhibition (IC_{50}) and D the minimum absorbance at infinite concentration [17].

2.1.10. Cross reactivity

The cross reactivity was determined for nine C_{18} carboxylic acid derivatives and an isomeric mixture of dihydroxyeicosatrienoic acid (DHET) methyl esters, all dissolved in acetonitrile. The cross reactivity was calculated relative to the IC_{50} of LTXD/*iso*-LTXD.

2.1.11. Urine tolerance

The effect of urine on the ELISA was determined by preparing standard solutions of LTXD/*iso*-LTXD in buffer containing 0, 2, 4, 10, 20 and 50% urine of a representative human urine sample on three different days. A single experiment using 100% urine was also performed.

2.1.12. Recovery of target analyte from urine

Urine from one human subject on five different days was spiked with four different concentrations of LTXD/*iso*-LTXD (3, 8, 17 and 35 μg/l) to investigate the matrix influence on the ELISA. These samples were then analyzed at dilutions of 10 and 50% urine. Seven determinations were carried out.

2.1.13. Glucuronidase assay

To optimize conditions for the hydrolysis of LTXD/*iso*-LTXD glucuronide conjugates in human urine, a sample was adjusted to pH 5.5 with citrate buffer (100 mM) and incubated at 37 °C with 0, 10, 25, 50, 100 and 250 units glucuronidase from *Helix pomatia* per milliliter urine. Samples were taken after incubation times of 0, 30, 60, 120 and 240 min and were analyzed with the developed immunoassay.

2.1.14. Urine samples

To confirm the presence of glucuronide conjugates of LTXD/*iso*-LTXD, urine samples from three people were analyzed by ELISA with and without

glucuronidase treatment. Samples were incubated for 4 h at 37 °C with 850 units glucuronidase per milliliter urine. After incubation, samples were diluted with assay buffer for analysis by ELISA.

2.1.15. HPLC conditions

The mobile phase consisted of a binary acetonitrile/water mixture including 0.1% HCOOH at a flow rate of 300 μl/min. The gradient consisted of the following steps: 30% acetonitrile (B) for 1 min, from 30% B to 100% B in 21 min, 100% B for 5 min, from 100% B to 30% in 1 min.

2.1.16. MS conditions

The mass spectra were recorded using ESI in the negative mode. The following parameters were used for all measurements: capillary voltage −3.25 kV; cone voltage −30 V; cone gas (N_2) 100 l/h; desolvation gas (N_2) 700 l/h; source temperature 120 °C; desolvation temperature 430 °C. SCAN mode measurements were performed in a mass range from 150 to 650 *m/z*. In the multiple reaction monitoring (MRM) mode, the collision voltage was set to −22 V, the collision gas (Argon) pressure was 2.5*e*–3 mbar, dwell time was 0.8 s. The respective MRM transitions are given in the Section 3.

2.1.17. Sample preparation for HPLC/MS

Urine samples (4 ml) were extracted twice with ethyl acetate after glucuronidase incubation. Ethyl acetate was evaporated under nitrogen and the sample was reconstituted in 1 ml methanol. The injection volume was 10 μl.

3. Results and discussion

3.1. Screening of the antisera

Antiserum 13465 was the only one exhibiting considerable binding to the coating antigen in a non-competitive ELISA and was therefore chosen for further assay development.

3.2. Coating antigen development

A key to immunoassay development is the design of suitable coating antigens. Heterology in the hapten structure compared to the original immunizing antigen often improves the selectivity and sensitivity of the assay [18]. Ricinoleic acid, ricelaidic acid, dihydroxystearic acid and 12-hydroxystearic acid were selected as structurally similar compounds compared to LTXD/*iso*-LTXD. All substances contain the 18-carbon backbone and the free acid function with varying numbers of hydroxyl groups and/or double bonds. The coupling reaction was carried out between the acid function of the hapten and free amine groups of the carrier proteins. The acid function was activated with EDAC and sulfo-NHS prior to reaction with the amine [15,16]. The activation reaction was slightly varied compared to the preparation of the immunogen since the immunogens were coupled with EDAC only. Variation of coupling reagents may reduce background interferences due to haptens and reagents that are non-covalently bound to the carrier protein.

The synthesized coating antigens were subsequently screened with antiserum 13465 in a two-dimensional titration experiment using a coating antigen concentration of 2.5 μg/ml and an antibody dilution of 1/4000. All coating antigens exhibited specific binding to antiserum 13465. The OVA ricinoleic acid conjugate (OVA–OLE) yielded the highest recognition.

An initial competitive inhibition experiment was carried out with the coating antigens. All tested combinations except OVA–12-hydroxystearic acid showed inhibition (see Table 1). The IC_{50} values of LTXD/*iso*-LTXD ranged from 1 to 10 μg/l. The BSA conjugates exhibited the lowest IC_{50} values, but the substrate incubation time to reach reasonable absorbances was 40 min. The screening assays performed with the OVA conjugates showed IC_{50} values between 1.5 and 5.5 μg/l with higher absorbance values at shorter substrate incubation time (20 min). Within the OVA coating antigen series (as listed in Table 1), the structural variation of the haptens affected the apparent IC_{50} values by as much as a factor of 4, although lower maximum absorbances have to be taken into account. These data showed that several coating antigens held the potential for a sensitive ELISA for LTXD/*iso*-LTXD. The OVA–OLE conjugate was selected for further assay development due to the low IC_{50} combined with a high absorbance.

Table 1
Screening data for competitive inhibition with antiserum 13465

Coating antigen	A[a]	B	C	D	r^2
BSA–LTXD/*iso*-LTXD	0.61	0.91	1.61	0.01	0.99
BSA–ricinoleic acid	0.81	1.18	1.77	0.03	0.99
BSA–ricelaidic acid	0.51	1.40	1.47	0.03	0.99
BSA–dihydroxystearic acid	0.23	1.74	1.29	0.01	0.99
BSA–12-hydroxystearic acid	0.18	9.78	1.08	0.10	0.80
OVA–LTXD/*iso*-LTXD	1.1	0.88	5.48	0.05	0.99
OVA–ricinoleic acid	0.93	1.12	4.41	0.05	0.99
OVA–ricelaidic acid	0.69	1.23	2.36	0.03	0.99
OVA–dihydroxystearic acid	0.30	1.36	1.49	0.03	0.99
OVA–12-hydroxystearic acid	No inhibition				

[a] *A*, *B*, *C* and *D* are values derived from fitting the standard curve data to a four-parameter equation (see text). The r^2 value reflects how well the data fit the derived equation.

3.3. Reproducibility of standard curve

The ELISA using OVA–OLE as a coating antigen proved sensitive and reproducible. Over a period of 1 month, 21 calibration curves were generated, a normalized average of which is represented in Fig. 2. The average IC_{50} of this curve was 8 ± 1 μg/l. The day-to-day IC_{50} value from the 21 individual curves was 9 ± 2 μg/l. The standard curves had maximum absorbances of 0.86, the slopes were near 1 and minimum absorbances were 0.06 on an average. The dynamic range of this assay, determined empirically as the concentrations yielding 20 and 80% inhibition, was 2.5 to 50 μg/l LTXD/*iso*-LTXD.

3.4. Cross reactivities

Cross reactivity experiments to determine the selectivity of the assay for LTXD/*iso*-LTXD compared to similarly structured compounds were performed.

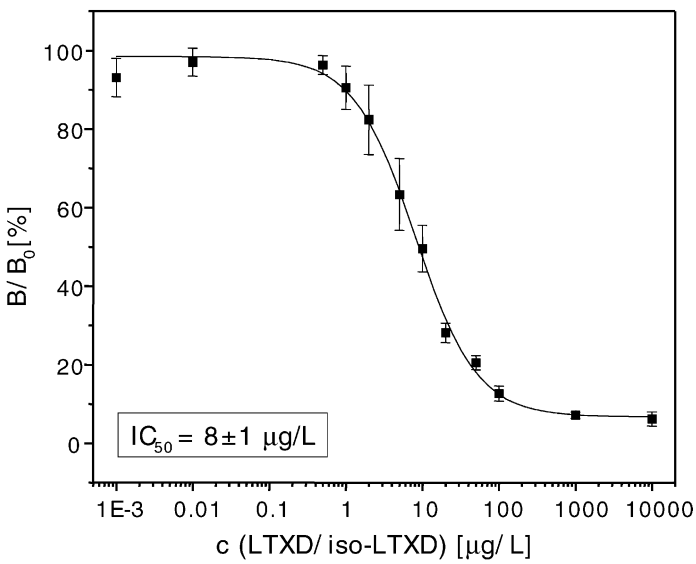

Fig. 2. Standard calibration curve for LTXD/*iso*-LTXD using OVA–ricinoleic acid (OVA–OLE) as a coating antigen and antiserum 13465 ($N = 21$).

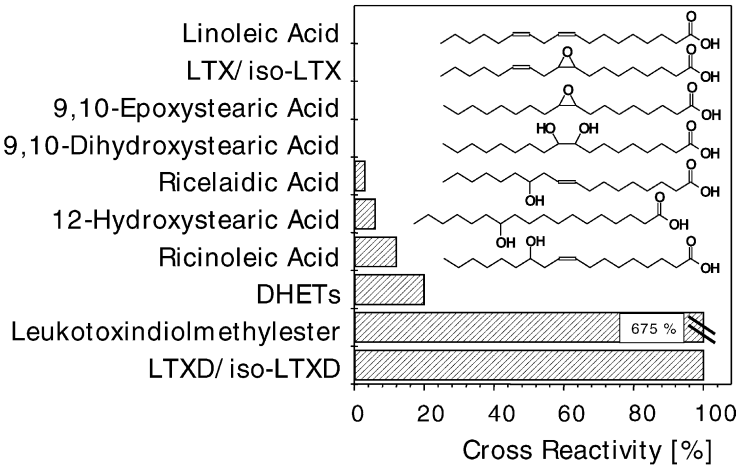

Fig. 3. Cross reactivity of various C_{18} carboxylic acids determined relative to the IC_{50} of LTXD/*iso*-LTXD.

Various 18-carbon fatty acids containing epoxide, hydroxyl functions and/or double bonds were selected. The four dihydroxyeicosatrienoic acid (DHET) isomers also are structurally related to LTXD/*iso*-LTXD due to the presence of the diol and olefin functions. The tested compounds including their structural formulas are presented in Fig. 3. It is of high importance that the assay does not cross react with the direct precursors of LTXD/*iso*-LTXD, linoleic acid and LTX/*iso*-LTX. The data showed that no cross reactivity was observed for these compounds, 9,10-epoxystearic acid and 9,10-hydroxystearic acid. Compounds containing a hydroxyl function at position 12 exhibited minor inhibition. The mixture of DHETs showed an inhibition of 20% compared to LTXD/*iso*-LTXD. Interferences are not expected from these compounds as the abundance of arachidonic acid is significantly lower compared to linoleic acid [19]. Consequently, arachidonic acid derivatives should occur in much lower concentrations. The methyl ester of LTXD/*iso*-LTXD is better recognized by antiserum 13465 compared to the free acid. This is due to the fact that the methyl ester mimics the hapten bound to the carrier protein better than the free fatty acid. Thus, the antibody may also recognize LTXD/*iso*-LTXD bound in other esterified forms, e.g. phospholipids. In case LTXD/*iso*-LTXD is present as an ester in a real sample, results should be given as free and bound LTXD/*iso*-LTXD equivalents.

Matrix effects need to be investigated to guarantee the applicability of the ELISA to real samples. Urine represents a complex matrix of varying salt content, organic compounds and proteins [20]. In many clinical immunoassays, the target analyte concentration is greater than the dynamic range of the assay. Dilution of the sample is therefore required prior to analysis. When urine concentrations in these assays are 2% or lower, matrix interferences are simply diluted out [21–23].

The following experiments were carried out under the assumption that any LTXD/*iso*-LTXD in the urine samples used is primarily excreted conjugated to glucuronic acid [5]. Since the glucuronic acid conjugate apparently does not cross react in the assay (see later), there should be no interference in the matrix and recovery studies.

To determine the degree of dilution needed to avoid matrix interferences for the LTXD/*iso*-LTXD assay, standard calibration curves were prepared in assay buffer with varying urine concentrations (Fig. 4). Urine concentrations of 10% or less exhibited little effect on the calibration curve, including the IC_{50}. At urine concentrations above 10%, the maximum absorbance values were decreased while the IC_{50} values remain stable. An experiment performed in 100% urine resulted in a standard curve that could not be evaluated because absorbances were near the background level.

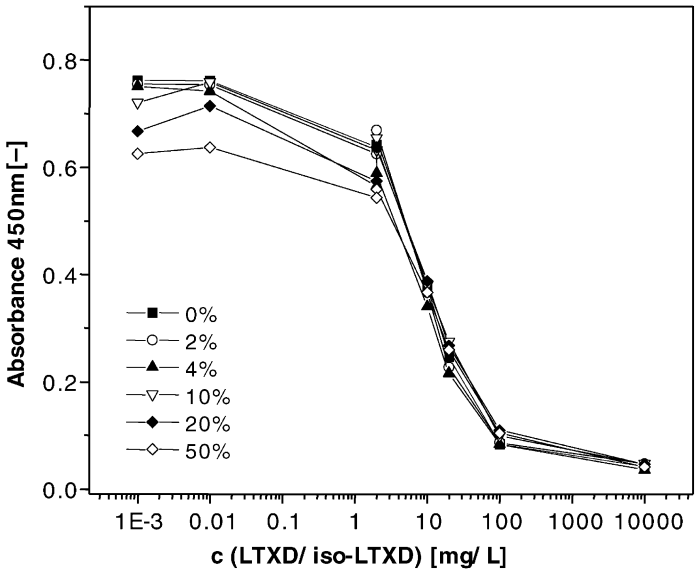

Fig. 4. LTXD/*iso*-LTXD calibration curves in varying concentrations of urine.

In order to simulate the analysis of real samples, a recovery experiment with two different urine dilutions and four different LTXD/*iso*-LTXD spiking concentrations was carried out. The results for a urine dilution of 10% in assay buffer corresponded well with the theoretical values. The standard deviations ranged from 7 to 14% for LTXD/*iso*-LTXD (Table 2). This is acceptable for the analysis of real samples, since first screening experiments will investigate primarily the concentration range of LTXD/*iso*-LTXD present in real samples and person-to-person variation is likely to be much greater. The 3 μg/l spiking concentration value for the 10% urine dilution showed a higher standard deviation of 25%. At urine concentrations of 50%, the recovery values consistently exceeded the theoretical values and are characterized by high standard deviations of 26–41%. The large determination error in the 50% samples and undiluted urine (data not shown) was likely due to matrix interferences, especially high salt concentrations. Nevertheless, these data indicate that the ELISA is well suited for the analysis of urine samples diluted to 10% in assay buffer.

Table 2
Percent recovery from urine spiked with LTXD/*iso*-LTXD[a]

Urine (%)	Spiking level (μg/l)			
	35	17	8	3
10	105 ± 14	101 ± 7	108 ± 13	127 ± 25
50	120 ± 35	135 ± 26	175 ± 34	310 ± 41

[a] One human urine sample was diluted to either 10 or 50% with water. Seven aliquots of each dilution were spiked at the concentrations given and analyzed for recovery.

As LTXD/*iso*-LTXD is mainly excreted as the glucuronic acid conjugate in urine [5], an enzymatic cleavage of the conjugate with glucuronidase was required prior to the determination by ELISA. The total amount of glucuronides in human urine excreted in 24 h can reach millimolar levels [24]. It is important that sufficient glucuronidase is present to cleave both the target analyte and other glucuronides present in the sample. A representative urine sample was incubated with different glucuronidase concentrations for up to 4 h to determine the minimum amount of enzyme and incubation time. In the control sample, no increase in the LTXD/*iso*-LTXD concentration was observed over the whole time range. This demonstrates that background hydrolysis at pH 5.5 does not significantly contribute to the overall result in this particular sample. Low enzyme concentrations of 10–50 units/ml urine yielded only incomplete hydrolysis of 32–71% within 4 h. For enzyme concentrations of 100

Table 3
Concentrations of LTXD/*iso*-LTXD in urine with and without glucuronidase treatment

	pH	LTXD/*iso*-LTXD (μg/l)	
		No glucuronidase (%)	With glucuronidase (%)
Urine A[a]	7	n.d.	412 ± 11
Urine B	5.5	72 ± 4	445 ± 7
Rat urine	8.5	122 ± 1	156 ± 1

[a] Urine A and B were human samples from a female and male, respectively. The rat urine sample was from a spontaneously hypertensive rat.

units/ml urine or higher, the hydrolysis rate reached a stable level of 85–100% after 2 h incubation time. Consequently, the minimum amount of enzyme required for a complete hydrolysis of glucuronic acid conjugates in the analyzed sample was 100 units/ml urine with an incubation time of 2 h. The concentration of glucuronic acid conjugates in urine is characterized by a high person-to-person variability [25]. To guarantee a complete enzymatic cleavage, an amount of 500 units or more glucuronidase/ml urine and an incubation time of 4 h were chosen for the analysis of real samples.

Three spot urine samples from two human subjects (A, female; B, male) and one rat (spontaneous hypertensive rat, SHR) were analyzed with the ELISA. LTXD/*iso*-LTXD was clearly detected in all samples after treatment with glucuronidase (see Table 3). In both human samples, a significant increase in the LTXD/*iso*-LTXD concentration to approximately 450 μg/l was observed after the enzymatic reaction. Urine sample B with an initial pH value of 5.5 exhibited hydrolysis without presence of glucuronidase. The LTXD/*iso*-LTXD concentrations of the rat urine were very similar in the control and in the glucuronidase treated sample. This result is in agreement with results from Maier et al. [25] who observed no increase in 20-hydroxyeicosatetraenoic acid concentrations in rat urine after glucuronidase incubation.

The presence of LTXD/*iso*-LTXD was furthermore confirmed by HPLC/MS/MS analysis of urine samples treated with glucuronidase. LXTD and *iso*-LTXD were separated by reversed phase liquid chromatography. ESI in the negative mode was applied for ionization of the fatty acid derivatives yielding deprotonation of the acid function and formation of a 313 *m*/*z* pseudomolecular anion. The isomers can be distinguished by their MS/MS spectra [26]. Characteristic MRM transitions for LTXD (313–201 *m*/*z*) and *iso*-LTXD (313–183 *m*/*z*) were selected for mass measurements. LTXD/*iso*-LTXD were clearly detected in the urine sample after glucuronidase treatment and identified by HPLC/MS/MS according to their mass spectra and retention times.

4. Conclusions

An ELISA for LTXD/*iso*-LTXD yielding detection limits in the low micrograms per liter range was successfully developed. The assay exhibited little cross reactivity to other linoleic acid derivatives. Due to the sensitivity of the assay and the tolerance for the urine matrix, only a simple dilution step was required for analysis. Urine samples treated with glucuronidase showed a significant increase in LTXD/*iso*-LTXD levels. HPLC/MS/MS was used to confirm the presence of LTXD/*iso*-LTXD in urine. For large numbers of samples, the ELISA will provide an excellent screening tool for the detection and semi-quantitative determination of LTXD/*iso*-LTXD. This method is the first assay suitable for large-scale clinical investigation of a tentative linoleic acid cascade and to develop and test hypotheses that suggest the LTXD/*iso*-LTXD may be useful biomarkers of certain disease states.

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