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Determination of telmisartan in human blood plasma Part I: Immunoassay development

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Abstract

Telmisartan is an angiotensin II receptor antagonist and a known drug against high blood pressure. In this report, the development of a new and rapid analytical technique, an enzyme-linked immunosorbent assay (ELISA) for the determination of telmisartan in human blood plasma is described. The immunoassay is based on a conversion of 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) to 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA), which is detected by fluorescence spectroscopy. The limit of detection was 0.1 ng/mL, the limit of quantification was 0.3 ng/mL and the working range extended from 0.3 ng/mL to 300 ng/mL.

Keywords: Telmisartan; ELISA; MNBDH; Glucose oxidase; MNBDA

1. Introduction

High blood pressure (hypertension) is a widespread disease and an important risk factor for vascular disease, renal and cardiac insufficiency [1]. The drug telmisartan, 4-[(2-*n*-propyl-4-methyl-6-(1-methylbenzimidazole-2-yl)-benzimidazole-1-yl)-methyl]-biphenyl-2-carboxylic acid (Fig. 1), is an angiotensin II receptor antagonist, which is highly selective for angiotensin II (AT_1) receptors. Angiotensin II is generated from angiotensin I and affects the blood pressure [2,3] owing to different mechanisms: by increasing the activity of the sympathetic nervous system, by causing a boosted sodium revertive resorption in the kidneys and by promotion of the secretion of aldosterone in the adrenal glands. Telmisartan is a benzimidazole derivative, which selectively inhibits the receptor of angiotensin II. Therefore, the effect of angiotensin II is blocked thus resulting in a steady vascular dilatation and abated blood pressure [4].

The need for drugs which abate high blood pressure is evident and is still increasing due to the growing number of humans in the western society who fulfill the risk factors for hypertension (e.g. stress, wrong food, overweight, alcohol). Regarding this, the pharmacokinetic investigation of telmisartan in human blood plasma or in urine is important.

However, already many publications discuss the pharmacokinetic behavior and the properties of telmisartan [5,6]. Due to its high lipophilicity and a high volume distribution, telmisartan shows a good tissue penetration. It is highly bound to plasma proteins (99.5%) [6]. A long half-life results in a recommended dose of usually 40 mg once per day. Telmisartan is subject to a very limited phase-I-metabolism so that the potential of interaction of this pharmaceutical is also low with respect to other drugs (e.g. paracetamol). Ebner et al., among others, investigated the chemical stability of telmisartan in the human body [7]. It undergoes only minimal biotransformation in the liver to form its major inactive metabolite, telmisartan acylglucuronide. However, only few studies on the decomposition of telmisartan in the human body, on the dose as well as on the combination of telmisartan with other drugs [6], have been published to date.

In 2002, Gonzalez et al. screened several angiotensin II receptor antagonists, amongst them telmisartan, by means of capillary zone electrophoresis (CZE) with UV/vis detection [8]. They were able to separate seven angiotensin II receptor antagonists within 8 min. Comparative HPLC measurements took 25 min to obtain the same resolution. In the same year, similar

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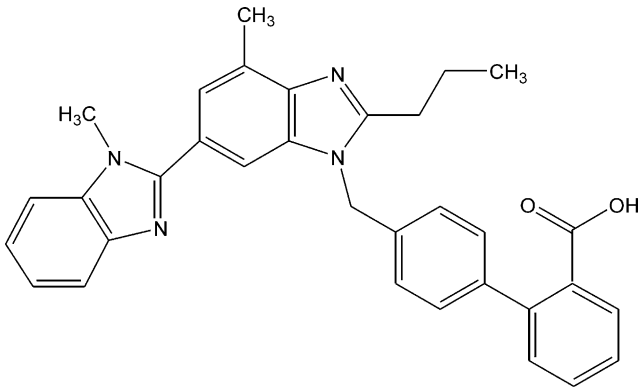

Fig. 1. Chemical structure of telmisartan.

CZE/UV/vis investigations were performed by Hillaert et al. [9]. They described the separation of candesartan, eprosartan, irbesartan, losartan potassium, telmisartan and valsartan. The limit of quantification was in the range of 0.05–0.07 mg/mL for the determined drugs. In 2003, they transferred the separation of these angiotensin II receptor antagonists to micellar electrokinetic chromatography [10]. The observed sensitivity was comparable with the results of the CZE investigations.

Maotin et al. developed a polarographic method for the determination of telmisartan [11,12], which was applied to its determination in pharmaceutical preparations and in biological samples such as human serum. They achieved a detection limit of 0.05 mg/mL.

In 2003, Torrealday et al. published investigations on the fluorescence detection of the native telmisartan after LC separation of urine samples, which were cleaned up previously by means of solid-phase extraction (SPE) [13]. However, although both selectivity and sensitivity of fluorescence detection (approximately 1 ng/mL) are superior to those of UV/vis absorbance, it is doubtful if the analytical figures of merit, which can be achieved with this method, are sufficient for highly complex samples.

Although telmisartan has already been available on the market since the late 1990s, these few publications regarding the analysis of this drug have mainly been published later than the year 2000. However, HPLC-based methods with either UV/vis [14] or fluorescence [15,16] detection were published for some other angiotensin II receptor antagonists as e.g. losartan potassium, candesartan or irbesartan. Kondo et al. presented an LC/electrospray–tandem MS method applying an off-line sample extraction procedure for the characterization of metabolites of the angiotensin II receptor antagonist candesartan cilexetil in rat blood plasma in 1996 [17]. Recently, Chen et al. published a paper on the determination of telmisartan in human blood plasma samples by a LC/ESI–MS method in the selected-ion monitoring (SIM) mode. They worked with an off-line procedure for sample preparation and used valsartan as internal standard [18]. The limit of quantification was in this case 1 ng/mL.

Enzyme-linked immunosorbent assays (ELISAs) find widespread application in clinical chemistry and related fields. They are characterized by low limits of detection and high selectivity for the analyte and, depending on the antibodies used, a limited number of very closely related compounds. Additional advantages are the possibilities of high sample throughput and low operating costs. Thus, we decided to develop an ELISA method based on the glucose oxidase-catalyzed conversion of 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) to the fluorescent 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA) for the determination of telmisartan in human plasma samples [19,20]. This is the first time that MNBDH has been used as substrate in an immunoassay. This reaction system, yielding MNBDA as product, was chosen due to usually higher sensitivity and selectivity of fluorescent detection schemes in comparison to UV/vis detection. It is the first time that such an approach is described in literature for the analysis of telmisartan or related compounds.

2. Experimental

2.1. Materials

Telmisartan reference substance (4-[(2-*n*-propyl-4-methyl-6-(1-methylbenzimidazole-2-yl)-benzimidazole-1-yl)methyl]-biphenyl-2-carboxylic acid), the hapten telmisartan-(2-amino acetic acid) (Tel-AAA) (see also Fig. 2), which is coupled to the enzyme glucose oxidase and the biotin-labeled polyclonal rabbit anti-telmisartan IgG were kindly provided by Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach, Germany). Bovine serum albumin (BSA) was purchased from Serva (Heidelberg, Germany). The enzymes glucose oxidase (GOD, E.C. 1.1.3.4) and horseradish peroxidase (POD, E.C. 1.11.1.7) as well as avidin were purchased from Sigma (Deisenhofen, Germany). The synthesis of 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole was performed as described in literature [21]. All other chemicals were purchased from Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany) in the highest quality available.

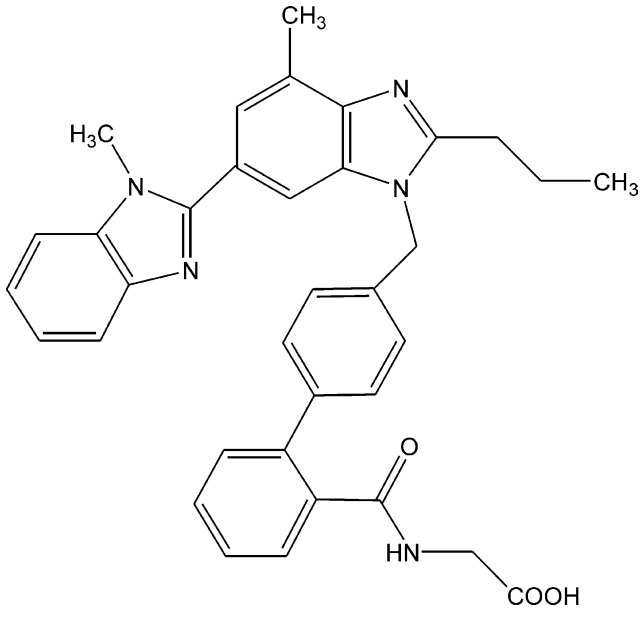

Fig. 2. Chemical structure of the hapten used for tracer synthesis (Tel-AAA).

2.1.1. Buffers

Buffer 1: 0.05 mol/L Na_2HPO_4/KH_2PO_4, 0.15 mol/L NaCl, pH 7.4;
Buffer 2: 0.05 mol/L Na_2HPO_4/KH_2PO_4, 0.15 mol/L NaCl, 5 g/L BSA, pH 7.4;
Buffer 3: 0.05 mol/L Na_2HPO_4/KH_2PO_4, 0.15 mol/L NaCl, 5 g/L BSA, 0.5 g/L NaN_3, pH 7.4.

2.2. Enzyme-tracer synthesis

The telmisartan-GOD conjugate was synthesized according to the following procedure: a solution of 65 mg GOD in 0.75 mL $NaHCO_3$ solution (0.13 mol/L) was mixed under stirring with 0.75 mL DMF (resulting pH ∼ 9.6). The pH was adjusted to 7.1 and the solution was cooled in an ice bath ($T = 0\,^{\circ}C$). A solution of 31.3 mg of the hapten Tel-AAA in 1 mL DMF (anhydrous) was cooled under stirring to −25 °C. *N*-Methylmorpholine (11 μL) and isobutyl-chloroformate (6.5 μL) were added to the latter, and the mixture was incubated for 10 min at −25 °C. Afterwards, 15.3 μL of this solution were slowly pipetted under stirring at 0 °C to the GOD solution. The pH was kept at 7.0. The mixture was incubated under stirring at 0 °C for 2.5 h.

The synthesized GOD-conjugate was purified by means of size-exclusion chromatography. The conjugate was added onto a PD10 Sephadex G25 column and purified with Tris buffer at pH 7.5 (0.05 mol/L Tris, 0.15 mol/L NaCl). $NH_2OH{\cdot}HCl$ (42.9 mg for each milliliter of purified conjugate) was added to the conjugate fraction. The mixture was stirred for 30 min at room temperature. Then, the conjugate was cleaned for a second time on a Sephadex G25 column (buffer: 0.1 mol/L phosphate, pH 7.0). For storage at −20 °C, 1.125 mL glycerin, 10.6 mg BSA and 1 mg thymol (per milliliter conjugate) were added to the GOD-tracer fraction.

2.3. Instruments

The ELISA detection was performed using a FLUOstar microplate reader from BMG LabTechnologies (Offenburg, Germany) with FLUOstar Galaxy software version 4.30-0. For the measurements, the following filters were used: 470 nm (bandwidth ±15 nm) for excitation and 545 nm (bandwidth ±10 nm) for emission. Nunc 96-well microtitration plates (Maxisorp, black) used for these measurements were purchased from VWR International B.V. (Roden, The Netherlands) and Corning (Costar No. 3915, black) microtitration plates were purchased from Diagonal (Münster, Germany).

2.4. Optimization of ELISA parameters

In order to find the best parameters for the performance of the immunoassay, the ELISA was carried out as described below for different substrate concentrations. The avidin concentration was varied between 0.05 μg/mL, 0.5 μg/mL and 5 μg/mL, the biotin-labeled anti-telmisartan IgG concentration between 0.15 ng/mL, 1.5 ng/mL, 45 ng/mL, 150 ng/mL and 1500 ng/mL and the GOD-conjugate dilution between 1:100, 1:300, 1:1000, 1:3000, 1:10,000 and 1:90,000. The immunoassays were performed for calibration standards in the range from 0.01 ng/mL to 3000 ng/ml. Additionally, two different types of microtitration plates were tested: the black Maxisorp plate from Nunc and the black Costar 96 microtitration plate from Corning. The different calibration curves were compared.

2.5. ELISA procedure

The microtitration plates were coated with avidin (100 μL/well, 0.5 μg/mL, in buffer 1) and incubated overnight at ambient temperature. Afterwards, the microtiter plates were washed three times (each time 300 μL/well, washing solution: 0.5 g/L Tween 20 in H_2O), blocked with 250 μL buffer 3, sealed with adhesive foil and stored for at least 2 weeks at approximately 4 °C. Then, the microtitration plates were washed three times again (each time 300 μL/well, washing solution), coated with biotin-labeled anti-telmisartan IgG (100 μL/well, 150 ng/mL in buffer 3), sealed with adhesive foil and incubated overnight at ambient temperature.

Calibration standards in the range of 0.01–3000 ng/mL were prepared in buffer 2 containing 10% pooled plasma. Quality control samples were diluted 1:10 with buffer 2. Unknown samples were diluted 1:10 at least. For higher dilutions pooled plasma was added to a final concentration of 10%. The microplates were washed four times (each time 300 μL/well, washing solution). Then immediately, 25 μL buffer 2 was pipetted into each well in order to prevent the coated surface from drying. Afterwards, 50 μL of the calibration solutions, quality controls (500 ng/mL, 20 ng/mL) or unknown samples (all diluted in buffer 2) was added in triplicate into wells of the microtitration plate. Then, 50 μL of the synthesized telmisartan–GOD-conjugate was added to each well. The plate was incubated on a shaker at room temperature for 4 h and afterwards washed four times (each time 300 μL/well, washing solution).

In the last step, 75 μL glucose (2×10^{-3} mol/L, in acetate buffer, pH 5.5; 0.01 mol/L) was added to each well of the microtitration plate. After an incubation time of 15 min, 30 μL of a mixture of 1.4 mL MNBDH (5×10^{-4} mol/L in acetonitrile) added to 10 mL POD (2.65 mg dissolved in 10 mL phosphate buffer, pH 5.8; 0.01 mol/L), were pipetted to each well. After a reaction time of 10 min, the fluorescence intensity of the fluorescent reaction product MNBDA was determined as described above.

2.6. Plasma test

The influence of different human plasma samples on the GOD-catalyzed reaction, which was used for detection of the immunoassay, was tested by executing this reaction on microtitration plates without added plasma and, comparatively, with 13 different plasma samples (see above). Therefore, 25 μL plasma (diluted 1:10 in buffer 2) were pipetted in six-fold into the wells of 10 rows of a microtitration plate. In the wells of one other row, 25 μL blank solution was added (buffer 2). Afterwards, 50 μL glucose (2×10^{-3} mol/L, in H_2O) were added

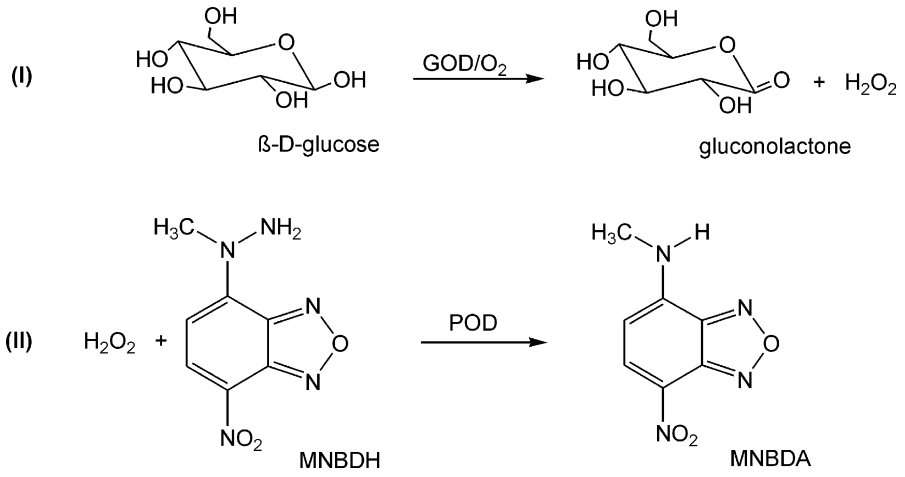

Fig. 3. POD-catalyzed reaction of MNBDH to the fluorescent MNBDA as detection scheme for a coupled precursor reaction. (I) Reaction of glucose with GOD and atmospheric oxygen to gluconolactone and H_2O_2. (II) Reaction of H_2O_2 with MNBDH under catalysis of POD to MNBDA.

to each well of the microtitration plate and 25 μL GOD solution (buffer 1) were added in two different concentrations (10 u/L and 30 u/L), in a manner that every plasma sample and the blank were determined in triplicate for each concentration. After an incubation time of 15 min, 20 μL of a mixture of 1.4 mL MNBDH (5×10^{-4} mol/L in acetonitrile) added to 10 mL POD (2.65 mg dissolved in buffer 1), were pipetted to each well. After a reaction time of 10 min, the fluorescence intensity of the strongly fluorescent 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole was determined as described above. The scheme of the GOD-catalyzed reaction is shown in Fig. 3.

3. Results and discussion

The MNBDH reaction has frequently been applied in many different fields: it was used for the determination of aldehydes and ketones [21] and nitrite [22], but also in combination with the enzymes glucose oxidase and/or peroxidase [20], e.g. for the determination of glucose in beverages [19]. As the reaction described in Fig. 3 yields the fluorescent MNBDA, which usually enables a sensitive and selective detection, it would also be advantageous to be able to use this assay in ELISAs. Therefore, the aim of this work was to develop an immunoassay for the determination of the drug telmisartan and to apply the MNBDH reaction for the first time in the presence of human blood plasma.

3.1. Plasma test

The results regarding the investigations on the stability of GOD and MNBDH in human plasma are shown in Fig. 4. The development of the fluorescence intensity over a reaction time of 420 min is shown for two different GOD concentrations (10 u/L part A; 30 u/L part B). In both cases, the GOD assay performed without added plasma is analyzed and compared with 13 different plasma-added assays. These measurements are all carried out in triplicate. For the reaction with a GOD concentration of 10 u/L, the mean R.S.D. over all determined fluorescence intensities ($n = 630$) was 5.6%. In case of a GOD concentration of 30 u/L, the mean R.S.D. was 4.8% ($n = 630$). As it can be seen in Fig. 4, the development of the reaction with time is for all reaction solutions, either without or with added plasma, very similar. This indicates that there is almost no disturbance or inhibition of the enzyme GOD or of the substrates MNBDH, glucose or POD by added plasma. Therefore, the enzyme-tracer synthesis was performed, and the ELISA based on fluorescence detection was applied for the investigation of telmisartan.

3.2. Enzyme-tracer synthesis

The synthesis of the GOD-tracer for the immunoassay was performed by means of the mixed-anhydride synthesis described above. The product was investigated by means of activity test

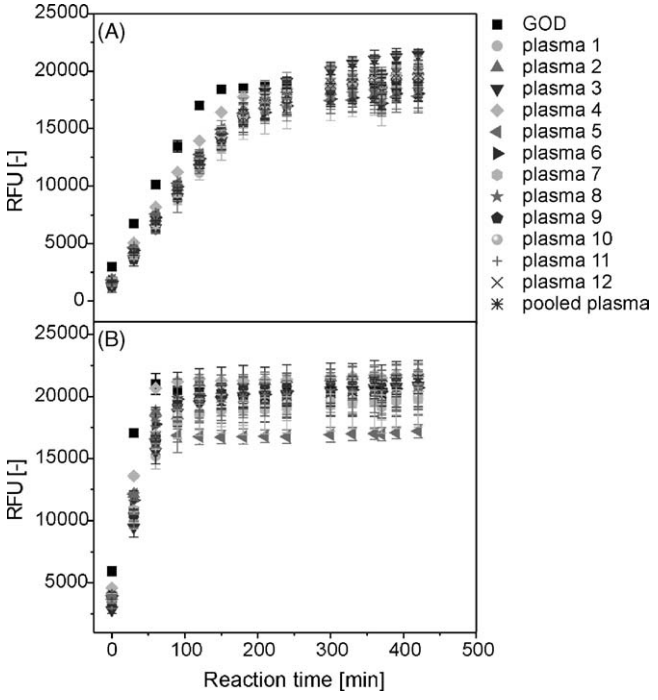

Fig. 4. Investigations regarding the stability of GOD and MNBDH in 13 different plasma samples: (A) GOD 10 u/L and (B) GOD 30 u/L.

measurements. The MNBDH reaction was performed by using the synthesized enzyme tracer in comparison to the commercially available GOD (educt of synthesis). For determining the number of telmisartan-hapten molecules, which were bound to the enzyme during the synthesis, a MALDI characterization of the product in comparison to the educt GOD was tried, but failed due to the chemical variability of the carbohydrate part within the GOD structure (different types and numbers of sugar molecules are attached).

3.3. ELISA

The pipetting/assay scheme of the ELISA is provided in Fig. 5. For the optimization of the ELISA conditions, several parameters were varied on two different types of microtitration plates (see above). The immunoassay was performed with avidin concentrations between 0.05 μg/mL and 5 μg/mL and biotin-labeled anti-telmisartan IgG concentrations between 0.15 ng/mL and 1500 ng/mL. The GOD-conjugate was diluted in the range of 1:100 to 1:90,000 and the calibration standards were used in the concentration range between 0.01 ng/mL and 3000 ng/mL. Initially, these investigations led to the result that the black Maxisorp microtitration plates from Nunc are favorable for the immunoassay. Best suited for the ELISA is an avidin concentration of 0.5 μg/mL, a biotin-labeled anti-telmisartan IgG concentration of 150 ng/mL and a GOD-tracer dilution of 1:300.

For calibration, 11 telmisartan standard solutions (0–1000 ng/mL) were analyzed on each microtitration plate together with the quality control and unknown samples. Due to the influence of human blood plasma on the immunoassay, all calibration standards were prepared in 10% pooled plasma. A typical calibration curve is provided in Fig. 6. The calculation of the mean B/B_0 values for all standards (four calibrations each with $n = 3$) resulted in $0.95B/B_0$ for 0.03 ng/mL, $0.5B/B_0$ for 3 ng/mL and $0.05B/B_0$ for 1000 ng/mL. The LOD of the ELISA, the concentration corresponding to a signal 3S.D. above the mean of the blank, was determined as 0.1 ng/mL, the LOQ (10S.D. above the mean of the blank) as 0.3 ng/mL and the range for the measurements was between 0.3 ng/mL and 300 ng/mL. The mean R.S.D. was 2.8% including the lowest R.S.D. of 0.8% and the highest R.S.D. of 10.8% of all measurements ($n = 84$).

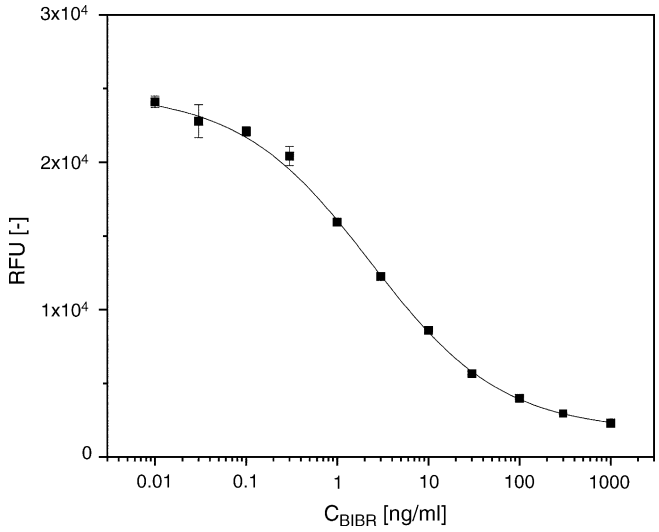

Fig. 6. Typical ELISA-calibration curve of the telmisartan determination.

The investigations show that the development of the immunoassay for the determination of telmisartan applying the MNBDH reaction was successful. The next step was to find out if this ELISA also allows the determination of the drug in real human blood plasma samples without an influence of possible telmisartan metabolites. Therefore, it was also important to develop an independent method, which would allow validation of the results. The procedures regarding these tasks are presented in part II of this publication.

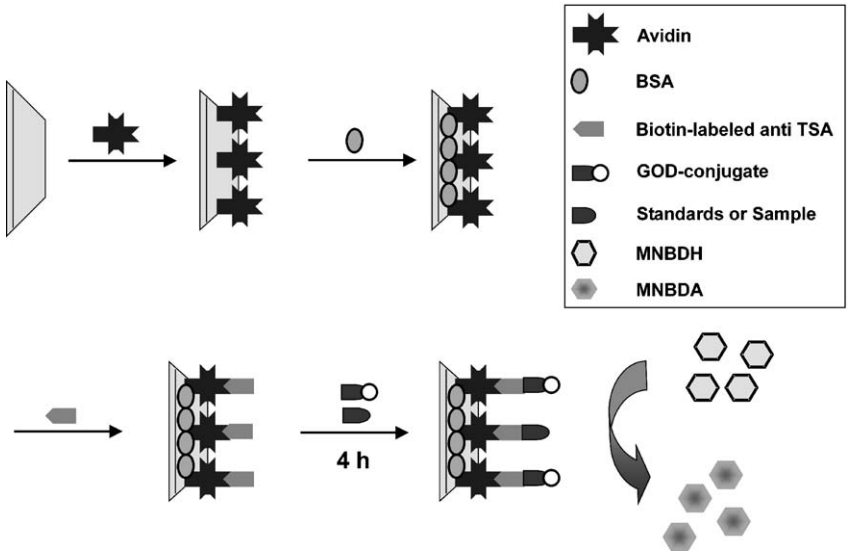

Fig. 5. Principle of the competitive ELISA. The synthesized glucose–oxidase (GOD) conjugate and telmisartan (sample or external standard) compete for the biotin-labeled anti-telmisartan (TSA) binding sides. In the last reaction step, which is catalyzed by GOD, the non-fluorescent MNBDH reacts to the highly fluorescent MNBDA. The emission is detected at 545 nm.

4. Conclusions

The first immunoassay for the determination of telmisartan has been developed. Telmisartan was successfully determined in pooled human plasma samples. For the first time, the MNBDH/MNBDA-system based on glucose oxidase as marker-enzyme and yielding fluorescence detection was used in an ELISA. The technique enables a fast, sensitive and selective determination of telmisartan. The development of a validation method and the application to telmisartan determinations in human blood plasma samples are presented in part II.

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References

[1] G.E. McVeigh, J. Flack, R. Grimm, Drugs 49 (1995) 161–175.
[2] B. Pitt, M.A. Konstam, Am. J. Cardiol. 82 (1998) 47S–49S.
[3] T. Unger, Am. J. Cardiol. 84 (1999) 9S–15S.
[4] R. Willenheimer, B. Dahlöf, E. Rydberg, L. Erhardt, Eur. Heart J. 20 (1999) 997–1008.
[5] W. Wienen, M. Entzeroth, J.C.A. van Meel, J. Stangier, U. Busch, T. Ebner, J. Schmid, H. Lehmann, K. Matzek, J. Kempthorne-Rawson, V. Gladigau, N.H. Hauel, Cardiovasc. Drug Rev. 18 (2000) 127–156.
[6] M. Sharpe, B. Jarvis, K.L. Goa, Drugs 61 (2001) 1501–1529.
[7] T. Ebner, G. Heinzel, A. Prox, K. Beschke, H. Wachsmuth, Drug Metab. Dispos. 27 (1999) 1143–1149.
[8] L. Gonzalez, U. Akesolo, R.M. Jimenez, R.M. Alonso, Electrophoresis 23 (2002) 223–229.
[9] S. Hillaert, W.J. van den Bossche, J. Chromatogr. A 979 (2002) 323–333.
[10] S. Hillaert, T.R.M. de Beer, J.O. de Beer, W. van den Bossche, J. Chromatogr. A 984 (2003) 135–146.
[11] T.X. Maotian, J.F. Song, N. Li, Anal. Bioanal. Chem. 377 (2003) 1184–1189.
[12] T.X. Maotian, J. Song, Y. Liang, J. Pharm. Biomed. Anal. 34 (2004) 681–687.
[13] N. Torrealday, L. Gonzalez, R.M. Alonso, R.M. Jimenez, E.O. Lastra, J. Pharm. Biomed. Anal. 32 (2003) 847–857.
[14] S.A. Ozkan, J. Liquid Chromatogr. Rel. Technol. 24 (2001) 2337–2346.
[15] L. Pastore, A. Tessitore, S. Martinotti, E. Toniato, E. Alesse, M.C. Bravi, C. Ferri, G. Desidri, A. Gulino, A. Santucci, Circulation 100 (1999) 1646–1652.
[16] N. Daneshtalab, R.Z. Lewanczuk, F. Jamali, J. Chromatogr. B 766 (2002) 345–349.
[17] T. Kondo, K. Yoshida, Y. Yoshimura, M. Motohashi, S. Tanayama, J. Mass Spectrom. 31 (1996) 873–878.
[18] B. Chen, Y. Liang, Y. Wang, F.L. Deng, P. Zhou, F. Guo, L. Huang, Anal. Chim. Acta 540 (2005) 367–373.
[19] J. Meyer, A. Büldt, M. Vogel, U. Karst, Angew Chem. Int. Ed. 39 (2000) 1453–1455.
[20] C. Hempen, U. Karst, Anal. Chim. Acta 521 (2004) 117–122.
[21] A. Büldt, U. Karst, Anal. Chem. 71 (1999) 1893–1898.
[22] A. Büldt, U. Karst, Anal. Chem. 71 (1999) 3003–3007.