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# Immunoenzymatic Method for Midkine Determination in Serum

## Immunoenzymatyczna metoda oznaczania midkiny w surowicy krwi

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## Abstract

**Background.** Midkine is a secreted protein originally engaged in neurogenesis and recently reported to be implicated in tumorigenesis. Midkine expression, both at the level of protein and mRNA, is elevated in various types of cancer. A potential significance of midkine level determination as a marker of tumour presence and progression is intensively studied. However, at the time when presented studies were conducted, no commercial ELISA kits for the assessment of serum midkine level were available.
**Objectives.** Developing a sensitive method for midkine determination in serum with application of commercially available antibodies and chromogenic substrate. The other goal of presented studies was practical evaluation of invented assay in serum samples from apparently healthy subjects and establishment of the reference range and test sensitivity.
**Material and Methods.** The studies were conducted on serum samples from 43 apparently healthy blood donors. In the described assay, double antibody sandwich indirect ELISA (DASI-ELISA) with two policlonal antibodies against recombinant human midkine raised in different hosts, was applied. For the enhancement of test sensitivity, the authors used biotin-streptavidin amplification system. Horseradish peroxidase and tetramethylbenzidine were applied for colour development.
**Results.** The sensitivity of the assay was 3–4 pg of midkine per ml of serum. The median midkine value, determined for the studied group, was 141 pg/ml and mean ± SD was 220 ± 320.6 pg/ml. The upper normal range limit, determined as 82$^{nd}$ percentile, was 541 pg/ml.
**Conclusions.** The invented ELISA proved to be a sensitive method for determination of serum midkine concentration and can be applied in studies on the serum level of this circulating growth factor in various physiological and pathological conditions (**Adv Clin Exp Med 2006, 15, 2, 247–252**).

**Key words:** midkine, ELISA, tumour marker, growth factor.

## Streszczenie

**Wprowadzenie.** Midkina jest peptydem wydzielniczym zaangażowanym przede wszystkim w neurogenezę. W ostatnich latach wykazano jednak udział midkiny w karcynogenezie, a jej zwiększoną ekspresję zarówno na poziomie białka, jak i mRNA potwierdzono w wielu typach nowotworów. Podjęto więc badania nad możliwościami zastosowania midkiny jako markera obecności i progresji choroby nowotworowej. Mimo tak intensywnie prowadzonych doświadczeń, w czasie gdy prowadzono badania własne, nie były dostępne komercyjne testy pozwalające na oznaczenie stężenia midkiny w surowicy.
**Cel pracy.** Opracowanie czułej metody immunoenzymatycznej pozwalającej na oznaczanie stężenia midkiny w surowicy, wykorzystującej komercyjnie dostępne przeciwciała. Opracowany test poddano ocenie praktycznej, ustalając zakres referencyjny i czułość przez oznaczenie stężenia midkiny w grupie honorowych krwiodawców.
**Materiał i metody.** Badania przeprowadzono na surowicach pozyskanych z krwi 43 honorowych krwiodawców bez widocznych objawów chorób. W teście wykorzystano technikę immunoenzymatycznej detekcji typu *sandwich* z dwoma rodzajami przeciwciał (DASI-ELISA): króliczymi przeciwciałami skierowanymi przeciwko midkinie jako przeciwciałami opłaszczającymi oraz skierowanymi przeciwko midkinie przeciwciałami kozimi jako przeciwciałami wykrywającymi. Przeciwciała wykrywające były znakowane biotyną, a do ich detekcji wykorzystano streptawidynę skoniugowaną z peroksydazą z chrzanu. Jako substratu użyto tetrametylobenzydyny.

**Wyniki.** Czułość opracowanego testu została wyliczona na 3–4 pg/ml. Mediana stężenia midkiny w grupie referencyjnej wyniosła 141 pg/ml, zaś średnia 220 ± 320,6 pg/ml. Górna granica zakresu normalnego, definiowana jako średnia + odchylenie standardowe, została wyliczona na 541 pg/ml.
**Wnioski.** Opracowano czuły test do oznaczania stężenia midkiny w surowicy, który można wykorzystać w badaniach nad sekrecją tego czynnika wzrostu w warunkach fizjologicznych i patologicznych (**Adv Clin Exp Med 2006, 15, 2, 247–252**).

**Słowa kluczowe:** midkina, ELISA, marker nowotworowy, czynnik wzrostu.

Midkine is a secreted, heparin-binding protein of great functional diversity, strongly expressed during embryogenesis [1]. Together with pleiotrophin, midkine constitutes a distinct family of heparin-binding growth factors not related to fibroblast growth factor (FGF) family. Midkine is, first of all, implicated in neurogenesis as it exhibits neurite-promoting activity, promotes survival and migration of neurons and induces synapse formation at neuro-muscular junction [2]. Besides, it has been shown to promote fibrinolytic activity of vascular endothelial cells as well as neutrophil, macrophage and osteoblast migration [2]. In studies conducted by Yu et al. [3] midkine suppressed cytotoxic activity of amyloid β-peptide while Callebaut et al. [4] demonstrated that this chemokine inhibits HIV infection. It has also been found that this growth factor plays an angiogenic role in carcinogenesis [5]. Midkine, which is, in some tissues under physiological conditions, expressed at low level, has been overexpressed in tissue sections from lung [6], breast [7], gastrointestinal [8], thyroid [9] and brain tumours [10]. The midkine-induced enhancement of tumour cells resistance to anti-cancer drugs has also been observed [11].

Midkine proved to be useful as a prognostic marker. Its overexpression has been correlated with worse prognosis in patients with neuroblastoma [12], urinary bladder carcinoma [13] and glioblastoma [14] and has been connected with tumour progression in case of prostate [15] and colon carcinomas [16]. Most of the reports deal with midkine at mRNA level or protein expression in tissue sections. Recently, the studies on serum midkine levels in various cancers and its correlation with tumour histopathological features, like type, size, grade and ability to form metastasis are in progress [17–20]. Although two sandwich ELISA tests for determination of serum midkine have been already described [17, 21], still there is no commercial ELISA kit available.

# Materials and Methods

## Serum Samples

Sera of apparently healthy blood donors (n = 43) were kindly provided by Regional Centre of Blood Donation and Therapeutics in Wrocław, Poland. Age and sex distribution of blood donors included in the presented studies is summarized in Table 1. Samples were kept frozen (–25°C) until examination. Prior to assay, the serum samples were brought to room temperature and mixed completely. The authors used sera ten times diluted with 0.05% Tween-20 in PBS. All samples were run in duplicates. Local Medical Ethics Committee approved the project presented in this paper.

**Table 1.** Age and sex distribution of blood donors included in the studies

**Tabela 1.** Charakterystyka badanej grupy pod względem wieku i płci

| Sex (Płeć) | Age (Wiek) | n |
|---|---|---|
| Female (Kobiety) | 25–40<br>> 40 | 9<br>1 |
| Male (Mężczyźni) | 25–40<br>> 40 | 22<br>11 |

## Enzyme-Linked Immunoassay

The authors applied the double antibody sandwich indirect ELISA (DASI-ELISA) method, where, in one test, two antigen-specific antibodies, raised in different hosts are used. In the described assay, rabbit anti-human recombinant midkine polyclonal antibodies were applied as capture antibodies while goat anti-human recombinant midkine polyclonal antibodies conjugated with biotin were used as detection antibodies. The wells of flat-bottom microtiter plate (Nunc MaxiSorp) were coated with 50 µl of anti-human midkine polyclonal rabbit antibodies (Gentaur, Belgium, cat. no.: 5479–100, lot no.: P8119) at concentration of 4 µg/ml in 50 mM carbonate buffer, pH 9.6 by overnight incubation. All incubation steps, unless otherwise stated, were conducted at room temperature on microplate rotation table at 100 rpm. After intensive washing with five changes of 300 µl of washing buffer (0.05% Tween-20 in PBS), the wells were blocked with 250 µl of buffered SuperBlock Protein with 0.05% Tween-20 (Pierce,

USA, cat no.: 37545, lot no.: FA66348 and cat. no.: 28320, lot. no.: GC94248, respectively) in a manner suggested by manufacturer. After washing, 50 µl of serum samples or standards were added and incubated for six hours. The emptied and washed wells were next filled with 50 µl of biotinylated goat anti-human midkine polyclonal antibodies (RnD Systems, USA, cat no.: BAF258, lot no.: UT01) at concentration of 0.5 µg/ml in PBS with 0.05% Tween-20. After overnight incubation at 4°C, the amplification and detection system of 50 ml of 0.2 µg/ml streptavidin conjugated with horseradish peroxidase (Jackson Immunoresearch, USA, cat. no.: 016-030-084, lot. no.: 63794) in PBS with 0.05% Tween-20 was applied and allowed to react for 30 minutes. The wells were next washed thoroughly with six changes of washing buffer and subsequently 50 µl of the substrate solution (1-Step ultra TMB-ELISA, Pierce, USA, cat. no.: 34028, lot no.: FD801109) was added for 30 minutes incubation in the dark. The colour development was stopped by acidification with equal volume of 2 M $H_2SO_4$. The amount of oxidized 3,3',5,5'-tetramethylbenzidine was measured on a microplate colorimetric reader (Multiscan MS, Labsystems) with 450 nm filter. Recombinant human midkine (Peprotech, USA, cat. no.: 450-16, lot. no.: 119190-B) dissolved in ten times diluted human serum depleted of midkine by affinity chromatography served as a standard. The references were used in the concentration range of 10–2000 pg/ml. Ten times diluted midkine-deprived serum served as a test sample blank in order to control the non-specific interactions of sera with a plate. Additionally, a reagent blank (a negative control of the test) was prepared by replacing serum samples with serum diluent (0.05% Tween-20 in PBS).

## Midkine-Depleted Serum

Midkine-depleted human serum was obtained by affinity chromatography procedure on heparin coupled to Sepharose column as described by Muramatsu et al. [21]. Serum samples from five healthy individuals were pooled together and serum in total volume of 5 ml was passed, in 20 mM Tris-HCl buffer (pH = 7.5) with 0.2 M NaCl, through 1 ml Hi-Trap Heparin HP column (Amersham Biosciences, Sweden). Collected unbound fraction was then dialyzed against PBS with 0.05% Tween-20 and concentrated to the initial volume of 5 ml by centrifugation at 5,500 rpm, 4°C using vivaspin 20 ml concentrator with 3,000 MWCO PES membranes (Vivascience, Germany).

## Estimation of Non-Specific Interactions

15 out of 43 studied sera were tested for the presence of non-specific interactions of sera components with a plate. In the assay, randomly chosen serum samples were accompanied by individual controls, where PBS with 0.05% Tween-20 was added instead of detection antibody.

## Calculation of Test Results

The mean values from two measurements were calculated for test samples, standards, test sample blanks and reagent blanks. The absorbance of the test sample blank (ten times diluted midkine-deprived serum) was subtracted from standards and examined samples. Absorbancies of standards, obtained in this way, were plotted against their concentration on log-log scale. Linear regression analysis was applied to construct the best-fitted standard curve. From the reference curve, the measured values of tested samples were converted into concentrations in picograms per millilitre (pg/ml) and multiplied by serum dilution factor.

# Results

The optimal signal was achieved with coating antibody concentration of 4 µg/ml in carbonate buffer, pH = 9.6 and concentration of detection antibody of 0.5 µg/ml. The biotin-steptavidin detection and amplification system was applied with optimal streptavidin concentration of 0.2 µg/ml. Different blocking systems were tested and the lowest signal to noise ratio combined with the shortest time required for blocking step was obtained with SuperBlock Protein from Pierce with 0.05% Tween-20. The background was significantly lowered by the addition of Tween-20 in concentration of 0.05% to antibody and streptavidin diluents. When incubation time and temperature regime were obeyed, the absorbancies of mean test sample blank varied between tests from 0.118 to 0.124 and their SD from 0.001 to 0.003, while those of reagent blank from 0.052 to 0.054 and SD from 0.001 to 0.003.

A serial dilution of serum was performed and ten times dilution proved to be optimal. Room temperature and gentle agitation of plates on a rotation table seemed to result in a better signal than stationary incubation at 37°C. Standard curve was based on recombinant, *E. coli*-derived, human midkine. In every assay the fit was achieved with

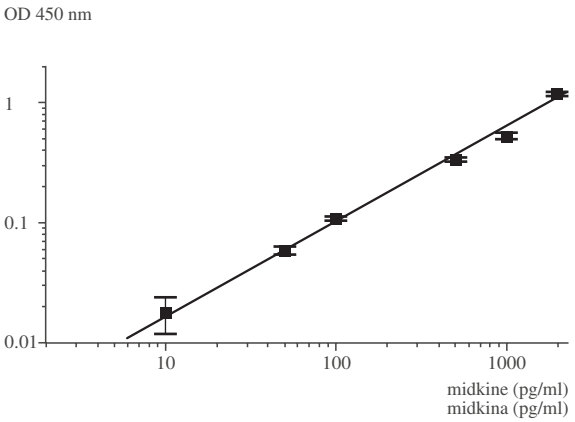


**Fig. 1.** Standard curve of the concentration of recombinant human midkine suspended in diluted serum. The mean values of two measurements with standard deviations are presented on log-log scale. The reference curve was fitted by linear regression with correlation coefficient of 0.997

**Ryc. 1.** Krzywa standardowa stężenia ludzkiej midkiny zawieszonej w rozcieńczonej surowicy krwi. Wartości średnie dwóch pomiarów z odchyleniem standardowym zaprezentowano na skali logarytmicznej. Krzywą wzorcową dopasowano za pomocą regresji liniowej ze współczynnikiem korelacji 0,997

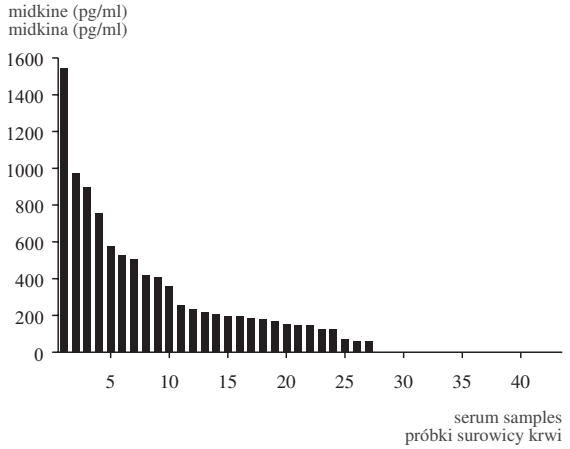


**Fig. 2.** The midkine concentration in serum samples of individual blood donors in the descending sorting. In the serum samples 28–43 midkine concentrations were non-detectable

**Ryc. 2.** Stężenie midkiny w surowicy krwi pochodzącej od indywidualnych krwiodawców uporządkowano malejąco. Stężenie midkiny w próbkach 28–43 było niewykrywalne

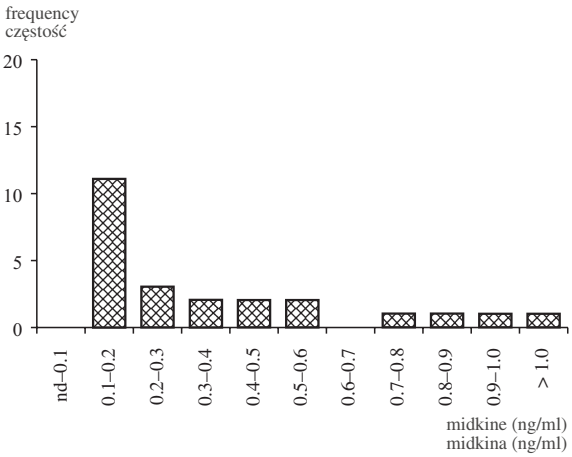


**Fig. 3.** Distribution of serum midkine levels of apparently healthy individuals (n = 43); nd – non-detectable

**Ryc. 3.** Rozkład częstości stężeń midkiny w surowicy krwi osób uznanych za zdrowe (n = 43); nd – wartość niewykrywalna

correlation coefficient ≥ 0.996. A typical reference curve, with standard deviations from the mean values, is presented in Figure 1.

The optimised test was used for quantification of serum midkine levels of 43 apparently healthy subjects to establish reference range of normal serum midkine concentration. The median value of serum midkine level was 141 pg/ml while mean value was 220 ± 320.6 pg/ml. The midkine values for individual serum samples of blood donors are presented in Figure 2, while the distribution of midkine levels in normal serum is summarized in Figure 3. Most of the examined cases were in the subrange from non-detectable to 100 pg/ml, followed by 100–200 pg/ml. The upper normal range limit, determined as 82$^{nd}$ percentile, was 541 pg/ml. The sensitivity of the assay was 3–4 pg of midkine per ml of serum.

Non-specific interactions of sera components with a plate were estimated and the mean absorbance of individually established controls for randomly selected 15 cases was 0.121 ± 0.008, which is a comparable value with those obtained for test sample blanks (from 0.118 ± 0.001 to 0.124 ± 0.003 in different assays).

# Discussion

The serum midkine assay presented in this paper utilizes, in one test, two kinds of polyclonal anti-human midkine antibodies, in contradistinction to midkine ELISA described by Muramatsu et al. [21], where only rabbit anti-human midkine antibodies were used for antigen capture and detection. Application of only one type of antibodies results in low test sensitivity as the same type of epitopes is recognized in capture and detection steps. To improve sensitivity of this method, the authors used detection system with fluorescent substrate, which, however, requires fluorescence microplate reader. Compared to one antibody system, sandwich ELISA with antibodies from two different species exhibits much higher sensitivity, as various antigen epitopes are utilized. Moreover,

as pointed out by Ikematsu et al. [17], this type of ELISA can be used with colorimetric system of antigen detection. In his improved midkine assay, rabbit and chicken anti-midkine antibodies were used. Detection antibodies were directly labelled with horseradish peroxidase and tetramethylbenzidine was utilized as a substrate. Application of chicken antibodies increased diversity of recognized antigen epitopes. However, their availability only on order is highly disadvantageous. The goal was to develop ELISA test equally sensitive but utilizing commercially available antibodies. The authors propose application of antibodies against human recombinant midkine from rabbit and goat, both of which are offered by main antibody manufacturers. In the assay, detection antibodies were biotin-conjugated and streptavidin coupled to horseradish peroxidase was used for antigen detection and signal amplification. The assay is linear in tested range of 10–2,000 pg of midkine per millilitre of serum, which is a better result than obtained by Muramatsu (50–10,000 pg/ml) [21]. Although, Ikematsu et al. [17] did not give the details concerning the test sensitivity, comparison of standard curves implies that the assay is even slightly more sensitive than the one described by them. It is probably due to the application of biotin-streptavidin amplification system in presented method. The sensitivity of presented test, calculated as mean of noise signal absorbancies plus three times standard deviation (mean + 3 SD), was estimated for 3–4 pg of midkine per ml of serum. The authors used serum depleted of midkine, by the means of affinity chromatography, as a standard solution in order to mimic the composition of serum samples. This, besides application of "sandwich" system of ELISA with saturating concentrations of capture antibody and both separate blocking step and non-ionic detergent as a diluent, minimalized the effect of non-specific adsorption to the plate. The absorbancies of individual controls for randomly chosen sera were found to be comparable with that of midkine-deprived serum. It validated the application of serum depleted of midkine as test sample blank.

The range of reference midkine concentrations is similar to that reported by Ikematsu et al. [17], although in the presently studied group the authors observed some individuals with midkine level elevated above 500 pg/ml (Figure 2). The value of 500 pg of midkine per millilitre of serum was established by Ikematsu [17] as a cut-off value and although in the case of described test most of the measured midkine levels were below 200 pg/ml, 541 pg/ml corresponded to 82$^{nd}$ percentile.

The newly developed method for immunodetection of midkine in serum proved to be sensitive and reliable. The further investigation on its application in the studies on potential use of midkine as a marker of tumour presence and progression are under way.

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