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Original research article

Stability of erythrocyte membrane and overall hemostasis potential – A biocompatibility study of mebrofenin and other iminodiacetic acid derivatives

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ABSTRACT

Background: Intravenous injection seems to be the most convenient way of administering drugs and contrast agents, which makes components of the blood the first and usually unwanted target of their action. Binding of intravenously administered compounds to erythrocytes, blood platelets and vascular wall may have serious clinical implications. The aim of this study was to examine the influence of four iminodiacetic acid derivatives, potential ligands for gadolinium complexation, on the process of coagulation and fibrinolysis, activity of thrombin and hemolysis.

Methods: Kinetic parameters of coagulation and fibrinolysis process were determined during an optical CL-test based on measurement of transmittance alterations. Thrombin (0.5 IU/mL) and t-PA (240 ng/mL) were used to obtain a clotting and lysis curve. The activity of thrombin was determined with a chromogenic substrate S-2238. Hemolysis was examined spectrophotometrically and expressed as a percentage of released hemoglobin.

Results: Exposure to iminodiacetic acid derivatives resulted in a significant increase in the overall potential of clotting and lysis (CL_{AUC}), as well as with the significant changes in the key parameters of these processes (thrombin time, initial plasma clotting velocity, clot stabilization time). Furthermore, iminodiacetic acid derivatives caused a significant decrease in the amidolytic activity of thrombin and enhanced hemolysis in a concentration-dependent manner.

Conclusion: Despite their influence on the process of coagulation and fibrinolysis, amidolytic activity of thrombin and hemolysis, iminodiacetic acid derivatives should be generally considered safe as the significant effects were observed mostly at 4 μmol/mL, which is about 10-fold higher than the theoretical plasma concentration of these compounds.

Introduction

Previous studies of nuclear magnetic resonance showed that Magnetic Resonance Imaging (MRI) can provide a three-dimensional, high-resolution visualization of specific tissues and organs of the body, and as such constitutes a breakthrough in diagnostic medicine [1,2].

As the sensitivity of MRI to differentiate between various tissue types is relatively low, contrast agents (CAs), such as low-molecular-weight gadolinium (III) chelates [3], are usually required to enhance the MRI images [4]. However, the gadolinium-based MRI CAs that have been approved for clinical use, such as gadopentetate dimeglumine (Magnevist) and gadodiamide (Omniscan), are not sufficiently specific and selective [5]. Therefore, we still need MRI CAs with sufficiently high affinity for hepatocytes, that could be used for identification and differentiation of focal changes in the liver and biliary ducts [6,7].

Iminodiacetic acid derivatives, such as mebrofenin complexed with gadolinium, show high affinity to hepatocytes and therefore provide high-resolution MRI of the liver [6,8].

Moreover, after their radiolabelling with technetium (^{99m}Tc), the iminodiacetic acid derivatives can be used as radiopharmaceuticals (^{99m}Tc-diosopropyl-IDA [DISIDA], ^{99m}Tc-bromotriethyl-IDA [mebrofenin]) during cholescintigraphy [9–11]. The results of many previous studies point to (99m)Tc-mebrofenin as the most valuable quantitative radiotracer for functional examination of the liver, suitable for determination of various aspects of its function [12].

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CAs are typically administered intravenously and ideally should exert only a minimum effect on the blood and vascular endothelium. However, some CAs were shown to interact with the blood components (*e.g.* red blood cells [RBCs]) and coagulation system. Although these effects were a subject of extensive *in vitro* and *in vivo* research, it mostly centered around the iodinated CAs used in clinical practice [13]. Morphological changes of RBCs may also affect their capacity for oxygen delivery and pH buffering [14]. However, these effects were not shown to be of clinical importance [15].

Several previous studies showed that coagulation of blood mixed with CA occurs faster than in the control sample [16,17]. The anticoagulant effect of CAs was demonstrated to result either from the inhibition of thrombin or from the inhibition of fibrin polymerization. Several studies showed that CAs may affect the coagulation cascade at any point (*e.g.* inhibition of factor Xa and thrombin generation) [18]. Furthermore, CAs were found to interfere with the assembly of fibrin monomers, which resulted in worse stabilization of a clot [19].

CAs may impede fibrinolysis and cause delay in the onset of clot lysis [20]. Furthermore, they may contribute to the formation of long/thin fibrils, which have a lower mass/length ratio and are more resistant to fibrinolysis [21].

To the best of our knowledge, none of the previous studies analyzed the biocompatibility of CAs based on iminodiacetic acid derivatives. Therefore, we decided to examine the influence of four iminodiacetic acid derivatives on the process of clot formation, stabilization and lysis. In order to identify a mechanism through which they interfere with coagulation and fibrinolysis, we analyzed the effect of iminodiacetic acid derivatives on the amidolytic activity of thrombin. Furthermore, we evaluated the influence of iminodiacetic acid analogs on RBC hemolysis.

Materials and methods

Reagents

The substrates for synthesis of iminodiacetic acid derivatives, namely nitrilotriacetic acid (cat. no. N9877), 4-methylaniline (cat. no. 236314), 2,4-dimethylaniline (cat. no. 240915), 2,4,6-trimethylaniline (cat. no. 92290) and 3-bromo-2,4,6-trimethyloaniline (cat. no. 458880), were purchased from Sigma–Aldrich (Poland). Acetic anhydride (cat. no. 242845) and pyridine (cat. no. 270970) were provided by Sigma–Aldrich (Poland), and the solvents, *i.e.* methyl alcohol (cat. no. 621990110), ethyl alcohol (cat. no. 396480427), dichloromethane (cat. no. 628410421), sodium hydroxide (cat. no. 810981118) and hydrochloric acid (cat. no. 575283115) by Polish Chemical Reagents (Poland). All the chemicals were used without further purification.

We used thrombin produced by Biomed (Poland) and recombinant tissue plasminogen activator (t-PA) manufactured by Boehringer-Ingelheim (Germany). Tris-buffered saline (TBS, cat. no. SRE0032) was purchased from Sigma–Aldrich. Calcium chloride (cat. no. 875010112) and sodium chloride (cat. no. 794121116) were provided by Polish Chemical Reagents (Poland). Chromogenic substrate S-2238 (cat. no. 82 0324 39) for determination of amidolytic activity of thrombin was purchased from Chromogenix (Italy). Triton X-100 (cat. no. 841810492) was obtained from Polish Chemical Reagents (Poland).

Synthesis of N-(acetanilide)-iminodiacetic acid derivatives

Iminodiacetic acid derivatives were synthesized as described previously [8]. Briefly, N-(4-methylacetanilide)iminodiacetic acid (Met-IDA; MW = 280.28 g/mol; pK_a = 3.24), N-(2,4-dimethylacetanilide)-iminodiacetic acid (DMet-IDA; MW = 294.30 g/mol; pK_a = 3.36), N-(2,4,6-trimethylacetanilide)iminodiacetic acid (TMet-IDA; MW = 308.33 g/mol; pK_a = 3.47) and N-(3-bromo-2,4,6-trimethylacetanilide)-iminodiacetic acid (mebrofenin, MBR; MW = 387.23 g/mol; pK_a = 2.81) were obtained during the reactions between the *in situ* obtained nitrilotriacetic acid anhydride and appropriate aniline derivative (Fig. 1).

The structure of the obtained compounds was verified based on ^{1}H NMR, ^{13}C NMR, elemental analysis and IR spectra. Melting points were estimated with an Electrothermal apparatus (all data presented in Supplementary Materials). The synthesized compounds were of chromatographic grade purity. The log *p* values for the IDA derivatives were calculated with a ChemSketch program: Met-IDA 0.93 ± 0.46; DMet-IDA 1.39 ± 0.46; TMet-IDA 1.85 ± 0.47; MBR 3.09 ± 0.59.

Test of clot formation and lysis (CL-test)

Plasma preparation

Blood samples were obtained from healthy donors from the blood donation center in Łódź. The blood was collected to vacuum tubes containing 3.2% buffered sodium citrate. Poor platelet plasma (PPP) was obtained by centrifugation (3000 × *g*, 20 min, room temperature) with a Micro 22R centrifuge (Hettich ZENTRIFUGEN). Subsequently, PPP was pooled and stored in small portions at −30° C until analysis. Immediately prior to each measurement, PPP was restored in a water bath at 37° C for 15 min. Once thawed, the PPP was not frozen again nor used for retesting.

CL-test

The kinetic parameters of clot formation and lysis were determined during the CL-test (described previously [22]) and analyzed with a dedicated software [22]. The test is based on the evaluation of the global assay of coagulation and fibrinolysis by continuous measurements of the optical transmittance alterations. The method constitutes a modification of the optical measurement of coagulation and blood fibrinolysis, presented by Glover et al. [23] and He et al. [24,25].

The accuracy of the assay was confirmed in both hypercoagulability (associated with normal pregnancy, preeclampsia, some thrombophilias, diabetes mellitus, stroke and coronary artery disease) and hypocoagulability (in patients with hemophilia). Furthermore, it proved accurate in the monitoring of anticoagulant treatment which points to the assay as a good candidate for clinical purposes [26].

General experimental conditions were defined previously and described elsewhere [27,28]. The measurements were taken at λ = 405 nm wavelength, in semi-micro cuvettes (Medlab Products, Poland), using a spectrophotometer Cecil CE 2021 with circulating thermostated water (37 °C) and a magnetic stirrer (Electronic Stirrer Model 300 Rank Brothers Ltd, England). Iminodiacetic acid derivatives at appropriate concentrations (in a 10-μL volume) and t-PA (240 ng/mL) were added to plasma diluted three times with TBS buffer. In the control samples, 10 μL of IDA solution was replaced with 10 μL of TBS buffer. Subsequently, the samples were incubated at 37 °C for 3 min, and then thrombin (0.5 IU/mL) was added to initiate clot formation. The final volume of the sample was 500 μL. During the experiments evaluating generation of endogenous thrombin, small amounts of thrombin (0.025 IU/mL) and calcium chloride (0.01 mmol/mL) were added. The addition of small amounts of thrombin and calcium chloride does not result in the initiation of coagulation process, but induces a feedback reaction which leads to generation of endogenous thrombin and resultant coagulation.

The obtained curves were evaluated using a computer software. The software enabled us to estimate the parameters of clot formation (phase I), stabilization (phase II) and fibrinolysis (phase