ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 983–988

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Mangafodipir trisodium injection, a new contrast medium for magnetic resonance imaging: in vitro metabolism and protein binding studies of the active component MnDPDP in human blood

Kim Gunnar Toft *, Grete Mørk Kindberg, Tore Skotland

Nycomed Imaging AS, Research and Development, PO Box 4220 Torshov, N-0401 Oslo, Norway

Received 22 May 1996; accepted 27 August 1996

Abstract

The binding to human serum proteins of MnDPDP (manganese(II) dipyridoxyl diphosphate), the active component of the magnetic resonance imaging contrast medium mangafodipir trisodium injection (Teslascan®) was studied in ultrafiltration experiments. Sera from three males and three females were incubated with 86 μM [^{14}C]MnDPDP for 60 min at room temperature (20–23°C), followed by centrifugation through filters with a cut-off of 30 kDa. Analysis of the filtrates and the initial incubation mixtures for manganese, by ICP-AES, and for DPDP and its dephosphorylated metabolites DPMP (dipyridoxyl monophosphate) and PLED (dipyridoxyl ethylenediamine diacetate) by liquid scintillation counting, showed a clear difference in protein binding of manganese and the ligands under these conditions. Only 2.2 ± 1.8% (mean ± S.E.; $n = 6$) of DPDP, DPMP and PLED were bound to protein, whereas 26.9 ± 2.9% (mean ± S.E.; $n = 6$) of manganese was bound to protein. No binding of DPDP, DPMP or PLED to blood cells was observed when whole blood, containing either heparin or EDTA as anticoagulant, was spiked with [^{14}C]MnDPDP and the cell-free fraction and the lysed cell fraction analysed by liquid scintillation counting. The extent of protein binding of manganese corresponded well with results from an in vitro metabolism study, in which MnDPDP was added to heparinized human whole blood, showing that approximately 25% of DPDP, DPMP or PLED were not bound to manganese. The in vitro metabolism study revealed that transmetallation with zinc was nearly complete within 1 min, and that dephosphorylation is a sequential process going from DPDP to the monophosphate DPMP, and then to the fully dephosphorylated compound PLED. © 1997 Elsevier Science B.V.

Keywords: Protein binding; In vitro metabolism; Mangafodipir trisodium; MnDPDP; Blood

Abbreviations: DPDP, *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetate-5,5'-bis(phosphate), dipyridoxyl diphosphate; DPMP, *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetate-5-phosphate, dipyridoxyl monophosphate; PLED, *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetate, dipyridoxyl ethylenediamine diacetate; ICP-AES, inductively-coupled plasma-atomic emission spectroscopy.

* Corresponding author. Tel.: +47 22 891010; fax: +47 22 891200.

PII S0731-7085(96)01944-9

1. Introduction

MnDPDP (manganese(II) *N,N'*-dipyridoxylethylenediamine-*N,N'*-diacetate-5,5',bis(phosphate) is the active component of mangafodipir trisodium injection (Teslascan®), a new contrast medium for magnetic resonance imaging (MRI) of the liver and hepatobiliary system [1]. Biodistribution studies in rats and dogs with ^{54}MnDPDP and [^{14}C]MnDPDP have shown that the plasma clearance, biodistribution and excretion pattern of the manganese and the ligand are clearly different [2]. The ligand is excreted mainly in the urine, while the Mn is distributed largely to the liver, pancreas and kidney and excreted mainly in the bile. A rapid uptake of Mn into bovine liver has been reported after intravenous injection of $^{54}MnCl_2$ or ^{54}Mn-α_2-macroglobulin. The uptake of $^{54}MnCl_2$ was explained by binding of Mn to α_2-macroglobulin in blood and transportation of the protein-bound Mn to the liver [3]. It is assumed that the Mn released during metabolism of MnDPDP is taken up by the liver in a similar way.

In plasma or serum, Mn(II) has been shown to bind mainly to the proteins α_2-macroglobulin and albumin [3]. Mn(III), which may be formed through oxidation of Mn(II) by ceruloplasmin [4] or by air oxidation of the samples, is bound mainly to transferrin [3,5]. In the present study, protein binding of MnDPDP was studied after incubation of human serum with [^{14}C]MnDPDP and subsequent filtration through a filter with a 30 kDa cut-off. The sum of [^{14}C]labelled ligands was analysed by liquid scintillation counting, and the Mn was analysed by ICP-AES (Inductively-Coupled Plasma—Atomic Emission Spectroscopy). Moreover, protein binding of Mn was studied indirectly in an in vitro metabolism study in which MnDPDP was added to heparinized human whole blood followed by quantitation of MnDPDP and metabolites by high performance liquid chromatography (HPLC). The amount of chelates not bound to Mn then correspond to the protein binding of Mn. The objective of this in vitro metabolism study was to further assess the blood sample handling method described in the previous paper [6] and to study the metabolic pathways of MnDPDP.

2. Materials and methods

2.1. Chemicals and reagents

MnDPDP, MnDPMP, MnPLED, ZnDPDP, ZnDPMP, ZnPLED, CaDPDP, DPDP and [^{14}C]MnDPDP were from Nycomed Imaging AS, Oslo, Norway. The synthesis and characterization of MnDPDP has been described by Rocklage et al. [7]. The structure of MnDPDP and the sites of [^{14}C]labelling are shown in Fig. 1; the specific activity was 10.4 μCi μmol^{-1}, and the radiochemical purity was 96.8%, as analyzed by HPLC. Human blood was collected from healthy Caucasian male and female volunteers. Water was purified by reversed osmosis, ion exchange and filtration through a 0.45 μm filter using a Milli-Q system (Millipore, Bedford, MA). To minimize air oxidation of the Mn compounds water used to prepare calibration standards and control samples was degassed with N_2-gas for 30 min. Other chemicals were of analytical grade.

2.2. Protein binding studies

Blood collected in vacutainers containing heparin from three males (A, B, C) and three females (D, E, F) was left to coagulate at room temperature for 15 min and then centrifuged for 10 min at $1800 \times g$ at room temperature (20–23°C). Aliquots (2 ml) of serum were mixed with 90 μl of freshly diluted [^{14}C]MnDPDP giving a final concentration of 86 μM. The samples were incubated at room temperature for 60 min. Ultrafiltration was performed in tubes with a filter cut-off of 30 kDa (Millipore Ultrafree^R-MC, Millipore, Bedford, MA). A sample from each incubation mix-

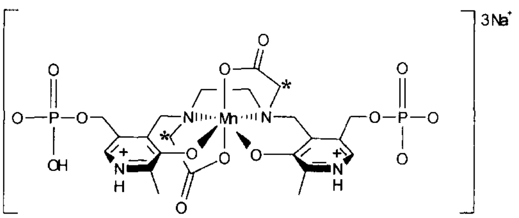

Fig. 1. Structural formula of the sodium salt of MnDPDP with sites of ^{14}C-labeling marked with asterisks.

ture was poured into a pre-cooled filter cup and centrifuged at $2500 \times g$ for 60 min at 4°C. Aliquots (6 replicates) were pipetted from the filtrate and the initial incubation mixture, and counted for radioactivity by liquid scintillation counting. The content of Mn in two replicates of the filtrate and the initial incubation mixture was measured by ICP-AES.

2.3. Distribution of ligand in whole blood

Blood from one female donor was collected in vacutainers containing sodium heparin or potassium EDTA, respectively. Of each blood sample 1 ml was transferred to microcentrifuge tubes and spiked with 10 μl [^{14}C]MnDPDP. The tubes were incubated for 10 min at room temperature, inverted gently each minute and subsequently centrifuged for 5 min at 2000 rpm ($725 \times g$) in a Heraeus Christ Labofuge^{GL} at 23°C. Plasma blanks were obtained from non-spiked blood samples. After collecting 500 μl of plasma, the remaining blood cells were washed 3 times in 500 μl phosphate buffered saline (PBS), and 500 μl supernatant was collected each time. The washed blood cells were lysed with Soluene-350 (Packard, Downers Grove, IL). Aliquots (10 μl) of the lysed blood cells, plasma and supernatants were counted for radioactivity by liquid scintillation counting.

2.4. In vitro metabolism

Approximately 100 ml blood was collected from one female donor in vacutainers containing sodium heparin. The blood was pooled and the pH was measured as 7.5.

Approximately 80 ml of blood was used for preparation of stock solution, calibration standards and control samples. The pH was adjusted to 10 by the addition of solid trisodium phosphate dodecahydrate (TSP; 17.0 mg ml^{-1} blood) using a magnetic stirrer at low speed. The blood was centrifuged at $1500 \times g$ for 20 min at 4°C and plasma was collected and transferred to 4 Centriprep 30 ultrafiltration devices (15 ml) with a cut-off of 30 kDa (Amicon, Beverly, MA). Ultrafiltration was performed at $1500 \times g$ for 90 min in a fixed-angle rotor at 4°C. The collected filtrate (called processed plasma) was pooled in a plastic bottle and purged with N_2-gas. Processed plasma that were not used for the preparation of calibration standards, and control samples, were purged with N_2-gas and frozen at −20°C.

Approximately 20 ml of blood was transferred to a 50 ml centrifuge tube. Three samples of 1.5 ml (zero time samples) were immediately transferred to microcentrifuge tubes containing 24.7–26.8 mg TSP to obtain a pH of 10.0 ± 0.2. The tubes were inverted several times to dissolve the TSP, spiked with 75 μl of a 2 mM MnDPDP solution and kept on ice before centrifugation. The rest of the blood (15.5 ml) was pre-incubated for 5 min at 37°C, spiked with 775 μl of a 2 mM MnDPDP solution and incubated at 37°C. Samples of 1.5 ml blood were taken after 1, 2, 5, 10, 20, 40 and 60 min, and triplicate samples of 1.5 ml were taken after 90 min directly into microcentrifuge tubes containing TSP. They were inverted several times and kept on ice until centrifuged at $1500 \times g$ for 10 min at 4°C. Plasma was collected and ultrafiltrated in Centrisart 20 tubes with a cut-off of 20 kDa (Sartorius, Göttingen, Germany) at $2500 \times g$ for 90 min at 4°C in a fixed-angle rotor. The ultrafiltrated plasma was transferred to HPLC tubes and frozen in a −20°C freezer. The samples were thawed at 4°C two days later and analysed by HPLC.

2.5. HPLC method

The HPLC method was based on separation on a mixed-bed column with both anion exchange and reversed-phase functions and are described in detail elsewhere [6]. Mn(III)DPDP was quantitated by the calibration curve for Mn(II)DPDP. The retention times for CaDPDP and free ligand (DPDP) were determined by three separate calibration standards of each compound in processed plasma covering the concentration range 5–25 μM.

2.6. ICP-AES

Mn was determined using a Perkin Elmer Plasma 2000 ICP-AES; the 257.610 nm emission

line and a multipoint standard were used. Duplicate samples of 30 μl were mixed with 125 μl 1000 μg ml^{-1} of scandium (internal standard) and 2345 μl 1 M HCl before analysis. The limit of quantitation (LOQ) of Mn, defined as 10 × S.D. of the response in the blank sample, was 9.2 μM.

2.7. Scintillation counting

In the protein binding study, aliquots of 30 μl of the filtrate and the initial incubation mixture were mixed with 2 ml scintillation fluid (Pico Fluor 40; Packard, Downers Grove, IL) in low-background scintillation vials. The radioactivity of the samples (five replicates) was counted for 2 min using a LKB Wallac, 1217 Rackbeta liquid scintillation counter. The initial incubation mixture showed an activity of approximately 55 000 cpm. No difference in quenching was observed in a control experiment by comparing water and serum samples containing the same amount of [^{14}C]MnDPDP. In the distribution volume study, aliquots of 10 μl were mixed with 2 ml scintillation fluid and counted as described above. The activity of a [^{14}C]MnDPDP solution used in the distribution volume study was 186519 ± 243 cpm 10 μl^{-1} (mean ± S.D.; $n = 5$).

2.8. Calculation and statistics

The amount of protein binding was calculated by the following formula:

$$\text{Protein binding (\%)} = \frac{(S - F) \times 100}{S}$$

where S is the concentration of the substance in the initial incubation mixture and F is the concentration of the substance in the filtrate.

PE Nelson ACCESS*CHROM GC/LC data sampling system, v. 1.8, was used for sampling and integration of the chromatograms. GraphPad Inplot, v. 4.0, was used for preparation of the calibration curves and GraphPad Prism, v. 2.0, was used for preparation of the figures. Excel v. 5.0, was used for calculating the mean, standard deviation (S.D.), standard error of the mean (S.E.) and relative standard deviation (R.S.D.).

Table 1
Protein binding of MnDPDP in human serum

Sex	Binding of [^{14}C]chelates: mean ± S.E.[a]	Binding of Mn: mean ± S.E.[b]
Male ($n = 3$)	2.7 ± 2.1	29.5 ± 0.4
Female ($n = 3$)	1.7 ± 1.6	24.3 ± 0.7
Total ($n = 6$)	2.2 ± 1.8	26.9 ± 2.9

The human sera were incubated with 86 μM [^{14}C]MnDPDP for 60 min at room temperature, and the amount of protein binding determined as described in Materials and methods. Figures are given as % binding (mean ± S.E.).
[a]The S.D. of the replicates ($n = 6$) were in the range 1.5–3.1%.
[b]The differences between the mean and the two replicates were in the range 0.04–3.0%.

Significance testing was performed by using a two-tailed t-test at a 5% significance level.

3. Results and discussion

The binding of MnDPDP to human serum proteins was studied in sera from six volunteers incubated with 86 μM [^{14}C]MnDPDP for 60 min at room temperature (Table 1). The radioactivity of the initial incubation mixture and that of the low molecular weight fraction indicated a small (2.2 ± 1.8%) but statistically significant ($P < 0.05$) amount of protein binding of the [^{14}C]chelates (sum of DPDP, DPMP and PLED) in human serum. The Mn analyses of the different fractions indicated that 26.9 ± 2.9% of the Mn was bound to proteins, thus clearly demonstrating some dissociation and subsequent protein binding of Mn from the chelates. This is in agreement with previous results from rats and dogs which showed that the tissue distribution and excretion patterns of the Mn and ligand were qualitatively and quantitatively different [2]. The data indicated a slightly higher degree of Mn binding to serum proteins in the three males (29.5 ± 0.4%) compared to three females (24.3 ± 0.7%). However, although the difference was statistically significant ($P < 0.05$), the small size of the samples ($n = 3$) does not allow this to be interpreted as evidence of a definite sex difference.

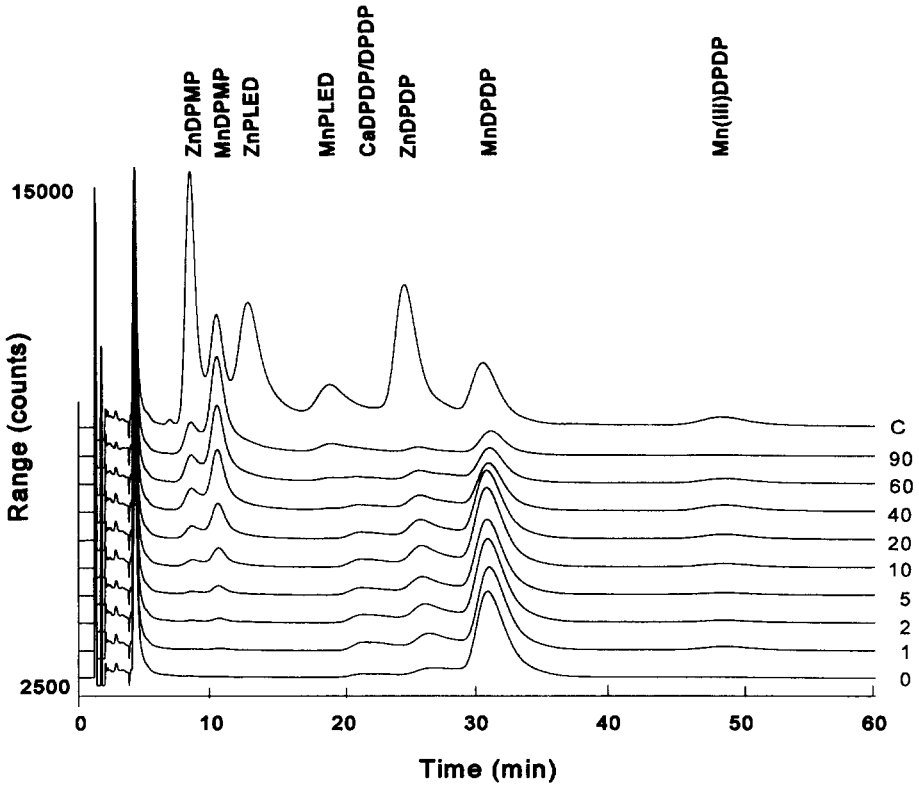

Fig. 2. Chromatograms of a control sample (C) in processed plasma containing 50 μM of each of MnDPDP, MnDPMP, MnPLED, ZnDPDP, ZnDPMP and ZnPLED, and processed plasma samples prepared from blood spiked with 97 μM MnDPDP and incubated at 37°C for 0, 1, 2, 5, 10, 20, 40, 60 and 90 min. Analysis was performed by isocratic elution in 0.2 M sodium phosphate pH 9.12 containing 3% (v/v) acetonitrile and 1.5 mM tetrabutylammonium phosphate at a flow rate of 1.2 ml min^{-1} and UV detection at 310 nm.

The amounts of radioactivity recovered in the cell-free fractions (sum of plasma and supernatants after washing with PBS) after spiking whole blood with [^{14}C]MnDPDP was 99.8% in spiked heparin-blood and 98.1% in spiked EDTA-blood. The activity in lysed blood cells was approximately the same as in the plasma blank. The results indicate that the [^{14}C]chelates do not bind to or enter the blood cells.

Fig. 2 shows the chromatograms of ultrafiltrated plasma samples prepared from blood spiked with 97 μM MnDPDP and incubated for 0. 1, 2, 5, 10, 20, 40, 60 and 90 min. Within 1 min of incubation a significant amount of ZnDPDP appeared showing that very rapid transmetallation with Zn to ZnDPDP had occurred. The minor peak eluting at approximately 21 min represents free ligand or CaDPDP. These compounds might arise as a result of protein binding of Mn and subsequent release of free ligand which in turn could chelate Ca. CaDPDP and DPDP co-chromatograph and have approximately the same area response factor, so it was not possible to discriminate between these two substances in the present chromatographic system. Small amounts of Mn(III)DPDP (R_t = 50 min) were observed. This compound was, however, probably not a real metabolite, but was more likely to be an artefact resulting from a combination of high pH and air oxidation [6,8].

The relative amounts of metabolites are shown in Fig. 3. The sum of Mn(II)DPDP and Mn(III)DPDP decreased from 82.6 to 14.8% of the total amount of metabolites over 90 min,

while MnDPMP increased from 0–56.4% MnPLED appeared after 60 min and increased to 10.8% after 90 min of incubation. The sum of Zn metabolites and DPDP/CaDPDP represents the amount of chelates in which Mn has been displaced, and was almost unchanged from 1 to 60 min of incubation with a mean value corresponding to $25.2 \pm 1.7\%$ (mean $\pm$ S.D.; $n = 7$) of the total amount of chelates (Fig. 3). This figure represents indirectly protein binding of Mn and is in close agreement with the 27% protein binding of Mn estimated in the ultrafiltration experiment described above in which [^{14}C]MnDPDP was added to human serum.

The results indicate a rapid transmetallation with available Zn that is nearly complete after 1 min, and a relatively slow dephosphorylation process. Although the intracellular concentration of Zn in red blood cells is approximately 10 times higher than the plasma concentration, this Zn does not appear to be in a form that is available to bind to free DPDP in the incubation mixture. This is in contrast to the results from in vivo metabolism studies in dogs and humans (to be published) where no free DPDP/CaDPDP was observed. While the transmetallation rapidly levels off in vitro, probably because of lack of available Zn, the transmetallation in vivo continues because Zn might be removed from available tissue stores or extracellular fluids during the process.

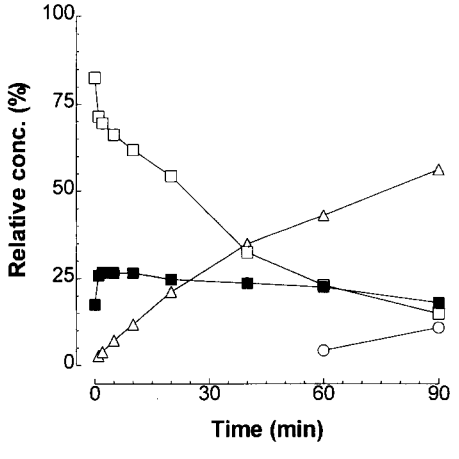

Fig. 3. Relative concentration of metabolites after in vitro incubation of 97 μM MnDPDP in human blood at 37°C. Sum of Mn(II)DPDP and Mn(III)DPDP (□), MnDPMP (△), MnPLED (○) and sum of Zn metabolites and DPDP/CaDPDP (■).

The present in vitro metabolism study shows that the dephosphorylation of MnDPDP is a sequential process going from MnDPDP to the monophosphate MnDPMP and then to the fully dephosphorylated compound MnPLED. The main metabolite after incubation of MnDPDP in human whole blood for 90 min at 37°C was MnDPMP. A considerable amount of MnDPDP was, however, still present at the end of incubation. This is in contrast to the in vivo observation where the parent compound was virtually non-existent in plasma 10 min after an intravenous bolus injection of 5 μmol mangafodipir trisodium injection kg^{-1} b.w (to be published). The reason for the slower dephosphorylation rate in vitro than in vivo may be due to the additional activity of tissue bound phosphatases in vivo or inactivation/inhibition of the available phosphatases in vitro.

Acknowledgements

The contributions made by Trine B. Haugen in the early phase of the ultrafiltration experiments and the technical assistance by Grete A. Friisk are greatly appreciated. We thank Ivar Martinsen for doing the ICP-AES analyses.

References

[1] E. Rummeny, Ch. Ehrenheim, H.B. Gehl, B. Hamm, M. Laniado, K.P. Lodemann, E. Schmiedel, A. Steudel and Th. G. Vogl, Invest. Radiol., 26 (1991) S142–S145.
[2] D. Grant, K. Zech and E. Holtz, Invest. Radiol. 29, (2) (1994) S249–S250.
[3] R.A. Gibbons, S.N. Dixon, K. Hallis, A.M. Russell, B.F. Sansom and H.W. Symonds, Biochem. Biophys. Acta, 444 (1976) 1–10.
[4] J.W. Critchfield and C.L. Keen, Metabolism, 41 (1992) 1087–1092.
[5] A.M. Scheuhammer and M.G. Gherian, Biochem. Biophys. Acta, 840 (1985) 163–169.
[6] K.G. Toft, G.A. Friisk and T. Skotland, J. Pharmaceut. Biomed. Anal., 15 (1997) 973–981.
[7] S.M. Rocklage, W.P. cacheris, S.C. Quay, F.E. Hahn and K.N. Raymond, Inorg. Chem., 28 (1989) 477–485.
[8] P. Aisen, R. Asa and A.G. Redgfield, J. Biol. Chem., 244 (1969) 4628–4633.