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Detection and quantitation of gadolinium chelates in human serum and urine by high-performance liquid chromatography and post-column derivatization of gadolinium with Arsenazo III

Erlend Hvattum [a,*], Per Trygve Normann [a], Gene C. Jamieson [b], Jan-Ji Lai [b], Tore Skotland [a]

[a] *Nycomed Imaging AS, PO Box 4220, Torshov, N-0401 Oslo, Norway*
[b] *Nycomed Salutar Inc., 428 Oakmead Parkway, Sunnyvale, CA 94086, USA*

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Abstract

A narrow-bore high-performance liquid chromatography method was developed for simultaneous separation of gadolinium diethylenetriaminepentaacetic acid (GdDTPA), the monomethylamide (GdDTPA-MMA) and the bis-methylamide (GdDTPA-BMA) in human serum and urine. The Gd complexes were detected at 658 nm after post-column derivatization with Arsenazo III. The serum samples were ultrafiltrated, whereas the urine samples were centrifuged and diluted before analysis. With an injection volume of 10 μl on a 2.1 mm ID reversed-phase column, the limit of detection of GdDTPA-BMA was calculated as 0.3 μM and 1.1 μM in serum and urine, respectively. The method was validated with respect to GdDTPA-BMA with a limit of quantification set to 2 μM and 10 μM in serum and urine, respectively. The best fit of the calibration curve was obtained using non-linear regression according to the equation $Y = A + BX + CX^2$ in the concentration ranges 2–800 μM and 10–2000 μM of GdDTPA-BMA in serum and urine, respectively. The precision of the method was found to range from 1 to 4% RSD. The recoveries of GdDTPA-BMA spiked in serum and urine were higher than 95% with an RSD equal to or less than 4%. The serum samples were stable for at least 5 months when stored at −70 °C, and the urine samples were stable for a least 6 months when stored at −20 °C.

Keywords: Arsenazo III; GdDTPA-BMA; GdDTPA-MMA; HPLC; Serum; Urine

1. Introduction

Magnetic resonance imaging (MRI) is based on the signal from hydrogen nuclei in low molecular weight and hydrogen-rich compounds, especially water and liquids. Image contrast is based primarily on inherent properties of the different tissues. These inherent tissue properties are hydrogen content (or hydrogen spin density) and hydrogen nuclei relaxation times ($T1$, $T2$, and $T2^*$). MR contrast agents alter image contrast by selectively altering the hydrogen relaxation times of tissues containing the contrast agent. Elements with the highest number of unpaired electron spin relaxation times will have the strongest magnetic relaxation effects on hydrogen.

The gadolinium (Gd) ion is an effective water proton relaxation catalyst, but if administered as a simple salt it is inappropriate for use

* Corresponding author.

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as a diagnostic pharmaceutical because it can be precipitated as salts of biorelevant anions (e.g. phosphate, carbonate, hydroxyl). These precipitates are mainly deposited in liver and spleen and show a very slow excretion rate from the body [1,2]. To reduce the toxicity of the Gd ion it is incorporated into organic compounds, and these water soluble and stable Gd complexes are being increasingly used as MR contrast agents.

A wealth of information exists regarding the chromatographic separation of metal ions (for a review see Ref. [3]), but few analytical methods have previously appeared in the literature regarding the chromatographic separation of Gd-chelate complexes. The HPLC methods described are e.g. ion-pair chromatography in reverse-phase mode with on-line UV and radioactivity detection [4], reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection [5] and reverse-phase anion-exchange HPLC with UV detection [6]. A major disadvantage of UV detection is interference by endogenous compounds in biological samples. This is also to some extent valid for fluorescence detection.

Gadodiamide injection (OMNISCAN®, Nycomed Imaging AS, Norway) is a non-ionic, low osmolar Gd-chelate containing 500 mM Gd diethylenetriaminepentaacetic acid-bis-methylamide (GdDTPA-BMA) and 25 mM caldiamide sodium, a calcium complex of the same ligand. Drugs are mainly metabolized in the liver through oxidation, reduction, conjugation and hydrolysis. GdDTPA-BMA is, however, solely distributed in the extra-cellular fluid and biotransformations in plasma are mostly hydrolytic. GdDTPA-BMA with two amide bonds may consequently be hydrolyzed into its monomethylamide (GdDTPA-MMA) and into GdDTPA, i.e. where both amide bonds are hydrolyzed. As a general rule amides are hydrolyzed much more slowly than esters [7], and studies have shown that the biotransformation of GdDTPA-BMA is negligible such that the entire intravenous dose administered is excreted unchanged in urine [8].

An HPLC method for the quantification of GdDTPA-BMA in biological fluids has briefly been presented [8]. We have improved this method and present an HPLC method which separates GdDTPA-BMA from its two hydrolysis products, i.e. GdDTPA-MMA and GdDTPA. The HPLC method combines the use of ion-pair chromatography in reversed-phase mode and selective detection of Gd complexes by post-column derivatization with Arsenazo III. The method has been validated, according to the principles presented by Karnes et al. [9], with respect to GdDTPA-BMA in human serum and urine.

2. Experimental

2.1. Materials

2,7-Bis(*o*-arsenophenylazo)-1,8-dihydroxy-naphthalene-3,6-disulfonic acid (Arsenazo III) was purchased from Tokyo Kasei Kogyo Co., Japan, GdDTPA-BMA, GdDTPA-MMA and GdDTPA were produced by Nycomed Imaging AS, Oslo, Norway. Seronorm Trace Elements Urine (STE-urine) was from Nycomed Pharma AS, Oslo, Norway. Human urine was from healthy male volunteers. Human serum was commercially available from Red Cross Blood-centre, Oslo, Norway. Water was purified by reversed osmosis, ion exchanged and filtrated through 0.45 μm filter using a Millipore Milli-Q system. Triethylamine, nitric acid and acetic acid were of analytical grade.

2.2. Chromatographic system

The chromatographic system used in this investigation consisted of a Spectra-Physics P4000 mobile phase pump, equipped with Spectra-Physics SCM 400 degassing unit; a Spectra-Physics AS3000 autosampler, equipped with 100 μl (Rheodyne) sample loop; a Spectra-Physics P1500 post-column reagent pump with biocompatible polyetheretherketone (PEEK) tubing and tee; a Spectra-Physics Focus UV/vis detector with a 6 mm 9 μl PEEK flowcell. The column used for the chromatographic analysis was a Supelcosil™ LC-18-DB, 250 × 2.1 mm, 5 μm (Supelco, Inc., PA) reversed-phase column with a corresponding guard column, Supelguard™ LC-18-DB, 20 × 2.1 mm, 5 μm (Supelco, Inc., PA).

The mobile phase consisted of triethylammonium acetate (10 mM) and EDTA (2 mM) (pH 6.5–7.0). The pH of the mobile phase was adjusted with either acetic acid (1 M) or sodium hydroxide (1 M). The post-column reagent consisted of Arsenazo III (0.15 mM), nitric acid (0.1 M) and urea (0.01 M) as described [10]. Prior to use the mobile phase and the post-column reagent were passed through a

0.45 μm filter and were then degassed for 15 min, either under reduced pressure (mobile phase) or with sonication (Bandelin Sonorex, Bandelin Electronic, Berlin, Germany) in a water bath (post-column reagent). The solutions were kept at room temperature and were not used for more than two days.

The flow rate of the mobile phase and post-column reagent was 0.3 ml min^{-1} and they were mixed in a PEEK tee before being carried through to the detector. The separation was performed at 30 °C. The samples were kept at 4 °C and 10 μl was injected for each analysis. Detection of the Gd–Arsenazo III complex was at 658 nm according to Ref. [10]; this was close to λ-max.

2.3. Data handling

Spectra-Physics SpectraSYSTEM™ Software PC1000, v. 1.2, was used for sampling and integration of the chromatograms. GraphPad™ Inplot, v. 4.03, was used for regression analysis and for calculating the sample concentrations.

2.4. Preparation of samples

From a biopharmaceutical analytical point of view a commercial available urine is a more suitable matrix for calibration standards than urine from single volunteers [9] and, consequently, the HPLC method for analysis of Gd complexes has been validated using calibration and control samples in either commercially available human serum or STE-urine. In the cases where urine from single volunteers have been used this is emphasized, i.e. as human urine.

Calibration and control samples with the Gd complexes in the biological fluids were prepared from a stock solution containing GdDTPA-BMA (20 mM), GdNaDTPA-MMA (2 mM) and $GdNa_2DTPA$ (2 mM) in water. The stock solution was diluted in either STE-urine or human urine to a concentration of 2000 μM GdDTPA-BMA and in human serum to a concentration of 800 μM GdDTPA-BMA. The urine and serum samples were further diluted with the appropriate matrix.

After storage at −70 °C (serum samples) or −20 °C (urine samples), the samples were thawed in a refrigerator. The serum samples were subsequently ultrafiltrated at 4 °C with Millipore Ultrafree centrifuge tubes (Millipore Ultrafree-MC, type PTGC, 10 000 NMWL Filter Unit, Millipore Products Division, Bedford, MA, USA) at 5000*g* for 60 min before analysis. The urine samples were centrifuged at 4 °C in Eppendorf microtubes at 15 000*g* for 10 min, and then 100 μl of the supernatant was diluted with 400 μl water before analysis. The urine and serum samples were stable for at least 24 h at 4 °C and the whole procedure was performed within a 24 h period.

3. Results and discussion

The specificity of the method was verified by the high resolution of GdDTPA-BMA versus GdDTPA-MMA and GdDTPA in serum and urine as shown in Fig. 1. Table 1 shows that the capacity factors for GdDTPA-BMA, GdDTPA-MMA and GdDTPA were only slightly affected by the matrix. The resolution (R_s) between GdDTPA-BMA and GdDTPA-MMA in serum was determined to be, $R_s = 5.9 \pm 0.1$ (mean ± SD; $n = 3$), calculated with 800 μM GdDTPA-BMA and 80 μM GdDTPA-MMA. Corresponding values of the resolution of these compounds were also found in urine (results are not shown).

In urine a small peak, with capacity factor 0.85, elutes just before GdDTPA-BMA (see Fig. 1). It was most noticeable in urine spiked with low concentration of GdDTPA-BMA (see Fig. 1(E)) or GdDTPA (not shown). This peak is probably due to transchelation of GdDTPA-BMA, i.e. demetallation of GdDTPA-BMA followed by chelation of Gd with some physiological ligands, in urine. These weakly chelated Gd complexes are detected as GdEDTA under the present HPLC conditions with EDTA added to the mobile phase.

Figs. 1(A) and 1(D) show a small endogenous peak in both serum and urine with approximately the same capacity factor as GdDTPA-BMA, $k' = 1.22$. With an injection volume of 10 μl the limits of detection (LOD) of the assay determined in ultrafiltrated serum and centrifuged urine were 0.3 μM and 1.1 μM, respectively. The LOD was calculated from the mean area of the endogenous peak in the following manner: $LOD = X + 3SD_x$, where X is the mean area ($n = 6$) of the endogenous peak. The present HPLC method was developed to determine both serum pharmacokinetic parameters and urine excretion of GdDTPA-BMA after intravenous administration of

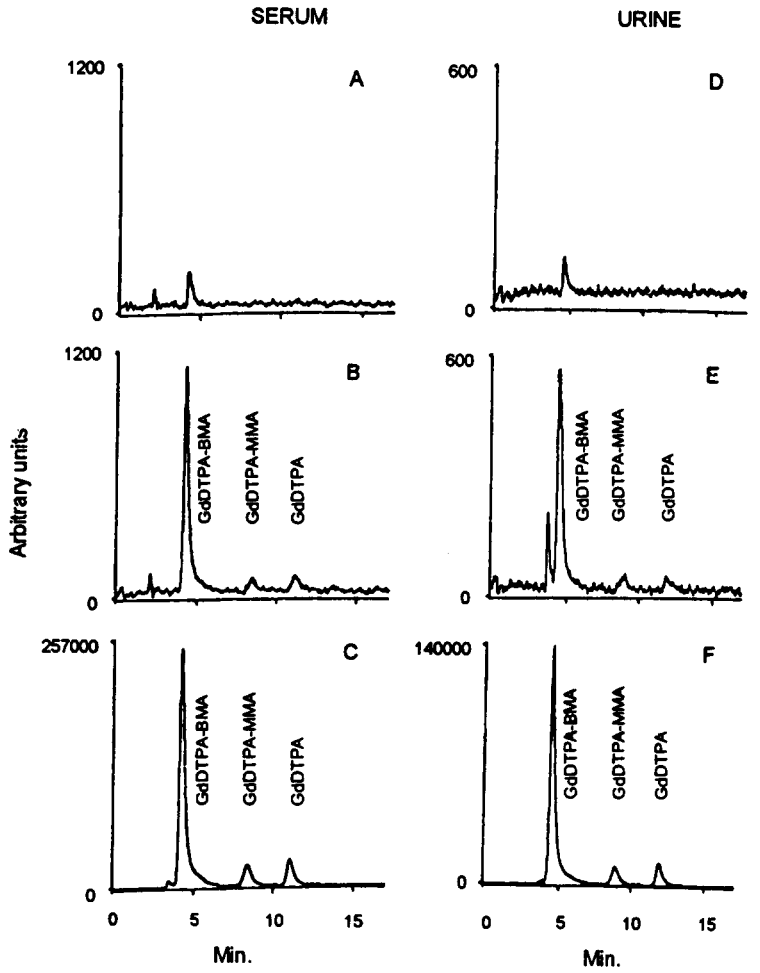

Fig. 1. Typical chromatograms of GdDTPA-BMA, GdDTPA-MMA and GdDTPA obtained from serum and STE-urine (the STE-urine samples have been diluted fivefold). (A) Blank human serum sample; (B) serum spiked with 2 μM GdDTPA-BMA, 0.2 μM GdNaDTPA-MMA and 0.2 μM $GdNa_2DTPA$; (C) serum spiked with 800 μM GdDTPA-BMA, 80 μM GdNaDTPA-MMA and 80 μM $GdNa_2DTPA$; (D) blank STE-urine sample; (E) STE-urine spiked with 10 μM GdDTPA-BMA, 1 μM GdNaDTPA-MMA and 1 μM $GdNa_2DTPA$; (F) STE-urine spiked with 2000 μM GdDTPA-BMA, 200 μM GdNaDTPA-MMA and 200 μM $GdNa_2DTPA$.

Table 1
The capacity factors for GdDTPA-BMA, GdDTPA-MMA and GdDTPA in water, human serum and STE-urine were estimated. The results are given as the mean capacity factor ± SD of a number of independent analyses within the same analytical series. The number of parallels are shown in parentheses

Matrix	GdDTPA-BMA	GdDTPA-MMA	GdDTPA
Water ($n = 9$)	1.25 ± 0.01	3.37 ± 0.02	4.74 ± 0.01
STE-urine ($n = 6$)	1.23 ± 0.01	3.36 ± 0.01	4.70 ± 0.02
Serum ($n = 7$)	1.22 ± 0.004	3.34 ± 0.02	4.68 ± 0.02

gadodiamide injection and therefore for practical reasons, the method was validated with a limit of quantification (LOQ) set to 2 μM in serum and 10 μM in urine. Although Arsenazo III is reported to be selective for lanthanides under the present conditions [10], endogenous metal ions react with the post-column reagent as illustrated by the endogenous peak (see Figs. 1(A) and 1(D)). The identity of this metal complex was not determined. The spectrum of the peak showed no conformity with that of the Gd complex (data not shown).

The stability of the GdDTPA-BMA complex is highly dependent of pH [11] and the post-column reagent, with 0.1 M nitric acid, renders the GdDTPA-BMA complex unstable, and trans- metallation of gadolinium to Arsenazo III is therefore favourable. This reaction is complete within a few seconds (results not shown).

The best fit of the calibration curves was obtained using non-linear regression according

Table 2
The estimated regression parameters of the equation $Y = A + BX + CX^2$ are based on the analysis of three different series with STE-urine spiked with 10, 25, 100, 200, 500, 1000 and 2000 μM GdDTPA-BMA, and four different series with human serum spiked with 2, 5, 20, 40, 100, 200, 400 and 800 μM GdDTPA-BMA

Matrix	A	B	C	r^2
STE-urine [a]	−35 000 ± 8400	8810 ± 310	−0.44 ± 0.13	1.000
Serum [b]	7400 ± 6100	40 050 ± 1210	−9.6 ± 1.6	1.000

[a] Mean ± SD of three different series.
[b] Mean ± SD of four different series.

Table 3
The within-run precision of the method was estimated from the analysis of five independent parallels with GdDTPA-BMA spiked in human urine and serum

Matrix	GdDTPA-BMA level [a] (μM)	RSD (%)
Urine	18.2 ± 0.7	3.9
	198 ± 4	2.0
	1513 ± 39	2.6
Serum	4.8 ± 0.1	2.1
	86 ± 2	2.3
	327 ± 2	0.6

[a] Mean ± SD of five independent parallels.

to the equation $Y = A + BX + CX^2$ with a weighting factor of $1/Y$ in the ranges of 2–800 μM and 10–2000 μM GdDTPA-BMA in serum and urine, respectively. The linearity of a calibration curve can be described by the equation $Y = DX^M$ [12]. For a linear calibration curve the M-value should be equal to unity, and deviation from unity is handled by an almost-linear approach in which a fractional exponent is used to impart some curvature to the calibration [12]. In the present study the values of M for the two calibration curves described above were: $M = 0.91 \pm 0.02$ (mean ± SD; $n = 4$) and $M = 0.96 \pm 0.02$ (mean ± SD; $n = 3$) for GdDTPA-BMA in serum and urine, respectively. In view of this, it was decided to fit the two calibration curves to a second-order polynomial. A weighting of $1/Y$ of the calibration curves increased the precision of the values in the low concentration range of GdDTPA-BMA, and we obtain an acceptable reverse calculated mean that was within ±10% in the whole concentration range of the serum and urine GdDTPA-BMA calibration curve. Table 2 shows the regressionparameters of the non-linear regression equation for a GdDTPA-BMA calibration curve in serum and urine. Calculation of the variations of the regression parameters revealed that the RSDs of coefficient B were 3.0% and 3.1% in serum and urine, respectively and the RSDs of coefficient C were 16.6% and 24.2% in serum and urine, respectively. The calibration curves are, consequently, highly reproducible and the calculated M-values (see above) of the equation $Y = DX^M$ show that the curves deviate only slightly from linearity.

The within-run precision of the method for detection of GdDTPA-BMA in serum and urine was found to be below 4% at the concentrations tested (Table 3).

Table 4
The accuracy of the method was estimated from the analysis of five independent parallels of GdDTPA-BMA spiked human urine and serum. The accuracy of the human urine samples were in proportion to a GdDTPA-BMA calibration curve in STE-urine and the serum samples in proportion to a GdDTPA-BMA calibration curve in serum

Matrix	Added (μM)	GdDTPA-BMA level [a] (μM)	Recovery (%)	RSD (%)
Urine	20	19.1 ± 0.4	95.3	2.1
	200	196 ± 4	98.0	2.0
	1500	1443 ± 24	96.2	1.7
Serum	10	9.9 ± 0.3	99.0	3.0
	75	76 ± 3	101.3	4.0
	300	303 ± 3	101.0	1.0

[a] Mean ± SD of five independent parallels.

Table 5
The STE-urine samples containing (A) 10, (B) 200 and (C) 2000 μM of GdDTPA-BMA were stored at −20 °C for the number of days indicated. The stability of the samples were estimated as mean ± SD in percentage of the concentration measured at zero-time. The number of independent parallels are shown in parentheses

Storage period (days)	Mean conc. ± SD		
	A	B	C
0 ($n = 6$)	100 ± 6.7	100 ± 1.1	100 ± 3.0
13 ($n = 3$)	96.7 ± 5.0	92.0 ± 5.7	95.4 ± 2.9
28 ($n = 3$)	93.3 ± 1.7	100.6 ± 2.3	106.2 ± 0.5
99 ($n = 3$)	85.0 ± 3.3	99.4 ± 1.1	98.9 ± 0.6
175 ($n = 3$)	101.7 ± 3.4	92.0 ± 2.3	92.3 ± 1.1

Table 6
The serum samples containing (A) 5, (B) 100 and (C) 400 μM of GdDTPA-BMA were stored at −70 °C for the number of days indicated. The stability of the samples was estimated as mean ± SD in percentage of the concentration measured at zero-time. The number of independent parallels are shown in parentheses

Storage period (days)	Mean conc. ± SD		
	A	B	C
0 ($n = 6$)	100 ± 1.1	100 ± 2.5	100 ± 1.2
7 ($n = 3$)	91.6 ± 0.5	96.0 ± 2.5	101.2 ± 1.2
44 ($n = 3$)	85.5 ± 1.7	89.1 ± 0.7	92.6 ± 0.7
99 ($n = 3$)	103.2 ± 1.5	102.5 ± 0.9	108.6 ± 2.5
142 ($n = 3$)	105.0 ± 1.9	102.2 ± 3.9	110.4 ± 0.3

The accuracy of the method was evaluated by measuring the recovery of known amounts of GdDTPA-BMA added to human serum and urine prior to sample preparation in proportion to a calibration curve in either water or the respective matrix. The accuracy for GdDTPA-BMA spiked in serum was 87.8 ± 5.9% and 100.3 ± 3.6% (mean ± SD; $n = 18$) in proportion to aqueous and serum calibration standards, respectively (see also Table 4). In urine, the relative recovery of GdDTPA-BMA in proportion to standards in water varied depending on the concentration of the compound due to the transchelation of Gd (see above), which was large at small concentrations of GdDTPA-BMA (see Fig. 1(E)). The accuracy of GdDTPA-BMA spiked urine from single volunteers in proportion to a calibration curve with GdDTPA-BMA in STE-urine was 96.5 ± 2.2% (mean ± SD; $n = 15$) (see also Table 4). Consequently, there is no matrix effect of urine compared to STE-urine. These data show that the quantification of GdDTPA-BMA in serum and urine is most accurate with GdDTPA-BMA calibration curves in serum and STE-urine, respectively.

Table 5 shows that the urine samples are stable for at least 6 months when stored at −20 °C. The recovery of GdDTPA-BMA in the serum samples declined, however, with time when stored at −20 °C (not shown). However, they were stable for at least 5 months when stored at −70 °C (Table 6).

In conclusion, an HPLC method for the analysis and separation of GdDTPA-BMA, GdDTPA-MMA and GdDTPA in human urine and serum has been developed and provides a sufficiently sensitive, accurate and reproducible analytical procedure. The method is applicable to the analysis of GdDTPA-BMA following clinical use of gadodiamide injection.

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