Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 835 (2006) 1–15
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

High-performance liquid chromatographic determination of the magnetic resonance imaging contrast agent Gadocoletate ion in human plasma, urine and faeces

Ilaria Setti [a,*], Roberto Celeste [a], Giancarlo Mariani [b], Daniela Dal Fiume [a], Alberto Morisetti [a], Vito Lorusso [a]

[a] *Milan Research Center, Bracco Imaging SpA, Via E. Folli 50, 20134 Milan, Italy*
[b] *LabAnalysis s.r.l., Via Europa 5, 27041 Casanova Lonati, Italy*

Received 7 October 2005; accepted 11 February 2006
Available online 30 March 2006

Abstract

Gadocoletate ion is a new paramagnetic intravascular contrast agent for magnetic resonance imaging (MRI). An high-performance liquid chromatographic method for assaying Gadocoletate ion in human plasma, urine and faecal samples is described. The analysis is based on the reversed-phase chromatographic separation of Gadocoletate ion from the endogenous components of the biological matrices and its detection during elution by ultraviolet light absorption at 200 nm. The selectivity of the method was satisfactory. The mean absolute recovery during the analytical sample preparation was greater than 87%. The precision, expressed as coefficient of variation (CV%) ranged from 0.29 to 5.90% and the accuracy, expressed as mean relative error (R.E.%) of the analytical method ranged from −3.7 to +7.1%. The detection limit in plasma and urine was 2.01 and 10.0 μg/mL (0.00203 and 0.0101 μmol/mL), respectively. The detection limit in homogenized faecal samples was 17.7 μg/g (0.0179 μmol/g). Stability studies were performed in human plasma and urine samples during the analytical cycle. Gadocoletate ion was shown to be stable in human plasma and in human urine when stored at about +4 °C for up 24 h, and after three freeze-thaw cycles. In addition, it was shown to be stable in samples of processed plasma and in diluted urine at about +4 °C for 48 h, and at room temperature for at least 24 h. As regards the long-term stability of Gadocoletate ion, the results of dedicated studies showed that Gadocoletate ion is stable in human plasma samples when stored at +4 °C for up to 30 days and at −80 °C for up to 90 days. Gadocoletate ion is stable in samples of human urine when stored at +4 °C for up to 30 days, and when stored at −20 °C and at −80 °C for up to 90 days. The method has been successfully validated in human plasma, urine and faeces and it has been shown to be precise, accurate and reliable.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Gadocoletate trisodium; MRI; HPLC analysis; Gadolinium complex; ICP–AES

1. Introduction

Gadocoletate trisodium (Lab. Code B22956/1) is a derivative of gadopentetate bearing on the methylene group of the centrally located acetate group in *S*-configuration, a propionic acid linker to the amino group of the 3β-amino-analogue of deoxycholic acid [1]. Its chemical name according to CAS is trisodium [(3β, 5β,12α)-3-[[(4*S*)-4-[bis[2-[bis[(carboxy-k*O*)methyl]amino-k*N*]ethyl]aminok*N*]-4-(carboxy-k*O*)-1-oxobutyl]amino]-12hydroxycholan-24-oato(6-)]gadolinate(3-) (Fig. 1) and the proposed non-proprietary name (INN) is gadocoletic acid trisodium salt.

Gadocoletate trisodium belongs to the class of intravascular (blood pool) paramagnetic contrast agent for magnetic resonance imaging (MRI). Gadocoletate trisodium is a gadolinium complex containing a lipophilic moiety, which makes it particularly favourable towards a high protein binding. The results of in vivo and in vitro studies showed that Gadocoletate trisodium binds strongly but reversibly to human serum albumin, characterised by a prolonged residence in the vascular compartment.

Gadocoletate ion is the active moiety in Gadocoletate trisodium, responsible for MR contrast enhancement [2].

Concentrations in biological samples of diagnostic agents containing gadolinium can occasionally be determined by using

* Corresponding author. Tel.: +39 0221772699; fax: +39 0221772794.
E-mail address: ilaria.setti@bracco.com (I. Setti).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.02.020

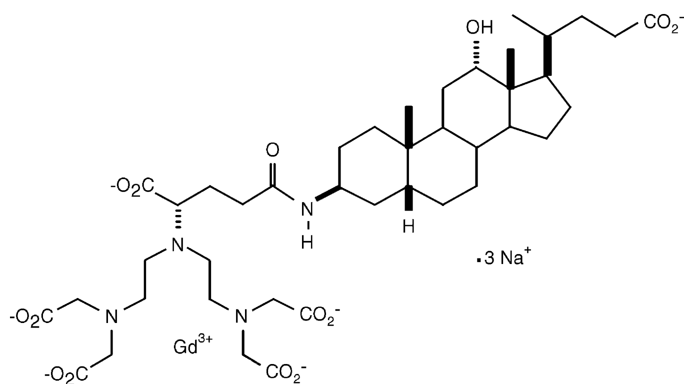

Fig. 1. Structural formula of Gadocoletate trisodium. Gadocoletate trisodium relative molecular mass 1059.17, Gadocoletate ion relative molecular mass 990.2.

radiotracer techniques [3] or by spectrometric techniques such as atomic absorption spectrometry [4], inductively coupled plasma atomic emission spectrometry [5] and X-ray fluorescence [6]. However, these techniques are unable to distinguish the contrast agent and the various chemical species of gadolinium potentially present in the sample (parent compounds and metabolites).

The purpose of this study was to develop and to validate an high-performance liquid chromatographic (HPLC) assay procedure for the measurement of Gadocoletate ion in human plasma, urine and faecal samples.

The analysis is based on the reversed-phase chromatographic separation of Gadocoletate ion from the endogenous components of the biological fluids and its detection during elution by ultraviolet light absorption at 200 nm.

The validation process was performed on human plasma, urine and faecal samples. For all assays an external standard method of calibration was adopted. The validation parameters were evaluated according to FDA guidance for bioanalytical method validation [7].

The described procedure has been used to study the pharmacokinetics of Gadocoletate ion after intravenous administration of Gadocoletate trisodium during a Phase I Clinical Study. A cross-validation has been performed with a non-specific procedure during the analysis of the clinical samples. Plasma, urine and faecal samples were analysed by inductively coupled plasma–atomic emission spectrometry (ICP–AES) for total gadolinium content. The results were compared with the HPLC assay data in order to verify the correspondence between the results and to detect any possible in vivo transmetalation effects.

2. Experimental

2.1. Apparatus

The method validation was performed on two chromatographic systems. The first system was a Merck-Hitachi (Tokyo, Japan) liquid chromatograph, which consisted of a Model L-7100 pump and a Model L-7200 autosampler. The chromatographic system was fitted with a Model L-7455 diode array UV–vis detector (10 mm flow-cell path-length) linked to a Merck-Hitachi work station.

The second system was an HP1100, Agilent Technology (Waldbronn, Germany) liquid chromatograph, which consisted of a Model G1311A pump, a Model G1313A autosampler, a Model G1316A thermostated column compartment and a Model G1322A degasser. The chromatographic system was fitted with a Model G1314A UV–vis detector linked to a HP1100 work station.

Analyses were performed on a Lichrospher 100 RP-8 reversed-phase column (25 cm × 4 mm I.D., particle size 5 μm) (Merck, Darmstadt, Germany) housed in a thermostated oven. A LiChrosorb RP-8 precolumn (2.5 cm × 4 mm I.D., particle size 5 μm) (Merck, Darmstadt, Germany) was used to prevent contamination of the analytical column.

The ICP–AES cross-validation was performed by assaying the gadolinium content in plasma, urine and faecal samples by using an ICP–AES system Perkin-Elmer Italy mod. Optima 3300 DX. Biological samples were mineralized in HNO_3 (65%, v/v) on a microwave oven MARS 5 (C.E.M., USA) before the analysis. The instrumental parameters were 17 mL/min of Argon primary flow, 0.5 mL/min of Argon auxiliary flow, 0.70 mL/min of Argon nebulizer flow, 1380 W of plasma power, 1.2 mL/min of speed injection and detection at a wavelength of 342.246 nm.

2.2. Materials

Gadocoletate trisodium aqueous solution (0.25 M) was prepared by Bracco Imaging SpA (Milan, Italy). Purified water was obtained by Millipore Milli-Q water purification system (Bedford, MA, USA). Analytical grade glacial acetic acid, potassium dihydrogen phosphate, hydrochloric acid (5%, w/w), nitric acid (65%, w/w) and HPLC-grade acetonitrile were obtained from Merck KGaA, (Darmstadt, Germany). Sodium heparin solution (Liquemin®, 5000 IU/mL) was obtained from Hoffman-La Roche (Wyhlen, Germany).

2.3. Biological samples

Blood blank samples were collected from healthy donors and supplied by Hospital Policlinico (Milan, Italy). Plasma samples were obtained by centrifugation of heparinized whole blood (1:50, v/v) for 15 min at about 3500 × g at room temperature. The supernatant plasma was separated, and samples obtained were stored at −20 °C until analysed.

Urine blank samples were obtained from male and female donors of Bracco Imaging laboratories (Milan, Italy) and LabAnalysis laboratories (Casanova Lonati, Italy).

Faecal blank samples were obtained from male and female donors of the LabAnalysis laboratories (Casanova Lonati, Italy).

Urine and faecal samples were stored at −20 °C until analysed.

2.4. Stock standard solutions

Gadocoletate ion stock standard solutions were prepared by diluting Gadocoletate trisodium 0.25 M solution in purified

water. For assay in plasma the concentrations of the stock standard solutions ranged from 60.1 to 14318 μg/mL of Gadocoletate ion; for assay in urine from 30.1 to 5966 μg/mL; for assay in faeces from 59.1 to 14779 μg/mL.

Stock standard solutions were stored in the dark at room temperature. Under these conditions, they were stable for at least 1 month.

2.5. Preparation of plasma samples

Calibrators (CAL) and quality controls (QC) were prepared by mixing 10 μL of each Gadocoletate ion standard solution with 100 μL of blank plasma. To these samples, 100 μL of a mixture (acetonitrile:acetic acid 1000:1, v/v) were added to precipitate the plasma proteins. The tubes were vortex-mixed for 1 min and centrifuged at about 15000 × *g* for 15 min at room temperature; 140 μL of the supernatant were mixed with 60 μL of water and were transferred to autosampler vials for HPLC analysis.

Blank plasma samples were prepared by adding 10 μL of purified water or the equivalent volume of Gadocoletate ion standard solution to 100 μL of plasma. The samples were then processed as described above.

2.6. Preparation of urine samples

Calibrators and quality controls were prepared by mixing 10 μL of each Gadocoletate ion standard solution with 100 μL of blank urine previously diluted 1:10 (v/v) with purified water into Eppendorf tubes. The tubes were vortex-mixed for 1 min. The solution was transferred to autosampler vials for HPLC analysis.

Blank urine samples were prepared by mixing 10 μL of purified water or the equivalent volume of Gadocoletate ion standard solution with 100 μL of 1:10 (v/v) diluted urine. The samples were then processed as described above.

2.7. Preparation of faecal samples

Faecal extract samples were homogenized with water in the ratio 1:3 (100 g of faeces with 300 g of water). Twenty milliliters of purified water were added to 2.5 g of homogenized faecal samples and the suspensions obtained were mixed with rolling shaker for approximately 15 min at room temperature and centrifuged for 15 min at 3500 × *g*. One milliliter of the supernatant (faecal extract) was added with 100 μL of each Gadocoletate ion stock standard solution to obtain Calibrators and quality controls.

Blank faecal samples were prepared by mixing 100 μL of purified water or the equivalent volume of Gadocoletate ion standard solution with 1 mL of faecal extracts prepared as described above.

2.8. Preparation of clinical samples

The preparation of the samples related to the analytical part of the Phase I Clinical Study was as follows:

- Clinical plasma samples were thawed at room temperature under stirring and then 10 μL of purified water were added to an aliquot of 100 μL of plasma. Plasma samples were stored at +4 °C and analysed within 1 month from the collection date. The samples were then processed as described in Section 2.5.
- Clinical urine samples were thawed at room temperature under stirring and then diluted 1:10 (v/v) with purified water. To an aliquot of 100 μL of diluted urine 10 μL of purified water were added. Urine samples were stored at −20 °C and analysed within 90 days from the collection date. The samples were then processed as described in Section 2.6.
- Clinical faecal samples were mixed with water in the ratio 1:3 (w/w) (i.e. 100 g faeces plus 300 g of water) and collected into 50 mL test tubes. Faecal samples were stored at −20 °C and analysed within 1 week from the collection date (homogenized samples).

The samples were then processed as described in Section 2.7.

2.9. Chromatographic conditions

Chromatographic conditions were developed first for in vivo preliminary studies, then for GLP studies where human biological fluids have been analyzed. These studies were carried out at the Pharmatox Laboratory of Milan Research Center, Bracco Imaging SpA, Milan, Italy and archived in the GLP archive of the test facility.

Elution was carried out in plasma, urine and faeces samples by gradient profile using KH_2PO_4 0.01 M with acetonitrile 75:25 (v/v) and acetonitrile at a flow rate of 1 mL/min.

The gradient profile for plasma and urine analysis is described in the following Table:

Time (min)	KH_2PO_4:CH_3CN (75:25, v/v)	% Acetonitrile
0	100	0
10	64	36
10.5	64	36
15	100	0
30	100	0

The gradient profile for faeces analysis is described in the following Table:

Time (min)	KH_2PO_4:CH_3CN (75:25, v/v)	% Acetonitrile
0	78	22
3	78	22
7	45	55
8	45	55
13	78	22
18	78	22

The temperature of the thermostated oven containing the column was set at 45 °C. The UV detection wavelength was 200 nm. The injection volume was 10 μL. The area of the chromatographic peak relative to Gadocoletate ion was integrated and used as analytical response.

2.10. Data processing for HPLC analysis

2.10.1. System suitability test (SST)

A standard solution containing 200 μg/mL of Gadocoletate ion for plasma and urine samples and 39.4 μg/mL of Gadocoletate ion for faecal samples was used for system suitability test. An aliquot (10 μL) was injected daily, before each chromatographic run, to check: retention time, asymmetry, number of theoretical plates, peak area and the general performance of the analytical system. The first SST sample was the reference for the succeeding analytical series. The acceptability criteria of SST were: retention time ± 10%; asymmetry ± 30%, number of theoretical plates > 60%; peak area ± 10%.

2.10.2. Specificity

The specificity of the HPLC assay was assessed qualitatively for the presence of interfering peaks and changes in the analyte retention times. The specificity was checked by comparison of chromatograms from basal matrix and matrix spiked with Gadocoletate ion standard solution at a concentration of 20.0 μg/mL for plasma and urine samples and at a concentration of 98.5 μg/mL for faeces samples. Six independent sources of basal plasma, urine and faeces were checked.

2.10.3. Detection limit (LOD)

The mean noise, calculated in the acceptability range of the analyte retention time and referred to the six chromatogram profiles of basal matrix compared in the specificity analysis, was calculated as follows:

$$N(\text{mean}) = \left[\frac{\sum(\text{height of the chromatographic signals})}{6}\right] \times 3$$

the detection limit was defined by establishing the minimum level at which Gadocoletate ion can be detected. Accuracy and precision values were estimated by external standard.

2.10.4. Quantification limit (LOQ)

The quantification limit was determined by analysing matrix samples spiked with known concentration of analyte that can be quantified with acceptable accuracy and precision (±15%).

2.10.5. Linearity

Linearity was evaluated with five calibration standards (CAL) for each biological matrix on 3 separate days.

The calibration standards ranged from 6.01 to 1432 μg/mL, from 3.01 to 597 μg/mL and from 5.9 to 1478 μg/mL of Gadocoletate ion for plasma, urine (diluted 1:10, v/v with water) and faecal samples (homogenized 1:3, w/w with water), respectively.

The calibration standards expressed as μmol/mL ranged from 0.0061 to 1.446 μmol/mL, from 0.0304 to 6.029 μmol/mL and from 0.00596 to 1.493 μmol/mL of Gadocoletate ion for plasma, urine (undiluted) and faeces samples, respectively.

Ten replicates were analysed for the lowest calibration standard that defined the LOQ, and the highest calibration standard. Six replicates were analysed for the intermediate concentration levels.

For the analysis of each biological matrix the HPLC peak areas of Gadocoletate ion (y) was measured and plotted against each nominal concentrations (x) and a least-squares linear regression was performed by using the equation $y = ax + b$. The variances of the peak areas given by the lowest and highest calibration standards of Gadocoletate ion showed strong heterogeneity thus a natural logarithm transformation was applied to both y and x data to give the equation $\ln(y) = a\ln(x) + b$.

The regression parameters (correlation coefficient, y-intercept and slope of the line) were tabulated for each analysis data.

The area responses for each calibration level were used to back-calculate the Gadocoletate ion concentration, based on the corresponding regression curve. Furthermore, it was determined: the mean, the standard deviation, and the coefficient of variation (CV%) [CV% = (S.D./mean value) × 100] of the back-calculated concentrations.

The following criteria were applied to define the acceptability and linearity of standard curves:

- 15% deviation of the lowest standard calibrator from nominal concentration;
- 10% deviation of the other standard calibrators from nominal concentration;
- coefficient of correlation: ≥0.990 (r);
- Punctual estimates of concentration were determined by inverse interpolation.

2.10.6. Precision

The precision of the assay was evaluated by determining the coefficient of variation occurring intra- and inter-assay analyses [8]. Five QC samples were analysed in replicate ($n = 6$) on 3 separate days and the concentrations of Gadocoletate ion were determined from the daily plasma, urine and faeces standard curve.

2.10.7. Accuracy

The accuracy of the assay was evaluated in 3 separate days by measuring the concentrations of Gadocoletate ion in the QC samples and by comparing them with the nominal concentrations of Gadocoletate ion in the samples.

The accuracy was computed by dividing the mean concentration found ($n = 6$) by the nominal concentration in the sample prepared for the analysis and it was expressed in terms of relative error (R.E.%) of measurement [8]:

$$\text{R.E. (\%)} = \frac{(\text{Mean calculated concentration} - \text{Nominal concentration})}{\text{Nominal concentration}} \times 100$$

2.10.8. Carry-over effect

The carry-over effect was demonstrated by injecting in sequence:

(1) one water sample;

Table 1
Retention times of Gadocoletate ion in human plasma, urine and faeces

Biological matrix	Retention times range (t_R) (min)
Plasma	5.45–6.84
Urine	5.32–6.98
Faeces	7.205–8.154

(2) an aqueous solution of Gadocoletate ion at a concentration of 14.3 mg/mL for plasma and urine samples and of 14.78 mg/mL for faecal samples;
(3) one water sample.

And then comparing the HPLC profiles of the first and last water samples.

2.10.9. Recovery

The absolute recovery of Gadocoletate ion along the sample preparation was determined by adding known amounts of Gadocoletate ion to blank matrix (plasma, urine and faeces) prior to extraction and by comparing the peak area of these standards (matrix samples) with those of blank matrix spiked with the same concentration of Gadocoletate ion, after extraction (reference matrix samples). For urine samples, the absolute recovery was determined by comparing the HPLC area response for the analyte in diluted urine (matrix sample) with the response of Gadocoletate ion in water (reference matrix samples). To assess recovery, plasma, urine and faecal samples were spiked with Gadocoletate ion to obtain solutions at three concentration levels (low, medium and high). For plasma samples the concentrations were: 47.7, 477 and 1193 μg/mL; for urine samples the concentrations were: 47.7, 201 and 477 μg/mL; for faecal samples the concentrations were: 7.88, 197 and 1232 μg/mL. Three replicates were analysed for each concentration level, both for matrix samples and for reference matrix samples.

The matrix samples and the reference matrix samples were analysed by HPLC. Recovery was assessed by comparing the peak areas of the Gadocoletate ion in plasma and urine samples (matrix samples) to the peak areas of the reference matrix samples that represented the 100% recovery.

Recovery was assessed by comparing the calculated concentrations of the Gadocoletate ion in faecal samples (matrix samples) to the calculated concentrations of the reference matrix samples that represented the 100% recovery.

2.10.10. Dilution effect

The dilution effect was evaluated by analyzing samples with a concentration higher than the highest standard calibrator of the standard curve. To validate the dilution procedure one sample of plasma, one sample of urine and one samples of faeces were spiked with Gadocoletate ion to obtain a solution at the concentration of 28637 μg/mL for plasma, 23864 μg/mL for urine and 3941 μg/mL for faecal suspension (see Section 2.7).

Aliquots of plasma and urine samples were diluted with blank human plasma and urine in the ratio 1:50 (v/v).

Aliquots of faecal suspensions were diluted with Milli-Q water in the ratio 1:45 (v/v).

Six replicates of the diluted matrix samples were processed as previously described and analysed by HPLC and the found concentrations were compared against nominal concentrations.

2.10.11. Short-term stability

The short-term stability during the analytical process was checked only for plasma and urine samples.

The stability was evaluated at three concentrations (low, intermediate and high) on both processed and non-processed biological samples during sample collection and handling, during different storage conditions, through the freeze and thaw cycles, during the whole analytical process. Six replicates were injected for each concentration level; calculated concentrations were compared against the initial concentrations at time 0.

(1) Stability at +4 °C in plasma and urine. First HPLC analysis was performed at time 0 and the second after storing at about +4 °C for 24 h.

Table 2
Absolute recoveries of Gadocoletate ion in plasma, urine and faeces

Biological matrix	Nominal concentration (μg/mL)	Unextracted samples (peak area values)			Extracted samples (peak area values)			Mean absolute recovery ($n = 3$) (%)
		Mean ($n = 3$)	S.D.	CV%	Mean ($n = 3$)	S.D.	CV%	
Plasma	47.7	40739	864	2.12	37664	273	0.72	92.5
	477	406260	4251	1.05	375061[a]	(–)	(–)	92.3
	1193	1066987	70928	6.65	931760	4773	0.51	87.3
Urine	47.7	113423	665	0.59	114861	732	0.64	101.3
	201	473352	3498	0.74	477171	1321	0.28	100.8
	477	1112377	7934	0.71	1107115	22740	2.05	99.5
Faeces	7.88	7.80[b]	0.10	1.3	7.70[b]	0.17	2.3	98.7
	197	194.83[b]	0.15	0.08	198.0[b]	1.1	0.57	101.6
	1232	1198[b]	17	1.4	1230.4[b]	7.1	0.58	102.7

(–) Not calculated.
[a] $n = 2$.
[b] Calculated concentration (μg/mL).

Table 3
Linearity of standard curves of Gadocoletate ion in plasma, urine and faeces by HPLC method validation

Biological matrix	Day	*a*	S.D.	*b*	S.D.	*r*
Plasma	1	6.656	0.035	1.0127	0.0065	0.9998
	2	6.724		1.0008		0.9998
	3	6.680		1.0024		0.9998
Urine	1	7.723	0.052	1.0016	0.0089	0.9999
	2	7.774		0.9882		0.9998
	3	7.826		0.9849		0.9998
Faeces	1	1.443	0.039	1.0115	0.0041	0.9998
	2	1.516		1.0034		0.9998
	3	1.500		1.0063		0.9998

Equation from linear regression with a natural logarithm transformation: $\ln(y) = a \ln(x) + b$, where a: intercept, b: slope, S.D.: standard deviation, r: correlation coefficient.

Table 4
Linearity of standard curves of Gadocoletate ion in plasma, urine and faeces by ICP–AES method validation

Biological matrix	Day	*a*	S.D.	*b*	S.D.	*r*
Plasma	1	10.861	0.047	0.986	0.015	0.9998
	2	10.767		0.993		0.9992
	3	10.813		1.015		0.9990
Urine	1	8.8592	0.0056	0.9915	0.0077	0.9998
	2	8.8287		0.9789		0.9997
	3	8.9367		0.9928		0.9996
Faeces	1	0.049	0.021	0.135	0.0026	0.9968
	2	0.016		0.134		0.9958
	3	0.056		0.130		0.9982

Equation from linear regression with a natural logarithm transformation: $\ln(y) = a \ln(x) + b$ where a: intercept, b: slope, S.D.: standard deviation, r: correlation coefficient.

(2) Freeze/thaw stability. First HPLC analysis was performed at time 0 and the second after three freeze-thaw cycles of the samples (each cycle is defined as frozen time followed by thawing at room temperature).
(3) Stability at +4 °C in the processed biological matrix. First HPLC analysis was performed at time 0 and the second after storing at about +4 °C for 48 h
(4) Stability at room temperature in the processed biological matrix. First HPLC analysis was performed at time 0 and the second after storing at room temperature (about +22 °C) for 24 h.

Table 5
Detection limits (LOD) of Gadocoletate ion in plasma, urine (undiluted) and faecal extract (homogenized 1:3, w/w with purified water; 2.5 g suspended in 20 mL of purified water)

Biological matrix	LOD (μg/mL)	LOD (μmol/mL)
Plasma	2.01	0.00203
Urine	10.0	0.0101
Faeces	17.7[a]	0.01788[a]

[a] (μg/g) or (μmol/g).

2.10.12. Long-term stability

The long-term stability was investigated at four concentrations (in the range of the CAL curve) in samples of human plasma and urine. Stability tests were performed after storing the samples under the following conditions in the dark: 30 days at +4 °C; 30 and 90 days at −20 °C; 90 days at −80 °C. For each concentration the difference between the instrumental responses of the compound and of the fresh standard was evaluated by calculating the 90% confidence interval for the relative difference between the geometric means of the two responses according to Timm et al. [9]. Using this 90% confidence interval, the following decision criteria was applied:

- if the interval lies within the limits −0.1 and +0.1 the compound is evaluated as stable;
- if the interval includes −0.1 the stability of the compound is questionable;
- if the interval includes values lower than −0.1 only the compound is evaluated as unstable;

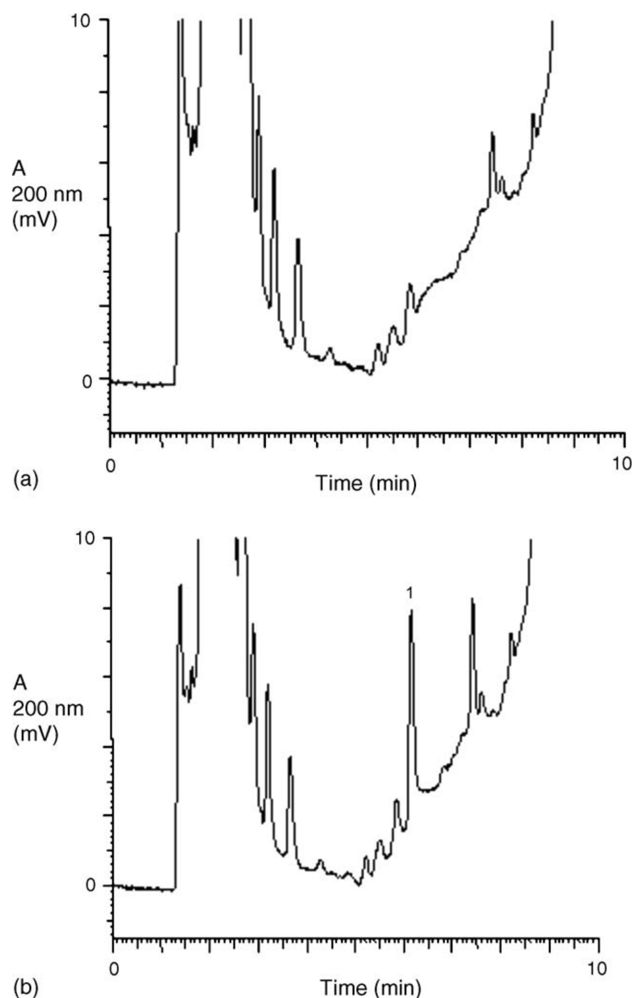

Fig. 2. High performance liquid chromatograms of human plasma extract monitored at 200 nm. Peak 1 is Gadocoletate ion and all other peaks are due to undefined substances remaining in plasma after sample preparation: (a) drug-free plasma extract from human non-patient volunteer and (b) extract of a human plasma sample spiked with 20.0 μg/mL of B22956 ion. The Gadocoletate ion concentration was estimated by interpolation on the standard curve.

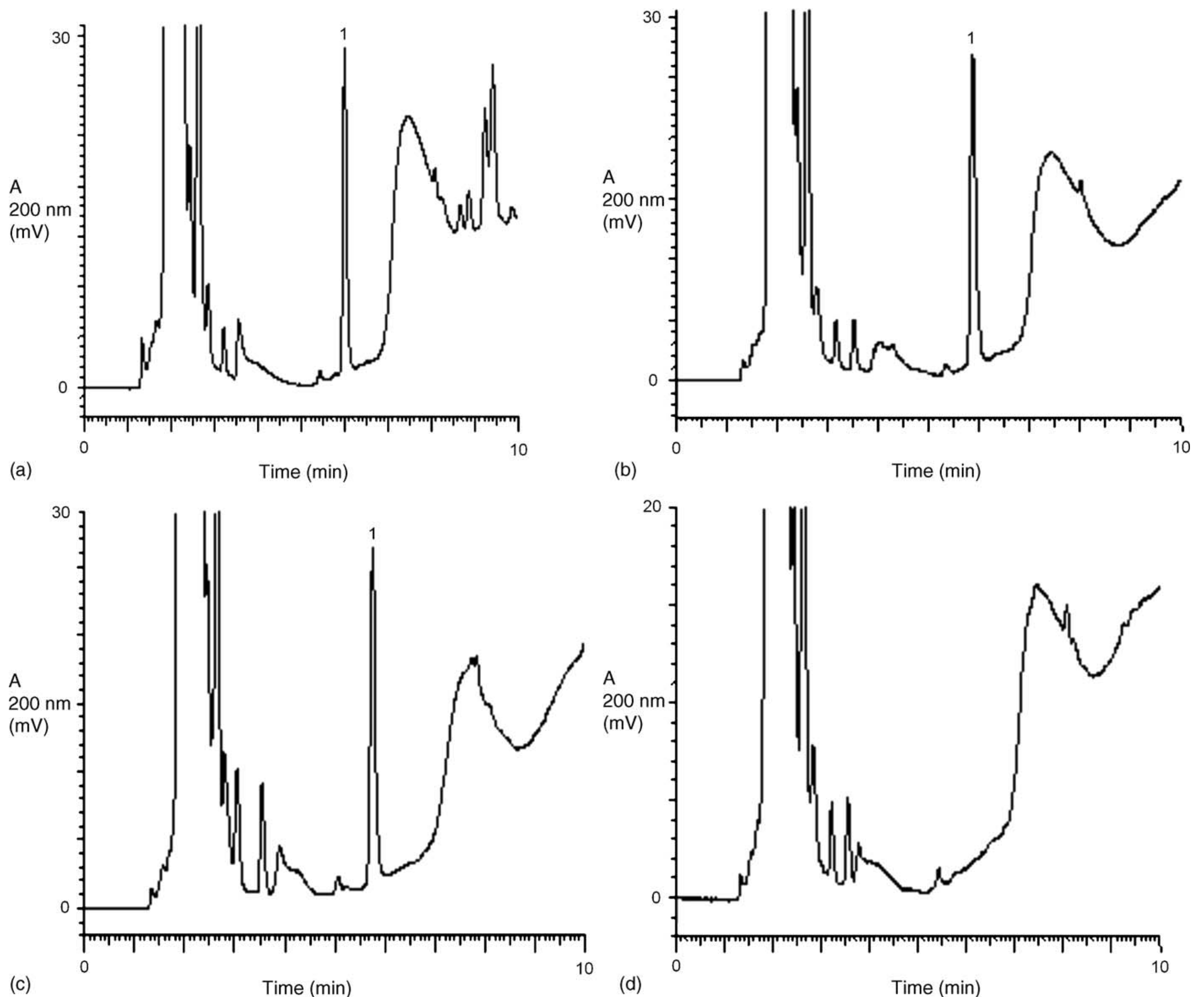

Fig. 3. Long-term stability of Gadocoletate ion in human plasma. Peak 1 is Gadocoletate ion: (a) human plasma sample spiked with 108 μg/mL of Gadocoletate ion (reference); (b) human plasma (spiked with 108 μg/mL) after 30 days at +4 °C; (c) after 90 days at −80 °C and (d) blank plasma sample stored for 90 days at −20 °C.

- if the interval includes +0.1 the possible reasons of the increase in the response of the sample must be indagated.

For each test and comparison the assumed significant level was $\alpha = 0.05$.

2.11. Cross-validation with ICP–AES data on the analyses of clinical samples

The spectroscopic methods for total gadolinium, such as inductively coupled plasma–atomic emission spectrometry, are a valid alternative to chromatographic methods for gadolinium based contrast agent [10].

To this end, an ICP–AES assay on human plasma, urine and faeces samples was developed and validated, in order to compare data obtained in the HPLC analyses with those of the ICP–AES assays.

The ICP–AES analysis is based on the digestion of the biological sample matrix with nitric acid in a microwave oven and the detection of Gadolinium at 342.246 nm. The analytical performances of the ICP–AES method are described below.

The selectivity of the method was satisfactory. The detection limit was 0.51 μg/mL (corresponding to 0.00324 μmol/mL) and 0.09 μg/mL (corresponding to 0.000573 μmol/mL) in plasma and urine (undiluted), respectively. The detection limit in homogenized faecal (homogenized with water in the ratio 1:3, w/w) samples was 0.15 μg/g (corresponding to 0.000954 μmol/g). The quantification limit was 1.9 μg/mL (corresponding to 0.012 μmol/mL) and 0.28 μg/mL (corresponding to 0.00178 μmol/mL) in plasma and urine (undiluted), respectively. The detection limit in homogenized faecal (homogenized with water in the ratio 1:3 w/w) samples was 0.56 μg/g (corresponding to 0.00356 μmol/g). A linear correlation was found between the natural logarithm of ICP–AES area responses

and the corresponding natural logarithm of gadolinium concentrations. The regression parameters (correlation coefficient, *y*-intercept and slope of the line) were tabulated for each analysis data.

Thus, the bioanalytical method described for the measurement of gadolinium in human plasma, urine and faeces samples has been shown to be precise, accurate and reliable and was applied for the cross-validation of the HPLC method for the assay of clinical sample.

3. Results

Table 1 shows the retention times for human plasma, urine and faecal samples. Table 2 shows data for the absolute recoveries. The coefficient of correlation (r) and the regression parameters of the standard curves, which were fitted to data on 3 different days, are shown in Tables 3 and 4 for the ICP–AES analysis. Detection limits of Gadocoletate ion are reported in Table 5.

3.1. Assay of Gadocoletate ion in plasma

Fig. 2 shows representative chromatograms of: (a) drug-free plasma extract from human non-patient volunteer and (b) extract of human plasma sample spiked with Gadocoletate ion.

No interfering peaks at the retention time of Gadocoletate ion were detected in the chromatograms of six blank human plasma samples. Table 6 shows the short-term stability data, indicating no significant degradation of Gadocoletate ion in plasma processed samples stored 24 h at room temperature in darkness in the autosampler. Fig. 3 shows representative chromatograms of: (a) human plasma sample spiked with 108 μg/mL of Gadocoletate ion (reference); (b) human plasma (spiked with 108 μg/mL) after 30 days at +4 °C; (c) after 90 days at −80 °C and (d) blank plasma sample stored for 90 days at −20 °C. Table 7 shows the long-term stability data and Table 8 shows the results of the statistical test according to Timm et al. [9], indicating that Gadocoletate trisodium is stable in samples of human plasma when stored at +4 °C for up to 30 days and at −80 °C for up to 90 days whereas it has to be considered less stable when stored at −20 °C at concentrations close to 10 μg/mL based on the statistical test of Timm et al. [9].

Table 2 shows the absolute recovery of Gadocoletate ion, which was obtained in a range from 87.3 to 92.5%. The best correlation between the peak area and the concentration of Gadocoletate ion was obtained over the range from 6.01 to 1432 μg/mL. Table 9 reports data for the precision and the accuracy of some estimated concentrations for analyses performed on 3 separate days.

3.2. Assay of Gadocoletate ion in urine

Fig. 4 shows representative chromatograms of: (a) drug-free urine extract from human non-patient volunteer and (b) extract of urine plasma sample spiked with Gadocoletate ion.

No interfering peaks at the retention time of Gadocoletate ion were detected in the chromatograms of six blank human urine samples. Table 6 shows the short-term stability data, indicating no significant degradation of Gadocoletate ion in urine processed samples stored 24 h at room temperature in darkness in the autosampler. Fig. 5 shows representative chromatograms of: (a) human urine sample spiked with 45 μg/mL of Gadocoletate ion (reference); (b) human urine (spiked with 45 μg/mL) after 30 days at +4 °C; (c) after 90 days at −20 °C and (d) blank urine sample stored for 90 days at −20 °C. Table 7 shows the long-term stability data and Table 8 shows the results of the statistical test according to Timm, indicating that Gadocoletate trisodium is stable in samples of human urine at all tested storage conditions.

Table 2 shows the absolute recovery of Gadocoletate ion, which was obtained in a range from 99.5 to 101.3%. The best correlation between the peak area and the concentration of Gadocoletate ion was obtained over the range from 3.01 to 597 μg/mL. Table 10 reports data for the precision and the accuracy of some

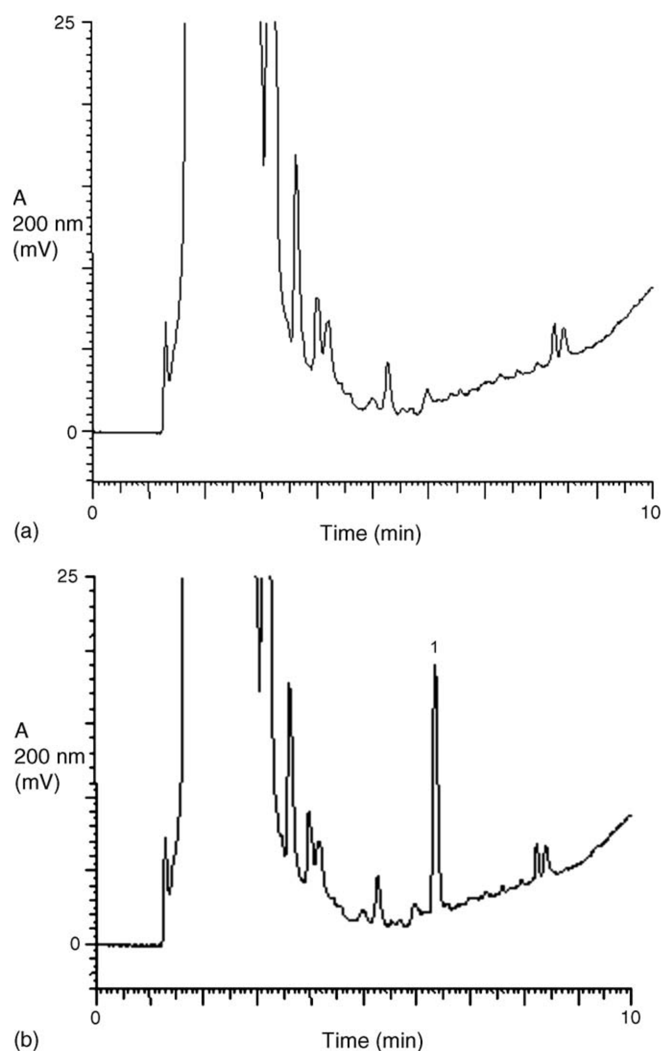

Fig. 4. High performance liquid chromatograms of human urine extract monitored at 200 nm. Peak 1 is Gadocoletate ion and all other peaks are due to undefined substances remaining in urine after sample preparation: (a) drug-free urine extract from human non-patient volunteer and (b) extract of a human urine sample (diluted 1:10) spiked with 20.0 μg/mL of Gadocoletate ion. The Gadocoletate ion concentration was estimated by interpolation on the standard curve.

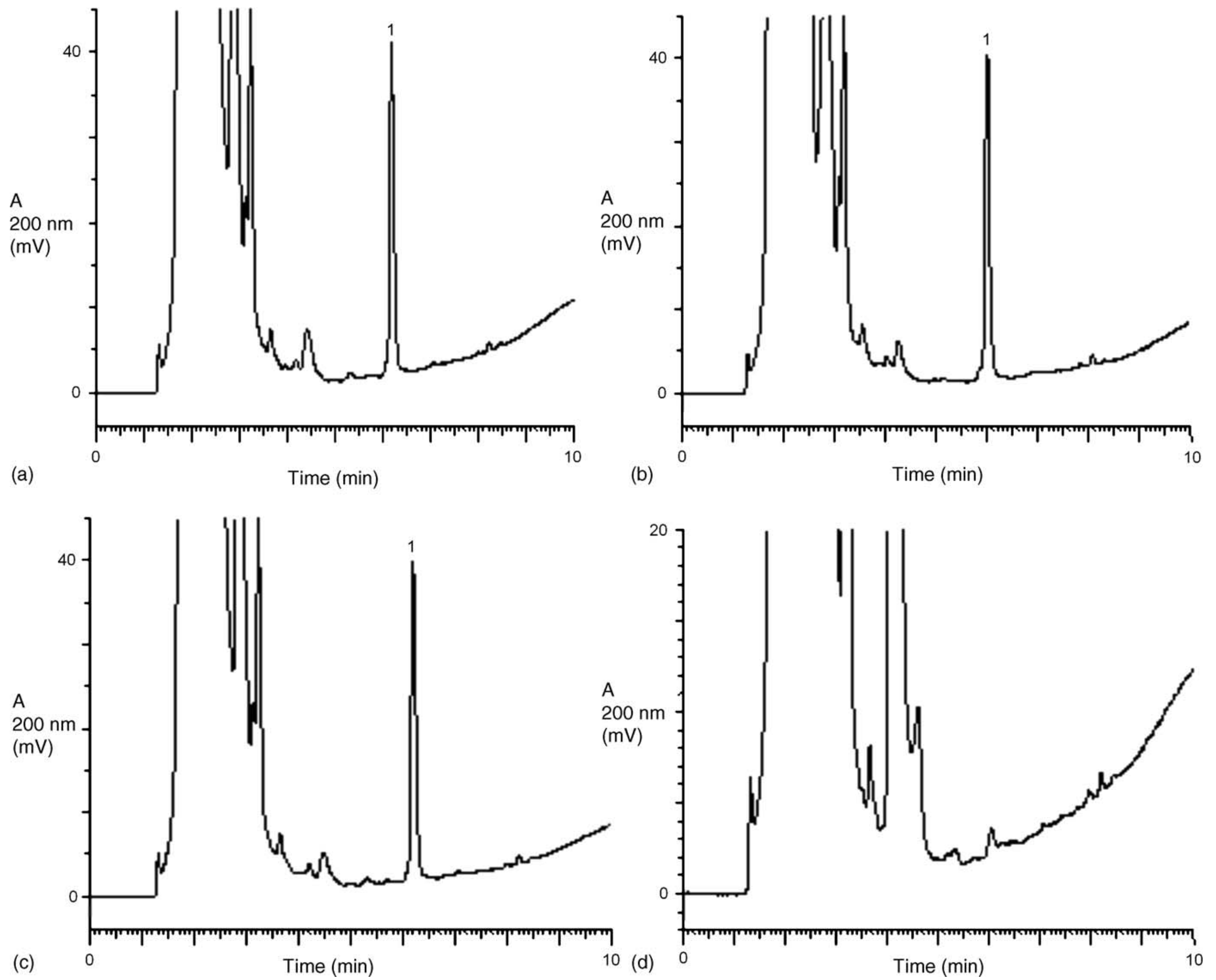

Fig. 5. Long-term stability of Gadocoletate ion in human urine. Peak 1 is Gadocoletate ion: (a) human urine sample spiked with 45 μg/mL of Gadocoletate ion (reference); (b) human urine (spiked with 45 μg/mL) after 30 days at +4 °C; (c) after 90 days at −20 °C and (d) blank urine sample stored for 90 days at −20 °C.

Table 6
Short-term stability

	Time = 0 average concentration (μg/mL)	4 °C for 24 h		Freeze/thaw	
		Average concentration (μg/mL)	Recovery %	Average concentration (μg/mL)	Recovery %
Untreated plasma	50.7	48.1	94.9	45.2	89.2
	497	480	96.5	450	90.6
	1229	1225	99.7	1144	93.1
Untreated urine	48.8	47.7	97.8	48.2	98.7
	206	205	99.5	203	98.4
	486	484	99.8	482	99.2
		4 °C for 48 h		Room temperature for 24 h	
Treated plasma	50.5	55.5	109.9	51.5	101.9
	488	519	106.3	495	101.4
	1209	1311	108.4	1240	102.6
Diluted urine	47.8	48.1	100.7	47.2	98.8
	202	202	99.8	203	100.2
	482	482	100.1	480	99.6

The average concentrations are rounded values.

Table 7
Long-term stability

Fresh[a] sample average peak area (LN)	30 days at +4 °C		Fresh[a] sample average peak area (LN)	30 days at −20 °C		Fresh sample average peak area (LN)	90 days at −20 °C		Fresh sample average peak area (LN)	90 days at −80 °C	
	Average peak area (LN)	Recovery %		Average peak area (LN)	Recovery %		Average peak area (LN)	Recovery %		Average peak area (LN)	Recovery %
Plasma											
9.07	9.08	100.1	9.07	8.85	97.5	9.13	8.74	95.7	9.09	9.08	99.9
11.4	11.5	100.3	11.4	11.4	99.4	11.5	11.4	99.0	11.4	11.4	99.8
13.0	13.0	99.7	13.0	13.0	99.6	13.0	13.0	99.6	13.0	13.0	99.7
14.0	14.0	100.4	14.0	14.0	99.8	14.0	14.0	99.4	14.0	14.0	99.2
Urine											
9.47	9.43	99.6	9.47	9.37	99.0	9.79	9.76	99.7	9.42	9.36	99.4
11.7	11.7	100.1	11.7	11.7	100.0	11.7	11.7	99.8	11.7	12.0	103.2
13.1	13.1	99.9	13.1	13.1	99.9	13.1	13.0	99.3	13.1	13.1	100.0
14.0	14.0	99.9	14.0	14.0	99.9	14.0	14.0	99.8	14.0	14.0	99.7

The average peak area (expressed as natural logarithm, LN) is referred to rounded values.
[a] Fresh samples for both 30 days at +4 and −20 °C.

Table 8
Long-term stability, Timm test: 90% Confidence limits for the relative difference (D) at four concentration levels for the stored and standard samples ($n = 6$) of human plasma and urine

Stored sample	Plasma								Urine							
	10.8 μg/mL		108 μg/mL		499 μg/mL		1347 μg/mL		4.50 μg/mL		45.0 μg/mL		180 μg/mL		450 μg/mL	
	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
30 days, +4 °C	−0.028	0.045	0.017	0.042	−0.057	−0.022	0.029	0.077	−0.073	−0.0011	−0.0058	0.027	−0.025	0.012	−0.012	−0.0020
30 days, −20 °C	−0.22	−0.18	−0.075	−0.048	−0.065	−0.038	−0.052	−0.0082	−0.114	−0.065	−0.018	0.015	−0.023	−0.0019	−0.017	−0.0034
90 days, −20 °C	−0.34	−0.31	−0.13	−0.094	−0.067	−0.044	−0.10	−0.064	−0.046	−0.014	−0.031	−0.020	−0.105	−0.063	−0.027	−0.018
90 days, −80 °C	−0.042	0.022	−0.044	−0.011	−0.059	−0.026	−0.14	−0.070	−0.091	−0.017	−0.015	0.0032	−0.019	0.0004	−0.055	−0.033

Interval of acceptability from −0.1 to +0.1 (values in bold are out of the acceptance criteria).

Table 9
Precision and accuracy for the assay of Gadocoletate ion in plasma

Concentration (μg/mL)	Day	Precision ($n=6$) (CV%)	Accuracy % ($n=6$) (R.E.%)
8.02	1	2.49	5.36
	2	3.93[a]	−1.75[a]
	3	2.68	7.11
47.7	1	2.03[a]	3.35[a]
	2	3.44	−2.52
	3	2.43	3.56
201	1	5.29	3.48
	2	2.71[a]	0.80[a]
	3	1.60	5.67
477	1	4.23	4.19
	2	3.33	0.84
	3	1.60	3.31
1193	1	5.90	−0.50
	2	3.36	2.35
	3	2.40	1.17

CV% = (S.D./mean) × 100; R.E. (%) = (mean calculated concentration − nominal concentration)/nominal concentration × 100.
[a] Values calculated on five replicates.

Table 10
Precision and accuracy for the assay of Gadocoletate ion in urine

Concentration (μg/mL)	Day	Precision ($n=6$) (CV%)	Accuracy % ($n=6$) (R.E.%)
6.01	1	1.13	2.83
	2	1.74	5.32
	3	1.32	−3.01
47.7	1	0.50	0.48
	2	0.29	1.13
	3	0.63	−3.65
201	1	0.88	1.24
	2	1.21	3.03
	3	0.95	−0.30
298	1	1.89	−2.42
	2	0.43	1.95
	3	0.68	−0.77
477	1	0.36	−0.73
	2	0.97	3.52
	3	0.77	0.46

CV% = (S.D./mean) × 100; R.E. (%) = (mean calculated concentration − nominal concentration)/nominal concentration × 100.

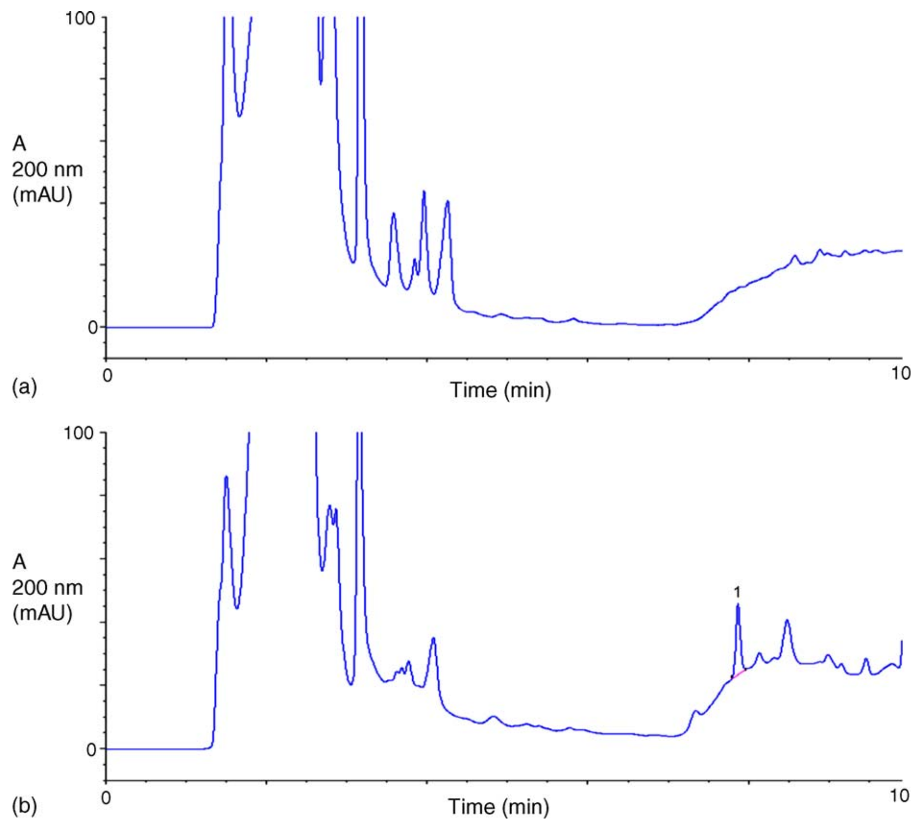

Fig. 6. High performance liquid chromatograms of human faeces extract monitored at 200 nm. Peak 1 is Gadocoletate ion and all other peaks are due to undefined substances remaining in faeces after sample preparation: (a) drug-free faeces extract from human non-patient volunteer and (b) extract of a human faeces sample spiked with 19.7 μg/mL of Gadocoletate ion. The Gadocoletate ion concentration was estimated by interpolation on the standard curve.

estimated concentrations for analyses performed on 3 separate days.

3.3. Assay of Gadocoletate ion in faeces

Fig. 6 shows representative chromatograms of: (a) drug-free faecal extract from human non-patient volunteer and (b) extract of faecal plasma sample spiked with Gadocoletate ion. No interfering peaks at the retention time of Gadocoletate ion were detected in the chromatograms of six blank human faecal samples. Table 2 shows the absolute recovery of Gadocoletate ion, which was obtained in a range from 98.7 to 102.7%. The best correlation between the peak area and the concentration of Gadocoletate ion was obtained over the range from 5.91 to 1478 μg/mL. Table 11 reports data for the precision and the accuracy of some estimated concentrations for analyses performed on 3 separate days.

3.4. Assay of gadolinium in clinical samples and cross-validation

The HPLC method was applied to the assay of Gadocoletate ion in samples collected during a Phase I Pharmacokinetic Study on human non-patient volunteers [11]. Fig. 7 shows representative chromatograms of: (a) blank human plasma sample; (b) plasma sample spiked with Gadocoletate ion and (c) Plasma sample from human non-patient volunteer after intravenous administration of Gadocoletate trisodium.

Fig. 8 shows representative chromatograms of: (a) blank human urine sample; (b) Urine sample spiked with Gadocoletate

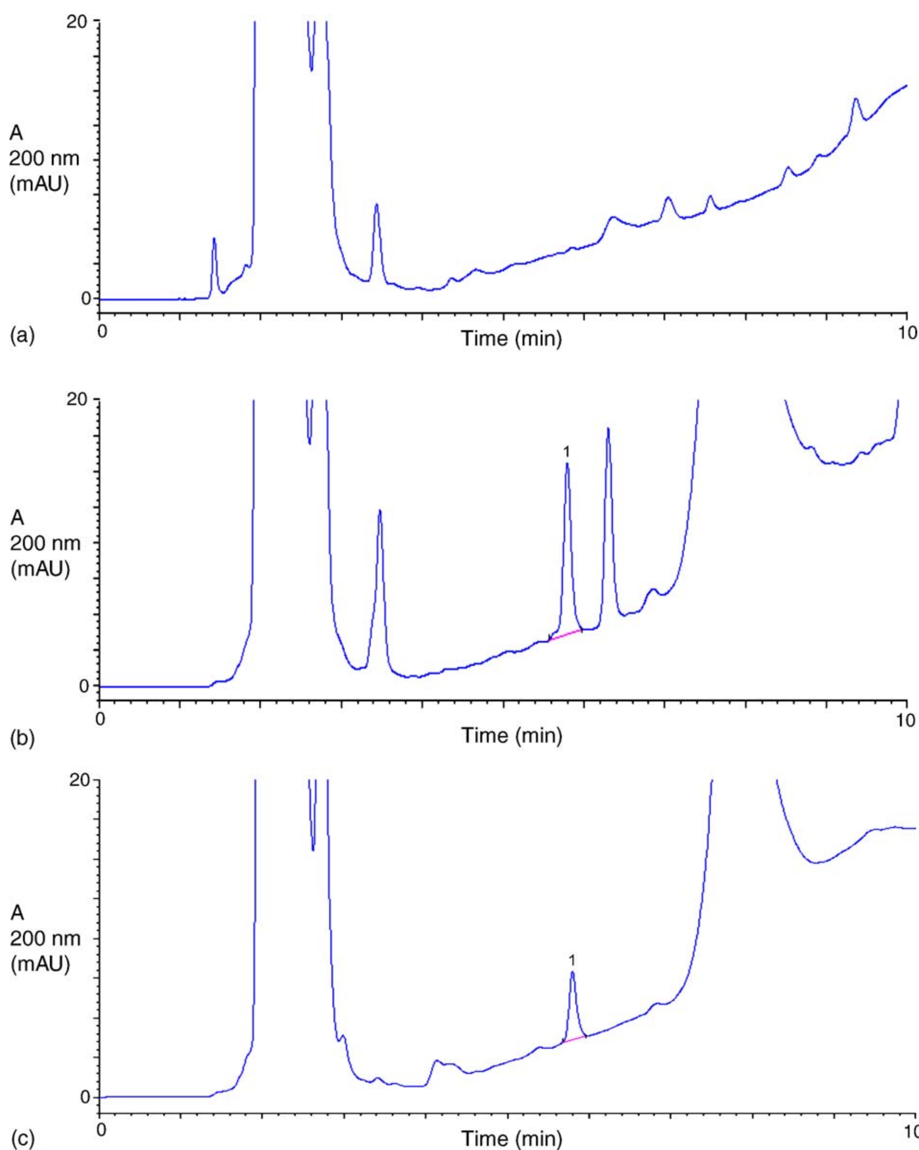

Fig. 7. High performance liquid chromatograms of human plasma extract monitored at 200 nm. Peak 1 is Gadocoletate ion and all other peaks are due to undefined substances remaining in plasma after sample preparation: (a) blank human plasma; (b) plasma sample spiked with Gadocoletate ion (20.0 μg/mL) and (c) plasma sample from human non-patient volunteer 24 h after intravenous administration of Gadocoletate trisodium, assayed concentration 16.1 μg/mL.

ion and (c) urine sample from human non-patient volunteer after intravenous administration of Gadocoletate trisodium.

Fig. 9 shows representative chromatograms of: (a) blank human faeces sample; (b) Faeces sample spiked with Gadocoletate ion and (c) Faeces sample from human non-patient volunteer after intravenous administration of Gadocoletate trisodium. For all biological matrices, the peak corresponding to Gadocoletate ion resulted well separated from the matrix endogenous compounds. The gadolinium assay performed by ICP–AES on the same Phase I clinical samples gave data consistent to those obtained by HPLC.

Fig. 10 shows the line obtained for the regression analysis showing the relationship between HPLC and ICP–AES concentration of Gadocoletate ion in human plasma samples as obtained by the analysis of the same clinical samples. As demonstrated by the regression data, the cross-validation between HPLC and ICP–AES results was satisfactory. Moreover, the correspondence between the assay expressed as mM of Gadocoletate (HPLC) or as mM gadolinium (ICP–AES) strongly demonstrates that the contrast agent did not undergone any transmetallation during the passage through the body and during the storage and analytical process.

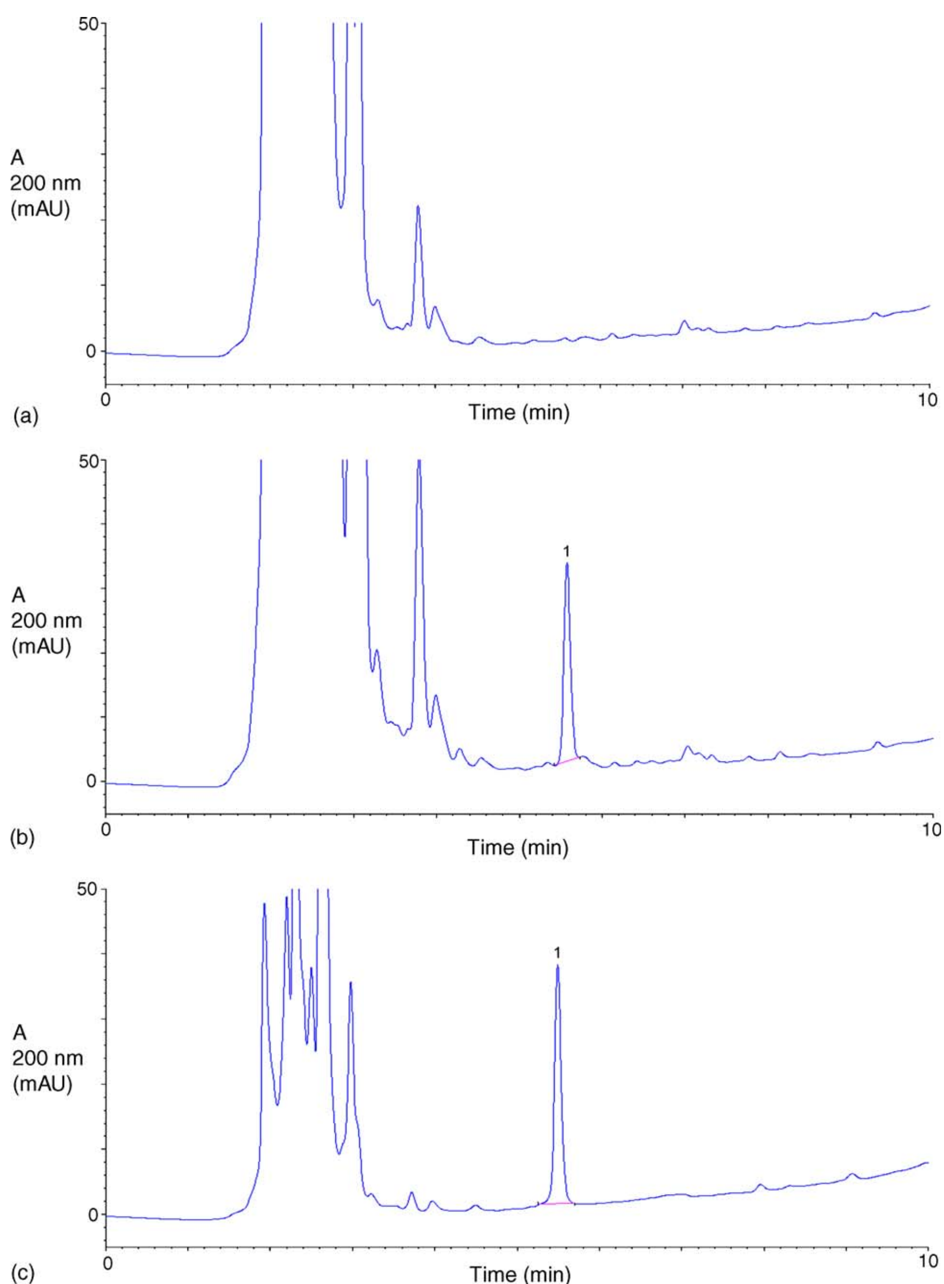

Fig. 8. HPLC chromatograms of human urine monitored at 200 nm. Peak 1 refers to Gadocoletate ion retention time about 6.0 min: (a) blank human urine; (b) urine sample spiked with Gadocoletate ion (48.9 μg/mL) and (c) urine sample from human non-patient volunteer 0–24 h after intravenous administration of Gadocoletate trisodium, assayed concentration 42.4 μg/mL.

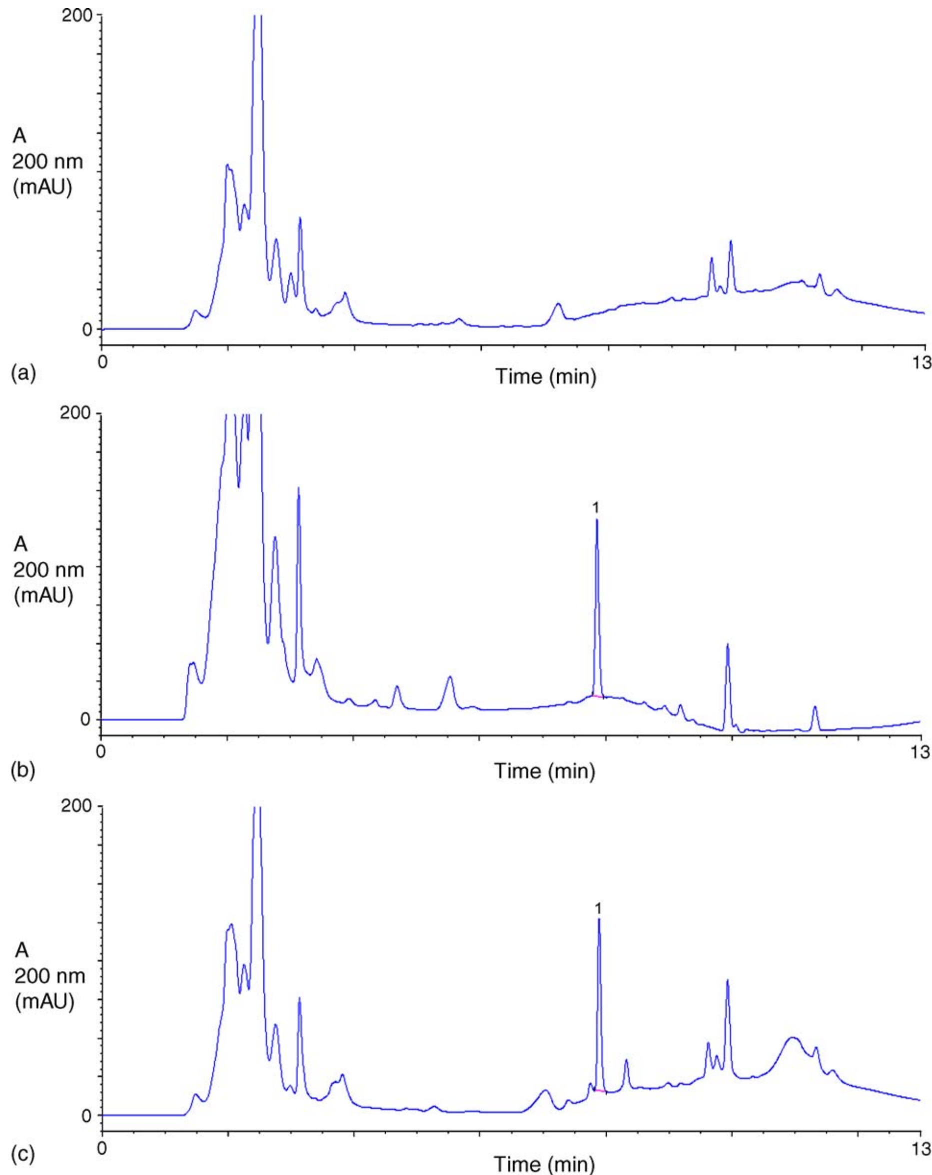

Fig. 9. HPLC chromatograms of human faecal samples monitored at 200 nm. Peak 1 refers to Gadocoletate ion retention time about 6.0 min: (a) blank human faeces; (b) faecal sample spiked with Gadocoletate ion (97.8 μg/mL) and (c) faecal sample from human non-patient volunteer 24–72 h after intravenous administration of Gadocoletate trisodium, assayed concentration 96.8 μg/mL of faecal suspension supernatant.

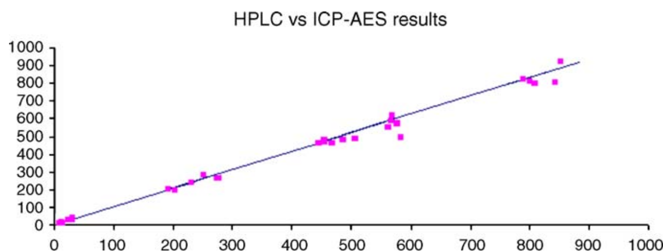

Fig. 10. Line obtained for the regression analysis showing the relationship between HPLC and ICP–AES concentration of Gadocoletate ion in human plasma samples as obtained by the analysis of the same clinical samples. Equation: $y = m \times x$; [HPLC] = 1.04 × [ICP–AES] ($y = mx$); r: 0.9903; $n = 30$.

4. Discussion

An accurate and precise bioanalytical method for the HPLC analysis of Gadocoletate ion in human plasma, urine and faeces has been successfully developed and validated.

The method is specific for the separation and quantification of the analyte from the endogeneous components of the biological matrices. Since the chromatographic peak of Gadocoletate ion is completely separated from any other peak in plasma, urine and faeces, the described assays can be said to have an excellent selectivity.

The detection limit of Gadocoletate ion was 2.01 and 10.0 μg/mL for plasma and urine (undiluted) samples, respectively and 17.7 μg/g for homogenized (1:3 with water, w/w) faecal samples.

Table 11
Precision and accuracy for the assay of Gadocoletate ion in faeces

Concentration (μg/mL)	Day	Precision ($n = 6$) (CV %)	Accuracy % ($n = 6$) (R.E.%)
7.88	1	1.66	7.02
	2	2.01	0.89
	3	2.50	−3.55
49.3	1	1.06	3.48
	2	1.05	−1.12
	3	0.78	1.49
197	1	0.60	2.00
	2	0.51	−1.44
	3	1.07	−0.31
493	1	0.60	4.79
	2	3.70	4.09
	3	4.38	1.87
1232	1	1.02	3.32
	2	0.68	3.29
	3	0.53	−0.09

CV% = (S.D./mean) × 100; R.E. (%) = (mean calculated concentration − nominal concentration)/nominal concentration × 100.

The quantification limit of Gadocoletate ion was 6.01 and 30.1 μg/mL for plasma and urine (undiluted) samples, respectively and 53.1 μg/g for homogenized (1:3 with water, w/w) faecal samples.

The method has been demonstrated to show acceptable precision and accuracy throughout the concentration range over which good linearity was observed.

The calibration curve of Gadocoletate ion in plasma ranged from 6.01 to 1432 μg/mL of Gadocoletate ion, corresponding to 0.00607/1.446 μmol/mL.

The calibration curve of Gadocoletate ion in 1:10 (v/v) diluted urine ranged from 3.01 to 597 μg/mL of Gadocoletate ion, corresponding to 0.00304 ÷ 0.6029 μmol/mL.

The calibration curve of Gadocoletate ion in faecal extract ranged from 5.91 to 1478 μg/mL of Gadocoletate ion corresponding to 0.00596/1.493 μmol/mL.

Plasma and urine samples containing concentrations of Gadocoletate ion in excess of the validated range can be precisely and accurately analysed after dilution with blank human plasma and urine. Faecal samples containing concentrations of Gadocoletate ion in excess of the validated range can be precisely and accurately analysed after dilution with Milli-Q water.

The stability in human plasma and urine was evaluated, and the Gadocoletate ion resulted stable for 24 h at about +4 °C. Gadocoletate ion is stable after three freeze-thaw cycles for both the matrices. In the processed plasma and diluted urine, Gadocoletate ion is stable at about +4 °C for 48 h and at room temperature for 24 h. The long-term stability data, indicated that Gadocoletate trisodium is stable in samples of human plasma when stored at +4 °C for up to 30 days and for up to 90 days at −80 °C whereas it has to be considered less stable when stored at −20 °C in particular at concentrations close to 10 μg/mL. The long-term stability in human urine indicated that Gadocoletate trisodium is stable at all tested storage conditions.

It is concluded that the bioanalytical method described for the measurement of Gadocoletate ion in human plasma, urine and faeces has been successfully validated and it has been shown to be precise, accurate and reliable.

References

[1] A. Preda, V. Novikov, M. Moglich, K. Turetschek, D.M. Shames, J. Magn. Reson. Imaging 20 (5) (2004) 865.
[2] F.M. Cavagna, V. Lorusso, P.L. Anelli, F. Maggioni, C. de Haen, Acad. Radiol. 9 (2) (2002) S491.
[3] B.L. Engelstad, D.L. White, J.P. Huberty, C.S. Wynne, E.C. Ramos, M.T. McNamara, H.I. Goldberg, Invest. Radiol. 22 (1987) 232.
[4] L. Liang, P.C. D'Haese, L.V. Lamberts, F.L. Van de Vyver, M.E. De Broe, Anal. Chem. 63 (1991) 423.
[5] J.G. Crock, F.E. Lichte, Anal. Chem. 54 (1982) 1329.
[6] M. Lal, R.K. Choudhury, R.M. Agrawal, X-ray Spectrom. 16 (1987) 23.
[7] FDA, Guidance For Industry, Bioanalytical method validation, 2001.
[8] IUPAC, Pure Appl. Chem. 66 (1994) 595.
[9] U. Timm, M. Wall, D. Dell, J. Pharm. Sci. 74 (1985) 972.
[10] E.M. Frame, E.E. Uzgiris, Analyst 123 (1998) 675.
[11] V. Lorusso, I. Setti, F. Cavagna, C. Wool, R. La Ferla, M.A. Kirchin, J.R. Parker, G. Pirovano, Proceedings of the 13th ISMRM Scientific Meeting in Miami Beach, FL, May 7–13, 2005.