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Journal of Chromatography B, 656 (1994) 415–422

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic assay of the magnetic resonance imaging contrast agent gadobenate in plasma, urine and bile

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(First received December 28th, 1993; revised manuscript received February 23rd, 1994)

Abstract

Gadobenate dimeglumine (Gd-BOPTA–Dimeg) is currently under evaluation as an intravascular paramagnetic contrast agent for magnetic resonance imaging. The anion Gd-BOPTA^{2-} is the moiety of Gd-BOPTA–Dimeg responsible for contrast enhancement. An HPLC method for assaying gadobenate (Gd-BOPTA^{2-}) in plasma, urine and bile samples is described. The analysis is based on the reversed-phase chromatographic separation of the ion pair Gd-BOPTA^{2-}–tetrabutylammonium from the endogenous components of biological fluids and its detection by UV absorption at 210 nm. The mean accuracy and precision of the method were in the range −3.4 to +5.0% and 0.2–3.5%, respectively. The method detection limits for Gd-BOPTA^{2-} in plasma (0.8 ml), urine (0.2 ml) and bile (1.0 ml) were 1.1, 7.6 and 1.7 μM (corresponding to 0.73, 5.1 and 1.1 μg/ml), respectively.

1. Introduction

Gadobenate dimeglumine (Gd-BOPTA–Dimeg) is (4*R*,*S*)-{[4-carboxy-5,8,11-tris(carboxymethyl)-1-phenyl-2-oxa- 5,8,11- triazatridecan-13-oato(5-)] gadolinate (2-)} dihydrogen compound with 1-deoxy-1-(methylamino)-D-glucitol (1:2) (Fig. 1). This compound is currently under evaluation as an intravascular paramagnetic contrast agent for magnetic resonance imaging [1,2]. The anion Gd-BOPTA^{2-} is the moiety of Gd-BOPTA–Dimeg responsible for contrast enhancement.

Concentrations of diagnostic agents containing gadolinium in biological samples can be determined by assaying the total gadolinium content by radiochemical methods [3] and spectrometric techniques such as atomic absorption spectrometry (AAS) [4], inductively coupled plasma atomic emission spectrometry (ICP-AES) [5,6]

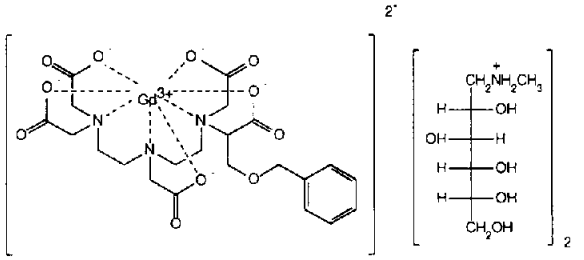

Fig. 1. Structure formula of gadobenate dimeglumine (Gd-BOPTA–Dimeg). Gd-BOPTA–Dimeg molecular mass, 1058.17 u; Gd-BOPTA^{2-} molecular mass, 665.72 u.

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0378-4347/94/$07.00
SSDI 0378-4347(94)00136-S

and X-ray fluorescence (XRF) spectrometry [7]. Despite their reliability, these techniques are not specific; they are unable to distinguish the various chemical species of gadolinium present in the sample (parent compound and metabolites) and therefore provide no precise information on the fate of the compound in the organism.

The high-performance liquid chromatographic (HPLC) method described here permits the selective determination of Gd-BOPTA^{2-}–tetrabutylammonium from the endogenous components of the biological fluids and its detection during elution by UV light absorption at 210 nm.

For plasma and bile assays, which required sample pretreatment, an internal standard method of calibration was employed. For assays on urine an external standard method was adopted.

2. Experimental

2.1. Apparatus

The assays were performed on a Merck–Hitachi (Tokyo, Japan) liquid chromatograph which consisted of a Model 655A-11 pump controlled by an L-5000 LC gradient programmer. The chromatographic system was fitted with a L-4250 variable-wavelength UV–Vis detector (5 mm flow cell path length) linked to a Model 450 MT2 data system (Kontron, Munich, Germany). Analyses were performed on a Hypersil-ODS reversed-phase column (20 cm × 4.6 mm I.D., particle size 5 μm) (Hewlett-Packard, Waldbronn, Germany) housed in a thermostated oven. A LiChrosorb RP-8 precolumn (3 cm × 4 mm I.D., particle size 7 μm) (Merck, Darmstadt, Germany) was used to prevent degradation of the analytical column.

2.2. Materials

Gd-BOPTA–Dimeg and (1′*R*,*S*) - {[1 - (1′ - carboxy - 2′ - phenoxyethyl) - 4,7,10 - tris(carboxymethyl) - 1,4,7,10 - tetraazacyclododecane(4-)]gadolinate(1-)} hydrogen compound with 1-deoxy-1-methylamino-D-glucitol (1:1) (coded B19106/7 and used as an internal standard) were synthesized by Bracco (Milan, Italy). B19106$^-$ (M_r 677.80) is the code for the anion in B19106/7 (M_r 874.02).

Purified water was obtained with a Millipore (Bedford, MA, USA) Milli-Q water-purification system. Tetrabutylammonium dihydrogenphosphate was obtained under the name of PIC A Reagent from Millipore–Waters (Milford, MA, USA). HPLC-grade acetonitrile and analytical-reagent grade glacial acetic acid and chloroform were obtained from Merck. Heparin solution (5000 U/ml) was obtained under the name of Liquemin from Hoffman–La Roche (Wyhlen, Germany).

2.3. Biological samples

Samples of human plasma and urine and rabbit bile were prepared. Samples of blank human plasma and urine were obtained from healthy subjects maintained on a normal diet. Test samples of human plasma and urine were taken from subjects previously administered a solution of 0.25 *M* Gd-BOPTA–Dimeg at a dose of 0.2 mmol/kg (0.13 g/kg). The plasma samples in both instances were prepared by collection of whole blood in test-tubes containing heparin solution (5000 U/ml) in a ratio of 10:1 (v/v) and subsequent centrifugation (10 min at 3500 *g*). The bile samples were obtained from control HY/Cr rabbits (Charles River, Como, Italy) and from HY/Cr rabbits to which a 0.25 *M* solution of Gd-BOPTA–Dimeg had been administered at a dose of 0.1 mmol/kg (0.067 g/kg). The samples were obtained in both instances after cannulation of the bile duct.

Method development and stability studies of gadobenate in biological fluids were carried out on plasma, urine and bile samples taken from rabbits.

2.4. Stock standard solutions

Gd-BOPTA^{2-} stock standard solutions for assays in each biological fluid were prepared by diluting a 0.25 *M* Gd-BOPTA–Dimeg solution in purified water. For assays in plasma and bile the stock Gd-BOPTA^{2-} concentration range was

0.30–37 m*M* (0.20–25 mg/ml); for assays in urine the range was 1.0–88 m*M* (0.67–59 mg/ml).

Aqueous solutions of B19106/7 at concentrations of 7.7 and 15 m*M* were prepared by diluting a 0.5 *M* solution. These were used as internal standards for the assay in plasma and bile, respectively. Stock standard solutions and biological samples were stored in the dark at 4°C. Under these conditions, they were stable for at least 1 month.

2.5. Preparation of plasma samples

Each calibration standard solution was prepared by adding 0.1 ml of the desired Gd-BOPTA^{2-} stock standard solution and 0.1 ml of 7.7 m*M* aqueous B19106^{-} (internal standard) to 0.8 ml of blank plasma taken from healthy subjects. To this sample 0.9 ml of acetonitrile was added to precipitate plasma proteins. After agitation and subsequent centrifugation (10 min at 2800 *g*), 20 μl of the clear supernatant were injected into the chromatograph. To determine the Gd-BOPTA^{2-} content in the plasma of healthy volunteers treated with Gd-BOPTA–Dimeg (0.2 mmol/kg), 0.1 ml of purified water rather than the equivalent volume of Gd-BOPTA^{2-} stock standard solution and 0.1 ml of the internal standard solution were added to 0.8 ml of plasma. The sample was then processed as described above.

2.6. Preparation of urine samples

Urine was filtered through Millipore HAWP filters (0.45 μm pore size) and 0.2 ml thereof was diluted to 1 ml with purified water. Each calibration standard solution was prepared by adding 0.1 ml of the desired Gd-BOPTA^{2-} stock standard solution to 1 ml of diluted urine. A 10-μl volume of the clear solution was used for chromatographic analysis. To determine the Gd-BOPTA^{2-} content in urine from healthy volunteers treated with Gd-BOPTA–Dimeg (0.2 mmol/kg), 0.1 ml of purified water rather than the equivalent volume of Gd-BOPTA^{2-} was added to 1 ml of filtered and diluted urine as described above.

2.7. Preparation of bile samples

Each calibration standard solution was prepared by adding 0.1 ml of the desired Gd-BOPTA^{2-} stock standard solution and 0.1 ml of 15 m*M* aqueous B19106^{-} (internal standard) to 1 ml of bile. To this sample 0.2 ml of glacial acetic acid and 1.5 ml of chloroform were added. The mixtures were shaken for 30 min at room temperature (20–22°C) and then centrifuged (10 min at 2800 *g*). Samples of 10 μl of the aqueous layer were taken for chromatographic analysis. To determine the Gd-BOPTA^{2-} content of bile from animals treated with Gd-BOPTA–Dimeg (0.1 mmol/kg), 0.1 ml of purified water rather than the equivalent volume of Gd-BOPTA^{2-} stock standard solution was added to 1 ml of bile plus 0.1 ml of internal standard. The sample was then processed as described above.

2.8. Chromatographic conditions

The chromatographic conditions were the same for all samples. Elution was carried out isocratically with a 74:26 (v/v) mixture of a $6.8 \cdot 10^{-3}$ *M* aqueous solution of tetrabutylammonium dihydrogenphosphate and acetonitrile at a flow-rate of 1 ml/min. The mobile phase was filtered by passing it through a 0.45-μm Millipore filter and degassed before use. The temperature of the thermostated oven containing the column was set at 40°C. The UV detection wavelength was 210 nm.

2.9. Stability

The stability of Gd-BOPTA–Dimeg was tested in plasma, urine and bile over the range of concentrations of pharmacokinetic relevance. Blank plasma, urine and bile samples were spiked with Gd-BOPTA–Dimeg stock standard solutions to give Gd-BOPTA^{2-} concentrations ranging from 0.075 to 2.5 m*M* (0.050–1.7 mg/ml).

The conditions tested were storage for 1 day in

light at 20°C, storage for 1 and 7 days in darkness at 4°C, storage for 1 month in darkness at 4°C and storage for 3 months in darkness at −19°C. The Gd-BOPTA^{2-} concentrations in the stored samples were compared with those in freshly prepared solutions.

2.10. Data processing

For plasma and bile analyses, the ratio of the Gd-BOPTA^{2-} peak area to the internal standard (B19106^{-}) peak area was calculated and plotted for each calibration standard solution and a least-squares linear regression was performed. For urine analysis the peak area of Gd-BOPTA^{2-} was calculated and plotted for each calibration standard solution and a least-squares linear regression was performed. Linearity was assessed by performing an analysis of variance and test of linearity on the calibration standard data. As the value of the *y*-intercept was not statistically different from zero, the data were fitted by origin-crossing lines. Sample concentrations were determined by interpolation from such origin-crossing calibration graphs.

The selectivity of the chromatographic method was evaluated by checking for interferences due to drug-free plasma, urine and bile. Further, the selectivity was verified from the peak purity analysis, which was performed by comparison of three UV spectra recorded at the beginning, apex and end of the Gd-BOPTA^{2-} peak present in the samples.

The evaluation of the precision and accuracy of the method was performed using two standard solutions for each biological fluid: 0.10 and 2.0 m*M* in plasma, 2.5 and 13 m*M* in urine and 0.075 and 1.6 m*M* in bile. The assays were repeated five times. The accuracy of the method was calculated from the percentage difference between the mean calculated concentrations and the known Gd-BOPTA^{2-} content of the solutions. Precision was determined from the relative standard deviation expressed as a percentage of the mean.

The method detection limits were determined by analysing fifteen replicate samples containing 0.012 m*M* (8.0 μg/ml) Gd-BOPTA^{2-}, corresponding to a concentration near the instrumental detection limit. For each biological fluid the mean concentration and the standard deviation (S_m) were calculated. Method detection limits (*MDL*) were estimated as described by Inman and Rickard [8] by the equation

$$MDL = \frac{K\sqrt{2}}{\sqrt{n_{\text{replicates}}}} \cdot S_m \quad (1)$$

where K is a constant corresponding to the Student's t distribution for the 95% confidence level and S_m is the standard deviation of the method, expressed as concentration. The value of $n_{\text{replicates}}$ was fixed at 2 because in routine analyses the assay was performed in duplicate.

The stability of Gd-BOPTA–Dimeg was assessed by means of the statistical procedure of Timm *et al.* [9], which is based on the percentage difference in concentration between stored and freshly prepared samples in terms of variability. A degradation of 10% is considered pharmacokinetically relevant.

3. Results

3.1. Assay of Gd-BOPTA^{2-} in plasma

Fig. 2 shows representative chromatograms of (a) blank human plasma, (b) a spiked sample (calibration standard) of human plasma (0.8 ml) containing 0.40 m*M* (0.27 mg/ml) Gd-BOPTA^{2-} and (c) a plasma sample from a healthy volunteer to which Gd-BOPTA–Dimeg had been administered. The mean retention times ± standard deviation for Gd-BOPTA^{2-} and the internal standard (B19106^{-}) obtained from determinations conducted on three days ($n = 20$) were 8.7 ± 0.1 and 4.5 ± 0.1 min, respectively. Although the region of the internal standard (peak 6 bis) is not clear in the blank chromatogram, integration of several blank samples indicated the peak area of interference (peak 6) to be relatively small and constant. As the concentration of B19106^{-} was also constant, it was considered justifiable to take the combined chromatographic signal arising from both B19106^{-}

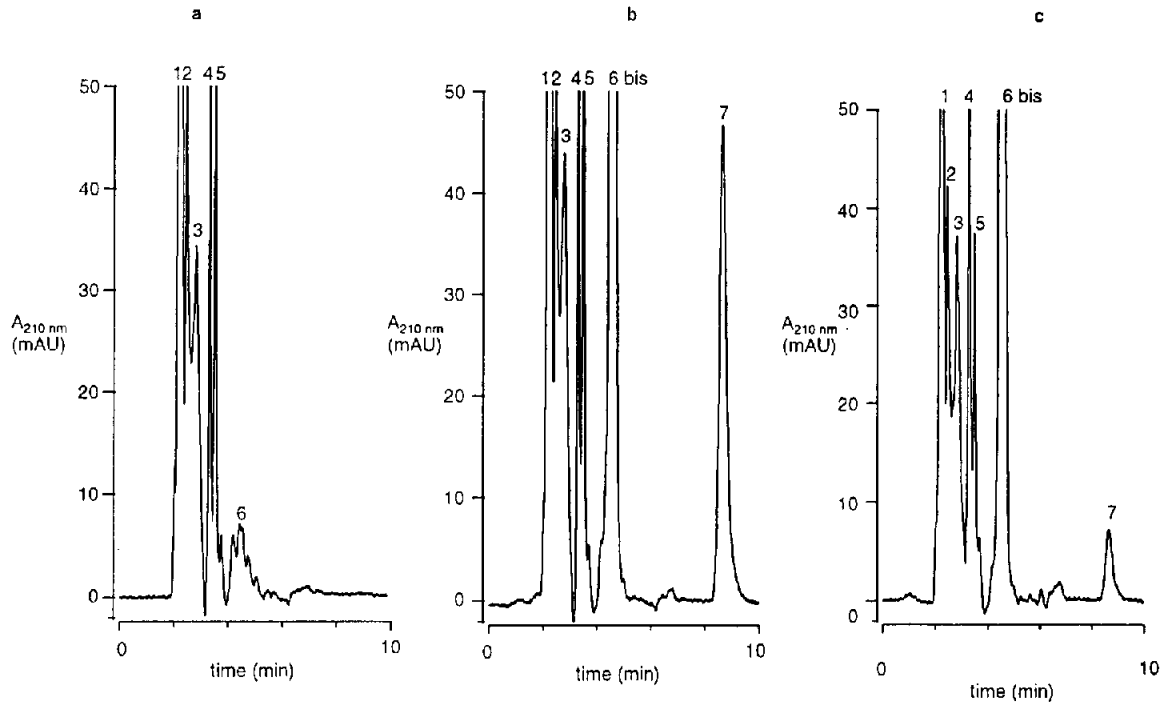

Fig. 2. High-performance liquid chromatograms of human plasma extract monitored at 210 nm. Peaks 1–6 are due to undefined substances remaining in plasma after sample preparation. Peak 6 bis is due to B19106$^-$ (internal standard) and peak 7 is Gd-BOPTA^{2-}. (a) Human plasma extract from a healthy subject maintained on a normal diet; (b) human plasma extract spiked with B19106$^-$ and Gd-BOPTA^{2-} (0.27 mg/ml); (c) extract of a plasma sample from a healthy volunteer 6 h after intravenous administration of Gd-BOPTA/dimeg (dose 0.13 g/kg body mass). Gd-BOPTA^{2-} concentration (0.047 mg/ml) was calculated by interpolation on the calibration graph.

and the interference as the internal standard. The best correlation between the peak-area ratio (Gd-BOPTA^{2-} to internal standard) and the analyte concentration was obtained over the range 0.037–4.6 mM (0.025–3.1 mg/ml). The coefficient of determination (r^2) and the regression coefficient (b) of the calibration graphs, which were fitted to data on three different days, are given in Table 1. Table 2 reports data for the precision and accuracy of the method for analyses performed on three different days. A method detection limit of 1.1 μM (0.73 μg/ml) was calculated for plasma ($\geq$0.8 ml) after introducing the values $S_m = 0.49$ μM (0.33 μg/ml) and $K = 2.145$ into Eq. 1. The stability study demonstrated no significant degradation of Gd-BOPTA^{2-} in plasma under any of the conditions tested.

Table 1
Linearity of Gd-BOPTA^{2-} calibration graphs

Biological fluid	Day	$b \pm s_b$ [a]	r^2 [b]
Plasma	1	0.7517 ± 0.0010	1.0000
	2	0.7553 ± 0.0008	1.0000
	3	0.7962 ± 0.0007	1.0000
Urine	1	2.632 ± 0.010	0.9997
	2	2.698 ± 0.004	1.0000
	3	2.752 ± 0.008	0.9998
Bile	1	0.4924 ± 0.0014	0.9999
	2	0.4715 ± 0.0014	0.9999
	3	0.4985 ± 0.0015	0.9999

[a] Equation from linear regression $y = bx$: regression coefficient ± standard error.
[b] Coefficient of determination.

Table 2
Variability of precision and accuracy in the assay of Gd-BOPTA^{2-} in plasma

Concentration tested		Day	Precision (C.V., $n = 5$) (%)	Accuracy (%)
m*M*	mg/ml			
0.10	0.067	1	3.10	+0.04
		2	2.36	+0.60
		3	3.50	+0.50
2.0	1.3	1	0.77	−0.24
		2	0.77	−0.20
		3	0.51	+0.78

3.2. Assay of Gd-BOPTA^{2-} in urine

Fig. 3 shows the chromatograms of (a) blank human urine, (b) a spiked sample (calibration standard) of human urine (0.2 ml) containing 13 m*M* (8.6 mg/ml) Gd-BOPTA^{2-} and (c) urine from a healthy volunteer to whom Gd-BOPTA–Dimeg had been administered. The mean retention time ± standard deviation of Gd-BOPTA^{2-} was 8.0 ± 0.2 min ($n = 20$). The best correlation between the peak area of Gd-BOPTA^{2-} and the analyte concentration was obtained over the range 0.50–44 m*M* (0.33–29 mg/ml). The determination and regression coefficients of the calibration graphs are given in Table 1. Table 3 reports data for the precision and accuracy of the method for analyses performed on three different days. For urine (≥0.2 ml), a method detection limit of 7.6 μM (5.1 μg/ml) was calculated using Eq. 1. The values for S_m and K were 3.52 μM (2.3 μg/ml) and 2.145, respectively. There was no significant degradation of Gd-BOPTA^{2-} in urine under any of the conditions tested.

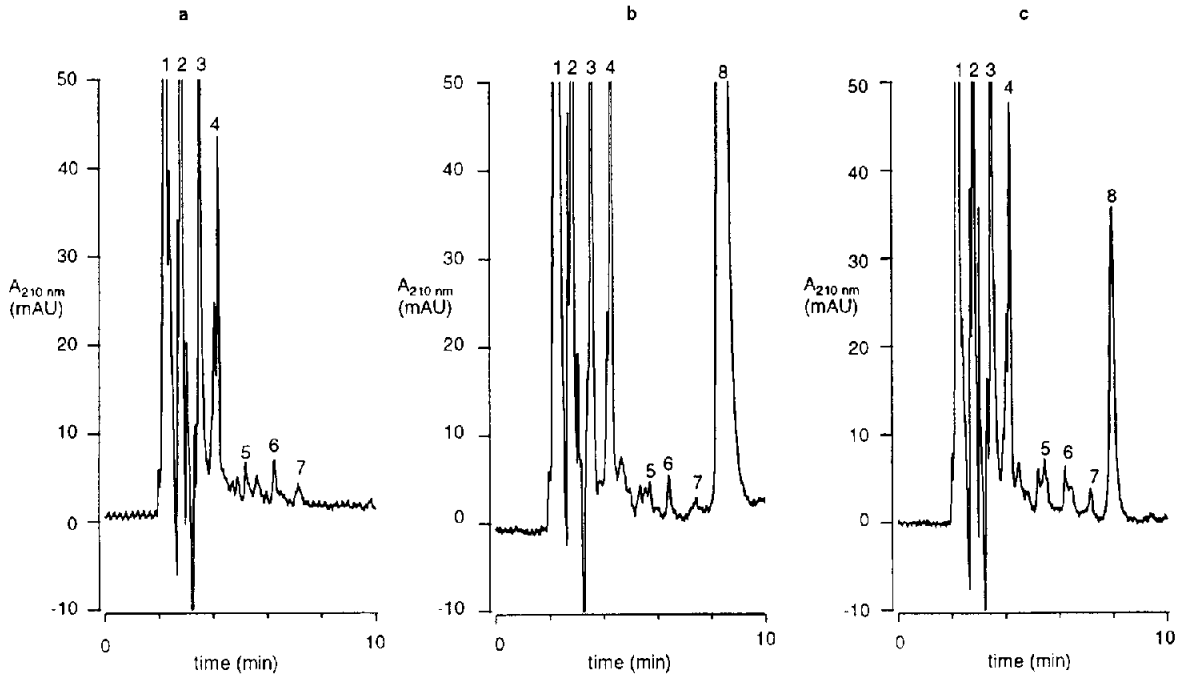

Fig. 3. High-performance liquid chromatograms of human urine monitored at 210 nm. Peaks 1–7 are due to undefined substances in the urine. Peak 8 is Gd-BOPTA^{2-}. (a) Human urine from a healthy subject maintained on a normal diet; (b) human urine spiked with Gd-BOPTA^{2-} (8.6 mg/ml); (c) urine sample from a healthy volunteer 6 h after intravenous administration of Gd-BOPTA–Dimeg (dose 0.13 g/kg body mass). Gd-BOPTA^{2-} concentration (0.66 mg/ml) was calculated by interpolation on the calibration graph.

Table 3
Variability of precision and accuracy in the assay of Gd-BOPTA^{2-} in urine

Concentration tested		Day	Precision (C.V., $n = 5$) (%)	Accuracy (%)
mM	mg/ml			
2.5	1.7	1	0.89	−0.04
		2	1.39	−2.52
		3	3.06	+5.02
13	8.6	1	0.70	+2.74
		2	0.82	+0.32
		3	1.49	+0.82

3.3. Assay of Gd-BOPTA^{2-} in bile

Fig. 4 shows the chromatograms of (a) blank rabbit bile, (b) a spiked sample (calibration standard) of rabbit bile (1 ml) containing 0.32 mM (0.21 mg/ml) Gd-BOPTA^{2-} and (c) bile from a rabbit to which Gd-BOPTA–Dimeg had been administered. The mean retention times ± standard deviation for Gd-BOPTA^{2-} and the internal standard (B19106^{-}) obtained from determinations conducted on three days ($n = 20$) were 6.4 ± 0.1 and 5.1 ± 0.0 min, respectively.

The best correlation between the peak-area ratio (Gd-BOPTA^{2-} to B19106^{-}) and the analyte concentration was obtained over the range 0.030–3.7 mM (0.020–2.5 mg/ml). The determination and regression coefficients of the calibration graphs are given in Table 1. Table 4 reports data for the precision and accuracy of the method for analyses performed on three different days. A method detection limit of 1.7 μM (1.1 μg/ml) for bile (≥1 ml) was calculated as described above. The values for S_m and K were 0.79 μM (0.53 μg/ml) and 2.145, respectively.

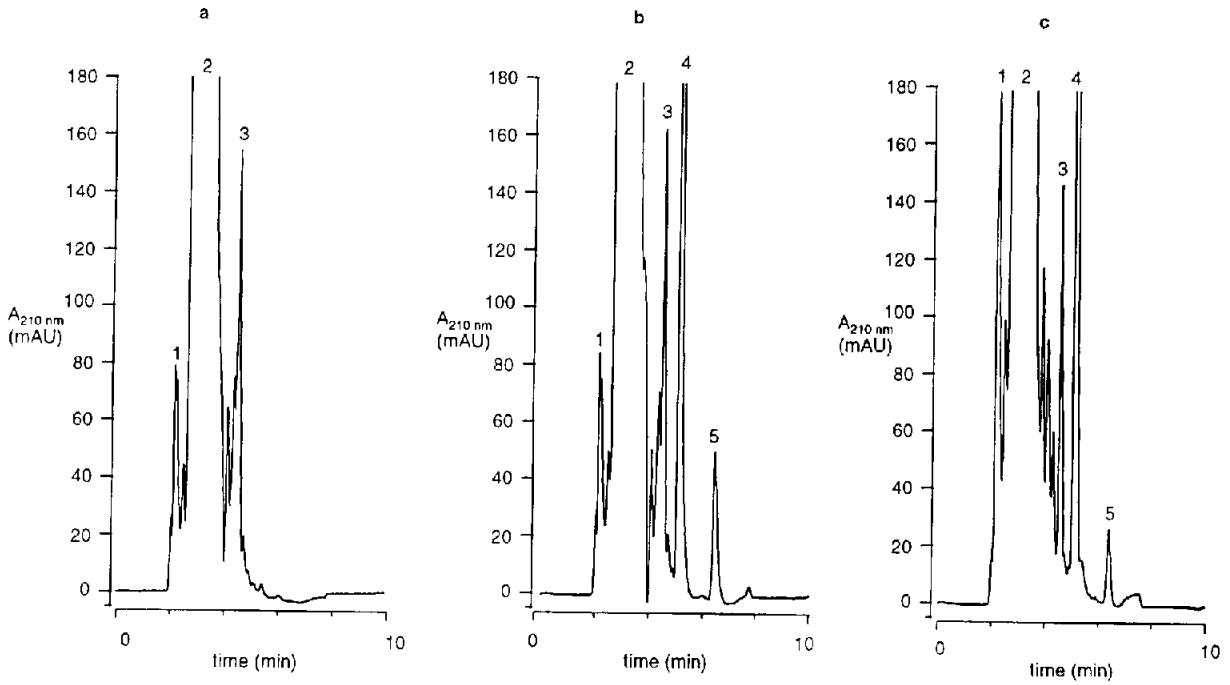

Fig. 4. High performance liquid chromatograms of rabbit bile monitored at 210 nm. Peaks 1–3 are due to undefined substances remaining in bile after sample preparation. Peak 4 is due to B19106^{-} (internal standard) and peak 5 is Gd-BOPTA^{2-}. (a) Rabbit bile extract; (b) rabbit bile extract spiked with B19106^{-} and Gd-BOPTA^{2-} (0.21 mg/ml); (c) extract of a bile sample from a rabbit 6 h after intravenous administration of Gd-BOPTA–Dimeg (dose 0.067 g/kg body mass). Gd-BOPTA^{2} concentration (0.093 mg/ml) was calculated by interpolation on the calibration graph.

Table 4
Variability of precision and accuracy in the assay of Gd-BOPTA^{2-} in bile

Concentration tested		Day	Precision (C.V., $n = 5$) (%)	Accuracy (%)
m*M*	μg/ml			
0.075	0.050	1	2.37	+0.24
		2	1.45	−3.38
		3	2.15	−3.42
1.6	1.1	1	0.15	+0.88
		2	0.51	+0.32
		3	0.49	+0.64

There was no significant degradation of Gd-BOPTA^{2-} in bile under any of the conditions tested.

4. Discussion

As the chromatographic peak of Gd-BOPTA^{2-} is completely separated from all other peaks in plasma, urine and bile samples, the described assays have excellent specificity.

Although with plasma the blank chromatogram in the region of the internal standard peak is not clean, the interference can be considered negligible in the determination of Gd-BOPTA^{2-} concentration.

The precision, accuracy and detection limits of the described methods are satisfactory for pharmacokinetic studies of Gd-BOPTA–Dimeg in large animals and man at clinically relevant doses. The methods are also suitable for similar studies in smaller animals but the amount of plasma and bile required limits the number of data points obtainable from individual animals.

The fact that the proposed techniques can be performed on readily available equipment and are rapid makes them suitable for routine use.

5. Acknowledgement

We are grateful to Professor C. de Haën for his kind encouragement and helpful discussions.

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