Monitoring the elimination of gadolinium-based pharmaceuticals. Cloud point preconcentration and spectrophotometric determination of Gd(III)-2-(3,5-dichloro-2-pyridylazo)-5-dimethylaminophenol in urine

The Analyst

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An extraction methodology based on cloud point phase separation of non-ionic surfactants has been developed for the preconcentration of ppb amounts of gadolinium in urine as a prior step to its determination by an absorptiometric procedure. A method based on the formation of complexes with 2-(3,5-dichloro-2-pyridylazo)-5-dimethylaminophenol was used for the extraction of Gd(III) in the surfactant-rich phase of non-ionic surfactant polyethyleneglycolmono-*p*-nonylphenylether (PONPE 7.5). The variables affecting the combined preconcentration-absorptiometric method have been evaluated and optimised. The extraction efficiency, linearity, and the limit of detection (LOD) of the method were determined. The optimised procedure was applied to determine total and free Gd(III) contents in real urine samples of patients after the NMR imaging diagnostic examination with contrast agent.

Introduction

During the last three decades, the use of rare earth elements (REEs) in manufactured goods have resulted in a wide variety of electromechanical and metallurgical devices to glasses, superconductors, lasers and electronic components. More recently, their application in medicinal chemistry has been evaluated.[1–4] Due to the strong tendency of the paramagnetic lanthanide cations to complex with naturally occurring agents, Gd-diethylenetriaminepentaacetate (Gd-DTPA) and 1,4,7,10-tetraazacycledodecane-1,4,7,10-tetraacetic acid (Gd-DOTA) chelates have been introduced as contrast agents in magnetic resonance imaging (MRI) and computer tomography scanning.[5–8]

The use of NMR contrast agents is an active area of research.[9–11] Specific extraneous resonanting nuclei can be introduced to label particular parts of a molecule. Measurement of proton relaxation times is a valuable aspect of NMR studies with lanthanides. The longitudinal relaxation rates of the protons of water are much greater in the presence of Gd(III) and the enhancement of protons at the Gd-binding site is sensitive to conformational changes and other environmental perturbations.

Physical chemical and biological results support the use of strongly chelated Gd complexes as NMR contrast agents. These agents are distributed in extracellular water (ECW) but not in intracellular water (ICW). The ECW : ICW ratio is often different in lesions than in normal tissue, leading to differential tissue concentration of contrast agent in lesion and normal.

A rapid and complete excretion of the contrast agent is desired when a diagnostic examination of a patient is being carried out. $[Gd(DTPA)]^{2-}$ and $[Gd(DOTA)]^{2-}$ are rapidly excreted mainly into urine, while free Gd(III) is retained, with liver being the main repository.[12] The excretion half-life of free Gd is enormously different from that of the Gd complexes: 7 d *versus* 5 min, respectively. Free Gd(III) ion has very poor acute tolerance and its long-term tolerance is unknown. It is therefore very important to determine free Gd(III) content in a Gd-based pharmaceutical. Analytical analyses are thus necessary to determine with confidence whether or not Gd was injected as [Gd(L), where L = ligands], or together with unchelated Gd(III), and if it was completely eliminated. In addition to uncomplexed Gd in the injected solutions, free metal can arise as a product of the reaction of endogenous elements with the metal complex.[13] $[Gd(DTPA]^{2-}$ reacts rapidly with Cu(II) and Zn(II) and free Gd is detected even some minutes after injection.[14] Such a situation is not observed with DOTA being the chelating agent. It is therefore very important to point out that kinetics of dissociation *in vivo* are more important than thermodynamic stability. After testing contrasting agents tolerance in small rodents, free Gd(III) and free DTPA ligand were found to be 25 to 100 times more toxic than the $[Gd(DTPA)]^{2-}$ complex (Gd(OH) LD_{50} 0.1 mmol kg^{-1}).

The use of micellar solutions in different areas of analytical chemistry has attracted much attention in recent years. Among other micelle-based separation methods, the cloud point extraction is an efficient extractive step for the enrichment of REEs, allowing the quantification of such metals at ppb levels.[15,16] Aqueous solutions of many surfactant micellar systems, when subjected to temperature alterations, exhibit critical phenomenon. Non-ionic surfactant solutions have the property of separating into two liquid phases (a surfactant-rich phase and an essentially bulk aqueous phase) when they are heated above a given temperature, called the cloud point. Any analyte solubilized in the hydrophobic core of the micelles, will separate and become concentrated in the small volume of the surfactant-rich phase.[17,18] The mechanism by which phase separation occurs is yet to be fully explained.

In the present paper we have developed and optimised a high sensitive and low-cost method for the preconcentration and determination of total and free Gd(III) contents in urine in order to monitor the elimination of the metal after the injection of gadolinium-based pharmaceuticals. The results were contrasted against ICP. With the purpose of removing concomitant ions

and separate free Gd(III) from the chelated metal, the sample was loaded in a AG50-X8 cation exchange column and a chromatographic method[19] was performed. The preconcentration step, mediated by micelles of the non-ionic surfactant polyethyleneglycolmono-*p*-nonylphenylether (PONPE 7.5), is performed by means of the formation of a Gd(III)-2-(3,5-dichloro-2-pyridylazo)-5-dimethylaminophenol [Gd(III)-3,5-diClDMPAP] complex. The optimised procedure was successfully applied to determine total and free Gd(III) contents in urine samples of patients after the NMR imaging diagnostic examination with contrast agent.

Experimental

Apparatus

A Gilford Response II spectrophotometer with 10 mm-optical path cells was used to perform the absorptiometric measurements. The ICP measures were made with a sequential inductively coupled plasma spectrometer (Baird ICP 2070, Baird, Bedford, MA, USA).

Reagents and solutions

A 1 mg ml^{-1} standard solution of Gd(III) was prepared from acidic dissolution of its oxide of analytical-reagent grade (Aldrich, Milwaukee, WI, USA). Stock solutions were standardised by a chelatometric method.[20] A 3.75×10^{-3} mol l^{-1} solution of purified 2-(2,5-dichloro-2-pyridylazo)-5-dimethylaminophenol (3,5-diClDMPAP) was prepared dissolving the reagent, and made up to 50 ml with distilled ethanol. A 3×10^{-3} mol l^{-1} solution of 2-(2-pyridylazo)-5-dimethylaminophenol (Tokio Kasei Industries), DMPAP, was prepared dissolving the reagent, and made up to 50 ml with distilled ethanol. As for the extracting solution, as it is not possible to obtain a real aqueous solution of surfactant polyethyleneglycolmono-*p*-nonylphenylether (Toko Kasei Industries, Tokyo, Japan), PONPE 7.5 (cloud point below room temperature) it was experimentally convenient to prepare a mother solution (solution A) as follows: 10 ml of PONPE 7.5, 10 ml of $NaClO_4$ (1 mol l^{-1}), and 40 ml of distilled ethanol, were mixed and made up to 100 ml with doubly distilled water. In this way the ionic strength was adjusted to 0.1 mol l^{-1} and adequate cloud point temperature (higher than 293 K) and accurate surfactant concentration (0.01%) could be reached. Under these conditions an optimal preconcentration factor was obtained. Other reagents used were octylphenylpoly(ethyleneglycol)ether (E. Merck, Darmstadt, Germany), TX-100, (Merck), Gd-diethylenetriaminepentaacetate ($[Gd\text{-}(DTPA)]^{-2}$, 469.0 mg ml^{-1}, Opacite, (Shering AG, Germany).

Experimental procedure

Determination of total gadolinium content. 1. 10 ml of urine [patient urine samples or normal urine samples spiked with proper amounts of Opacite and Gd(III)] were collected in a laboratory dish and dried in a water bath at 90 °C.

2. 1 ml of concentrated nitric acid and 1.5 ml of concentrated hydrochloric acid were cautiously added, the mixture was quantitatively transferred to a crucible and heated and boiled until all the nitric acid fumes were spelled and white fumes were evolved.

3. The sample was dried again and step 2 was repeated. At this point all the organic matter was decomposed.

4. 10 ml of doubly distilled water were added. The resultant clear solution was loaded in a AG50-X8 cation-exchange column, and the chromatographic procedure recommended by Crock *et al.*[19] was performed in order to remove Ca(II) and other possible concomitants present in the sample. The elution procedure was as follows: (a) load 10 ml of sample solution; (b) elute with 10 ml of 2 mol l^{-1} hydrochloric acid, followed by 10 ml of 2 mol l^{-1} nitric acid, discarding both eluates; (c) elute with 10 ml of 7.5 mol l^{-1} nitric acid, and collect the eluate for subsequent Gd determination; (d) regenerate the column by washing with 50 ml of 8 mol l^{-1} nitric acid.

5. Finally, Gd(III) was quantified following the developed CPE-absorptiometric methodology.

Determination of free Gd(III) content. 1. 10 ml of urine [patient urine samples or normal urine samples spiked with proper amounts of Opacite and Gd(III)] were collected. The sample was previously adjusted to pH 1 with hydrochloric acid. Then it was loaded in the AG50-X8 cation-exchange column and a chromatographic procedure was performed in order to remove Ca(II), other possible concomitants present in the sample and chelated Gd ($[Gd\text{-}(DTPA)]^{-2}$).

The elution procedure was as follows: (a) load 10 ml of sample solution; (b) elute with 10 ml of 0.5 mol l^{-1} hydrochloric acid, the eluate contains the Gd ($[Gd\text{-}(DTPA)]^{-2}$); (c) elute with 10 ml of 2 mol l^{-1} of hydrochloric acid, followed by 10 ml of 2 mol l^{-1} nitric acid, discarding both eluates; (d) elute with 10 ml of 7.5 mol l^{-1} nitric acid, and collect the eluate for subsequent Gd determination; and (e) regenerate the column by washing with 50 ml of 8 mol l^{-1} nitric acid.

2. Free Gd(III) was quantified following the developed CPE-absorptiometric methodology.

All absorptiometric measurements were performed against a blank prepared identically, but from a non-gadolinium spiked urine sample.

CPE-absorptiometric determination procedure. 1 ml of solution A, the eluate containing the metal, chelating reagent and buffer solution were placed in a centrifuge tube. The solution prepared was kept at 315 K for 10 min for equilibration and then centrifuged (graded Corning plastic centrifuge tube 15 ml capacity with a plastic cap) for 5 min at 2000 rpm (606.06*g*). After being cooled at 255 K for 5 min the surfactant phase which had separated became a viscous gel and the aqueous phase could be poured off.

1. Standard scale (cuvettes, 3.5 ml capacity). The surfactant phase (0.4 ml) in the tube was then dissolved at ambient temperature by adding 1 ml of ethanol and made up to 3 ml with doubly distilled water. The absorbance at 592 nm of the resultant clear solution was measured in a standard cuvette against a blank of reagents prepared identically.

2. Semi-micro scale (cuvettes with frosted thick wall, 1.4 ml capacity). The surfactant phase (0.4 ml) in the tube was dissolved by adding 0.3 ml of ethanol and made up to 1 ml with doubly distilled water. The absorbance of the resultant clear solution was measured in a semi-micro cuvette against a blank of reagents prepared identically at 592 nm.

3. Micro scale (cuvettes with frosted thick wall, 0.7 ml capacity). The absorbance of the surfactant-rich phase (0.4 ml) was directly measured in a micro cuvette against a blank of reagents prepared identically at 592 nm.

Results and discussion

Selection of surfactant extracting solution

Experiments were carried out in order to verify the Gd(III)-3,5-diClDMPAP-TX-100 system thermal stability. Above 333

K, complete decoloration was observed, so a complex decomposition was assumed. For this system, the cloud point was 343 K. This fact indicated that it is not possible to use the surfactant TX-100.

The cloud point of the studied system with PONPE 7.5 as the extracting solvent was near room temperature. Besides, although regular solution theory predicted that partition constants of the metal chelates would be almost independent of the metal ion nature, they varied with the kind of extracted metal in the case of CPE with PONPE 7.5.[21] The mechanism in the variation of the partition constants could be explained in terms of the presence of microscopically ordered structures in the surfactant phase, such as those in liquid crystals.[22]

The critical temperature values for the phase separation (cloud point) of the system PONPE 7.5–Gd(III)-3,5-diClDMPAP–buffer agent–sodium perchlorate with increasing amounts of ethanol were studied in order to set up the optimal composition of the extracting solution. The ethanol concentration prior to CPE step varied from 0 to 10% (v/v), concluding that the optimal preconcentration efficiency, convenient cloud point and a favourable kinetics of extraction were achieved with a 4% (v/v) ethanol concentration.

Characterization of the chelating reagent

Among other reagents used for REEs spectrophotometric determination,[23,24] pyridylazo reagents were the most sensitive. Halogenated derivatives of pyridylazo compounds were reported to give much more sensitive reaction than the unsubstituted ones DMPAP and 3,5-diClDMPAP were tested for the system under study; the best results regarding sensitivity, micelle-enhancement (solubilization, reaction rate, spectral effects and stability) and extraction efficiency were shown by the halogenated dye.

3,5-diClDMPAP has been synthesised in our laboratory and successfully used to develop high sensitivity direct absorptiometric methodologies[25–27] and conventional extraction-spectrophotometric determination of trace metals.[28] The binding constants of pyridylazo reagents to non-ionic micelles have already been determined.[25] The cloud point partition and dissociation constants for the chelating agents have also been calculated.[16]

Effect of experimental variables on CPE parameters and optimisation of system

The effect of several experimental parameters upon the extraction parameters and sensitivity have been thoroughly evaluated and optimised.

Buffer concentration and ionic strength. Several buffer agents were tested. Sodium tetraborate showed the best performance: higher extraction percentage; optimal stability; lower equilibration time and ease of phase separation. Above a buffer concentration of 2×10^{-3} mol l^{-1} a decrease of the complex absorbance was observed. This behaviour could be explained in terms of a competitive reaction of the metal ion with tetraborate in the micelle microenvironment. Within experimental error, the ionic strength had no appreciable effect upon extraction efficiency and sensitivity, for the interval 0.02–0.8 mol l^{-1}.

pH. The effect of pH upon sensitivity and extraction parameters was tested within the range of pH 5–12. The absorbance *vs*. pH profile is shown in Fig. 1(I). As can be seen, the complex extraction begins at pH 5.8 and starts to decrease at pH 10.8, showing a plateau for the range pH 8.8–10.5.

Equilibration time. Our data, taken after equilibration times of 2, 5, 10, 20 min and 2 days showed a very slight increase in the extraction efficiency and a modest decrease in the phase volume ratio (V_s/V_w, the subscripts s and w being surfactant and aqueous phase, respectively). All the experiments were conducted at a temperature of 315 K, well above the cloud point of the system PONPE 7.5–water.

Chelating reagent concentration. Fig. 1(II) shows the results of the experiments carried out in order to determine the optimal reagent–metal ion relation. Above a reagent to metal ion excess of 7 : 1, no variation took place in the sensitivity of the method.

Surfactant concentration. The effect of PONPE 7.5 concentration upon sensitivity and extraction parameters was studied within the surfactant concentration range 0.1–1.1% (m/m). The results are shown in Fig. 1(III). Quantitative extraction was

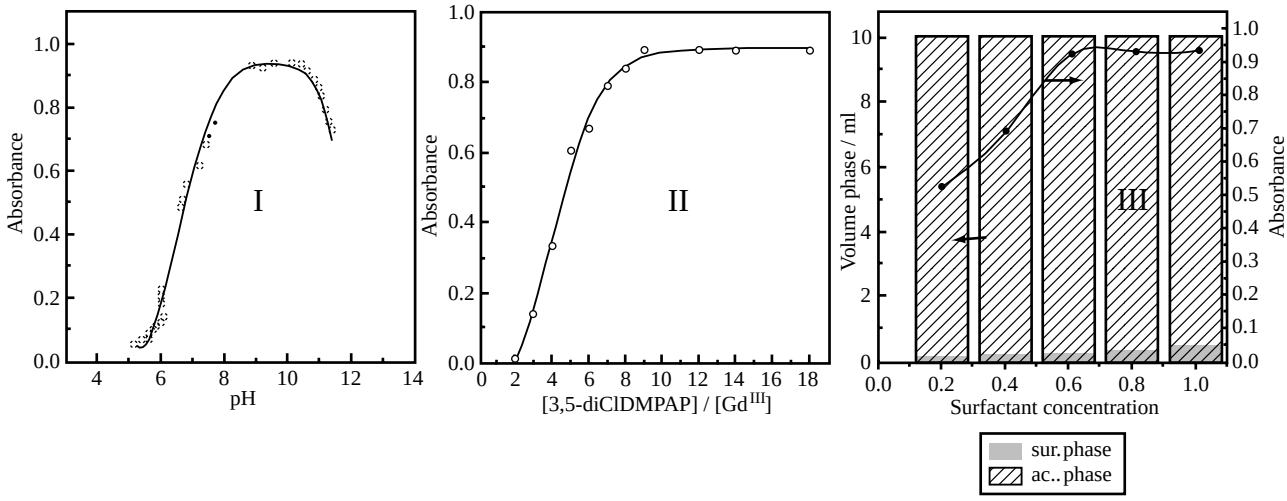

Fig. 1 I, Effect of pH. Conditions: [PONPE 7.5] = 1% m/m; [Gd(III)] = 2×10^{-6} mol l^{-1}; [3,5-diClDMPAP] = 2×10^{-5} mol l^{-1}; equilibration temperature = 315 K; equilibration time = 10 min; each desired pH was obtained with additions of suitable amounts of diluted HCl or NaOH. II, Effect of reagent excess. Conditions: [PONPE 7.5] = 1% m/m; [Gd(III)] = 2×10^{-6} mol l^{-1}; [$Na_2B_4O_7$] = 1×10^{-3} mol l^{-1}; equilibration temperature = 315 K; working pH = 9.5; ionic strength = 0.1 mol l^{-1}; equilibration time = 10 min. III, Effect of surfactant concentration. Volumes calculated by the height measurement of each phase after 48 h equilibration time; n = 3. Conditions: [PONPE 7.5] = 1% w/w; [Gd(III)] = 2.2×10^{-6} mol l^{-1}; [$Na_2B_4O_7$] = 1×10^{-3} mol l^{-1}; [3,5-diClDMPAP] = 3×10^{-5} mol l^{-1}; equilibration temperature = 315 K; working pH 9.5; ionic strength = 0.1 mol l^{-1}.

observed for an amphiphile concentration higher than 0.6% (m/m). In order to achieve a good preconcentration factor, 1% (m/m) was chosen as optimal.

Centrifugation time. No effect was observed upon extraction parameters when centrifugation time was increased from 1 up to 30 min.

Table 1 summarises the optimal experimental conditions for Gd(III) cloud point extraction-absorptiometric determination with 3.5-diClDMPAP and PONPE 7.5. Successive CPE procedures were performed to the aqueous phase in order to verify the extraction efficiency. Quantitative cloud point extraction for 3,5-diClDMPAP-gadolinium chelate was observed under the optimal experimental conditions (extraction percentage higher than 99.9%).

Table 1 Experimental conditions for the CPE-absorptiometric determination of gadolinium

Equilibration temperature	315 K
Equilibration time	10 min
Centrifugation time	5 min
Cooling time	5 min
Working pH	9.50
Buffer solution	sodium tetraborate 10^{-3} mol l^{-1}
Ionic strength	0.1 mol l^{-1} (sodium perchlorate)
Surfactant	PONPE 7.5 (1% m/m)
Maximum of reagent absorption	450 nm
Maximum of complex absorption	592 nm
%E[a]	99.98%

[a] Percentage extracted by the successive extraction method.

Beer's Law

The calibration curves for the standard, semi-micro and micro scales were measured under the optimal experimental conditions. The results are shown in Table 2.

Determination of free Gd(III) and [Gd(DTPA)]$^{-2}$ in urine samples

Real (patient) urine samples. Application of the proposed methodology to the analysis of urine samples of patients after the NMR imaging diagnostic examination with contrast agent led to the results given in Table 3.

Spiked urine samples. Normal urine samples spiked with proper amounts of Opacite and Gd(III) were prepared reproducing the expected samples for a patient injected with the contrast agent. The sample compositions were calculated considering the following factors: kinetics of dissociation *in vivo*, excretion mechanism, dosage and original concentration of the pharmaceutical. Application of the proposed methodology to the analysis of spiked samples led to the results given in Table 4.

Analytical performance of the method

Comparing and contrasting the analytical performance, it is clear that the developed method is superior (LOD 5.8×10^{-9} mol l^{-1}) to existing ICP (LOD 6×10^{-8} mol l^{-1})[29,30] and GFAAS (LOD 1×10^{-7} mol l^{-1})[31,32] procedures currently employed.

Table 2 Beer's Law

Scale	P[a]	Apparent molar absorptivity[b]	LOD[c]/ mol l^{-1}	Beer's Law fulfilment
Standard	3.33	4.60×10^5 mol l^{-1} cm^{-1}	4.35×10^{-8}	3.42–510 μg l^{-1}
Semi-micro	10	1.38×10^6 mol l^{-1} cm^{-1}	1.45×10^{-8}	1.13–171 μg l^{-1}
Micro	25	3.45×10^6 mol l^{-1} cm^{-1}	5.80×10^{-9}	0.45–68 μg l^{-1}

[a] Preconcentration factor = micellar phase volume (ml)/Aqueous phase volume (ml). [b] Referred to 10 ml. [c] Lower limit of detection.

Table 3 Determination of $[Gd(DTPA)]^{2-}$ and free Gd(III) in real (patient) urine

Sample	Scale	Total Gd (s)/mol l^{-1}[a]	Free Gd(III) (s)/mol l^{-1}[a]
1[b]	Standard	6.78×10^{-3} (5×10^{-4})	1.02×10^{-5} (9.2×10^{-7})
2[c]	Micro	3.36×10^{-7} (2.42×10^{-8})	2.55×10^{-7} (1.74×10^{-8})

[a] Mean value of six patients, three splits each (n = 27). [b] Urine samples taken 5 min after injection of 10 ml of Opacite. [c] Urine samples taken 7 d after injection of 10 ml of Opacite.

Table 4 Determination of $[Gd(DTPA)]^{2-}$ and free Gd(III) in urine

Sample composition[a]	Scale	$[Gd(DTPA)]^{2-}$				Free Gd(III)			
		Added/μg	Found/μg	RE(%)[b]	s[c]	Added/μg	Found/μg	RE(%)[b]	s[c]
P[d] = 5	Standard	9.35	9.39	0.4	0.022	0.53	0.54	1.9	0.013
P = 0.5	Semi-micro	1.60	1.63	1.8	0.017	0.91	0.93	2.2	0.008
P = 0.1	Micro	0.160	0.163	1.9	0.009	0.45	0.46	2.2	0.004

[a] Referred to 10 ml sample volume. [b] Relative percentage error. [c] Standard deviation (n = 6). [d] $P = \frac{[Gd(L)]^{2-}}{Gd^{3+}}$: relative concentration respect to Gd content. Note: The present results were contrasted against ICP following a direct methodology; synthetic aqueous samples were prepared reproducing the total Gd contents in the urine samples. Standard scale: added 9.89 μg; found 9.92 μg. Semi-micro scale: added 2.51 μg; found 2.48 μg. Micro scale: added 0.61 μg; not found.

Conclusions

Cloud point extraction offers an interesting possibility for preconcentrating Gd in urine samples. A safe, low-cost and highly sensitive methodology for monitoring Gd in urine has been developed. Studies given above have demonstrated quantitative cloud point extraction of the metal chelate, high efficiency of the chromatographic step at separating concomitant ions and chelated Gd, and consequently, the possibility to determine total gadolinium content as well as free Gd(III) in a wide concentration range. The results demonstrate the usefulness of the proposed method to effectively determine the speciation of gadolinium. The proposed method can also be applied to the determination of free Gd(III) for quality control of gadolinium-based pharmaceuticals.

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