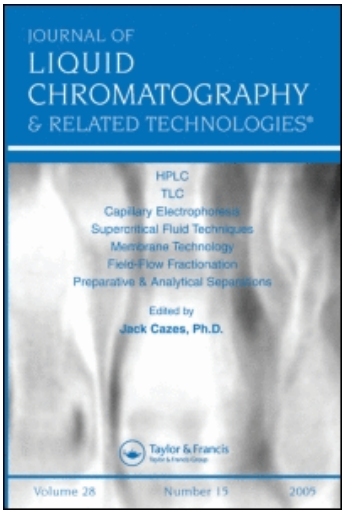

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THE DETERMINATION OF CHARGE OF CATIONIC ^{99m}Tc-RADIOPHARMACEUTICALS

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ABSTRACT

Cation exchange HPLC methods for the determination of charge of cationic ^{99m}Tc-complexes were developed, based on a method described previously for anions. Non-specific retention of lipophilic complexes was reduced by the addition of acetonitrile. The method was validated using a number of metal salts, and was used to confirm the charges on several lipophilic technetium cationic complexes.

INTRODUCTION

The discovery that cationic, lipophilic ^{99m}Tc complexes demonstrate perfusion-dominated heart uptake in several species [1] has prompted extensive research into related compounds with properties suitable for planar and SPECT myocardial perfusion imaging in man [2]. Attempts have been made to develop structure-distribution relationships (SDRs) in order to identify compounds with optimum properties in a series [3], with the primary properties studied being lipophilicity and molecular weight. The

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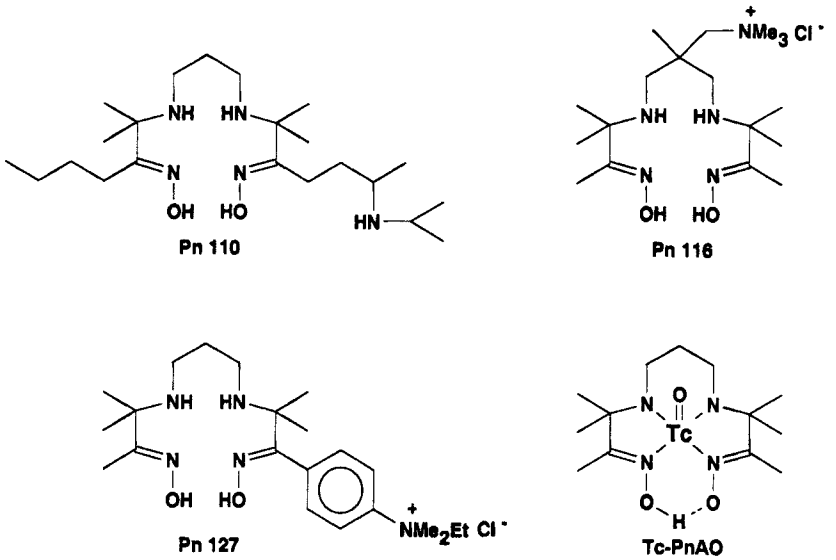
absolute charges of complexes will also have a profound influence on their biodistribution, but this has not been the subject of a systematic study

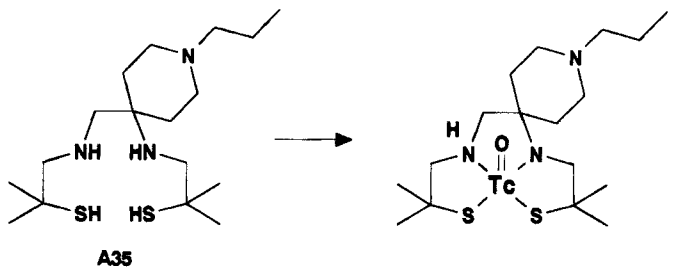
The majority of cationic technetium complexes prepared and studied as potential myocardial perfusion tracers belong to the group termed "technetium-essential" [4]. For these complexes, the overall charge results from the difference between the oxidation state of technetium and the number of negative charges generated on the ligand(s) as a result of metal complexation. As technetium has a wide variety of available oxidation states and coordination numbers/geometries [5,6], it is often difficult to predict the net charge of a complex from knowledge of its ligand structure. As most ^{99m}Tc preparations are at concentrations of $10^{-6}M$, or less, the charge of a ^{99m}Tc complex is usually assigned from knowledge of the structure of its ^{99}Tc counterpart [7]. Ideally, the charge on ^{99m}Tc complexes should be determined directly from measurements of these complexes, as the chemistry of ^{99m}Tc complexes cannot be predicted with certainty from knowledge of the ^{99}Tc chemistry [5,6].

We recently described an HPLC method for the determination of charge of anionic complexes [8], which was based upon methods described previously [9-11]. The method involved anion exchange chromatography, and the net charge of an anionic complex was determined from a relationship which links retention time with the aqueous eluent competing ion activity. In this procedure, the observed retention time had to be corrected for the system dead-time (R_O) and for any retention of the complex due to mechanisms other than ion-exchange (R_C).

We now report on studies to adapt the method for anion charge determination for the determination of charge of cationic complexes. Several cation-exchange columns were examined for this purpose, and the Partisil and TSK SCX columns were selected for further study. Using these columns, the charge determination method was validated by the determination of charges of uncomplexed metal ions, and the HPLC systems are used to determine the charges on previously reported technetium DMPE [12] and TBI [13,14] complexes. The charges on several new technetium complexes were also determined.



a. Structures of the PnAO ligands and Tc-PnAO



b. Structure of the ligand A35, and its technetium complex

Figure 1. Structures of the PnAO and DADT ligands, and their ^{99m}Tc complexes.

These were based on the ligands PnAO [15] and DADT [16] (which form neutral technetium complexes) in which the ligand is derivatized with a cationic group. The structures of these ligands are shown in figure 1.

EXPERIMENTAL

Theory

In our studies on the chromatographic determination of the charge of anions [8] by anion exchange HPLC, the following relationship was used

$$\log (R_i) = \text{constant} - \frac{m}{a} \log (A) \qquad \text{---------- (1)}$$

where R_i is the corrected retention time ($R_i = R_t - (R_O + R_C)$; R_t is the observed retention time, and R_C and R_O are as described, above) and A is the activity of the competing ion, m is the charge of the test compound, and a is the charge of the competing ion in the mobile phase. The charge on the test compound was determined by plotting log (R_i) against log (A) by measuring R_t over a range of competing ion concentrations, and applying corrections for R_O and R_C. The slope of this linear plot equals the ratio of charges carried by the test compound and the eluate competing ion. The relationship in equation 1 is also valid for the behaviour of cations in cation exchange chromatograqphy. In this study, a variety of cation exchange column/competing ion combinations were examined for the determination of the charge of cations. R_O was determined by using a non-retained marker, while R_C was minimised by the addition of an organic modifier to the eluent.

Materials

A preliminary investigation was conducted using a number of cation exchange columns, as summarised in table 1.

Table 1. Preliminary evaluation of candidate Strong cation exchange (SCX) columns

Column	Retention mechanisms*	Base	Solvent compatability	Comments
TSK SCX	Strong hydrophobic	copolymer	Wide tolerance to buffer types, pH, and organic solvents	Only useful with an organic modifier. Its high hydrophobicity limits compounds to low (<2) log P values
TSK SP5PW	Moderately hydrophobic	polymer	- ditto -	- ditto -
MONO-S	Moderately hydrophobic	Hydrophilic polymer	- ditto -	- ditto -
Waters ion column	very high hydrophobic	polymer	- ditto -	Hydrophobicity gives excessive R_c values.
AMINEX	Strong hydrophobic	polmer	High back pressure	Unpredicatable performance
Partisil	Low hydrophobicity. Possible interference from free silanols	silica	Best competing ion is NH_4^+. pH 2.5-8.0. Good tolerance to organic modifiers	Organic modifier required for lipophilic cations, but R_c can be reduced to negligible values by using a modifier.

Aqueous solutions containing K^+ and NH_4^+ as competing ions were prepared as the mobile phases for the validation of the method using metal cations. Ammonium hydroxide or potassium hydroxide was dissolved in HPLC grade water, and the pH was adjusted to pH 7.4 with glacial acetic acid. The solution was then diluted to the required maximum competing ion strength. For the studies involving aqueous acetonitrile HPLC mobile phases, solutions were prepared as described above, using a mixture of either 30:70 or 70:30 acetonitrile/water. Solvents were degassed and filtered prior to use.

Solutions of the radioactive metal cations (^{201}Tl(I), ^{24}Na(I), ^{54}Mn(II)), used to validate the HPLC systems, were obtained from Amersham International plc.

Dimethylphosphinoethane (DMPE) was obtained from Lancaster Synthesis. t-Butylisonitrile (TBI) was synthesized in-house by the reported method [14]. The ligands Pn110, Pn 116, Pn 127 and HM-PAO were synthesized in-house, using methods

based on those described elsewhere [17-20]. Formation of the ^{99m}Tc-complexes of these ligands follows the procedures described previously for the formation of ^{99m}Tc-PnAO complexes [15,21]. The ^{99}Tc(I)(DMPE)$_3$, $^{99/99m}$Tc(III)(DMPE)$_2$Cl$_2$ and $^{99/99m}$Tc(V)O$_2$(DMPE)$_2$ complexes were prepared by methods similar to those described previously [22-24]. The synthesis and technetium labeling of the ligand A35 were described elsewhere [25].

Chromatographic Procedure

A two-pump Gilson system (controlled by an Apple IIe with Gilson 702/3 software) was used in conjunction with a Rikadenki chart recorder, Gilson 802 manometric module and an Anachem mixer. A Gilson Holochrome UV/visible detector was fitted for detection of Co(II) (540nm) and ^{99}Tc samples (280 nm), while a radioisotope detection system (Mini-instrument gamma scintilation detector and ESI 5140 ratemeter) was used for the detection of all other radioactive samples. A third pump was used for automatic sample injection. Peripheral equipment were automated with the aid of a Gilson 501 contact module plus AC accessory, controlled by the 702/3 software. Sample retention times were provided by the system software.

The determination of system dead times (R_0) and estimation of R_c

The system dead-times (R_0) of the TSK and Partisil SAX columns were determined using nitrate. As the main contributor to R_c (the portion of substrate retention by mechanisms other than ion exchange) on the polymer column is likely to be hydrophobic, the retention of a series of neutral, lipophilic compounds (benzyl alcohol, benzaldehyde, nitrobenzene, benzene) were determined. The lipophilicities (Log P values) of these compounds were obtained from the MedChem database [26].

Procedures for charge determination

As the majority of cations examined were radioactive, standard procedures and precautions were used for the safely handling of these materials. Solutions (2-10mL) containing the radioactive cations were contained in a sealed 10mL vial, encased within a lead container. Vials were stoppered with a rubber septum, and sealed with a metal fitting. The vial was vented with a hypodermic needle, and connected via a six inch length of narrow bore PTFE tubing, pushed through a pucture in the septum, to the injection pump of the HPLC system. Automatic sample injection was controlled by the system software.

Either a TSK or Partisil SCX column was used with a mobile phase containing either K^+ or NH_4^+ as the competing ion. A mobile phase containing no organic modifier was used with metal ions to validate the system. The use of an organic modifier (to reduce non-specific retention) was validated by the determination of charge of ^{201}Tl using mobile phases composed of 30:70 and 70:30 acetonitrile: water. The charges on several $^{99m/99}$Tc-complexes were determined using a mobile phase based on 30:70 acetonitrile: water. An appropriate maximum competing ion concentration for a given test compound was selected by some preliminary studies, and this was selected as solvent 'A' for the HPLC system. Solvent 'B' was either water (for the 100% aqueous system) or the same acetonitrile/water mixture as used for solvent 'A'. All measurements of solute retention time were conducted with isocratic elution using predetermined proportions of solvents 'A' and 'B', at a flow rate of 1 mL/min, unless stated otherwise. Columns were equilibrated with the desired mobile phase prior to the start of the determination. Details of the column and mobile phase used with individual test compounds are given in the results section.

Observed retentions times were corrected for system dead time. The competing ion activities were determined from published data [27,28], shown in table 2. Using these

Table 2. Cation activities for the mobile phases used in this study

	Cation activities				
% A	KOAc 1M	NH_4OAc 0.1M	KOAc 1.1M	KOAc 0.07M	NH_4OAc 0.07M
100	0.7810	0.0800	0.8690	0.0700	0.0581
75	0.5700	0.0619	0.6353	0.0525	0.0444
50	0.3750	0.0425	0.4136	0.0350	0.0305
25	0.1886	0.0221	0.2076	0.0175	0.0158
10	0.0800	0.0092	0.0869	0.0070	0.0065

data, linear regression analyses were performed using a programmed Casio FX 300 calculator of $\log_{10}$ (activity) vs $\log_{10}$ (corrected retention volume), from which the charges of the test substrates were determined using equation 1.

RESULTS AND DISCUSSION

Preliminary evaluation of columns and competing ions

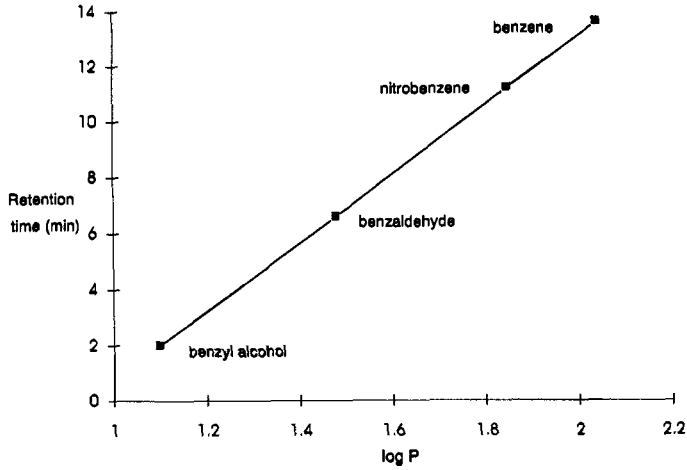
The basis for the HPLC method reported previously [8] for the determination of charge of anions was that retention due to mechanisms other than ion-exchange (R_C) and the system dead-time (R_O) should remain constant in determinations involving only a change in competing ion activity (A). Therefore, from equation 1, the relationship between log (R_t) and log (A) will be curvilinear, but it will convert to a linear relationship by subtracting a value from all observed R_ts which equals R_C and R_O. An iterative method was used to obtain the optimum linear relationship between $\log(R_t - x)$ vs log(A). The slope of this plot provided the charge of the anion.

While equation 1 will be applicable to the determination of charge of cations by cation exchange HPLC, the method developed previously for anion charge determination may not be suitable (when converted to an analogous cation exchange system) for the

highly lipophilic cations which have been studied as myocardial perfusion tracers. By using a purely aqueous eluent (as was the case in the anion charge method [8]), there is the possibility that $R_C >> R_i$ (particularly at low competing ion concentrations) for the more lipophilic compounds in this study. High R_C values reduce the precision in charge determination, as was indicated by the determination of charge of the moderately lipophilic Tc-EHIDA complex [8]) and observed by others [29]. Therefore, we decided to employ an alternative strategy. This relied upon the addition of an organic modifier to the eluent in sufficient quantities to minimise the contribution of hydrophobic interactions to R_C. We did not conduct a systematic study to select the most appropriate organic modifier; acetonitrile was selected as it appeared to have little effect on the activity coefficient of the chosen competing ion and it had previously been used successfully as the organic modifier in an HPLC method for the determination of pK_a of technetium chelates [30]. The column dead-time (R_O) was determined using a non-retained marker (nitrate). Thus, R_i could be determined directly from measurements of retention time.

While there are many possible choices for competing ion, K^+ or NH_4^+ were favoured as they have moderate-to-good solubility with common anions, and activity coefficients were known (table 2). Preliminary studies demonstrated that they generally provided a suitable range of retention times with test cations. In addition, the NH_4^+ competing ion suppresed tailing in amine-containing ligands on the silica-based columns.

The summary of the preliminary evaluation of columns for charge determination are shown in table 1. The columns evaluated were restricted to the strong cation exchange (SCX) type, based on sulphonic acids, as the weak exchange systems are generally based on carboxylic acids, a functional group which might give rise to metal retention through coordination. The primary variable in this evaluation is the column base, and columns with polymer, resin, and silica bases were included in the study. Our intial preference was the polymer base. Underivatised polymer-based columns have been



Conditions: TSK SCX (15 x 0.5 cm) column, eluted with 0.1M NH_4OAc in acetonitrile-water (30:70) at 1 mL/min, ambient temperature.

Figure 2. The retention on the TSK SCX column of neutral, lipophilic compounds.

used, for example, for the determination of lipophilicity, as their mode of retention is almost exclusively hydrophobic [31,32]. Thus, we felt that an SCX column with a polmer base will have hydrophobicity as the sole contributor to R_C, which might be eliminated through the use of a organic modifier. However, we found that for the polymer and resin SCX columns hydrophobic interactions remain a problem. Figure 2 shows the relationship between retention times and lipophilicity (log P) of some neutral compounds on the TSK SCX column. These data demonstrate that even for moderately lipophilic compounds, hydrophobic retention is likely to be a major contributor to R_C for polymer and resin-based columns, so these columns may be suitable only for the determination of charge of relatively hydrophilic compounds.

Inorganic cations

Initial validation of the technique was performed by analyses on simple inorganic cations. Unipositive and bipositive ions ($^{24}Na^+$, $^{201}Tl^+$, $^{54}Mn^{2+}$, Co^{2+}) were

Table 3. Observed retention volumes and determined charges for the inorganic cations

	Retention volumes (mL)						
Column	TSK	TSK	TSK	TSK	SCX	SCX	SCX
Cation	Tl-201	Na-24	Mn-54	Co^{2+}	Tl-201	Tl-201	Tl-201
Eluate (A)	1M K^+ aqueous	1M K^+ aqueous	1.1 K^+ aqueous	1M K^+ aqueous	0.1M NH_4^+ aqueous	0.1M NH_4^+ 30% ACN	0.1M NH_4^+ 70% ACN
% A :							
100	24.6	10.6	67.5	36.0	9.0	9.3	7.8
75	34.0	21.0	93.0	54.0	11.1	11.7	9.6
50	58.3	33.0	200.0	100.8	14.4	17.1	14.4
25	-	58.0	-	-	27.0	33.6	28.8
10	-	-	-	-	67.8	80.4	75.0
charge	1.16	1.04	2.18	2.10	0.95	1.02	1.07

investigated using 100% aqueous mobile phases. Co^{2+} was detected by UV absorption (550nm), while the other ions were detected by their γ-emissions. Detection of ^{54}Mn proved difficult, because of the high energy of its γ-photon (840 keV). Results of these studies are shown in table 3. The calculated values of cation charges were in good agreement with the expected values.

To validate the use of acetonitrile as a mobile phase modifier, studies with $^{201}Tl^+$ were extended to include the use of the acetonitrile/water (3:7 and 7:3) mobile phases. These results are shown in table 3. The determined charge is essentially unaffected by the use of the organic modifier, indicating that competing ion activity is unaltered by the use of acetonitrile in the mobile phase. For the remaining studies, an acetonitrile/water ratio of 3:7 was selected. This proportion of organic modifier appeared to be adequate to minimize the contribution of R_c to overall retention for the majority of the cations studied.

Technetium-chelates

Charge determinations were conducted on a series of known "technetium-essential" cations, and cationic derivatives of Tc-PnAO and Tc-DADT complexes. These studies were conducted on the Partisil SCX and TSK-styrene-SCX columns with mobile phases

Table 4 Observed retention volumes and determined charges for the "technetium-essential" cations

	Retention volumes (mL)						
Cation	^{99m}Tc BIN	^{99m}Tc BIN	$^{99}Tc(I)$ DMPE	$^{99}Tc(V)$ DMPE	$^{99m}Tc(V)$ DMPE	$^{99m}Tc(III)$ DMPE	$^{99}Tc(III)$ DMPE
% A:							
100	n/d	n/d	3.6	44	40	7.2	4.0
75	1.8	3.9	5.2	56	56	9.0	8.0
50	6.0	6.0	8.0	80	82	12.6	13.2
25	9.9	10.5	15.6	150	154	22.6	24.8
10	22.2	23.4	39.2	n/d	n/d	48.8	57.2
charge	1.20	0.93	1.08	0.98	1.04	0.90	1.17

In all cases, the column is Partisil SCX, and eluate (A) is 0.1M NH_4OAc in 30% ACN
n/d = not determined.

Table 5. Observed retention volumes and determined charges for the ^{99m}Tc-PnAO and DADT complexes.

	Retention volumes (mL)				
Column	SCX	SCX	SCX	TSK	TSK
Cation	Tc-A35	Tc-Pn110	Tc-Pn110	Tc-Pn116	Tc-Pn127
Eluate (A)	1M K^+	0.07M K^+	0.07M K^+	1M K^+	1M K^+
% A:					
100	3.60	5.40	6.60	2.40	5.70
75	6.00	6.90	7.50	3.00	7.10
50	7.20	9.45	11.40	4.50	10.20
25	12.60	18.00	20.10	8.25	18.30
10	26.40	43.80	49.80	19.35	44.00
charge	0.93	0.96	0.94	0.94	0.92

containing 30% acetonitrile. The results for the technetium-essential cations are given in table 4; those for PnAO and DADT complexes are given in table 5. In general, the calculated charges on all complexes were in good agreement with the expected values.

The ^{99m}Tc PnAO complexes derivatised with either amine or quaternary ammonium substituents provided sharp chromatograms on the silica-based column when eluted with a mobile phase containing NH_4^+. ^{99m}Tc HM-PAO [33], a neutral ^{99m}Tc

PnAO complex was also studied to examine non-specific retention. This complex displayed very slight (approximately 1 mL) retention which was independent of mobile phase competing ion concentration. Thus, this HPLC technique provided no evidence of charge in a metal complex known to be neutral.

Of the technetium-essential cations, only ^{99m}Tc(I) $(dmpe)_3$ demonstrated little non-specific retention. Poor peak shapes were observed for the highly lipophilic cation ^{99m}Tc(I) $(TBI)_6$, indicating either instability or some non-specific interaction. The ^{99m}Tc(V)O_2 $(dmpe)_2$ complex showed considerable non-specific retention. These observations suggest that there is a strong interaction between the TcO_2 metal core and the silanol-based column, as noted previously for this complex [12]. The ^{99m}Tc(III)Cl_2 $(dmpe)_2$ complex displayed intermediate retention, also indicating some interaction between the metal core and free silanol groups. The ^{99}Tc-counterparts of the Tc(V) and Tc(III) dmpe complexes were also examined; the observed retention times for the ^{99}Tc-complexes were similar to the corresponding ^{99m}Tc-complexes, providing additional evidence that the complexes are the same at both concentration levels.

CONCLUSIONS

The absolute charge of cations can be determined readily using a simple HPLC method based on SCX columns. Both the Partisil (silica-based) and TSK (polymer-based) SCX proved to be satisfactory for this purpose, although both columns demonstrated some limitations. The Partisil column should be avoided for test compounds subject to strong interactions with free silanols, while the hydrophobic base of the TSK column may place a limit on its use when examining hydrophobic cations. Through the use of an organic modifier to reduce or eliminate column retention (R_c) due to mechanisms other than ion-exchange, the charge on test compounds could be determined directly from retention data, eliminating the need for an interative approach [8] to correct for R_c. The method confirmed the charge on several "technetium-essential"

cations reported previously, and was used to verify the charge on several new technetium complexes.

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