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International Journal of Pharmaceutics 190 (1999) 197–205

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Development of a lyophilized kit formulation for labeling of DNA probes with ^{99m}Tc

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Received 26 July 1999; accepted 16 August 1999

Abstract

DNA fragments such as oligodeoxynucleotides (ODNs) are under investigation for a possible utilization in nuclear medicine. Until now, experiments on ^{99m}Tc-labeled ODNs in vitro or in vivo have required the application of time-consuming procedures to obtain and control the purity of the radiolabeled compound. A lyophilized labeling kit would ease and improve the reproducibility in further investigations with this class of promising biomolecules; therefore a study was initiated to evaluate the suitability of conjugates of ODNs and a bifunctional chelating agent to be part of lyophilized kit formulations. We report here the development of the first kit for one-step labeling of oligonucleotides with ^{99m}Tc. The formulation comprises 250–500 pmol *S*-benzoyl-mercaptoacetyldiglycine (MAG2)-ODN phosphorothioate conjugate, 5 mg potassium sodium tartrate tetrahydrate and 100 μg stannous chloride dihydrate in a lyophilized kit. Labeling yields above 90% were reproducibly achieved after addition of 0.1–1 GBq pertechnetate and subsequent heating in a boiling water bath. Once formed, the ^{99m}Tc-MAG2-ODN complexes were stable for at least 24 h. The shelf life of the kits is at least 10 weeks when stored protected from light at room temperature, but even kits stored at 40°C gave labeling yields above 90% after 10 weeks.

Keywords: Lyophilization; Oligonucleotides; Radiopharmaceutical; Stability; Technetium-99m

1. Introduction

Nuclear medicine is a diagnostic imaging modality that is particularly well suited for targeting. The use of a radioactive tracer agent to disclose the extent of a disease or the function of a particular body process has already for several

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0378-5173/99/$ - see front matter
PII: S0378-5173(99)00293-8

years been used in conjunction with antibodies, peptides and other biomolecules arising from advances in biochemistry (Griffiths et al., 1992; Fischman et al., 1993; Biassoni et al., 1997; Chianelli et al., 1997; Tewson and Krohn, 1998). In the last few years, molecular biology has shown rapid progress, with particular focus on the human genome project and potential medical spin-offs (Uhlmann and Peyman, 1990; Dykes, 1996; Romano et al., 1998). One of the characteristics of this field is small-scale chemistry with large-scale impact, a characteristic that is well suited for a possible exploitation in radiopharmacy and nuclear medicine.

The power of the specific hybridization properties of nucleotides and the ease of complementary strand design has attracted several scientists to work with DNA or RNA fragments for potential use in diagnostic and therapeutic nuclear medicine (Dewanjee et al., 1994; Hnatowich et al., 1995; Schering Aktiengesellschaft, 1995; Winnard et al., 1997; Zhang et al., 1997; Hjelstuen et al., 1998a; Tavitian et al., 1998; Hilger et al., 1999). It has been demonstrated that a bifunctional chelating agent can be conjugated to natural or modified oligodeoxynucleotides (ODNs) and the resulting conjugate can be labeled with diagnostic radionuclides such as ^{111}In or ^{99m}Tc (Dewanjee et al., 1994; Hnatowich et al., 1995). The ODNs modified with a metal chelate moiety were still able to hybridize to the RNA target (Hjelstuen et al., 1998b; Stalteri et al., 1999). The in vitro and in vivo stability of the resulting radiolabeled complexes has been studied, as well as the distribution in animals (Hnatowich et al., 1995; Tavitian et al., 1998; Hjelstuen et al., 1999).

Until now, the radiolabeled ODNs have been made for each individual experiment, starting from the ODN and the bifunctional chelating agent and using wet-chemistry labeling techniques. In all examples described in the literature, at least one purification step was involved in this time-consuming process, which eventually influences the reproducibility of the final outcome. To be able to further investigate the prospects and limitations of the use of DNA fragments in nuclear medicine, a one-step labeling kit for introduction of ^{99m}Tc into the ODN derivative would provide the research scientist with a useful tool. In addition, the method of preparation of a ^{99m}Tc-chelate from such a kit formulation would be similar to the methods used in nuclear medicine departments for commercially available labeling kits, thus allowing new groups of scientists access to ^{99m}Tc-labeled DNA probes.

The aim of the study was to evaluate the possibility to make lyophilized ^{99m}Tc-labeling kit formulations of conjugates of 20-nucleotide (nt) phosphorothioate ODNs and the *S*-benzoyl (Bz)-MAG2 chelating agent, as evaluated by the qualitative and quantitative composition of the kits, the labeling characteristics before and after lyophilization and the on-shelf stability of the kits.

2. Materials and methods

2.1. Oligodeoxynucleotides

The DNA probes used in this study were the phosphorothioate (PS) backbone modified 20-nt antisense ODN GX-1 and the scrambled control CTRL2. GX-1 has been selected among several antisense ODNs due to its superior access to a site on the mRNA of the CAPL gene as demonstrated by a decrease in the protein synthesis and has later proven efficient in the form of a hammerhead ribozyme (Mælandsmo et al., 1996). The sequence of GX-1 is 5′-GGA AGG TGG ACA CCA TCA CA-3′. The sequence of CTRL2 is 5′-AGT GAC CGA GTA GGC ACC AA-3′. GX-1 and CTRL2 with 1-aminohexyl linkers on the 3′-end were synthesized by Oligon AS (Oslo, Norway).

2.2. Conjugation and purification

Lyophilized ODNs (20–30 nmol) were conjugated to an excess of *S*-benzoyl-mercaptoacetyldiglycine-*N*-hydroxysuccinimide (*S*-benzoyl-MAG2-NHS) ester as described previously (Hjelstuen et al., 1999). After conjugation, the S-Bz-MAG2-ODNs were initially purified by high-performance liquid chromatography (HPLC). Isolated peaks containing the conjugate

were evaporated to dryness under reduced pressure and the residue was used for labeling with ^{99m}Tc.

2.2.1. High-performance liquid chromatography

HP System 1050 (Hewlett Packard, Palo Alto, CA) equipped with autoinjector, on-line degassing, gradient pump and variable wavelength detector set at 254 nm. Column: a SynChropack RP-P C18 6.5 μm column, 4.6 × 250 mm (SynChrom, Lafayette, IN). Mobile phase at a flow rate of 1 ml/min. Gradient mixture: 0.025 M phosphate buffer (PB) pH 5.85 (A), distilled water (B) and methanol (C). Linear gradient: 0 min 100% A; 15 min 80% A–20% C; 20 min 50% A–50% C; 20.1 min 50% B–50% C; 25 min 10% B–90% C; 28 min 10% B–90% C; 30 min 100% B.

To be able to scale up and speed up the purification process, a study was initiated to develop a mini-column purification method. A Sep-Pak C18 Light (Waters, Milford, MA) mini-column was chosen and the following procedure applied: preconditioning of the column with successively 5 ml 96% (v/v) ethanol and 5 ml 0.05 M PB pH 7.5, application of the conjugation reaction mixture (110 μl), elution with successively 1 ml 0.05 M PB pH 7.5, 9 ml distilled water and, finally, 5 ml CH_3CN. Fractions of 1 ml of the aqueous phases and 0.1 ml of the organic phase were collected and analyzed by HPLC (method as above) to determine the degree of retention of each individual component on the mini-column.

The 5-ml CH_3CN fraction containing the S-Bz-MAG2-ODN conjugate was in most cases dispensed in 0.2-ml aliquots into 25 vials (10 ml size). The liquid in the vials was evaporated to dryness under reduced pressure to obtain a residue of the S-Bz-MAG2-ODNs.

2.3. Kit formulation and labeling with technetium-99m

For the wet-chemistry experiments, the dried contents in a vial with an aliquot of the S-Bz-MAG2-ODN obtained from HPLC or Sep-Pak purification was dissolved in 100 μl 1 M carbonate buffer (CB) pH 9.3. Subsequently were added 5 mg potassium sodium tartrate tetrahydrate (KNaT), 100 μg $SnCl_2{\cdot}2H_2O$ and 1 ml eluate from a commercial ^{99m}Tc-generator (IFETEC, Isopharma AS, Norway) containing 0.1–1 GBq ^{99m}Tc in the form of sodium pertechnetate ($^{99m}TcO_4^-$). The mixture was heated in a boiling water bath for 10 min. After cooling to ambient temperature, the radiochemical purity was determined using the reversed phase HPLC as described above, with the addition of a radiometric detector.

In a preformulation study on the kit production process, the 5-ml acetonitrile fraction obtained from the purification process was split into three vials before evaporation of the solvent. Each of the residues was redissolved in 100 μl 1 M CB pH 9.3. Three procedures of kit production were then evaluated:

1. A mixture of 5 mg KNaT in 50 μl water and 100 μg $SnCl_2{\cdot}2H_2O$ in 50 μl 0.05 M HCl was incubated at room temperature in the dark for 15 min and then added to vial A with the conjugate in CB. The vial was kept at −18°C for at least 1 h prior to lyophilization.
2. A solution of 5 mg KNaT in 50 μl water was added to vial B and this mixture was kept at −18°C for 15 min. Then 100 μg $SnCl_2{\cdot}2H_2O$ in 50 μl 0.05 M HCl was added and the vial was again kept at −18°C for at least 1 h before lyophilization.
3. 5 mg KNaT in 50 μl water and 100 μg $SnCl_2.2H_2O$ in 50 μl 0.05 M HCl were added at once to vial C which was then kept at −18°C for at least 1 h before lyophilization.

The vials were lyophilized overnight in a benchtop lyophilizer (Leybold-Heraeus, Hanau, Germany). Labeling with ^{99m}Tc was performed by addition of 0.8–1 GBq $^{99m}TcO_4^-$ in 1 ml eluate and subsequent heating in a boiling water bath for 10 min.

Several studies were initiated to investigate the importance of the amount of each of the kit components. In the first study, the quantities of KNaT and stannous chloride were kept constant at 5 mg and 100 μg, respectively, and the amount of S-Bz-MAG2-ODN conjugate was varied. In a next series of experiments, the amount of conjugate was kept constant at 200 μl of the CH_3CN eluate containing the S-Bz-MAG2-ODN (corre-

sponding to 250–500 pmol conjugate) and the quantities of KNaT and stannous chloride were simultaneously decreased (maintaining the molar ratio between KNaT and stannous chloride constant). In the final two studies, the stannous chloride quantity was first varied while the quantity of the other constituents was kept constant, and finally the tartrate quantity was varied while the quantities of the other constituents were kept constant.

Labeling kits were prepared according to the selected kit formula by dissolving the residue (from a 0.2-ml CH_3CN fraction) in each vial in 100 μl 1 M CB pH 9.3. To this solution was added 5 mg KNaT in 50 μl water and 100 μg $SnCl_2 \cdot 2H_2O$ in 50 μl 0.05 M HCl. The vials were kept at −18°C for 1 h and lyophilized overnight. Labeling studies using the lyophilized kits were performed by adding 0.1–1 GBq ^{99m}Tc followed by heating as described above.

A GeneQuant spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK) measuring absorbance at 260 nm was used for determination of oligonucleotide concentration. The radioactivity in the sample was quantified using a Capintec ionization chamber (Capintec, Ramsey, NJ).

2.4. Stability of kit labeled ^{99m}Tc-MAG2-ODNs

After labeling, the reaction mixtures were stored at room temperature. Samples were withdrawn for HPLC analysis at fixed time points up to 24 h after labeling.

Table 1
Labeling yields of ^{99m}Tc-MAG2-GX-1 from wet-chemistry labeling of S-Bz-MAG2-GX-1 obtained by either HPLC or Sep-Pak C18 light mini-column purification

	Purification method[a]	
	HPLC	Mini-column
Labeling yield (%)	91.4	92.8
Range	89.5–94.7	89.6–95.3

[a] ($n = 3$).

2.5. Shelf life of the kits

Kits containing conjugates of GX-1 or CTRL2 were stored at 2–8°C, 23°C and 40°C. The 40°C storage condition was chosen to mimic an accelerated stability situation. Two kits from each conjugate at each storage condition were labeled at 2, 4 or 10 weeks after production, respectively. The shelf life was determined according to the labeling yield as analyzed by HPLC 30 min after labeling.

3. Results

Using the procedure described, the Sep-Pak mini-column was able to separate S-Bz-MAG2 and other polar components from the desired S-Bz-MAG2-ODN conjugate. After elution of the mini-column with 1 ml PB and 9 ml water, no S-Bz-MAG2 could be detected by HPLC analysis of the further fractions. The bulk of the S-Bz-MAG2-ODN conjugate eluted with the unconjugated ODN in the acetonitrile fractions. More than 80% of the ODN and conjugate eluted with the first milliliter of acetonitrile, but to improve the yields, we decided to collect the total acetonitrile fraction of 5 ml before splitting into vials.

S-Bz-MAG2-ODNs purified by the mini-column procedure or by HPLC and labeled according to the wet-chemistry procedure routinely gave high labeling yields as presented in Table 1 for ^{99m}Tc-MAG2-GX-1.

In the study of three different methods for formulating the first lyophilized kits, none of the procedures A, B or C appeared to be superior in terms of labeling yields after reconstitution with pertechnetate solution. The obtained labeling yields were 95.9%, 98.5% and 98.7% for methods A, B and C, respectively. Due to the minor differences in labeling yields, the experiments were not repeated and the procedure C was applied for the manufacture of multiple kits.

In order to determine the minimum quantity of S-Bz-MAG2-ODN required for a labeling kit to give a sufficiently high labeling yield, the amount of the conjugate was decreased in a series of wet-chemistry labeling experiments. The results are summarized in Fig. 1 and show a consistently

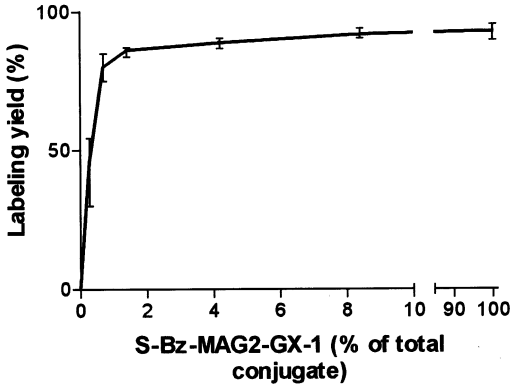

Fig. 1. Labeling yield of ^{99m}Tc-MAG2-GX-1 as a function of the quantity of S-Bz-MAG2-GX-1 put into the reaction. The stannous chloride and potassium sodium tartrate quantities were kept constant at 100 μg and 5 mg, respectively. Ranges of observations are indicated ($n = 4$).

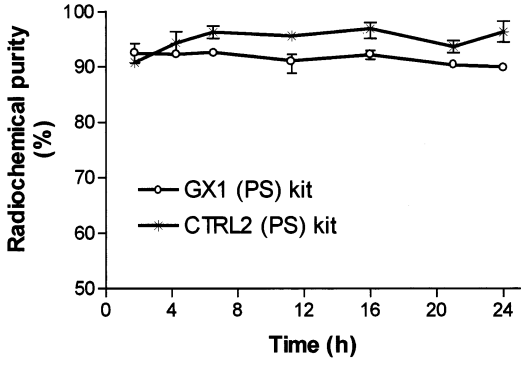

Fig. 2. Stability testing (24 h) of labeled kits, as analyzed by HPLC. Ranges of observations are indicated ($n = 2$).

high, but slowly decreasing labeling yield down to 2% of the purified S-Bz-MAG2-ODN obtained from one conjugation reaction. From 1.5% and below, a sharp drop in the labeling yield was observed.

Labeling of the first kits with the final formula gave labeling yields of 92.4% and 90.8% for the two conjugates S-Bz-MAG2-GX-1 and S-Bz-MAG2-CTRL2, respectively. The stability was then analyzed by HPLC for the next 24 h. As shown in Fig. 2, the radiochemical purity for both ^{99m}Tc-MAG2-ODNs stayed above 90% for the period studied.

Kits stored at different conditions were analyzed 2, 4 and 10 weeks after manufacture. As shown in Fig. 3, the labeling yields after 10 weeks at 2–8°C or 23°C were still above 90%. The lower average labeling yield of 88.4% for the GX-1 kits stored at 40°C at 4 weeks did not reoccur after 10 weeks. A 24-h stability study of ^{99m}Tc-MAG2-GX-1 obtained by labeling the kits from all storage conditions 4 weeks after manufacture is presented in Fig. 4. The stability of the radiolabeled conjugates showed the same pattern as when the conjugates were labeled immediately after lyophilization.

The influence of the quantity of stannous chloride in the labeling of S-Bz-MAG2-ODN is presented in Fig. 5. High labeling yields were observed down to 5 μg $SnCl_2 \cdot 2H_2O$. Below 5 μg $SnCl_2 \cdot 2H_2O$, a sharp decrease in labeling yields was observed. At 1 μg $SnCl_2 \cdot 2H_2O$, a labeling yield of 25% was achieved.

The influence of the quantity of potassium sodium tartrate tetrahydrate in the labeling of S-Bz-MAG2-ODN is presented in Fig. 6. No noticeable changes in the labeling yields were observed down to 0.25 mg KNaT. Even without any KNaT, a labeling yield of 88% was achieved.

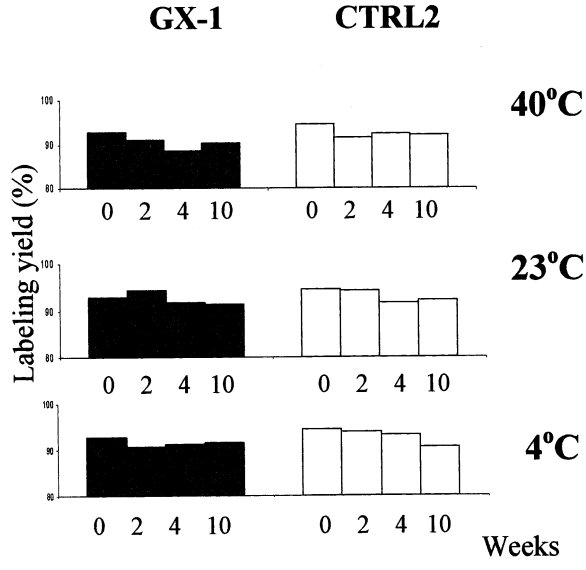

Fig. 3. Labeling yields of S-Bz-MAG2-ODN kits stored at 4°C, 23°C and 40°C for up to 10 weeks as a measure of the shelf life of the kits ($n = 2$).

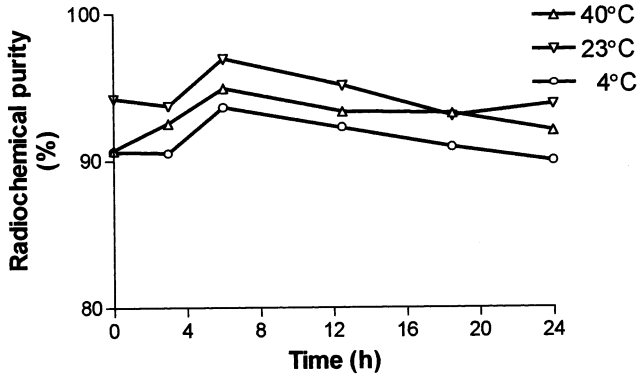

Fig. 4. Stability (24 h) of ^{99m}Tc-MAG2-GX-1, reconstituted from labeling kits stored under different conditions for 4 weeks after manufacture. Single observations.

In a further series of experiments, the quantities of stannous chloride and KNaT were decreased with respect to the conjugate, but in a constant ratio to each other (which means that the amount of $SnCl_2 \cdot 2H_2O$ was 2% of the amount of KNaT in each test). As presented in Fig. 7, higher individual quantities of the two components were required when both were decreased simultaneously, as compared to when the amount of only one component was decreased at the time.

4. Discussion

Wet-chemistry labeling of HPLC-purified S-Bz-MAG2-ODN conjugates have already been reported to give labeling yields above 90% (Hjelstuen et al., 1999). By accepting an increase in the amount of unconjugated ODN present in the purified product, it was possible to pass to a mini-column purification method that could handle larger volumes in a shorter time. By using the described purification method, a conjugate free of unconjugated chelating agent was obtained.

It was possible to formulate lyophilized labeling kits of the S-benzoyl-MAG2-PS-ODNs GX-1 and CTRL2, and qualitatively transfer the components from the wet-chemistry labeling. Even the carbonate buffer, which is not ideal for lyophilization, served its purpose in the kits. The resulting pH after labeling was measured to be the same as in the solution before lyophilization.

The quantity of S-Bz-MAG2-ODN in the kit necessary to chelate more than 90% of the ^{99m}Tc activity has been shown to be very low. Two percent of the conjugate obtained after a conjugation reaction starting from 20–30 nmol ODN was sufficient to give reproducible labeling yields above 90% using pertechnetate activities up to 1 GBq. This corresponds to 100–250 pmol S-Bz-MAG2-ODN on the assumption that the conjugation yield was approximately 30% (Hjelstuen et al., 1999) and that all the conjugate could be collected in the acetonitrile fraction after purification. In the kit formulation, 4% of the conjugate was used as a standard quantity.

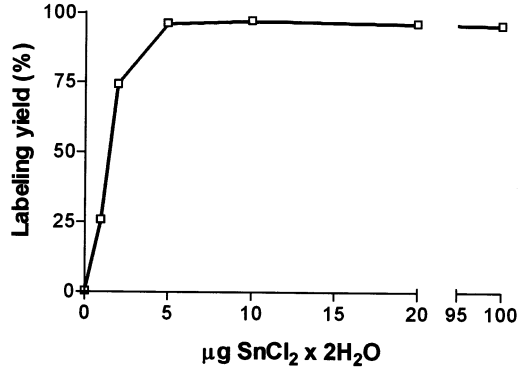

Fig. 5. Labeling yield of ^{99m}Tc-MAG2-CTRL2 as a function of the quantity of $SnCl_2 \cdot 2H_2O$ put into the reaction. The S-Bz-MAG2-ODN and potassium sodium tartrate quantities were kept constant. Single observations.

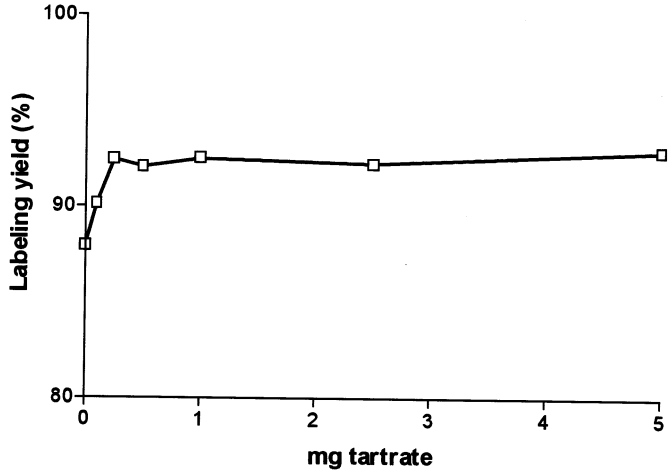

Fig. 6. Labeling yield of ^{99m}Tc-MAG2-ODN as a function of the quantity of potassium sodium tartrate tetrahydrate put into the reaction. The S-Bz-MAG2-ODN and $SnCl_2 \cdot 2H_2O$ quantities were kept constant. Single observations.

The described kit has high stability during storage. After storage for 10 weeks at 2–8°C, 23°C or 40°C, the kits perform well, with labeling yields still above 90%.

After labeling, the ^{99m}Tc-MAG2-ODN complex obtained using the kit was shown to be as stable as the previously reported complex from the wet-chemistry labeling. Kits labeled 4 weeks after manufacture show a similar 24-h stability of the radiolabeled complex.

The study on the manufacturing process was initiated with the aim to preserve the oxidation sensitive stannous chloride during the preparation of the kits and the subsequent lyophilization. Our experience is that, even under inert gas, stannous chloride in stock solution and in dispensed kits has a tendency to degrade. Therefore, care was taken in order to minimize the exposure to air before freezing. In one procedure, the weak chelating agent KNaT was allowed to associate with the stannous ions for 15 min in the dark before the mixture was added to the alkaline conjugate solution and immediately frozen. The intention was to utilize the potentially preservative effect of KNaT as a metal chelating agent on the stannous ions. In the second procedure, KNaT and the alkaline conjugate solution were mixed and frozen before addition of stannous chloride. The intention was to promote rapid freezing of the stannous chloride solution upon addition to an already frozen pellet. In the third procedure, all solutions were simply mixed and frozen. The time of air exposure was only the time it took to mix the ingredients and for freezing to occur in the 200-μl solution, i.e. a few minutes.

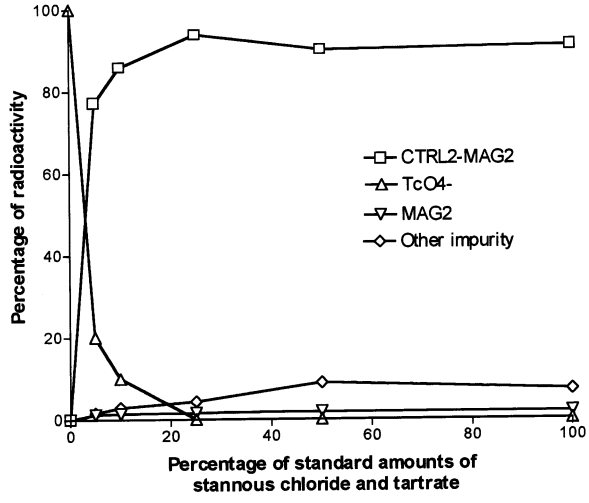

Fig. 7. Percentage of the radioactive components in the reaction mixture as a function of a simultaneous change in the quantity of potassium sodium tartrate tetrahydrate (KNaT) and $SnCl_2 \cdot 2H_2O$ put into the reaction. The standard amounts of $SnCl_2 \cdot 2H_2O$ and KNaT were 100 μg and 5 mg, respectively. The S-Bz-MAG2-ODN quantity was kept constant. Single observations.

Inert gas was not used during the manufacture. Using any of the three procedures, the resulting labeling yields of the kits were between 95.9% and 98.7%. These differences were not large enough to indicate that the manufacturing process and the care taken to preserve the stannous chloride were of particular importance to this kit. Since the simplest procedure gave satisfactory results, this was chosen as the most appropriate manufacturing process for the DNA labeling kits. The production procedure was optimized and the final formula allowed the use of an even lower content of S-Bz-MAG2-ODN conjugate than was used in the preformulation studies.

The amount of stannous chloride and KNaT in the final kit is in the same range as used in other technetium labeling kits, for instance the commercially available MAG3 kit (Mallinckrodt Medical, Petten, The Netherlands). However, with the very low content of the chelating agent S-Bz-MAG2-ODN in the DNA kit, the molar excesses of stannous chloride and KNaT are quite high and could possibly be lowered. In an experiment in which the stannous chloride and KNaT quantities were decreased simultaneously, no change in labeling yields were observed down to 25% of the standard amounts of the two components. At 10% of the standard stannous chloride/KNaT amount, the labeling yield was 85%, and it continued to decrease at lower stannous chloride/KNaT quantities. Surprisingly, when the quantity of the reducing agent stannous chloride was decreased alone, 5 μg (5% of the standard amount) $SnCl_2 \cdot 2H_2O$ promoted an equally efficient labeling reaction as 100 μg. Only 2 μg $SnCl_2 \cdot 2H_2O$ was required to give a labeling yield of 74%, and even 1 μg stannous chloride was sufficient to obtain 25% of ^{99m}Tc-MAG2-ODN. As expected, when no $SnCl_2 \cdot 2H_2O$ was added, no labeling occurred.

Potassium sodium tartrate is present in the kit as a weak chelating agent to promote exchange labeling from pertechnetate, via a possible ^{99m}Tc(tartrate)$_2$ intermediate into the more stable ^{99m}Tc-MAG2-ODN (de Kieviet, 1981). In one experiment, KNaT was shown to be superior to sodium gluconate as an exchange labeling agent in this particular labeling reaction (data not shown). However, in the present study, 88% ^{99m}Tc-MAG2-ODN could be obtained even without the presence of any tartrate. No change in labeling yield was observed for KNaT quantities ranging from 0.25 to 5 mg.

Even though these last experiments indicate that a kit formulation with lower quantities of stannous chloride and KNaT could be feasible, the influence of the decreased quantities on the shelf life of the kit has not been studied. We have decided to retain the present kit formula for further investigations with ^{99m}Tc-MAG2-ODNs.

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