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Evaluation of different formulations to label exogenous natural surfactant labeled with ^{99m}Tc (^{99m}Tc-ENS). Stability and toxicity studies

Abreviated title: ^{99m}Tc-ENS. Stability and Toxicity Studies

G. Calmanovic[(1*)], R. Caro[(1)], C. Goldman[(1)], J. Boccio[(1)], J. Nicolini [(2)], T. De Paoli[(3)], A. Hager[(3)] and M. Zubillaga[(1)]

(1)Radioisotope Laboratory and (3)Physics Department, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina. (2)Bacon Laboratories, Buenos Aires, Argentina.

Correspondencia:

Bioch. Pharm. Gabriela Calmanovici,

Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

Junín 956-Piso Bajo, 1113-Buenos Aires-Argentina.

E-mail: gcalmano@huemul.ffyb.uba.ar
Tel-Fax: +54-11-4964-8202.

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Abstract

Exogenous natural surfactant (ENS) labeled with ^{99m}Tc (^{99m}Tc-ENS) is a new ventilatory agent for aerial lung scintigraphy. To develop a stable formulation of 99mTc-ENS to be routinely used in Nuclear Medicine Centers, the properties of two freeze-dried formulations (ENS + stannous chloride + gentisic acid and ENS + stannous chloride + ascorbic acid) and a control freeze-dried formulation of ENS were analyzed monthly for 12 months. Their physicochemical properties, labeling yields and biodistribution were adequate during this period. Since ENS + stannous chloride + gentisic acid formulation has the lowest dispersion of the

Resumen:

El surfactante natural exógeno (ENS) marcado con ^{99m}Tc (^{99m}Tc-ENS) es un nuevo agente ventilatorio para la centellografía aérea pulmonar. Con el fin de elegir una formulación estable de ENS para ser marcado rutinariamente con ^{99m}Tc en Centros de Medicina Nuclear, las propiedades de dos formulaciones (ENS + cloruro estannoso + ácido gentísico y ENS + cloruro estannoso + ácido ascórbico) y una formulación control liofiolizada del ENS fueron analizadas

biodistribution results, it was chosen for a future production of 99mTc-ENS. Acute toxicity of the chosen formulation demonstrates that the toxic dose is at least 1000 times higher than the diagnostic dose.

KEY WORDS: surfactant, 99mTc, freeze-drying, ventilation scintigraphy, stability, radiopharmaceutical, toxicity.

mensualmente por un período de 12 meses. Sus propiedades fisicoquímicas, sus porcentajes de marcación y sus distribuciones biológicas fueron adecuadas durante este período. La formulación ENS + cloruro estannoso + ácido gentísico presenta la menor dispersión en los resultados de biodistribución, por lo cual esta formulación fue elegida para la futura producción de ^{99m}Tc-ENS. El estudio de toxicidad aguda de esta última formulación demostró que la dosis tóxica es al menos 1000 veces mayor que la dosis diagnóstica.

INTRODUCTION

Ventilation scintigraphy is a very important tool to diagnose pulmonary tromboembolism [1-3] or other abnormalities [4-5]. However, the radiopharmaceuticals currently used for this study (133Xe, 81Kr and radioactive aerosols such as ^{99m}Tc-Technegas and dietilen triamino penta acetic acid labeled with ^{99m}Tc, 99mTc-DTPA) have many limitations [6-9]. Several groups are studying the way to improve the ventilation imaging [10-12], such as more specific radioaerosols for aerial lung space [13-15]. With this aim, in our laboratory we are studying a radioaerosol, the exogenous natural surfactant (ENS) labeled with 99mTc (^{99m}Tc-ENS) [13-14]. Previous studies performed in rats demonstrated the high specificity of ^{99m}Tc-ENS for the lungs with a percentage of activity concentration of 98.7±1.3% [16]. Preliminary studies in human volunteers had demonstrated an ^{99m}Tc-ENS homogenous pattern of lung distribution with images of comparable quality to those obtained with ^{99m}Tc-DTPA, the radiopharmaceutical most commonly used for ventilation scintigraphy in our country. However, the radiation dose received by the patient

is lower with the ^{99m}Tc-ENS administration than with ^{99m}Tc-DTPA [13]. ^{99m}Tc-ENS images present also less interference of kidneys in the lung image in smokers volunteers than ^{99m}Tc-DTPA images [13].

In a previous work [17] we demonstrated that ENS freeze-dried alone with the addition of stannous chloride at the time of preparation is suitable to prepare 99mTc-ENS. In the present work, we studied formulations that include the ENS and the stannous chloride in the freeze-dried formulation, taking into account that the inclusion of all the ingredients in one flask has the advantage of minimal manipulation with less chance of error and the possibility to accelerate emergency lung procedures in Nuclear Medicine Centers. For this purpose and since in previous studies we demonstrated that ENS cannot be freeze-dried with stannous chloride alone [17], we studied two different techniques: we freeze-dried the ENS and stannous chloride placed in separate compartments in the same flask and we freeze-dried ENS and stannous chloride with a protective agent. To choose this agent many excipients were analyzed such as gentisic acid (2,5 dihidroxi benzoic acid), ascorbic acid and citric acid, which are commonly used to stabilize and increase the shelf life of freeze-dried compounds [18-20].

With the aim of defining a stable formulation of ENS to be routinely labeled with ^{99m}Tc in Nuclear Medicine Centers, the stability of two freeze-dried formulations and a control freeze-dried formulation of the non-radioactive precursor of ^{99m}Tc-ENS was studied in this work. Afterwards, the toxicity study of the chosen formulation was performed, since they are always required for the test of new formulations.

MATERIAL AND METHODS

GENERAL PROCEDURES

Freeze-Dried Formulations and Freeze-Drying Process Formulation 1 (F1): The sterile ENS suspension[1] was placed in a flask to be freeze-dried. Each flask contained 2.5 mg of ENS. This formulation was used as control. Formulation 2 (F2): Stannous chloride and gentisic acid were conveniently dissolved in 1N hydrochloric acid, sterilized by filtration and added to the sterile ENS suspension[1]. This solution was placed in a flask in order to be freeze-dried. Each flask contained 2.5 mg of ENS, 0.5 mg of stannous chloride and 1 mg of gentisic acid. Formulation 3 (F3): Stannous chloride and ascorbic acid were conveniently dissolved in 1N hydrochloric acid, sterilized by filtration and added to the sterile ENS suspension[1]. This solution was placed in a flask in order to be freeze-dried. Each flask contained 2.5 mg of ENS, 0.5 mg of stannous chloride and 1 mg of ascorbic acid.

In all the formulations the freeze-drying cycle was performed in the same way (freezing:-33°C, primary drying: 2°C, secondary drying: 7°C) in a freeze-drying equipment [17]. The freeze-dried pharmaceuticals were stored at 4 °C until the moment of their reconstitution.

Preparation of the Radiopharmaceuticals

$^{99m}TcO_4^-$, as sodium pertechnetate, eluted from a molybdenum generator was used to obtain the radiopharmaceuticals. 0.5 mg of stannous chloride and an activity between 630-740 MBq of $^{99m}TcO_4^-$ solution were added to reconstitute F1. The other two formulations were reconstituted adding an activity between 630-740 MBq of $^{99m}TcO_4^-$ solution only. The final volume of the three reconstituted formulations was 3 mL and the final activity concentration was approximately 210-250 MBq/mL. The absolute activity of all the radiopharmaceuticals was measured in an ionization chamber.

Animals
164 Sprague-Dawley male and female rats were placed in stainless steel cages (315 mm by 445mm by 240mm high) and maintained with standard food and water ad libitum with cycles of 12 hours of light and darkness.

STABILITY STUDY

The quality controls were performed monthly in 4 samples for each formulation during 12 months. These controls included:

Physicochemical Analysis
The physical characteristics before and after reconstitution of each formulation sample were examined and the pH was measured.

Radiochemical Purity
An ascending paper chromatography on Whatman 1 chromatography paper with acetone as solvent was carried out for each formulation sample and the radiochemical purity was given as the labeling yield percentage [6].

Biodistribution studies
Briefly, the rat was anesthetized with 300 mg/kg of chloral hydrate AR. Each radiopharmaceutical sample was placed in the chamber of a nebulizer compressed-air[2] in order to be nebulized to the rat. Twenty-five minutes after the aerosol inhalation, the animals were sacrificed and the lungs, blood, spleen, kidneys, liver and gastrointestinal system were extracted. The radioactivity of each organ was measured in a gamma counter with the same geometry for all the organs, using a monochannel gamma spectrometer with a 5 cm x 5 cm NaI (Tl) standard crystal, which was previously set to optimal electronic conditions. All measurements were carried out with constant geometry with an efficiency equal to 5% and a relative error less than 1%. The results of the biodistribution studies were given as percentage of activity concentration [16]. The statistical analysis was performed by the Kruskal-Wallis one-way analysis of variance by ranks, followed by the Dunns test. Significance was set at the 0.05 level[21] .

ACUTE TOXICITY STUDY

The toxicity study was designed according to the OECD guideline for the testing of chemicals [22]. Technical restrictions (eg: reconstitution volume, injected volumen, etc) and published toxicological data were taken into account to established the highest dose to be administered. Each test dose was prepared labeling the chosen formulation and storing them for 2 weeks at 4°C to test the toxicity due to the formulation itself but not to the radioactivity.
The tested doses and the administration procedures were:

Diagnostic Dose x 1000. Inhalatory Administration
(Tc: 80 ng/kg; gentisic acid: 14.3 mg/kg; stannous chloride: 7.1 mg/kg and ENS: 35.7 mg/kg). For the inhalatory administration, the rat was placed in a whole body exposure system specially designed for this case; each radiopharmaceutical was placed in the chamber of a nebulizer compressed-air. The dose was administered to 10 rats: 5 female (test group 1) and 5 males (test group 2)

Diagnostic Dose x 100. Intravenous Administration
(Tc: 8 ng/kg, gentisic acid: 1.4 mg/kg; stannous chloride: 0.7 mg/kg and ENS: 3.6 mg/kg). A volume of 0.2 ml was injected by retro orbital sinus puncture to a group of 10 rats: 5 females (test group 3) and 5 males (test group 4).

All the animals were randomized in 4 cages (test group 1 to 4) and were acclimatized to the laboratory conditions for 2 weeks prior to the study. Food intake, water intake and individual weights were controlled during this time.

During and following exposure individual observations were systematically recorded. Cageside observations included changes of the skin and fur, eyes, mucous membranes, respiratory system, somatomotor activity, behavior pattern and time of death. Food intake, water intake and individual weights were recorded. At the end of the test the surviving animals were weighed and necropsed in order to examine the physiopathology of the organs [22].

Data Analysis

The median lethal concentration (LC50) [22] or the non observable adverse effect level (NOAEL) was calculated.

NOAEL= A x SF

where A: highest quantity of agent that is expected not to produce adverse effect and SF: security factor equal to 1000 in the risk estimation of human dose by the same route of exposure which is the nebulization in the present study [23].

The food intake, water intake and weight of the rats of each group are given as the Mean±SD. Statistical analysis in each group comparing the results obtained before and after the administration of each tested dose were performed by the Mann-Whitney test for food intake and water intake. To compare the weight of each animal before and after the administration of each dose a Wilcoxon signed ranked test was carried out [24]. Significance was set at the 0.05 level.

Notas

1. Baby Fact P/ GEMEPE SA, Argentina
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2. Omron NE-C08 nebulizer comp-air™, USA.
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RESULTS

STABILITY STUDY

All the freeze-dried formulations were white powders, which could be reconstituted to white suspensions and with a radiochemical purity always higher than 97%. F1 and F2 presented a pH of 4.0, while the pH of F3 was 3.0. All these parameters were constant for each formulation during the 12 months of the stability study.

Table 1.
Stability studies: biodistribution results showing the percentage of activity concentration in lungs

		Months												
		0	1	2	3	4	5	6	7	8	9	10	11	12
F1	X	98.08	97.33	97.33	97.40	97.30	94.03	89.43	96.37	98.90	96.27	95.33	96.93	96.13
	SD	0.32	2.26	2.10	2.15	2.25	3.13	3.46	2.52	0.90	2.75	3.03	1.48	1.85
F2	X	91.85	95.40	93.25	88.55	96.07	92.17	91.63	92.90	93.87	96.13	95.27	97.40	94.03
	SD	3.33	2.39	3.52	2.29	1.76	0.60	4.21	0.79	8.06	1.00	1.37	0.98	3.33
F3	X	97.07	96.00	97.07	96.60	94.73	88.93	89.28	93.23	98.07	95.47	92.33	90.93	92.57
	SD	0.95	0.83	1.48	3.20	1.81	7.40	2.51	5.34	1.16	2.30	4.39	4.97	6.39

F1: Formulation 1 (ENS), F2: Formulation 2 (ENS + gentisic acid + stannous chloride), F3: Formulation 3 (ENS + ascorbic acid + stannous chloride). All the formulations were labeled with 99mTc.

Table 1 shows the lung activity concentration during one year. No significant variation was observed for each formulation. However, biodistribution results

Table 2.
Stability studies. Statistical analysis of biodistribution results

	F1			F2			F3		
	Media	SD	Median	Media	SD	Median	Media	SD	Median
Lungs	96.27	2.99*	97.30	93.73	3.61	93.80	94.02	6.20	95.40
Blood	1.10	1.05*	0.50	1.74	1.17	1.75	2.04	1.98	1.55
Spleen	1.53	1.42	0.95	2.1	1.53	1.70	2.81	2.29	1.35
Kidneys	0.71	0.51	0.65	1.69	0.99	1.65	1.46	1.10	1.05
Liver	0.34	0.29	0.20	0.50	0.42	0.40	0.41	0.39	0.30
Gastrointestinal system	0.43	0.37	0.25	0.37	0.35	0.20	0.28	0.27	0.20

F1: Formulation 1 (ENS), F2: Formulation 2 (ENS + gentisic acid + stannous chloride), F3: Formulation 3 (ENS + ascorbic acid + stannous chloride). Results are given as percentage of activity concentration. *$p<0.05$ respect to F2 and F3. All the formulations were labeled with 99mTc.

(Table 2) shows that in lungs and blood there are statistically differences between F1 and F2 and between F1 and F3, but there are no differences between F2 and F3. The results obtained for the remaining organs do not differ statistically among the 3 formulations. The mean and the median are similar for F2 in practically all the studied organs and not so similar for F1 and F3, indicating that F2 has the lowest dispersion of results of the three studied formulations. F3 has the highest standard deviation of the three formulations in most of the studied organs indicating a major variability of results. Therefore, F2 is chosen as the studied formulation to be used for routine production.

ACUTE TOXICITY STUDY

For this part of the study ^{99m}-Tc-F2 was stored at 4° C for 2 weeks to test the toxicity due to the formulation itself but not to the radioactivity. The toxicity results are shown in Table 3.

Table 3.
Acute toxicity study: food intake, water intake and weight per animal for each group.

Test group			Food intake (mg/animal)		Water intake (mL/animal)		Weight (mg)	
N°	Exp	Sex	Before	After	Before	After	Before	After
1	Inh	F	18.7±2.0	18.0±2.7	30.2±4.8	32.7±6.2	295.6±11.0	298.1±16.0
2	Inh	M	24.0±2.1	23.9±4.0	37.4±6.9	39.2±7.3	409.9±14.5	419.0±14.0
3	Iv	F	17.9±2.0	18.0±2.4	33.2±7.2	33.6±8.2	277.3±8.4	285.9±11.4
4	Iv	M	23.7±1.6	25.8±4.4	40.8±6.9	43.3±7.4	405.3±35.5	403.1±35.5

The results are given as Media±SD. Exp: route of exposure, iv: intravenous administration, inh: inhalatory administration, F: female, M: male. Before: before administration of the dose, After: after administration of the dose

The data obtained before and after the administration of each tested dose show no statistical significant differences for food intake, water intake and individual rat weight. No animal died during the observation period. None of the animals displayed signs of toxicity or any toxic effect and the anatomopathological findings were normal. For these reasons, the NOAEL was calculated. Results are shown in Table 4.

Table 4.
Acute toxicity study. LC50 and NOAEL expressed as individual doses.

	Experimental results			RTECS (26)		
Compound	**NOAEL (mg/kg)**	**Route of exposure**	**Specie**	**LC 50 (mg/kg)**	**Route of exposure**	**Specie**
ENS	35.7	inh	rat	NA	-	-
	3.6	iv	rat	NA	-	-
Stannous chloride	7.1	inh	rat	NA	-	-
	0.7	iv	rat	43	iv	rat
Gentisic acid	14.3	inh	rat	NA	-	-
	1.4	iv	rat	374	iv	mice
Technetium	8 x 10-5	inh	rat	NA	-	-
	8 x 10-6	iv	rat	NA	-	-

inh: inhalatory administration, iv: intravenous administration, NOAEL: no observable adverse effect level, LC50: median lethal concentration. NA: not available.

DISCUSSION

As it has been shown in previous works [13-14], the images obtained with ^{99m}Tc-ENS presents the same quality of those obtained with ^{99m}Tc-DTPA. However, the radiation dose received by the patient is lower with the ^{99m}Tc-ENS administration than with ^{99m}Tc-DTPA; and ^{99m}Tc-ENS images present less interference of kidneys in the lung image in smokers volunteers than ^{99m}Tc-DTPA images [13]. On the other hand, the cost of ^{99m}Tc-ENS is lower than those of 133Xe, 81Kr and ^{99m}Tc-Technegas; and ^{99m}Tc-ENS availability is higher since it is a radiopharmaceutical labeled with ^{99m}Tc.

In the present work, we studied several freeze dried formulations as a function of time with the aim of defining a stable formulation of ENS to be labeled routinely with ^{99m}Tc (^{99m}Tc-ENS) in Nuclear Medicine Centers. In preliminary attempts we studied Formulation 4 (F4) and Formulation 5 (F5) at time zero. F4 (ENS + stannous chloride in a separate compartment) showed adequate labeling yield and biodistribution pattern (Percentage of lung activity concentration: 92.64±2.35%) but its production technique is too complicated to be used as a routine procedure. F5 used ENS, stannous chloride and citric acid. However, even though its labeling yield was always higher than 85%, its biodistribution pattern was not adequate (Percentage of lung activity concentration: 75.70±4.56%. Percentage of activity concentration in the remaining organs: kidneys 16.27±4.13; liver 1.10±0.34; spleen 2.37±0.71; blood 3.53±0.28). For these reasons F4 and F5 were not followed up as a function of time (non-published data).

The remaining 3 formulations for which stability studies were performed presented adequate physical characteristics, labeling yields and biodistribution during the studied period so as to be routinely used for labeling ENS with 99mTc in Nuclear Medicine Centers. However, F1 limitations have been described above; F3 statistical analysis shows the highest dispersion of the results of the three studied formulations in most of the studied organs and a pH=3.0, that is in the lowest level of the pH range demonstrated to maintain the optimal surface activities of natural surfactants (pH= 3.0-7.5) [25]. Taking into account these results and the fact that F2 has the lowest total dispersion of the three studied formulation and a pH more similar to the pH range referred above, F2 is chosen to be labeled in Nuclear Medicine Centers with optimal labeling

yield and a shelf life of at least 12 months.

Toxicity studies of the chosen formulation were performed, since they are always required for the test of new formulations. In the present study, since the LC50 cannot be calculated due to technical restrictions, the highest amount that can be administered (A) is fixed from the radioactivity which is administered to the patient according to nuclear medicine regulations.

The results indicate that the radiopharmaceutical administered by nebulization can be safely used for ventilation scintigraphy since its NOAEL for inhalatory administration is 1000 times the diagnostic dose. The NOAEL for intravenous administration is 100 times the diagnostic dose; this result indicates that even in the case of a big mistake in which 99mTc-ENS is injected in place of being nebulized, no toxic effect would be observed.

As it is shown in Table 4, stannous chloride and gentisic acid have LC50 values [(26)] higher than the upper doses used in the present study. The LC50 for technetium is not available but even so, the level of 80 ng/kg precludes any toxic effect. The LC50 for ENS is not known, but the doses of exogenous surfactants used for neonatal respiratory distress syndrome therapy by tracheal instillation are generally between 25-200 mg/kg [(27)].

The foregoing discussion allow us to choose F2 (ENS + stannous chloride + gentisic acid) as the formulation to prepare the non-radioactive precursor of ^{99m}Tc-ENS with a shelf life of at least 12 months.

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