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LIMITATIONS OF THIN LAYER CHROMATOGRAPHY (TLC) IN THE EVALUATION OF RADIOLABELING OF NEW PEPTIDE COMPOUND

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Abstract: Studies shows that new radiopharmaceuticals can be obtained from radiolabeling peptides and proteins, such the lectin SteLL, encountered on leaves of a common medicinal plant, and which has antitumor activity. To assess the radiolabeling efficacy of this protein, literature and pharmacopoeias cites that thin layer chromatography (TLC) can be used. In this way, we investigated if it was possible to estimate the radiolabeling percentage of SteLL with technetium-99m, using TLC. Different TLC literature-cited methods were tested. Our results suggest the lack of TLC reproducibility and difficulty identifying and quantifying synthesis impurities, leading to false-positive results.
Keywords: protein, technetium, TLC.

INTRODUCTION

One of the focuses of Nuclear Medicine is the search for compounds and techniques for diagnosing and treating diseases, including malignant tumors [1]. Usually, the treatment of these tumors requires chemotherapy, radiotherapy, and surgery sessions. However, these processes have side effects and efficiency limits. Thus, the search for new, more effective, and safer diagnostic radiopharmaceuticals is essential [2].

In Cancerology, an ascending class of new radiopharmaceutical drugs is those obtained from natural products, such as natural peptides and proteins. Depending on the purpose, radioisotopes are coupled to a peptide or protein to damage cancer cells, signalize them, or even to exert dual activities: the protein will present an antitumor effect, while the radioisotope will be used for diagnosis. For those purposes, common carrier molecules are peptides, including lectins from vegetal species [3], [4].

Once the lectin SteLL, extracted from a folk medicine plant known as aroeira-da-praia (*Schinus terebinthifolia)*, presents *in vivo* antitumoral activity [5], we can assume that the radiolabeling of SteLL with a diagnostic gamma or positron emitter could lead to a new diagnostic agent for cancer.

Radiolabeling efficacy, or radiochemical purity, is shown as a percentage and should be determined on all radiolabeled products prior to use. For this purpose, thin layer chromatography is one of the most used techniques. Thin-layer chromatography (TLC) is an analytical methodology used to obtain quality control data from different radiopharmaceuticals and assess radiolabeling and purity coefficients. Using TLC it is possible to identify composites by its established retardation factor (RF). A reproducible TLC method can quantify free pertechnetate ($Na^{99m}TcO_4$) and hydrolyzed technetium ($^{99m}TcO_2$), the most common impurities in technetium-99m radiopharmaceuticals [6], [7].

The European and Brazilian Pharmacopoeias cite several TLC methods, seeking harmonization in the evaluation of the radiolabeling efficacy of radio conjugates with technetium-99m and other radioisotopes. Depending on the chemical composition of the product to be characterized, different mobile or stationary phases can be applied [8], [9]. In the case of the radiopharmaceutical technetium-99m colloidal sulfur injection, mobile phase of chromatographic paper and mobile phase of NaCl 0.9% are recommended, allowing quantification of the product (RF 0.0), as well as the pertechnetate ion impurities (RF 0.6) and other unidentified (RF 0.8 to 0.9) [8].

Despite its widespread use, it is noteworthy that TLC has some limitations, such as the deposition of more than one molecule in the same RF, inability to identify the compound of interest and impurities [10], [11]. Furthermore, in the case of new

compounds, there are still no established and recommended chromatographic methods, leaving it to the researcher to adapt methods used in molecules with similar characteristics and in established radiopharmaceuticals.

In this work, we reproduced TLC methods reported by scientific papers. Those methods are cited as reliable for the estimation of radiolabeling percentage using different peptidic compounds. Our objective was to verify if those methods could also be reliable to estimate the radiolabeling percentage of ^{99m}Tc-SteLL and impurities.

MATERIALS AND METHODS

Stannous chloride ($SnCl_2$ H_2O) solution in HCl 0.1 N at 1 mg/ml, isolated SteLL solution at 1mg/ml, obtained according Ramos et al. [5]; silica gel TLC F254 100 aluminum plates (10x2 cm diameter), Whatman® qualitative paper grade 1 (10 x 2 cm size), Whatman® cellulose chromatography papers 3MM (10 x 2cm size), NaCl 0.9%, acetone, 0.22 µm syringe filters and pH measuring stripes. A miniGITA TLC scanner (Raytest, Germany) was used for plate counting and Rf estimation. To obtain a ^{99m}Tc-SteLL, we synthesized samples with 100 µL of isolated SteLL and 100 µL of stannous chloride solution, stirred and preserved at room temperature, protected from light, for 20 minutes. Then one mCi of sodium pertechnetate eluate, donated from Hospital Português, was added to each sample, stirred, and held for 10 minutes until pH correction to 7, using 1 M NaOH and 0.01 N HCl. This radiolabeling method was adapted from Patricio *et al.* [12] and Koch e*t al.* [13].The total volume of samples was adjusted to 2 mL with NaCl 0.9%. Aliquots of 10 µL of each sample were submitted to different chromatographic methods (Table I).

METHOD	STATIONARY PHASE	MOBILE PHASE	PRODUCTS RF
Monteiro *et al.,* 2010 [10].	Silica gel	0.9% NaCl	$Na^{99m}TcO_4$ = 1.0.
	Whatman grade 1 paper	ethyl acetate: methanol (8:2)	$Na^{99m}TcO_4$ and $^{99m}TcO_2$ = 0.
Patrício *et al.,* 2011 [12].	Whatman grade 1 paper	Acetone	$Na^{99m}TcO_4$= 1.0. ^{99m}Tc-Lectin = 0.0.
Dias *et al.,* 2005 [14].	Whatman 3MM paper	Saline, acetone	$^{99m}TcO_2$ and ^{99m}Tc-MDP= 1.0.

Table I: Literature-cited chromatographic methods applied on ^{99m}Tc-SteLL radiolabeling efficacy.

In each reproduced method, the mobile phases were placed in glass chambers, and left for 15 minutes to rest for air saturation. Then, 5 µL of samples were applied on application line at 2 cm from the border of each stationary phases, and let dry for 15 min. Then, the stationary phases containing the samples were allocated in the cited chambers, with the volume of solvent below the application line of the samples, and left until the mobile phase ascended two centimeters before the end of the plate (total run of 8 cm). Stationary phases were dried at room temperature and counted by the TLC scanner, were products RF were calculated.

We also modified the radiolabeling method, employing different pH and temperature conditions to the samples, to verify if better radiolabeling efficacy could be obtained. An additional batch of pertechnetate and stannous chloride 1:1 was synthesized to verify if it would be possible to remove colloidal impurities, mainly $^{99m}TcO_2$, employing filtration, as reported by Diniz *et al.*(2005) [15]. For this, we used 0.22µm syringe filters.

In order to complement the findings of this article, we also made a brief bibliographic survey about the TLC technique and methods of separation and quantification of impurities. For this, the chosen database was PubMed. The

search keys used were "TLC chromatography ^{99m}Tc". Review articles and those written in languages other than English were excluded. Thirty articles were obtained. These were randomly selected and read in search of evidence on the separation and quantification of ^{99m}Tc product and impurities, by means of TLC. Results are shown in Supplementary File 1.

RESULTS AND DISCUSSION

Studies cite that, due to its higher molecular weight, the ^{99m}Tc-SteLL compound will remain at the point of application (RF 0). Other analytes, if present, should remain at RF 0 or 1, depending on the chromatographic method. These results are shown for established radiopharmaceuticals such as ^{99m}Tc-MIBI [10] and ^{99m}Tc-cefuroxime [16], as well the new radiolabeled compound ^{99m}Tc-Cramoll [12]. Under all conditions, our results showed that $Na^{99m}TcO_4$ remained at RF ± 1, separated from the other compounds. Otherwise, $^{99m}TcO_2$ remained at the point of application (RF ± 0), regardless of the chromatographic method, which is a problem since, accordingly similar studies, ^{99m}Tc-SteLL should have an RF close or equal to 0. The results are in Table II.

PRODUCT	STATIONARY PHASE	MOBILE PHASE	RF
Hydrolyzed Technetium ($^{99m}TcO_2$)	W1[a]	Acetone	0.033
		NaCl 0.9%	0.041
	3MM[b]	Acetone	0.041
		NaCl 0.9%	0.033
	SG[c]	Acetone	0.041
		NaCl 0.9%	0.133

a: qualitative Whatman® grade 1 paper; b: 3MM cellulose chromatography paper; c: silica gel TLC F254 100.

Table II: Retardation factors (RF) for hydrolyzed technetium and free pertechnetate under different chromatographic conditions.

The same results were observed in the system using acetate:methanol (8:2) mobile phase. These findings did not agree with published studies, in which it is possible to keep the separation of impurities from the radiolabeled molecule of interest.

In none of the batches it was possible to quantify if there was radiolabeling of SteLL, or only formation of impurities since it was only possible to separate the free pertechnetate. Submitting samples through 0.22 μm filtration also was not useful. In these cases, the attempt to pass only ^{99m}Tc-SteLL was unsuccessful, as the mixture did not pass through the filter, possibly due to the molecular size being greater than the supported limit.

Our results suggest that there may be flaws in the use of TLC to quantify the radiolabeling efficiency and purity of new compounds, especially when dealing with ^{99m}Tc-labeled peptides. Since it was impossible to identify different RFs for the radiolabeled peptide and impurities, many of the reported studies may overestimate the radiolabeling efficiency, ignoring the presence of impurities critical to the quality of the product. Many articles reporting radiolabeling with ^{99m}Tc do not cite methods for quantifying impurities.

In fact, our literature review shows that about 13.3% of studies citing TLC for quantification of impurities do not make it clear which method was used, and about 43.3% of these articles do not mention clear separation and/or quantification of $Na^{99m}TcO_4$ and $^{99m}TcO_2$. Among these studies, one of Bozkurt *et al.* shows a clear separation of the cited impurities and the ^{99m}Tc-MAG3, using a combination of silica gel, Whatman 1, and 3 stationary phases. Mobile phases chosen were 40% methyl ethyl ketone, 60% ethyl acetate, and 50% acetonitrile [17].

There is also a study from Proulx *et al.*, which is similar to our methods tests. They obtained separation and quantification of

^{99m}Tc-MIBI from $Na^{99m}TcO_4$ and $^{99m}TcO_{2,}$ using a combination of ITLC-SG (silica gel), saline, and acetone [18].

CONCLUSION

We conclude that the use of thin layer chromatography to assess radiolabeling of new molecules may be limited. Our results suggest the lack of reproducibility of methodologies and difficulty identifying and quantifying synthesis impurities, leading to false-positive results. New methods and protocols are necessary for evaluating radiolabeled peptide agents.

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