Thin-Layer Chromatographic Procedures for the Characterization of Technetium-99m Bicisate

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Objective: Technetium-99m (^{99m}Tc) bicisate is a radiopharmaceutical imaging agent useful for assessing regional cerebral perfusion. We will present the details of two procedures used to confirm the radiochemical purity (RCP) of ^{99m}Tc-bicisate.

Methods: After radiolabeling, the performance of a quality control procedure is recommended to confirm the RCP of ^{99m}Tc-bicisate. For routine quality control, a single thin-layer chromatographic (TLC) method is sufficient for determining the RCP of the kit. For research purposes, a second TLC method has been developed to provide quantification of individual radioimpurities.

Results: Information on the radiolabeling reaction and potential radioimpurities is provided to illustrate the factors that must be evaluated in the development of TLC procedures for this kit. Data are provided which demonstrate the specificity and ruggedness of each procedure.

Conclusion: Implementation of these two TLC procedures should assist the user in obtaining accurate RCP measurements.

Key Words: Thin-layer chromatography, ^{99m}Tc-bicisate, radiochemical purity (RCP).

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Neurolite (Du Pont Merck Pharmaceutical Company, N. Billerica, MA) is a kit for the preparation of technetium-99m (^{99m}Tc) labeled oxo N,N′-1,2-ethylenediylbis-L-cysteine diethyl ester (referred to hereafter as ^{99m}Tc-bicisate) which is useful for assessing regional cerebral perfusion (*1*). The ^{99m}Tc-bicisate kit consists of two vials; a lyophilized vial (Vial A) containing the chelating ligand (bicisate dihydrochloride) and a terminally sterilized vial (Vial B) containing phosphate buffer. The composition of each vial is described in Table 1.

Following the manufacturer's instructions for preparation of the kit will result in material of high radiochemical purity (RCP). We recommend that the purity be confirmed through the use of a quality control procedure. Two separate thin-layer chromatographic (TLC) procedures for radiolabeled ^{99m}Tc-bicisate kits are presented in this paper. For routine quality control, a single thin-layer chromatographic method (TLC) is required. For research purposes, a second TLC method has been developed to provide quantification of individual radioimpurities. The studies that were required to demonstrate the specificity of each method are described. A summary of other validation studies is presented and should provide a basis for evaluating future quality control methods for the kit. In addition, information on the ruggedness of the radiolabeling reaction and TLC procedures is presented as an aid to the nuclear medicine technologist.

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MATERIALS AND METHODS

Preparation of ^{99m}Tc-Bicisate

We prepared ^{99m}Tc-bicisate using the following procedure. The plastic discs from both vials were removed and the top of each vial closure was swabbed with alcohol to disinfect the surface. Vial B was then placed in a suitable radiation shield. Using a sterile shielded syringe, 3.70 GBq (100 mCi) of sterile, nonpyrogenic, oxidant-free sodium pertechnetate ^{99m}Tc injection, in approximately 2.0 ml, were added to Vial B. Using a sterile syringe, 3.0 ml of sodium chloride injection were added rapidly into Vial A to dissolve the contents. The contents of the vial were then shaken for a few seconds. One ml of this solution was then transferred immediately (within 30 sec) from Vial A to Vial B. The contents of Vial B were then swirled for a few seconds and allowed to stand for 30 min at room temperature. The reaction vial (Vial B) was then stored at room temperature (15–30°C) until use.

Quality Control TLC Procedure

The quality control procedure was performed using Bakerflex silica gel IB-F TLC plates (J. T. Baker, Phillipsburg, NJ), 2.5 × 7.5 cm, 250 μm and an ethyl acetate, high-performance-liquid-chromatography (HPLC) grade (J. T. Baker) mobile phase. Pencil lines were drawn across the TLC plates at heights of 2 cm, 4.5 cm, and 7 cm from the bottom of the plate. Approximately 5 μl of the final solution were spotted at the center of the 2-cm mark. This volume was delivered, using a syringe fitted with a 25- or 27-gauge needle, by allowing a drop to form while holding the syringe

TABLE 1. Composition of a Technetium-99m Bicisate Kit

Ingredient	Amount per Vial
Ligand Vial (Vial A)	
bicisate dihydrochloride (ECD·2HCl)	0.9 mg
stannous chloride dihydrate, ACS	0.072 mg
(ethylenedinitrilo)tetraacetic acid disodium salt dihydrate, ACS (EDTA)	0.36 mg
mannitol, USP	24 mg
nitrogen, NF (headspace)	
Buffer Vial (Vial B)	
sodium phosphate dibasic, heptahydrate, ACS	4.1 mg
sodium phosphate monobasic, monohydrate, ACS	0.46 mg
water for injection, USP	qs 1 ml

in a vertical position (*2*). (The diameter of the spot should not be greater than 10 mm.) The spot was allowed to dry for 5–10 min. The plates were then placed into a preequilibrated TLC tank and allowed to develop to the 7.0-cm line (about 15 min). After drying, the plates were cut with scissors at the 4.5-cm mark. The activity on each piece of the plate was counted using a dose calibrator or a gamma counter. The top portion of each plate contained the ^{99m}Tc-bicisate and the bottom portion contained all radioimpurities (see Fig. 1).

The radiochemical purity was calculated using the following equation.

$$\%^{99m}\text{Tc-bicisate} = \frac{A_t}{A_t + A_b} \times 100,$$

where A_t = activity of the top piece and A_b = activity of the bottom piece.

For quantification using a scanning detector, the RCP was calculated as the area percent of the ^{99m}Tc-bicisate peak, as shown below.

$$\%^{99m}\text{Tc-bicisate} = \frac{(\text{Peak area of } ^{99m}\text{Tc-bicisate peak})(100)}{\text{Total area of all peaks}}$$

Research TLC Procedure

The research procedure was performed using MKC18 TLC plates (Whatman, Clifton, NJ), 2.5 × 7.5 cm, 200 μm. The mobile phase was composed of 60% acetone, HPLC grade (Fisher Scientific, Pittsburgh, PA) and 40% 0.50 M ammonium acetate, HPLC grade (Fisher Scientific). The TLC plates were spotted with approximately 2 μl of sample near the bottom of the plates. The spot was allowed to dry for 5–10 min. The plates were then placed into a preequilibrated TLC tank and allowed to develop to a distance of at least 6 cm from the origin. The radioactivity distribution was determined by scanning the TLC plates with a radiation detector. The percent of each radioimpurity was calculated as the area percent of the impurity peak. A typical TLC chromatogram is shown in Figure 2.

RESULTS AND DISCUSSION

The radiolabeling of bicisate is analogous to that of many ^{99m}Tc-labeled radiopharmaceuticals in that labeling is accomplished using a lyophilized vial containing a chelating ligand and a reductant. The contents of the lyophilized vial are combined with sodium pertechnetate ^{99m}Tc solution in a manner that yields a final vial with a nearly neutral pH. In the case of ^{99m}Tc-bicisate, stannous ion serves as the reductant and bicisate as the chelating compound. A second vial containing phosphate buffer is used to obtain a final pH value of 7.0. Following the reconstitution instructions provided by the manufacturer assures that the radiolabeled product will be of high RCP.

When developing a procedure for determining the purity of a radiopharmaceutical such as ^{99m}Tc-bicisate, it is necessary to consider the possible radioimpurities that may arise both from normal side reactions and through deviations from the manufacturer's radiolabeling instructions. Demonstrating that no impurity coelutes with the desired radiochemical species is required for proving method specificity. The labeling reaction for a ^{99m}Tc-bicisate kit produces ^{99m}Tc-bicisate at ≥90% RCP and, potentially, five radioimpurities, as shown in Figure 3.

These radioimpurities are formed through different mechanisms and therefore are not likely to all be present at any one time. Since some of these impurities arise from incomplete reaction, it is useful to characterize the labeling reaction as a function of time (see Fig. 4) to determine the rate of formation and elimination of potential impurities. The use of kits which have not been completely reacted is a good source of impurities with which to evaluate chromatographic resolution. For each of the five potential impurities, the identity of each impurity in poorly labeled kits was confirmed by preparation of standard solutions of independently synthesized impurities and comparing the chromatographic retention using the quality control and research TLC procedures. All such studies were performed at the ^{99m}Tc level, with the exception of the Tc-(IV)ECD species (described below), which was confirmed using the analogous ^{99}Tc complex.

The R_f values obtained for each radioimpurity and for ^{99m}Tc-bicisate using both methods are listed in Table 2. Based on the R_f values for all potential radiolabeled components, the quality control procedure is specific for the quantification of ^{99m}Tc-bicisate. All radioimpurities are retained at the origin and are therefore on the bottom section of the plate. The ^{99m}Tc-bicisate peak moves near the solvent front and is therefore the only radiolabeled species on the upper portion of the plate.

In the research TLC procedure, several radiolabeled species are retained at approximately the same R_f value. This is the case for TcO_4^- and TcECM, where unless two peaks are

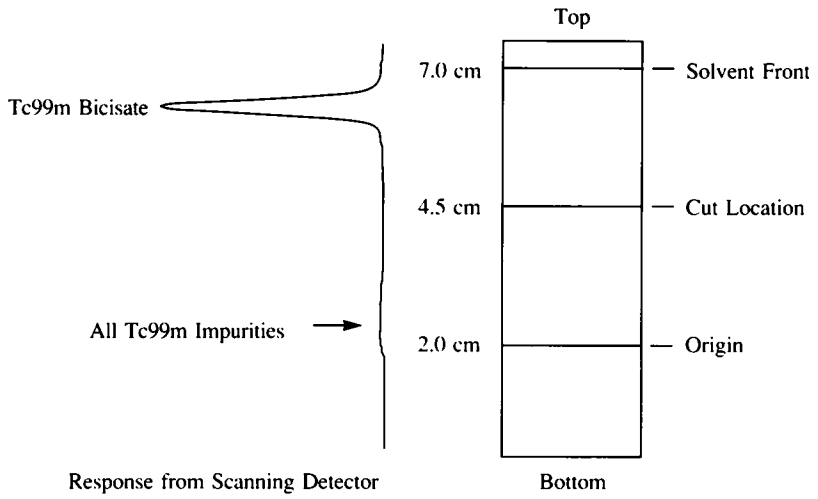


FIG. 1. Specificity of quality control TLC procedure demonstrated by cut location and elution profile.

detected in the R_f range of 0.7 to 0.8, it is not possible to identify which of the two species is present. The ^{99m}Tc-(IV)ECD elutes as a broad band beneath the ^{99m}Tc-bicisate peak. The combined ^{99m}Tc-bicisate/^{99m}Tc-(IV)ECD peak is separated from all other radioimpurities by this method. In the quality control TLC procedure, the ^{99m}Tc-bicisate peak is separated from ^{99m}Tc-(IV)ECD and all other radioimpurities. Therefore, using data from both procedures, it is possible to calculate the % ^{99m}Tc-(IV)ECD. A discussion of each potential radioimpurity is given below.

Hydrolyzed reduced technetium (Tc-R) has been characterized as including both ^{99m}Tc-dioxide and ^{99m}Tc-tin colloid (*2*). The ^{99m}Tc-dioxide is formed by hydrolysis of reduced ^{99m}Tc. The ^{99m}Tc-tin colloid is formed by the hydrolysis of stannous ion, which then complexes reduced ^{99m}Tc. Both forms of hydrolyzed reduced technetium are common to radiopharmaceuticals containing a stannous ion reductant.

Sodium pertechnetate ^{99m}Tc is present after the 30-min reaction time if the stannous ion content of the ligand vial is insufficient to reduce all the sodium pertechnetate ^{99m}Tc added to the vial. Unreacted sodium pertechnetate ^{99m}Tc can be detected during the first 20 min of the normal labeling reaction. Sodium pertechnetate ^{99m}Tc(VII) does not form a complex with bicisate.

The ^{99m}Tc-EDTA is an intermediate in the formation of ^{99m}Tc-bicisate and is detected in the first 20 min of the labeling reaction. After 20 min, the exchange of ^{99m}Tc from

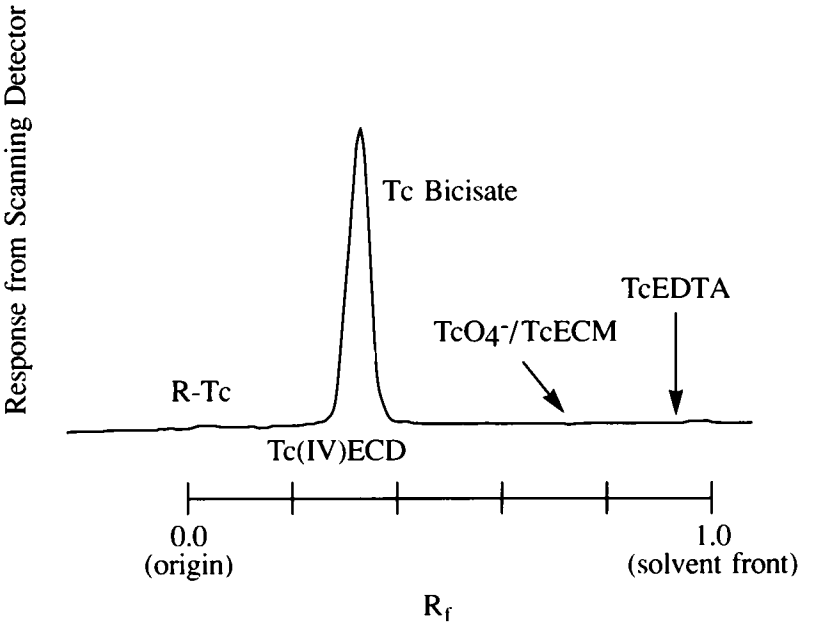


FIG. 2. Thin-layer chromatogram of radiolabeled ^{99m}Tc-bicisate kit obtained using the research TLC procedure.

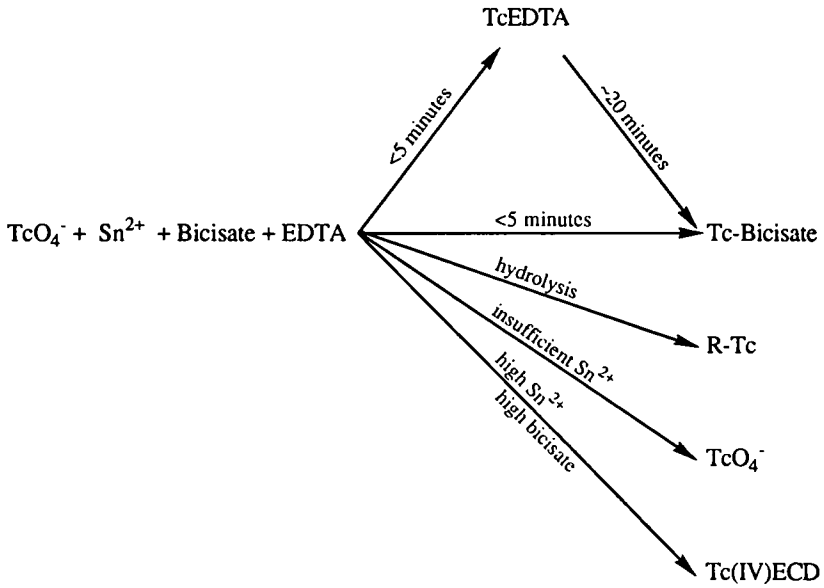


FIG. 3. Radiolabeling reaction and formation of radioimpurities.

EDTA to bicisate is complete and ^{99m}Tc-EDTA is no longer present in the solution.

ECM (ECD monoacid monoester) is a potential impurity in Vial A and is formed by hydrolysis of bicisate dihydrochloride. The ^{99m}Tc-ECM may be produced in the final vial by the radiolabeling of the trace level of ECM initially present in Vial A or by hydrolysis of ^{99m}Tc-bicisate after radiolabeling. The ^{99m}Tc-ECM is the metabolized form of ^{99m}Tc-bicisate which is retained in the brain (*3*).

The fifth potential impurity has been identified as a group of three related materials that are forms of ^{99m}Tc-(IV)ECD. Small quantities of these ^{99m}Tc-(IV)ECD species form during the 30-min reaction time when excess stannous ion and bicisate are present. They are not detected as distinct peaks by either of the two radio-TLC methods used. However, the percentage of ^{99m}Tc-(IV)ECD can be calculated using data from both methods, as described earlier. Identification of these species was not possible on the ^{99m}Tc level because of the excessive radiation dose of large quantities of material; however, reactions with ^{99}Tc provided sufficient material for characterization.

The Tc-(IV)ECD species are similar to one observed in the preparation of ^{99m}Tc-mertiatide, a renal function imaging agent. Technetium-99m mertiatide utilizes the Tc(V)-oxo complex of the tetradentate ligand designated MAG_3. The MAG_3 ligand consists of three amide donors and one thiol group compared to the two thiols, one amide and one amine donors in bicisate (see Fig. 5). When stannous levels are too high in this kit, a radiochemical impurity is formed, which is designated Tc(IV)-MAG_3 (*4*).

In addition to demonstrating specificity, the complete validation of an analytical procedure requires demonstrating accuracy, linearity, precision, and determination limits. The results of these studies for Tc-bicisate determination show near 100% recovery, precisions of 1–2%, limits of detection <1%, and limits of quantification of approximately 1%. A

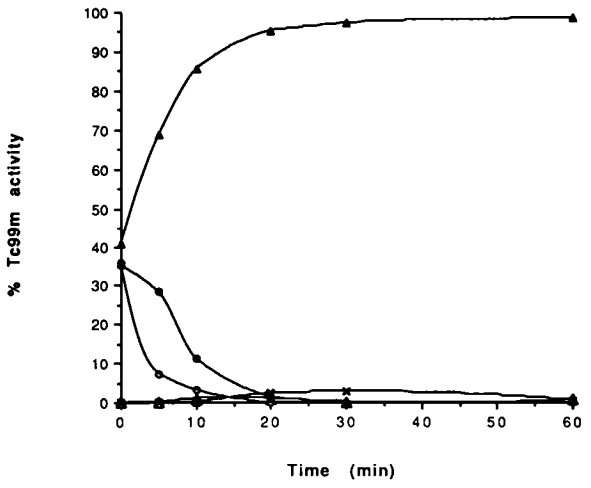
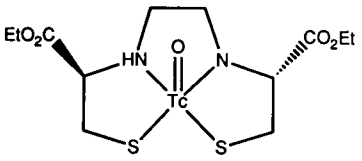


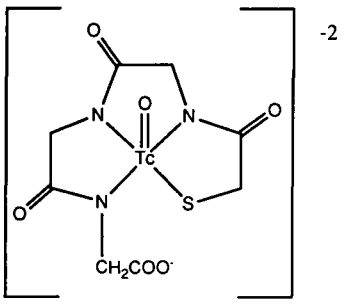
FIG. 4. ^{99m}Tc-bicisate and radioimpurity formation as a function of time: (▲ = ^{99m}Tc-bicisate, ○ = TcO_4^-, ● = Tc-EDTA, △ = Tc-R, and x = Tc-(IV)ECD.

TABLE 2. R_f Values for Technetium-99m Bicisate and Potential Radioimpurities

^{99m}Tc Complex	R_f Range: Quality Control Procedure	R_f Range: Research Procedure
^{99m}Tc-bicisate	0.8–1.0	0.1–0.4
Tc-R	0.0–0.2	0.0–0.1
Pertechnetate	0.0–0.2	0.7–0.8
Tc-EDTA	0.0–0.2	0.95–1.0
Tc-ECM	0.0–0.2	0.7–0.8
Tc-(IV)ECD	0.0–0.2	0.1–0.4



Technetium Tc99m Bicisate



Technetium Tc99m Mertiatide

FIG. 5. Structures of Tc(V) complexes of ^{99m}Tc-bicisate and ^{99m}Tc-mertiatide.

typical plot demonstrating the accuracy and linearity of the quality control procedure is shown in Figure 6.

Several additional studies were performed to evaluate the ruggedness of the radiolabeling and TLC procedures. The effect of temperature on the radiolabeling reaction was evaluated by performing the procedure at 15, 25, and 30°C. In each case, all materials (saline, Vial A, Vial B, pertechnetate) were maintained at the appropriate temperature, as was the final reaction vial. The radiolabeling results (Fig. 7) demonstrate that a 30-min reaction time is appropriate for room temperature labeling.

During the development of both TLC procedures, the stability of the sample after spotting on the plate, but prior to development, was evaluated. Any degradation that would occur on the plate during this stage of the procedure would result in artificially low RCP values. These experiments found evidence for degradation of ^{99m}Tc-bicisate on silica plates (quality control procedure) but not on C-18 plates (research procedure). Further experiments showed that the degradation was dependent on exposure of the plates to light. An experiment was performed to evaluate the extent of sample degradation as a function of time, using plates exposed to room light (50 FC) and plates stored in the dark. The results of this study are provided in Table 3. These data, obtained using a relatively low light intensity level, show the necessity of limiting the time between sample spotting and the development of plates.

The proper pretreatment and storage of TLC plates is critical to the reproducibility of many TLC-based separations. The effect of varying moisture levels on R_f values is well documented (5). The TLC plates required for both the quality control and research methods were evaluated by exposing the plates to a range of moisture levels and measuring the effect on the R_f value for the ^{99m}Tc-bicisate peak. The results of this study are given in Table 4. These results show that the retention of ^{99m}Tc-bicisate on both

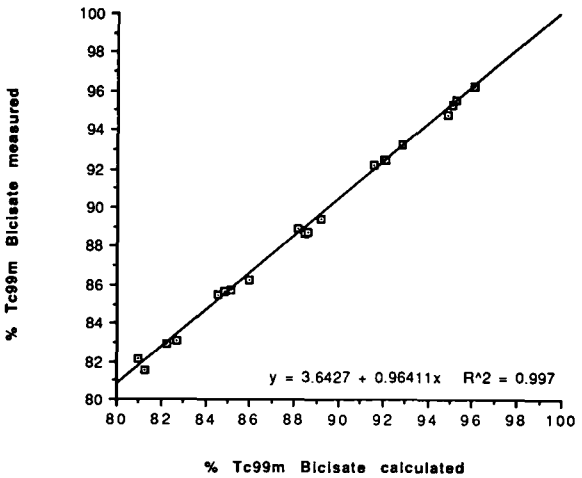


FIG. 6. Accuracy and linearity of response for ^{99m}Tc-bicisate in the presence of Tc-EDTA.

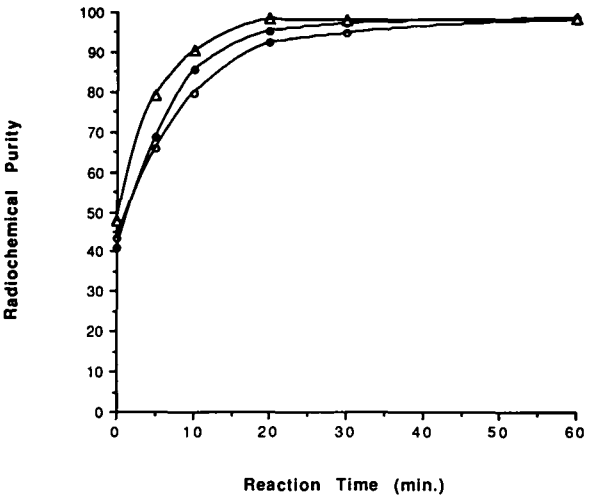


FIG. 7. Radiolabeling reaction as a function of time and temperature (○ = 15°C, ● = 25°C, △ = 30°C).

TABLE 3. The Effect of Light Exposure on Radiochemical Purity

Time after Spotting (min)	Radiochemical Purity (%)	
	Exposed to Light	Stored in Dark
5	98.0 ± 0.3	99.0 ± 0.9
10	100.0 ± 0.0	100.0 ± 0.0
20	98.4 ± 0.1	100.0 ± 0.0
30	95.6 ± 0.3	99.4 ± 0.6
60	89.2 ± 1.0	97.8 ± 0.1

TABLE 4. Effect of TLC Plate Moisture on R_f Value for Technetium-99m Bicisate

TLC Plate Treatment	^{99m}Tc-Bicisate R_f Value	
	Silica Plate (quality control)	^{18}C Plate (research)
Dried at 110°C for 30 min	0.94	0.43
As received from manufacturer, then stored in a desiccator	0.91	0.40
Stored at 55% relative humidity	0.82	0.25
Soaked in water, excess allowed to run off plate immediately prior to use	0.41	0.10

types of plates is sensitive to moisture. As long as the ^{99m}Tc-bicisate R_f value is significantly above 0.5, the cut and count quantitation procedure (quality control) will provide accurate results. However, since laboratories are often subject to a wide range of humidity levels and do not have access to a scanning detector to determine R_f values, we recommend that the TLC plates be stored in the presence of a desiccant.

The development and use of a chromatographic procedure for determining radiochemical purity requires knowledge of all potential radioimpurities and ruggedness parameters that can affect the final analytical results. The information provided in this paper should assist the user in obtaining accurate RCP measurements and also serve as a basis for evaluating future RCP methods for a ^{99m}Tc-bicisate kit.

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