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Equilibrium Dialysis and Ultrafiltration Compared for Determining the Protein Binding Rates of ^{99m}Tc-DTPA

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Abstract

Diethylene triaminopentaacetic acid (^{99m}Tc-DTPA) is one of the technetium radiopharmaceuticals mostly used in renal imaging for evaluation of glomera filtration rate. The ^{99m}Tc-DTPA binding rates on plasma proteins was investigated using two useful and reproducible methods. Equilibrium Dialysis (ED) and Ultrafiltration (UF) are described and there are performances compared. ^{99m}Tc-DTPA binds strongly to Human Serum (HS) than to Human Serum Albumin (HSA). In our assay, using UF technique, we found the binding rates were in HS 48.72 % ±2.58 and 51 % ±1.43 for respectively two ^{99m}Tc-DTPA concentrations [1mg/L] and [2mg/L]. Using ED method we found the binding rates were in HS 11.22 % ±2.17 and 14.94 % ±2.30 for respectively two ^{99m} Tc-DTPA concentrations [1mg/L] and [2mg/L]. Moreover, According to HSA concentrations, the ^{99m} Tc-DTPA binding rates increase using both techniques.

Keywords: ^{99m}Tc-DTPA; Protein Binding; Human Serum; Human Serum Albumin; Equilibrium Dialysis; Ultrafiltration.

1. Introduction

Plasma protein binding (PPB) plays an important role in the whole body disposition of drugs [1]. Pharmacokinetic properties such as rates of hepatic metabolism, renal excretion, membrane transport and distribution volume, depend on the free fraction ratio [2].

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Only free drugs can cross the plasma membrane and bind with the receptors for pharmacological actions [3]. The free fractions of the drugs can vary among individuals because of protein concentration variation and the drugs protein binding might change with a sudden change in the concentration level of plasma proteins either after surgical conditions, in geriatric or pediatric patients or because of certain disease states. In the case of radiopharmaceuticals, the biodistribution may be altered significantly because of medical procedures or unexpected problems [4] which can have a significant clinical impact on safety, interpretation of analysis and accuracy of diagnostic imaging [5], so it is very important to analyze the free fraction of the drug rather than total drug concentrations for a safe dosage.

Several methods have been used for determination of the free drug concentration in plasma as well as drug–protein binding ratio. These methods include affinity chromatography, ultrafiltration, ultracentrifugation, equilibrium dialysis, micro dialysis, capillary electrophoresis solvent micro-extraction and supported liquid membrane extraction. These methods differ in their rapidity, complexity and data quality. The two predominant techniques for studying the protein binding of drugs are equilibrium dialysis (ED) and ultrafiltration (UF). Ultrafiltration (UF) admitted as probably the most common method for determining unbound plasma concentrations (C_u), is a fast procedure (15-45 min, being a typical range) and a very simple method to use, in addition to the availability of variables commercial devices [6]. Otherwise the Equilibrium dialysis technique (ED) is commonly used and is generally accepted as a "standard method". Numerous studies have clarified some crucial aspects of this technique, namely the simplicity sample preparations and storage conditions, the effect of temperature, the interpretation of analytical data, and the importance of the buffer solution and the protein sample volume ratio [7].

The purpose of our study is to evaluate and compare the performances of the rapid Ultrafiltration approach with the “classical” and more time-consuming Equilibrium Dialysis method, to study proteins binding in human serum albumin and human serum of diethylene triaminopentaacetic acid (^{99m}Tc-DTPA), which is one of the technetium radiopharmaceuticals mostly used in renal imaging for evaluation of glomelar filtration rate [8].

2. Materials and Methods

2.1 Biological Materials

^{99m}Tc-DTPA protein binding was studied in vitro by using Human Serum (HS) obtained from fresh blood samples of normal healthy volunteers, collected in The National Transfusion Center of Rabat Morocco and Human Serum Albumin (HSA) (Vialebex®, 200mg/ml). Both Human serum and Human serum albumin was kept respectively at -20°C and 4°C. The HSA dilutions were performed in phosphate buffer adjusted to pH 7.4.

2.2 The ^{99m}Tc-DTPA Kit Reconstitution and Radiochemical purity control of the product

Technetium ^{99m}Tc is obtained from a Molybdenum^{99} / Technetium^{99} generator (Drytec-General Electric). The preparation of the radiopharmaceutical is carried out by reconstituting a kit of lyophilisate of diethylene triaminopentaacetic acid (DTPA-ROTOP) with 5 ml of sodium pertechnetate (^{99m}TcO-Na +). A solution (S) is prepared by diluting 9 mCi of the reconstituted solution to 8 ml. ^{99m}Tc-DTPA was studied at two concentrations

(1 mg / L and 2 mg / L): volume ratio (radiopharmaceutical / serum) approximately as clinical conditions. Two impurities are potentially present: free ^{99m}TcO-4 and reduced technetium Tc-R hydrolyzed. To determine these impurities, the Thin Layer Chromatography (TLC) technique with two systems was used (Figure 1).

System (1): For $^{99m}TcO^{-4}$ separation

- Stationary phase: Silica gel on glass plate (Gelman Sciences ITLC-SG).
- Mobile phase: Methyl ethyl ketone solution (Merck).

System (2): For Tc-R separation

- Stationary phase: Silica gel on glass plate (Gelman Sciences ITLC-SG).
- Mobile phase: NaCl solution.

Radiochemical purity of the radiopharmaceutical products is calculated according to the formula: ***% PRC = 100 - (% Tc-R +% $^{99m}TcO^{-4}$)***

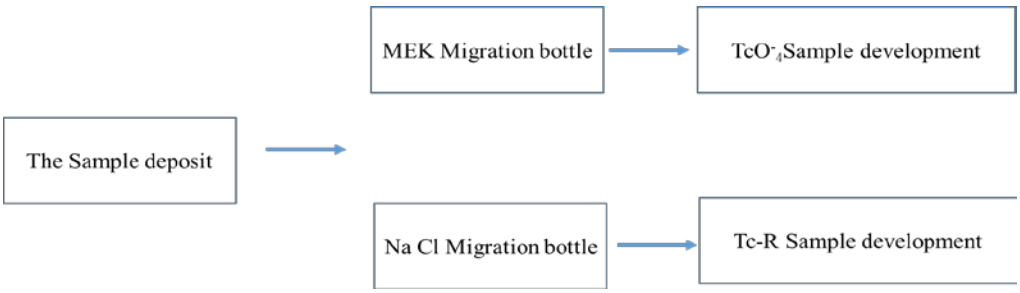

Figure 1: Determination of radiochemical impurities in a radiopharmaceutical by thin-layer chromatography.

2.3 Ultrafiltration

Ultrafiltration was performed using Ultracet YMT Ultrafiltration Devices (Centrifree® Ultrafiltration Device, Millipore Ireland Ltd.) to separate the free and bound fraction of the ^{99m}Tc-DTPA by plasma proteins. 3 ml of (SH / SAH: 20g/l; 40g/l and 60g/l) and 1 mL of ^{99m}Tc-DTPA are incubated for 60 minutes at 25°C. 200 µl of the incubated (SH / SAH) were transferred to a Centrifree ® Ultra-filtration device and centrifuged for 30 minutes at 2000 rpm. After centrifugation, the two parts of each filter: the filtrate (F) and the supernatant (S) are separated and then the radioactivities of both parts are measured by a gamma counter using a scintillation detector. The percentage of ^{99m}Tc-DTPA bound to the plasmatic proteins is determined as $\boldsymbol{F_b = F/ (F + S) * 100.}$

2.4 Equilibrium dialysis

Equilibrium dialysis was performed using a systems (DIANORM® Munich Germany) apparatus with 20 cells of the macro 1S type (1 ml/1 ml) with a high membrane surface area. Experiments were realized in a water-bath at 37°C and under constant stirring at 12 rpm. Each half-cell was filled with a volume of 1 ml. In all experiments, the drug was introduced in phosphate buffer compartment and the proteins in the other

compartments of the cell. Cell compartments were separated by Diachema Dialysis membranes (DIANORM GmbH Munich Germany), manufactured from natural cellulose, with a molecular weight cut-off of 5000. The membranes were rinsed with doubly distilled water for 15 min and incubated overnight in the buffer before use. We studied the time needed to reach dialysis equilibrium, and the equilibrium remained unchanged after 3 h. All subsequent experiments were performed for 5 h. After dialysis, the radioactivities are measured in each all half-cell compartments by a gamma counter using a scintillation detector. The percentage of bound ^{99m}Tc-DTPA in each all compartments was calculated by dividing the concentration of ^{99m}Tc-DTPA bound to the (HS / HAS) solution [MP] by the initial concentration [M_0] of ^{99m}Tc-DTPA. $\boldsymbol{F_b = [MP] / [M_0]*100\ \%.}$

3. Results

3.1 Radiochemical purity (RCP)

Several manipulations were realized to detect impurities and to ensure the radiochemical purity of our radiopharmaceutical product ^{99m}Tc-DTPA. This has been achieved through, the Thin Layer Chromatography technique with two systems, one to separate the pertechnetat $^{99m}TcO^{-4}$ and the other to separate the hydrolyzed reduced technetium Tc-R. Table 1 summarizes the tests performed on 5 series of 5 controlled radiopharmaceutical preparations. In all cases, the level of both impurities (^{99m}TcO-4 and Tc-R) is less than 1% and the rate of the radiochemical purity of ^{99m}Tc-DTPA is greater than 99%.

Table 1: Radiochemical purity of ^{99m}Tc-DTPA (in %)

	TcO^-_4	Tc-R	RCP %
^{99m}Tc-DTPA	0.4 ± 0.09	0.3 ± 0.4	99.3 ± 0.17

3.2 Ultrafiltration

Using the ultrafiltration method, protein binding is measured by separating the unbound fraction from the bound fraction, this procedure can influence the binding rate determination, therefore, the filtered volume of (SH / SAH) of all samples was minimized to 200 µl. As an analytical method, ultrafiltration could also have potential problems such as adsorption of the drug into the membrane and / or leakage of the drug attached through the filter membrane. In this sense, we carried out a verification of the non-specific fixation by filtration of a volume of 200µl of ^{99m}Tc-DTPA solution alone, and correction of the final results. As shown in Table 2, our results were:

- The ^{99m}Tc-DTPA binding rate of [1 mg/L] to the SH solution is averaged at 48.72% ± 2.58 (n=5). The binding rate of ^{99m}Tc-DTPA [2 mg/L] is averaged at 44.73% ± 7.12 (n=5).
- The ^{99m}Tc-DTPA binding rate of [1 mg/L] in the case of the HSA solution: 20g/L; 40g/L; 60g/L is estimated respectively to be 33.00% ± 2.96 (n=5); 35.14% ± 3.58 (n=6); 40.31% ± 4.50 (n=6). The ^{99m}Tc-DTPA binding rate of [2 mg/L] is respectively 37.42 % ± 3.10 (n=5); 39.54 % ± 6.25 (n=6); 44.73 % ± 7.12 (n=6).

Table 2: Fraction bounded of ^{99m}Tc-DTPA to HS and HAS (in %), studied by the Ultrafiltration technique.

[^{99m}Tc-DTPA]	**HSA (20g/L) (n=5)**	**HSA (40g/L) (n=6)**	**HSA (60g/L) (n=6)**	**HS *(n=5)***
1 mg / L	33.00 ± 2.96	35.14 ± 3.58	40.31 ± 4.50	48.72 ± 2.58
2 mg / L	37.42 ± 3.10	39.54 ± 6.25	44.73 ± 7.12	51.16 ± 1.43

3.3 Equilibrium Dialysis

As a pilot experiment, the time required to reach dialysis was studied, when the values of the total concentration (C_{tot}) on both sides of the dialysis membrane were identical and constant. In our case, the ^{99m}Tc-DTPA equilibrium time was reached after 3 hours. To determine the optimal dialysis time in this experiment, all samples were dialyzed at 37 ° C in the equilibrium dialysis system (DIANORM® Munich Germany), for 5 hours. The radioactivity in each half cell was determined with a gamma well counter. The ^{99m}Tc-DTPA binds strongly according to the concentrations of Human Albumin Serum (HSA) and Table 3 shows that the bound fraction of ^{99m}Tc-DTPA increases proportionally with (HSA) the concentrations.

- The 99mTc-DTPA binding rate [1 mg/L] to the human serum SH solution is averaged at 11.22 % ± 2.17 (n=11). The binding rate of 99mTc-DTPA [2 mg/L] is averaged at 14.94 % ± 2.30 (n=11).
- The 99mTc-DTPA binding rate [1 mg/L] in the case of the HSA solution: 20g/L ; 40g/L; 60g/L is estimated respectively to be 3.12 % ± 0.73 (n=10); 5.67 % ± 1.10 (n=10); 6.84 % ± 0.69 (n=13). The ^{99m}Tc-DTPA binding rate [2 mg/L] are respectively 4.85 % ± 0.94 (n=10); 7.64 % ± 1.36 (n=10); 9.98 % ± 1.22 (n=13).

Table 3: Fraction bounded of ^{99m}Tc-DTPA to HS and HAS (I %), studied by the Equilibrium Dialysis technique.

[^{99m}Tc-DTPA]	**HSA (20g/L) *(n=10)***	**HSA (40g/L) *(n=10)***	**HSA (60g/L) *(n=13)***	**HS *(n=11)***
1 mg / L	3.12 ± 0.73	5.67 ± 1.10	6.84 ± 0.69	11.22 ± 2.17
2 mg / L	4.85 ± 0.94	7.64 ± 1.36	9.98 ± 1.22	14.94 ± 2.30

4. Discussion

The radiochemical purity measurement is an essential step, it ensures that the radiochemical quality of the

radiopharmaceutical is in accordance with the monographs of the European Pharmacopoeia, it must be greater than 95% [9]. Radiopharmaceuticals must have, as injectable drugs, the criteria of physicochemical characteristics conformity, organoleptic characteristics and sterility. They must also meet the quality criteria related to their radioactive nature and radiochemical purity [10], which is defined as the total fraction of the radioactivity present in the form of a desired radiopharmaceutical, and depends on the quantities of radionuclide impurities and other radionuclides relative to half-lives of all radionuclides, and changes in the amounts of radionuclides over time. Examples of contaminants commonly found in ^{99m}Tc radiopharmaceuticals are ^{99}Mo contaminants. The radiochemical purity of a ^{99m}Tc-radiopharmaceutical has a remarkable effect on the behavior of radiopharmaceuticals in vivo, on the subsequent interpretation of the analysis, and on the accuracy of the diagnosis after imaging procedure [11]. Therefore, the purpose of radiochemical purity control is to confirm the absence of other radiochemical species with different physicochemical properties compared to the ^{99m}Tc-DTPA complex that can interfere with protein binding and eliminate all assumptions that may influence the outcome. In our case, the results of the radiochemical purity controls were greater than 99%, with are highly in accordance with the monographs of the European Pharmacopoeia.To evaluate the rate of protein binding of ^{99m}Tc-DTPA, we have focused in two analytical techniques: ultrafiltration and equilibrium dialysis. These methods were applied *in vitro* to determine ^{99m}Tc-DTPA free and bound fractions to plasma proteins and both techniques were applied sequentially to the human serum (SH) and the human serum albumin (HSA), we found the high values of ^{99m}Tc-DTPA binding in the SH compared to the HSA solutions. This could be explained by the presence, in addition to albumin, of other proteins that seem to also fix the radiopharmaceutical. Indeed, according to authors in [12], it is very important to note that in addition to albumin, which accounts for 40 to 50% of total proteins, there are other proteins capable to bind ^{99m}Tc-radiopharmaceuticals such as α and β globulin, transferrins and ceruloplasmin, and the ^{99m}Tc-DTPA radiopharmaceutical selectively binds to albumin and α- globulin with specific binding to α_1-antitrypsin.Ultrafiltration method seems to be a suitable method for the separation of substances bound to plasma proteins [13] it is a simple and rapid method in which centrifugation forces the buffer containing free drugs to cross the membrane and achieve a rapid separation [14] of the free molecule and bound to the protein in order to measure the protein binding of drugs. Besides, its advantages such as ease and short treatment time, several authors have raised questions about the adequacy of UF for the measurement of protein binding of drugs, due to nonspecific binding (FNS) drug on membrane filters and plastic devices [15], the main disadvantage of this method. When the drug binds strongly to the filtration membrane, the concentration of the drug retained may deviate from the true free concentration [16]. This influences the determination of the drugs protein binding rates [9], the reason why the filtered volume of (SH and SAH) has been minimized to 200µl in order to avoid saturation of the filter membrane [17]. Also, to verify the presence of nonspecific binding by adsorption of the drug to the membrane or the leakage of the drug fixed through the membrane of the filter, we proceeded to the correction of the results by a control of the non-specific fixation by filtration of a volume of 200 µl of only ^{99m}Tc-DTPA solution as a positive control.

Equilibrium dialysis is considered a more accurate method [18], providing us with a simple procedure for measuring the protein binding rate of ^{99m}Tc-DTPA. Numerous studies have clarified some crucial aspects of this technique, such as the simple sample preparations and storage, the temperature and buffer pH effects, the interpretation of analytical data and the importance of the buffer / protein sample volume ratio. The time

required to reach equilibrium during equilibrium dialysis studies limits the utility of the method, which is why dialysis cells with a high surface area to volume ratio, such as DIANORM, were used to shorten the time. dialysis at 4-6 hours or less [19]. The high specificity of this method makes it the suitable one for our purpose, because of the ^{99m}Tc-DTPA binding rates to plasma proteins is analyzed at equilibrium, eliminating the possible effect of nonspecific binding (NSF) [20] [21]. Currently, to evaluate the binding of drugs to proteins, most investigations have done this using equilibrium dialysis, mainly because this method offers the advantage that the free and bound fractions of the drug are not separated and therefore, the balance of the link will not be affected. This reason mainly explains the considerable amount of data currently available with this technique. Therefore, equilibrium dialysis can still be considered as the gold standard for the study of the binding characteristics of a new drug to the particular serum or protein, such as the determination of n (number of attachment sites) and K (affinity constant). This technique should also be used to validate other analytical assays [22].

5. Conclusion

The purpose of this study is to compare the reliability of two separation techniques (in vitro), Ultrafiltration and Equilibrium Dialysis, to evaluate the free and bound fraction of acid diethylene triaminopentaacetic ^{99m}Tc-DTPA, which is one of the technetium radiopharmaceuticals mainly used in renal imaging for evaluation of glomerular filtration rate. Comparing these two techniques, it can be concluded that the determination of the free fraction by ultrafiltration is much simpler, but the frequent observed leakage of proteins is a disadvantage of the UF system, in addition to the non-specific binding (NSB), which was consistent with several publications. The equilibrium dialysis technique, is a time consuming and preparative technique, however it's a very specific method allowing the determination of pharmacokinetic parameters easily under physiological conditions (equilibrium conditions, physiological temperature). So in this context, DE is considered as the reference method. For in vivo purposes, we validated two in vitro methods and we determined the percentage of ^{99m}Tc-DTPA binding to human serum (SH) and to human albumin serum (HSA). Our results showed that in all ranges studied, there was a very significant difference between equilibrium dialysis and ultrafiltration results. The results obtained by UF method were very different from the results obtained with equilibrium dialysis method. In addition, ultrafiltration requires expensive equipment, but the equilibrium dialysis technique is less expensive to study drugs protein binding in vitro and in vivo.

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Conflict of Interest

The authors declare that they have no conflicts of interest in relation to this article.

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