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Quality Assurance of Iodinated (^{125}I) Human Fibrinogen

by
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ABSTRACT

The radiopharmaceutical iodinated (^{125}I) human fibrinogen is currently used for the detection of deep vein thrombosis in the legs, a fairly common post - surgical complication.

A comprehensive quality assurance programme for ^{125}I - human fibrinogen has been determined for routine use at the Australian Radiation Laboratory, with adaptions necessary for hospital quality control testing.

INTRODUCTION

^{125}I - Human fibrinogen has been used in Melbourne metropolitan hospitals for several years for the detection of deep vein thrombosis of the legs. This condition can frequently develop after surgery, and early detection is important for the prevention of pulmonary embolism, a complication which can be fatal (1). ^{125}I - Human fibrinogen can also be used in the detection of renal transplant rejection and for fibrinogen turnover studies (2). So far, this test has been performed without any quality assurance or quality control being performed prior to injection. A testing method at present does not exist in either the British Pharmacopoeia (BP) or the United States Pharmacopoeia (USP).

PROPERTIES OF IODINATED (^{125}I) HUMAN FIBRINOGEN

Fibrinogen is a plasma protein essential in the blood clotting mechanism. It is present in human plasma at concentrations of approximately 300 mg/100 ml, the normal adult range being 200 - 400 mg/100 ml. It has a molecular weight of 341,000 g/mole(3,4), and is less soluble than other plasma proteins, being insoluble in the absence of salts and partially soluble in dilute salts.

Biosynthesis of fibrinogen occurs in the liver and it disappears from circulation with a half-life of 4 - 6 days. The protein is labile, being denatured readily by heating above 56^{o}C, by chemical agents such as salicylaldehyde and ninhydrin, and also by isolation, labelling and storage procedures (2,5).

The use of iodinated (^{125}I) human fibrinogen *in vivo* depends on its incorporation in the normal body clotting mechanism. It is deposited as insoluble iodinated fibrin during fibrinogenesis, which may be complete within three to four hours after initiation of thrombosis (2, 6). The clinical test assumes both this, and that a formed clot does not migrate from the area of detection, which would give falsely low results.

MATERIALS AND METHODS

Iodinated (^{125}I) human fibrinogen solution:

Iodinated (^{125}I) human fibrinogen injection, supplied by the Radiochemical Centre, Amersham, was used throughout as the source of iodinated (^{125}I) fibrinogen. Each vial contained 1.0 mg of white, freeze-dried iodinated fibrinogen.

Reconstitution was rendered with 1.1 ml distilled water, to result in a final specific activity per vial of 100 μCi/ml (3.70 MBq/ml) of ^{125}I. The total activity per vial was 100 μCi (4.07 MBq) at 1200 hours GMT 6-3-80. This activity was verified for radionuclidic content to be within the quoted limits of 80.0-120.0% on the stated reference date, using a Capintec Radioisotope calibrator CRC-2N.

The reconstituted solution also contained 0.65% sodium chloride, 0.75% sodium citrate as anticoagulant, and not more than 22 mg human albumin (6).

Bovine Thrombin Solution

Topical thrombin (USP), bovine origin, supplied by Parke-Davis as a 5000 US unit vial was used. The thrombin was in lyophilised form, and was accompanied by a vial containing 5 ml of isotonic saline diluent. The thrombin was reconstituted with 5 ml saline, and 5 ml glycerol as preservative, to become finally a 500 unit/ml solution. This solution is stable indefinitely in the refrigerator (7).

Human Albumin Solution

Iodinated (^{125}I) human albumin injection was used as an albumin reference throughout the testing, as supplied by the Radiochemical Centre, Amersham. Albumin concentration in the supplied injection was 20 mg/ml. It contained as preservative 0.9% benzyl alcohol, and sodium chloride for isotonicity.

Human albumin has a molecular weight of 69,000 g/mole and constitutes 55.2% of the total protein content of human plasma (compared with fibrinogen, which comprises 6.5%). Its isoelectric point is lower than that of fibrinogen (pH 4.7 compared with pH 5.5 for fibrinogen), which explains why its electrophoretic migration is more rapid than that of fibrinogen in neutral or slightly alkaline pH values (4).

Iodinated (^{131}I) Sodium Iodide Solution

As a reference standard throughout the testing procedures, sodium iodide (^{131}I) was used, as supplied by the Radiochemical Centre, Amersham. The solution used was carrier-free and contained 1 mg/ml sodium thiosulphate, phosphate buffer and sodium chloride.

This was used as a reference for detection of free radioactive iodide in the fibrinogen solution.

Radionuclide

Iodine-125 may be prepared by the neutron irradiation of xenon. It has a half-life of 60 days and decays by electron capture with the emission of gamma-rays. The major emission has an energy of 28 keV (138%) with 7% 35 keV gammas also emitted. The identification of the radionuclide bound to the fibrinogen was verified by measuring the gamma spectrum on a Packard Auto-gamma Scintillation Spectrometer. Not more than 1.0% of the total activity is due to iodine-126 at the date of calibration. This was performed by the metrology section.

Particulate Matter

No particles, visible to the human eye, were present in the reconstituted solution. The presence of particulate matter was checked by inverting the vial and visualising against white and black backgrounds, care being taken to shield the vial with lead glass.

pH

The pH of the iodinated-fibrinogen solution was within the range 5.0-7.0, determined by the use of narrow-range pH indicator papers.

Radiochemical Purity

Method 1

This method utilises the clotting mechanism of fibrinogen by thrombin in the normal clotting procedure in the body (8, 9).

0.3ml of reconstituted iodinated (^{125}I) human fibrinogen was counted in a suitable detector. The Capintec dose calibrator was used in this case, although a scintillation spectrometer would equally suffice. 0.1ml of bovine thrombin solution (500U strength) was added, the tube was shaken vigorously and allowed to stand for 20 minutes. This was found to be sufficient time to enable complete fibrin clot formation (clotting was incomplete after 10 minutes). The supernatant was then removed (but not discarded) with a pasteur pipette, and the clot washed twice with 0.5ml aliquots of distilled water. Each washing was added to the supernatant and the total was counted. The activity of the washed clot was also measured. The fraction of activity present in the clot was calculated as a percentage of the original ^{125}I-Fibrinogen activity.

If the test proved to be equivocal, with a high percentage of activity present in the supernatant instead of the fibrin clot, a further check could be made on the nature of the supernatant activity, by TCA precipitation using 5% TCA (8, 9). This would isolate other plasma proteins present, such as human

albumin, from free iodide. By estimating the activities of the TCA-precipitate and of the supernatant (containing free iodide but no proteins), a determination of whether the activity was due to protein iodination or breakdown of the fibrinogen itself could be reached.

Method 2

Aliquots of 5 µl of reconstituted iodinated (^{125}I) fibrinogen were applied to Whatman No. 1 paper strips and developed by descending chromatography, using 75% methanol and saline as eluents. Concurrently, 5 µl aliquots of iodinated (^{131}I) sodium iodide and iodinated (^{125}I) human albumin were applied, as references, to separate Whatman No. 1 paper strips and developed in these eluents. After drying, the strips were scanned using a Packard 7220/21 radiochromatogram scanner. They were then cut in half and counted in a Packard 5912 autogamma scintillation spectrometer.

Method 3

Aliquots of 5 µl of reconstituted iodinated (^{125}I) fibrinogen were applied to Whatman No. 1 paper strips, which had been soaked in 0.05M barbital buffer at pH 8.6. Aliquots of 5 µl of iodinated (^{131}I) sodium iodide and of iodinated (^{125}I) human albumin were separately applied to paper strips, as references. These were all submitted to low voltage electrophoresis at 500V for 30 minutes. After drying, the strips were scanned using a Packard 7220/21 radiochromatogram scanner. They were then segmented into different peak areas and counted in a Peckard 5912 autogamma scintillation spectrometer.

Nature of Protein

This was determined by low voltage electrophoresis, using 0.05M barbital buffer at pH 8.6. Triplicate strips of cellulose acetate, which had been soaked in the barbital buffer, were separately spotted with 10 µl of iodinated (^{125}I) fibrinogen and 10 µl 2.5% human albumin solution. These were run for 1 hour at 400V, stained in a solution of 0.2% w/v Ponceau S in 3% v/v acetic acid and washed three times with 5% v/v acetic acid. The strips were then soaked in ethanol for 1 minute and dried between sheets of blotting paper.

Total Protein

The Biuret test method for total protein was used (7). A limit of 80.0 to 120.0% of the stated values is imposed (10).

Chloride Test

To approximately 1 µl of 0.1% w/v silver nitrate solution, 2 drops of the iodinated fibrinogen were added. A white precipitate which forms

immediately and is insoluble in 10% nitric acid indicates the presence of chloride ions.

Citrate Test

The presence of an anticoagulant, in the form of sodium citrate, is determined by reaction of 1 drop of iodinated fibrinogen with 3 drops of pyridine and 1 drop of acetic anhydride, to form the characteristic yellow colour indicating citrate.

RESULTS

The purity level quoted is that not less than 80.0% of the total activity is bound to fibrinogen (6). All the analytical methods (methods 1,2 and 3) for radiochemical purity showed iodinated (^{125}I) fibrinogen values to be within this range.

Thrombin clotting resulted in a mean value of 92.6% of the initial activity being present as iodinated fibrinogen (see Table 1). After the expiry date of the injection had been exceeded by 4 days, the value of iodinated fibrinogen remaining in the clot dropped to a mean of 78.8%. However, the values of paper chromatography in 75% methanol did not alter beyond the expiry date. Chromatography in this solvent resulted in 96.8% of the activity remaining at the origin as iodinated fibrinogen, while 3.2% as free iodide (^{125}I) migrated to R_f 0.74 (see Table 2). Iodinated human albumin remained at the origin, therefore inseparable from the iodinated fibrinogen. Chromatography in saline separated fibrinogen from both human albumin and iodide; 92.8% of the activity was bound to ^{125}I-fibrinogen at R_f 0.01, while 7.2% migrated as free iodide (^{125}I) to R_f 0.92. Human albumin migrated to R_f 0.96.

In the paper electrophoresis, 99.6% of activity as iodinated fibrinogen migrated 3.8cm towards the anode (Table 3); 0.4% of activity as free iodide (^{125}I) migrated 14.5cm towards the anode. Human albumin migrated 5cm towards the anode under these conditions.

Cellulose electrophoresis revealed that human albumin migrated 0.7cm towards the anode, and human fibrinogen migrated 0.3cm towards the anode.

Total protein results revealed 21.5mg protein. This value was in agreement with the stated amount of fibrinogen (1 mg) and of human albumin (not more than 22 mg).

The tests for chloride and citrate both proved positive for these ions, and those for pH, particulate matter and radionuclidic purity all came within stated limits.

DISCUSSION

Among the various methods attempted for determination of radiochemical purity, two were selected for routine use in a quality assurance programme at the Australian Radiation Laboratory for iodinated (^{125}I) human fibrinogen - methods 1 and 3 (the clotting test procedure and paper electrophoresis).

For hospital quality control assessments, paper chromatography (75% methanol) could be substituted for paper electrophoresis.

Method 1 utilises a clotting characteristic with thrombin which is unique to the fibrinogen protein, thus separating it from any other proteins or free iodide present. Results were within the normal range (ie greater than 80% of activity was found to be due to iodinated (^{125}I) fibrinogen), thus rendering use of the TCA precipitation for an equivocal result unnecessary. If insufficient time is allowed for a fibrin clot to form, the results could be erroneous. A fibrin clot needs between 5 and 8 minutes to form at 37^{o}C (4), and should therefore be left for 15 to 20 minutes at room temperature after addition of bovine thrombin.

Both solvents used in the paper chromatography separate free iodide from iodinated fibrinogen quite satisfactorily. Human albumin, which would only be important in the unlikely event of false protein iodination, remained at the origin in 75% methanol, but migrated with the solvent front in saline. Since fibrinogen showed a tendency to streak in the saline, this solvent system was considered less efficient than 75% methanol.

Paper electrophoresis also separates free iodide from fibrinogen. Human albumin migrates further than fibrinogen, thus this procedure separates all three entities quite adequately, although the separation of albumin fibrinogen should normally not be important. Free iodide was the major contaminant present.

Cellulose electrophoresis merely verified the findings of the paper electrophoresis, and was deemed an unnecessary addition to the quality assurance programme.

SUMMARY

A quality assurance programme has been proposed and tested, and put into use as a routine testing procedure on iodinated (^{125}I) human fibrinogen injection.

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TABLE 1

RESULTS OF CLOTTING IODINATED (^{125}I) FIBRINOGEN WITH THROMBIN

	Initial activity (μCi)	Supernatant activity (μCi)	% Activity in clot
	55.0	6.3	88.5
	62.8	3.8	93.3
	66.4	0.9	98.7
	67.2	6.9	89.7
average	62.9	6.0	92.6

TABLE 2

RESULTS OF PAPER CHROMATOGRAMS

	75% Methanol Elution % activity		Saline Elution % activity	
	R_f 0.0	R_f 0.71 - 0.76	R_f 0.01 - 0.02	R_f 0.92 - 0.99
	96.9	3.1	93.6	6.4
	96.8	3.2	90.1	9.9
	96.8	3.2	94.7	5.3
average	96.9	3.2	92.8	7.2

TABLE 3

RESULTS OF LOW VOLTAGE ELECTROPHORESIS % activity

	3.5 - 4.1cm towards anode	14.3 - 14.7cm towards anode
	99.5	0.5
	99.6	0.4
	99.8	0.2
average	99.6	0.4