

Feb. 20, 1973

J. C. TURNER ET AL

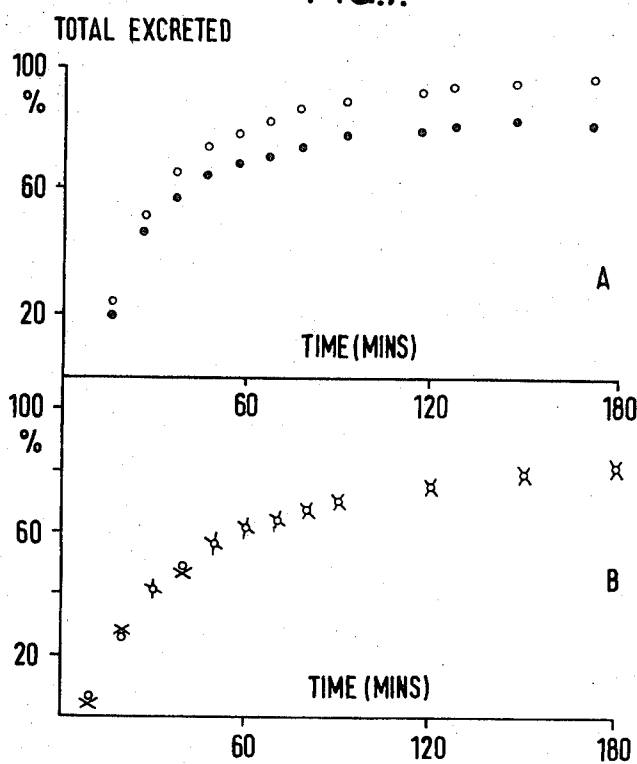
3,717,704

INULIN TYPE RADIOACTIVE DIAGNOSTIC MATERIALS

Filed Jan. 6, 1970

2 Sheets-Sheet 1

FIG. 1.



- UNLABELLED INULIN
- INULIN (CARBOXYLIC ACID - C14)
- × INULIN (HYDROXYMETHYL C14)

Feb. 20, 1973

J. C. TURNER ET AL

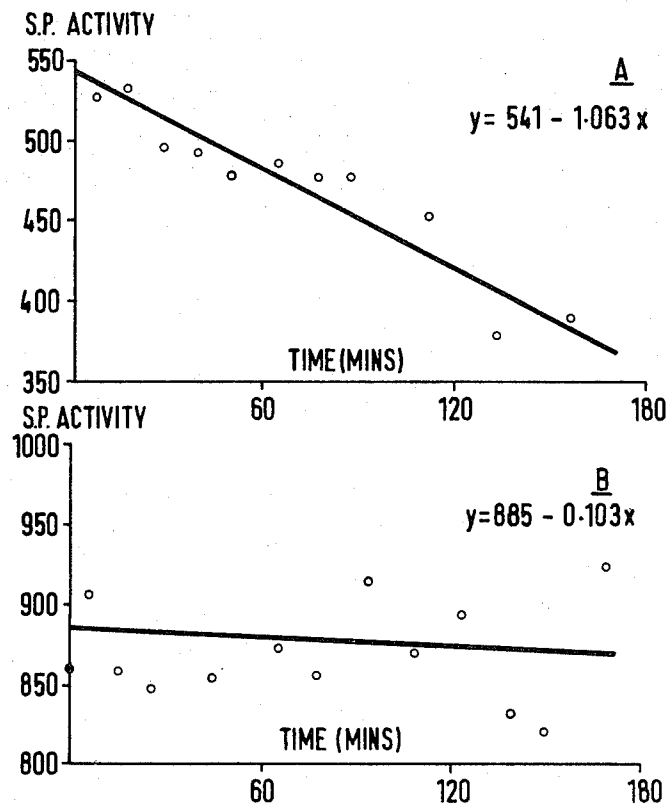
3,717,704

INULIN TYPE RADIOACTIVE DIAGNOSTIC MATERIALS

Filed Jan. 6, 1970

2 Sheets-Sheet 2

FIG. 2.



1

3,717,704 INULIN TYPE RADIOACTIVE DIAGNOSTIC MATERIALS

John Christopher Turner, Chesham, and Geoffrey Shephard, Amersham, England, assignors to United Kingdom Atomic Energy Authority, London, England
Filed Jan. 6, 1970, Ser. No. 929
Int. Cl. A61k 27/04

U.S. Cl. 424—1

4 Claims

ABSTRACT OF THE DISCLOSURE

For use as a diagnostic composition, there is provided inulin having a hydroxymethyl group of which the carbon atom is a carbon-14 atom. This material is prepared from inulin (carboxylic acid C-14) by methylating and reducing.

BACKGROUND OF THE INVENTION

The present invention relates to radioactive materials and more specifically to radioactive materials and compositions used for diagnostic purposes.

The compound inulin is a polysaccharide of molecular weight approximately equal to 5000 and consisting of some 30-35 fructose units linked end to end in a straight chain. The principal medical use of this compound has been in the measurement of glomerular filtration rate (G.F.R.) which can be used as an index of kidney function. It will be understood that the inulin is injected into the bloodstream and, being physiologically inert, it is freely filtered through the kidneys into the urine without change. The inulin in the urine has normally been estimated by a colorimetric method which involves hydrolysing the inulin to fructose. It will be readily appreciated that this estimation procedure is laborious and it is also complicated by the necessity to undertake blank estimations to allow for the presence of glucose which may be in the urine.

In order to avoid some of the difficulties above-mentioned it has been proposed to prepare and use a radioactive material based on inulin, i.e. "labelled" inulin. It will be well known that, provided the radioactivity is firmly bound, the use of a radioactive material for diagnostic purposes is a straightforward and safe procedure and the estimation of the material is relatively easy since it can be counted using conventional detectors for radioactivity and it may not be necessary to undertake any chemical separation. The problem of blank estimations is also minimised. A convenient radioactive label is carbon-14.

The labelled inulin that has been previously proposed and prepared has been made by reacting the inulin with hydrogen cyanide containing carbon-14 so as to give a nitrile. This nitrile is then hydrolysed to give a carboxylic acid group containing carbon-14. This particular compound is variously called "inulin carboxyl-C14," "carboxy inulin-C15" or "inulin (carboxylic acid-C14)" and the latter name will be used hereinafter. From the point of view of estimation, inulin (carboxylic acid-C14) has proved to be extremely satisfactory. However, some doubts have been expressed as to whether it may not behave in exactly the same way as the unmodified inulin owing to the presence of the carboxyl group. Obviously it is extremely desirable that there should be no difference in the physiological behavior of the labelled inulin as compared with the unlabelled material for this would render the previously accumulated knowledge useless.

OBJECTS OF THE INVENTION

It is one object of the present invention to provide a new or improved labelled inulin.

2

It is an alternative object of the present invention to provide a diagnostic composition including such labelled inulin.

It is a further alternative object of the present invention to provide a method for the manufacture of such labelled inulin.

OUTLINE OF THE INVENTION

According to one aspect of the present invention there is provided a labelled inulin comprising inulin having a hydroxymethyl group of which the carbon atom is a carbon-14 atom. Hereinafter this compound will be called "inulin (hydroxymethyl-C14)."

According to a further aspect of the present invention there is provided a diagnostic composition comprising a solution of inulin (hydroxymethyl-C14) in a physiologically inert saline solution.

It will be appreciated that the new labelled insulin contains no carboxylic acid groups and the chemical group which has been introduced by the labelling process is a hydroxymethyl group which is already present in fructose units so that no physiological difference should be present between the new labelled inulin and the inactive or unlabelled inulin. It will also be appreciated that the estimation of the new labelled inulin is essentially the same as the estimation of inulin (carboxylic acid-C14).

It should also be stated that inulin being physiologically inert, the inulin (hydroxymethyl-C14) is similarly inert, the small amount of radioactivity due to the soft β -emitting C-14 being totally without significance at the doses and concentrations involved. The preferred method of preparation of the material is such that it is of high, e.g. better than 98%, radio-chemical purity and a convenient concentration of the diagnostic composition for injection into the bloodstream is between 1.6 mg./ml. and 5.0 mg./ml. or more specifically 2 mg./ml. It will be appreciated that 20 mg. corresponds to 50 μ Ci. The amount to be injected or "dose" is not very dependent on body weight but only on achieving an adequate concentration of activity in the plasma. In the case of experimental animals such as rabbits, the dose is conveniently 1 μ Ci/kg. whilst for a man it could be 2 μ Ci/kg. These doses should be compared with the conventional inulin dosage of 200 mg./kg. and 500 mg./kg. respectively.

Accordingly to yet another aspect of the present invention, a method of preparing inulin (hydroxymethyl-C14) comprises taking inulin (carboxylic acid-C14), methylating the same to give a methyl ester and then reducing the ester to give an alcohol.

Very briefly, the preferred method of preparation is to methylate the acid with diazomethane in a mixture of methanol and ether and then to reduce the methyl ester with sodium borohydride to give the alcohol. The product can then be fractionated to yield a narrow molecular weight range around 5,000.

PREFERRED METHOD OF PREPARATION

In order that the present invention may more readily be understood, the detailed preparation of inulin (hydroxymethyl-C14) will now be described.

This preparation uses as its starting point inulin (carboxylic acid-C14) which is available from the Radiochemical Centre, Amersham, and the preparation of that material from unlabelled inulin will not be described. 0.43 gram of inulin (carboxylic acid-C14) of a specific activity of 1.4 microcuries per milligram was powdered and mixed with 1.5 millilitres of methanol. Two millilitres of a 0.14 molar solution of diazomethane in ether was added and the mixture was stirred in a flask at room temperature for twenty-minutes. The volatile contents of the flask were then removed by pumping under high vacuum. The non-volatile residue was dissolved at 40° C. in 15 millilitres of

water containing ammonium hydroxide (1 millilitre of ammonia of specific gravity .880 per 150 millilitres of water). A solution of 60 milligrams of sodium borohydride in 3 millilitres of water was added and the mixture was stirred at room temperature for forty-five minutes and then evaporated to dryness in a water bath of temperature not greater than 35° C. The resulting gel was dissolved in 3 millilitres of water and 10 millilitres of methanol was added to cause the precipitation of the product as a fine powder which was filtered off and dried. The treatments with diazomethane and sodium borohydride were repeated on the residue.

The dry powder product of these two preparations were mixed and dissolved in 5 millilitres of water and placed on a 200 millilitre column of "Sephadex G-25" and eluted with water in 5 millilitre fractions. Fractions numbered 12-20 of the eluate contained the major component which was of a similar molecular weight range to that of the starting material inulin (carboxylic acid-C14) and these fractions were pooled and concentrated to dryness on a water bath at a temperature not greater than 35° C. This procedure yielded 0.36 gram of solid material which is an 84% yield.

COMPARISON WITH THE PRIOR ART

Tests using infra-red spectroscopy have indicated that no carboxyl group is present in the new labelled inulin. In order to simulate glomerular filtration the new labelled inulin has been mixed with unlabelled inulin and an endeavour has been made to separate the two by electrophoresis and gel filtration but these attempts have been unsuccessful. It should be noted that it is possible to separate unlabelled inulin from inulin (carboxylic acid-C14) by electrophoresis.

BRIEF DESCRIPTION OF THE DRAWINGS

For a fuller understanding of the tests reference should be made to the accompanying drawings, wherein:

FIGS. 1A and 1B are graphs showing total excretion against time, and

FIGS. 2A and 2B are graphs showing specific activity of the excreted inulin against time.

In the following tests the following diagnostic compositions were used:

TABLE I

Abbreviation	Active ingredient	Dose
In.....	Unlabelled inulin.....	200 mg.
InCO ₂ H.....	Inulin (carboxylic acid-C14)....	4 mg.; 10 µCi.
InCH ₂ OH.....	Inulin (hydroxymethyl-C14)....	4 mg.; 10 µCi.

Rabbits were injected intravenously via an ear vein with a solution of native inulin and one of the labelled derivatives. Urine samples were taken at timed intervals after the injection and the inulin content and radioactivity determined. The specific activity of inulin in the urine could be compared with the specific activity of the starting material. This technique would reveal any intra- or extrarenal discrimination between the labelled and unlabelled inulin.

Urine samples were taken (every ten minutes for the first hour, then approximately every twenty minutes for the next two hours) by washing the bladder with two volumes of saline (20 ml. each). The time of each sample was noted at the removal of the second wash from the bladder.

Radioactive assay of the samples was by liquid scintillation counting using a Nuclear Chicago Mark I liquid scintillation counter. The urine samples were diluted to 100 ml. and duplicate samples (1 ml.) added to 10 ml. of Triton X-100 scintillant solution. All samples were cooled to 4° C. and stored in the dark for 30 minutes before counting. No chemiluminescence was observed. Suitable predetermined background counts were subtracted from each sample and the samples counted until a net sample count gave a standard deviation of less than

1%. Counting efficiency was determined by the channels ratio method and was routinely 60-80%.

The colorimetric determination of inulin was performed by the acid hydrolysis of inulin to fructose, the latter being estimated by Nakamura's modification of the method of Roe and Epstein (see Agr. Biol. Chem. (Tokyo), 32, 696 (1968)). This modification gives a fructose:glucose colour yield of 100:1 thus minimising the glucose background in the samples. Replicate samples were generally within 2.5% of the mean. Standard fructose samples were estimated simultaneously with the experimental samples; this technique allowed the inulin content of the samples to be expressed directly in µg. The fructose standards, which covered the range of experimental values, obeyed by the Lambert-Beer Law.

The cumulative totals of inactive inulin and the labelled inulin derivative cleared into the urine at each sampling time were calculated. Table II shows these totals one hour, two hours and three hours after the initial injection of inactive and active material. It is evident that a large proportion (about 50-60%) of inulin is cleared within the first hour of the experiment and that about 80% of it is cleared within three hours of the start.

TABLE II

Cumulative clearance totals for inulin, inulin (carboxylic acid-C14, and inulin (hydroxymethyl-C14), expressed as the percentage proportion cleared into the urine of the injected material at each time

Animal	Injected materials	Time (min.) after injection	Percentage of active material cleared	Percentage of inactive material cleared
R3.....	InCO ₂ H plus In.....	55	55.2	58.2
		120	71.9	75.8
		180	81.7	87.3
R4.....	InCO ₂ H plus In.....	55	51.0	54.4
		123	69.1	75.6
		178	74.5	83.7
R5.....	InCO ₂ H plus In.....	55	68.4	78.7
		125	80.5	94.0
		170	81.7	96.7
R6.....	InCO ₂ H plus In.....	65	35.0	37.2
		135	48.3	52.0
		180	63.0	68.9
R7.....	InCH ₂ OH plus In.....	60	50.6	49.2
		130	68.4	67.2
		175	72.7	71.6
R8.....	InCH ₂ OH plus In.....	65	37.1	31.9
		130	42.3	39.4
		170	45.0	43.7
R9.....	InCH ₂ OH plus In.....	60	61.3	61.4
		120	75.4	75.1
		180	81.9	82.0
R10.....	InCH ₂ OH plus In.....	65	59.7	60.0
		120	76.1	76.7
		170	81.6	82.6
R11.....	InCH ₂ OH plus In.....	30	49.5	49.5
		60	64.8	65.1

It will be observed that there is an obvious and continuously increasing discrimination between inulin (carboxylic acid-C14) and unlabelled inulin. The clearance of inulin (carboxylic acid-C14) falls markedly below that of unlabelled inulin, as can be seen in FIG. 1A, which shows this increasing discrimination between active and inactive material for a typical experiment. The clearance of inulin (carboxylic acid-C14) falls off so that there appears to be irreversible retention of active material within the body.

The other tracer inulin (hydroxymethyl-C14) shows a good correlation between the totals of active and inactive material excreted at each time (see Table II and FIG. 1B).

The ratio, labelled material excreted:inactive inulin excreted, was calculated for each experiment with each tracer for the final readings in the table of cumulative clearances (Table II). The mean value of this ratio and its standard deviation have been computed for the sets of experiments with each derivative, and are as follows:

Derivative	R ¹	S.D.
Inulin (carboxylic acid-C14)....	0.899	0.017
Inulin (hydroxymethyl-C14)....	1.006	0.007

¹ Mean ratio of labelled derivative cleared to native inulin cleared, for the final totals.

5

Application of Student's *t* test ($n=4$) shows that the deviation of the inulin (carboxylic acid-C14) from behaviour as native inulin ($R=1.000$) is highly significant ($p<0.01$). On the other hand, inulin (hydroxymethyl-C14) shows a high degree of correlation with native inulin.

The specific activity of inulin in the urine

The criterion of cumulative clearance totals is useful in establishing the existence of discrimination between inulin and a labelled inulin, since retention of the labelled tracer within the body is easily demonstrated. The cumulative totals may be unduly weighted by a small inaccuracy in the estimation of the samples taken early on in the experiment, which contain large amounts of tracer and inulin. Hence, a much more sensitive criterion which would show discrimination between the labelled compound and native inulin is the specific activity of the inulin in each emergent urine fraction.

This was calculated from the radioactivity and inulin content of each fraction and expressed as the specific activity of the fraction at the mid-point of each clearance time. The specific activity was compared with that of the injected material. These specific activities naturally show a scatter about a trend line and the slope *b* of the latter was computed for the least-squares line ($y=a+bx$), for the regression of specific activity upon time. The standard deviation of the slope and the value of the intercept (*a*) were also computed.

FIG. 2A shows a typical trend line for inulin (carboxylic acid-C14), whilst FIG. 2B shows a similar trend line for inulin (hydroxymethyl-C14). The data for each experiment are presented in Table III, which gives the slope of the regression line, expressed as a percentage change in the initial specific activity, the standard deviation of the slope, also expressed as a percentage, the computed intercept of the line, expressed as a percentage of the initial specific activity, and the significance level of the deviation of the slope from zero.

TABLE III

The variation in urine specific activity (S.A.) with time
[The intercept, *a*, and the slope, *b*, of the least-squares regression line, $y=a+bx$, are expressed respectively in terms of percentage of and percentage change per hour in the initial specific activity.]

Animal	Injected material	Change in S.A. <i>b</i> percent per hour	S.D. of <i>b</i> , percent hr.	Initial S.A. <i>a</i> , percent	<i>p</i> *
R3	InCO ₂ H	-6.0	2.2	98.0	<0.05
R4		-11.7	1.1	99.3	<0.001
R5		-12.7	2.7	95.6	<0.001
R6		-4.4	1.3	99.4	<0.01
R7		-0.7	1.3	102.9	N.S.
R8	In.CH ₂ OH	-4.2	2.4	108.4	N.S.
R9		+2.2	2.0	100.1	N.S.
R10		-2.1	1.3	101.3	N.S.
R11		-0.3	5.3	98.0	N.S.

**p* Represents the probability that the N.H. that $b=0$ is correct. It was obtained using the *F* test and Student's *t* test ($n=10-12$).

The specific activity results for inulin (carboxylic acid-C14) indicate that it cannot be regarded as a good tracer for native inulin in this system. There is a continuous

6

decline in the specific activity of the emergent urine throughout the experiment. This decline shows variation from animal to animal and is very highly significant in two experiments ($p<0.001$), highly significant in one ($p<0.01$) and significant in the fourth ($p<0.05$). The mean value of the slope, *b*, for the four experiments was -8.7% , ($\sigma=1.7\%$; $p<0.001$) which is very highly significant in its deviation from zero.

Inulin (hydroxymethyl-C14), in contrast, can be seen to be a good tracer for unlabelled inulin. No value of the slope, *b*, shows a significant variation from zero. It therefore shows no significant change in specific activity over the duration of the experiment, even at the end when the concentration level of inulin in the plasma is about 6 mg. percent. Moreover the standard deviation of the slope is small, which means that the 95% confidence limits for the magnitude of the slope cover a small range (about 4.5% either side of the mean value) (see FIG. 4).

The means value of *b* for inulin (hydroxymethyl-C14) is -1.0% ($\sigma=2.9\%$) which again indicates that this labelled inulin derivative is not separated from inulin in this system.

Any small difference in physiological treatment between the labelled inulin derivative and unlabelled inulin would be reflected by a relatively large change in the specific activity of the inulin in the urine especially towards the end of the experiment when the plasma concentration levels of each are low. For example, if the labelled derivative was discriminated against by, say, 5% per hour, by being either excreted more slowly by an intra-renal process or removed from circulation by an extra-renal one, the specific activity of inulin in the urine after three hours would have fallen to 75% of its original value. Even a 2% discrimination would give a specific activity of 88% of the original in the urine after three hours.

We claim:

1. A radioactively labelled diagnostic material comprising inulin having a hydroxymethyl group of which the carbon atom is a carbon-14 atom.

2. A diagnostic composition comprising a solution of inulin (hydroxymethyl-C14) in a physiologically inert saline solution.

3. A diagnostic composition according to claim 2, wherein the concentration of inulin (hydroxymethyl-C14) is between 1.6 mg./ml. and 5 mg./ml.

4. A method of preparing inulin (hydroxymethyl-C14) which comprises taking inulin (carboxylic acid-C14), methylating the same by treating with diazomethane to give a methyl ester and then reducing the methyl ester by treating the methyl ester with sodium borohydride to give an alcohol.

References Cited

Nuclear Science Abstracts, vol. 17, October 1963, item 31920 on p. 4256. Extracted from Proc. Soc. Exptl. Med. 113:584-8 (July 1963).

BENJAMIN R. PADGETT, Primary Examiner