Biochem. J. (1985) **226**, 319–322
Printed in Great Britain

An alternative procedure for incorporating radiolabelled cholesteryl ester into human plasma lipoproteins *in vitro*

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(*Received 8 October 1984/Accepted 22 October 1984*)

A simple method has been developed for labelling human plasma lipoproteins to high specific radioactivity with radioactive cholesteryl esters *in vitro*. After isolation by preparative ultracentrifugation, the selected lipoprotein was incubated for 30min at 4°C in human serum ($d > 1.215$) that had been prelabelled with [4-^{14}C]cholesteryl oleate or [1,2-^{3}H]cholesteryl linoleate, and was then re-isolated by ultracentrifugation. All major lipoprotein classes were labelled by the procedure. Specific radioactivities of up to 18d.p.m.·pmol^{-1} (46d.p.m.·ng^{-1}) were achieved. When radiolabelled high-density lipoprotein was infused intravenously, the radioactive cholesteryl ester behaved *in vivo* indistinguishably from endogenous cholesteryl esters produced by the lecithin (phosphatidylcholine): cholesterol acyltransferase reaction.

The labelling of plasma lipoproteins with radioactive cholesteryl esters *in vitro* has usually been achieved by incubating plasma with radioactive cholesterol at 37°C for several hours, during which time radioactive esters are formed by the LCAT reaction. After re-isolation of the lipoprotein, the residual radioactivity in non-esterified cholesterol is removed by incubation with erythrocytes (Barter & Jones, 1980). This approach has several problems: it induces changes in lipoprotein composition (Barter *et al.*, 1982); it does not permit labelling with specific cholesteryl esters; the removal of radioactive non-esterified cholesterol is sometimes incomplete; and losses of lipoprotein occur during the incubations with erythrocytes. Although methods have been described in which radioactive cholesteryl ester is incorporated directly into lipoproteins, none of these is ideal, one being of low efficiency (Thomas & Rudel, 1983) and the others involving exposure of the lipoprotein to heptane (Kruger *et al.*, 1978), dimethyl sulphoxide (Brown *et al.*, 1975) or artificial liposomes (Hough & Zilversmit, 1984). We have developed an alternative procedure which is simple, efficient, avoids exposure of the lipoproteins to laboratory reagents, and uses conditions compatible with subsequent infusion of the preparation into humans.

Abbreviations used: LCAT, lecithin (phosphatidylcholine): cholesterol acyltransferase; VLDL, LDL and HDL, very-low-density, low-density and high-density lipo-proteins respectively; LPDS, lipoprotein-deficient serum; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid) ('DTNB').

Materials and methods

Radiochemicals

[4-^{14}C]Cholesteryl oleate (59.4mCi·mmol^{-1}) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. [1,2-^{3}H]Cholesteryl linoleate (1.0Ci·mmol^{-1}), [4-^{14}C]cholesterol (55.7mCi·mmol^{-1}) and [1,2-^{3}H]cholesterol (47.7Ci·mmol^{-1}) were prepared by The Radiochemical Centre, Amersham, Bucks., U.K. Before use, all radiochemicals were purified by t.l.c. (Stokke & Norum, 1971).

Isolation of plasma lipoproteins

Venous blood was collected from fasted (14h) healthy subjects into Na_2EDTA (1mg·ml^{-1}), and the plasma isolated at 4°C by centrifugation. Lipoproteins were separated by sequential preparative ultracentrifugation (Mistry *et al.*, 1981): VLDL was isolated at $d < 1.006$, LDL at d 1.006–1.063 and HDL at d 1.063–1.210. LPDS, which contains the lipid-transfer protein (Zilversmit *et al.*, 1975), was recovered at $d > 1.215$g/ml. All lipoproteins and LPDS were dialysed at 4°C against NaCl

(0.15mmol·l^{-1})/Na_2EDTA (1mmol·l^{-1}), pH7.4, and filtered (0.22μm-pore-size filter; Millipore) before use.

Labelling procedure

A portion (100–500μCi) of radioactive cholesteryl ester in acetone (0.2–0.5ml) was added dropwise with swirling to 10ml of LPDS in a glass tube at room temperature. The acetone was then evaporated under a gentle stream of O_2-free N_2 for 45min. An equal volume of VLDL, LDL or HDL (up to 50mg of cholesterol) was added to the labelled LPDS, and the mixture incubated on a roller at 4°C for 30min. After adjustment of the background density with solid NaBr or NaCl as appropriate, the labelled lipoprotein was recovered by ultracentrifugation for 20h at 105000*g*, dialysed against NaCl (0.15mmol·l^{-1})/Na_2EDTA (1mmol·l^{-1}), pH7.4, and filtered.

All procedures were carried out under sterile conditions, a laminar-flow cabinet being used. All glassware and density solutions were autoclaved.

Other procedures

Lipids were extracted as described by Folch *et al.* (1957) or with propan-2-ol (37°C, 30min); by both methods, recovery of cholesteryl esters was essentially complete. Separation of lipids was by silicic acid t.l.c. (Stokke & Norum, 1971). Rialuma (LKB Instruments, Croydon, Surrey, U.K.) was used as scintillation solvent; counting efficiency (mean values: ^3H, 31%; ^{14}C, 64%) was determined with radiolabelled n-hexadecane (The Radiochemical Centre) as internal standard. Cholesterol mass was assayed by an enzymic procedure (Boehringer-Mannheim G.m.b.H., catalogue no. 187 313). Electrophoresis of lipoproteins on cellulose acetate was for 40min at 180–200V in barbitone buffer, pH8.6, followed by staining with Fat Red 7B. Precipitation of VLDL and LDL with heparin and $MnCl_2$ was performed as described by Warnick & Albers (1979).

Results and discussion

Timed incubations of LDL in [^{14}C]cholesteryl ester-labelled LPDS, followed by addition of heparin and $MnCl_2$ and measurement of radioactivity in a propan-2-ol extract of the precipitated LDL, demonstrated that 97.8±0.9% ($n = 6$) of the radiolabel was transferred from the LPDS to the lipoprotein within 30min. No radioactivity was precipitated in the absence of LDL.

The overall efficiency of the labelling procedure (with preparative ultracentrifugation to re-isolate the lipoproteins, as routinely practised), was determined by relating the total radioactivity recovered in six preparations of [^3H]cholesteryl ester-labelled lipoproteins (two each of VLDL, LDL and HDL) to the radioactivity originally added to the LPDS. The results were: VLDL, 59 and 64%; LDL, 36 and 42%; HDL, 42 and 45%.

The effect on efficiency of diluting the LPDS with NaCl (0.15mmol·l^{-1}) before addition of the radioactive ester was examined in further experiments with LDL. A dilution of 1:10 produced a further 27% loss of labelled ester by adsorption to the glass tube; with a 1:100 dilution the corresponding value was 43%. Accordingly, undiluted LPDS was used for all subsequent work.

In 15 different preparations of labelled VLDL, LDL and HDL, specific radioactivities ranged from 3 to 46d.p.m./ng of cholesteryl ester (1200–17700d.p.m.·nmol^{-1}). When the total lipids in VLDL, LDL and HDL were extracted by the method of Folch *et al.* (1957), separated by t.l.c., eluted with diethyl ether and counted for radioactivity, 94.1±0.6% of the radioactivity was recovered with the cholesteryl esters and 1.8±0.2% with the non-esterified cholesterol.

The physical association of the radioactive ester with the labelled lipoproteins was examined by electrophoresis (one experiment), ultracentrifugation (eleven experiments) and precipitation (three experiments). [^{14}C]Cholesteryl oleate-labelled preparations of LDL and HDL and normal human serum were electrophoresed in parallel on separate strips of cellulose acetate. The serum strip was then stained, and the sections of the other strips corresponding to the α-band (HDL), the β-band (LDL) and the unstained regions were placed in separate vials of scintillation solution and counted (counting error < 3%) for radioactivity. With labelled HDL, 77% of radioactivity was recovered from the α-band; with labelled LDL, 74% was recovered from the β-band.

When preparations of ^{14}C- or ^3H-labelled VLDL, LDL and HDL were separately recentrifuged at 105000*g* in an angle-head rotor for 24h at *d* 1.006, 1.063 or 1.21, 92–99% of the radioactivity (mean 96.7%, $n = 11$) was recovered with the sedimented or floating lipoprotein.

When heparin and $MnCl_2$ were added to [^{14}C]-cholesteryl oleate-labelled LDL, more than 97% of the radioactivity was recovered with the precipitated lipoprotein. When unlabelled LDL was mixed with labelled HDL, and heparin and $MnCl_2$ added immediately, 6.0% of the ^{14}C was recovered in the precipitated LDL.

To determine if the radioactive ester could be transferred from HDL to VLDL *in vitro* via the lipid-transfer protein (Zilversmit *et al.*, 1975), labelled HDL was incubated with unlabelled VLDL in human LPDS for 6h, and samples were taken at timed intervals for precipitation of VLDL with heparin and $MnCl_2$, and measurement of the

specific radioactivities of the cholesteryl esters of the two lipoproteins. Rapid transfer of radioactivity from HDL to VLDL was observed, the specific radioactivity in VLDL reaching that of HDL in about 3h (Table 1). When the same experiment was performed in NaCl (0.15mmol·l^{-1}) instead of LPDS, after 6h of incubation the specific radioactivity in VLDL was only 15% of that in HDL.

The transfer of exogenous [1,2-^{3}H]cholesteryl linoleate from a labelled HDL preparation to VLDL *in vivo* was compared with the simultaneous transfer in the same direction of endogenous [4-^{14}C]cholesteryl esters formed by the esterification of [4-^{14}C]cholesterol in HDL *in vivo*. A healthy male subject who had fasted for 14h overnight was given a continuous intravenous infusion (after a primary bolus injection) of two sterile autologous HDL preparations: one that had been labelled *in vitro* with [1,2-^{3}H]cholesteryl linoleate and another that had been labelled *in vitro* with [4-^{14}C]-cholesterol at 4°C (Stokke & Norum, 1971). At 30min intervals, blood was collected into Na_2EDTA (1mg/ml) and the LCAT inhibitor Nbs_2 (1.4mmol·l^{-1}). VLDL and HDL were isolated by preparative ultracentrifugation and the ^{3}H and ^{14}C specific radioactivities of their cholesteryl esters determined (Table 2). Between 30 and 120min after the start of the infusion, the ^{3}H and ^{14}C specific radioactivities of HDL cholesteryl esters were essentially constant, indicating the presence of steady-state conditions. Throughout this time the ^{3}H and ^{14}C specific radioactivities of VLDL cholesteryl ester increased exponentially towards the corresponding specific radioactivity in HDL. The exponential rate constants for the two isotopes were essentially identical (^{3}H, 0.0053min^{-1}; ^{14}C, 0.0050min^{-1}).

The ability of cholesteryl ester-labelled LDL to compete with ^{125}I-LDL for uptake by cultured human skin fibroblasts via apolipoprotein B,E receptors was compared with that of native LDL in two experiments. The procedures used were as described by Goldstein & Brown (1974). Derepressed near-confluent monolayers were incubated at 37°C in medium containing 5% (v/v) LPDS and ^{125}I-LDL (10μg of protein·ml^{-1}). To some dishes [^{3}H]cholesteryl linoleate-labelled LDL or unlabelled LDL was added at concentrations of 5–40μg of protein·ml^{-1}. After 6h the medium was assayed for non-iodide trichloroacetic acid-soluble radioactivity (i.e. degraded LDL), and the cells were washed (at 0–4°C) and assayed for heparin-

Table 1. *Transfer of [^{14}C]cholesteryl oleate with time from labelled HDL to unlabelled VLDL in vitro in the presence of LPDS*

Labelled human HDL and unlabelled VLDL were incubated together in LPDS (d>1.215) at 37°C. Samples were taken at timed intervals, and VLDL was precipitated with heparin and $MnCl_2$ (Warnick & Albers, 1979). The lipids were then extracted from the VLDL and from the supernatant (containing the HDL) as described by Folch *et al.* (1957), and the cholesteryl esters isolated by t.l.c. (Stokke & Norum, 1971).

	Specific radioactivity (dpm·ng^{-1})		
Time (min)	HDL cholesteryl ester	VLDL cholesteryl ester	VLDL / HDL
2	8.3	0.59	0.07
30	6.2	0.74	0.12
60	5.9	0.92	0.16
90	5.4	2.90	0.54
150	6.3	6.00	0.95
240	5.6	4.80	0.86
360	4.8	5.60	1.17

Table 2. *Specific radioactivities of VLDL and HDL cholesteryl esters in a healthy male subject during the simultaneous continuous intravenous infusion of two autologous HDL preparations, one labelled in vitro with [1,2-^{3}H]cholesteryl linoleate and another labelled in vitro with [4-^{14}C]cholesterol*

	Radioactivity in cholesteryl ester					
	(^{14}C d.p.m./mg)†			(^{3}H d.p.m./mg)		
Time (min)*	HDL	VLDL	Ratio‡	HDL	VLDL	Ratio‡
30	1891	1269	0.67	72986	44004	0.59
60	2150	1425	0.75	79487	57861	0.77
90	1761	1606	0.85	70914	67340	0.90
120	1787	2020	1.06	76638	75136	1.00

* Time after the start of the infusion.
† Produced *in vivo* by esterification of the infused [4-^{14}C]cholesterol.
‡ Ratio of VLDL specific radioactivity to mean HDL specific radioactivity.

Table 3. *Effects of unlabelled LDL (U-LDL) and [^{3}H]cholesteryl linoleate-labelled LDL (CE-LDL) on the binding, internalization and degradation of ^{125}I-LDL by cultured human fibroblasts*

The ^{125}I-LDL concentration in all dishes was 10 μg of protein·ml^{-1}; the specific radioactivity was 274 d.p.m./ng of protein. See the text for experimental details. Results are the means ± S.D. of triplicate determinations.

LDL added	Final concn. (μg of protein·ml^{-1})	^{125}I-LDL protein (ng/mg of cell protein)		
		Binding	Internalization	Degradation
None	—	39 ± 2	131 ± 5	362 ± 32
U-LDL	10	17 ± 3	71 ± 8	272 ± 6
CE-LDL	10	10*	75 ± 6	278 ± 10
U-LDL	20	12 ± 3	37 ± 1	189 ± 13
CE-LDL	20	10 ± 2	38 ± 12	159 ± 20

* Single measurement.

releasable (surface-bound) and heparin-resistant (internalized) radioactivity. Cholesteryl ester-labelled and unlabelled LDL were found to reduce the uptake and hydrolysis of ^{125}I-LDL to similar extents. Representative results appear in Table 3.

In summary, our observations demonstrate that the method can be used to label human VLDL, LDL or HDL to high specific radioactivity with radioactive cholesteryl oleate or cholesteryl linoleate; that when labelled HDL is infused intravenously into man, the exogenous radioactive cholesteryl ester behaves indistinguishably from endogenous cholesteryl esters formed *in vivo* by the LCAT reaction; and that the procedure does not alter the affinity of LDL for the apolipoprotein B,E receptor of cultured fibroblasts.

The procedure is 10–20-fold more efficient than that reported by Thomas & Rudel (1983), which is also based on the principle of incorporating the labelled ester into lipoproteins via the lipid-transfer protein. These workers incubated whole plasma (after addition of NaN_3 and Nbs_2) at 37°C in a glass tube that had been pre-coated with radioactive cholesteryl ester, and then isolated the required labelled lipoprotein by ultracentrifugation. After an incubation of 30 min, only 2.8, 2.6 and 5.5% of the added radioactivity was recovered in VLDL, LDL and HDL respectively; after 5 h the corresponding values were 5.4, 9.7 and 12.1%. Incubations beyond 5 h altered the compositions of LDL and HDL.

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