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RAPID METHOD FOR MEASURING PLASMA LOW-DENSITY LIPOPROTEIN TURNOVER USING HIGH-PERFORMANCE GEL EXCLUSION CHROMATOGRAPHY*

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SUMMARY

High-performance gel exclusion chromatography using flow-through radioactivity monitoring was employed for rapid measurement of low-density lipoprotein (LDL) turnover Iodinated LDL was injected into five fasting rabbits Serial blood samples were obtained and small aliquots (0 1—0 2 ml) were injected into the chromatographic system using 0 2 *M* Tris—acetate buffer (pH 7 0) Radioactivity in lipoproteins was measured by an on-line gamma counter (Flo-One) attached to the high-performance liquid chromatograph and in a regular gamma counter after collecting the fractions Sequential ultracentrifugation was also used to separate lipoproteins, and the radioactivity in each fraction was measured The Flo-One method was faster, however, the efficiency of Flo-One varied with the amount of radioactivity and therefore it was necessary to inject the same amount of radioactivity to get comparable values

INTRODUCTION

Low-density lipoproteins (LDL) are the major cholesterol-transporting lipoproteins of human plasma [1] Levels of plasma LDL and its major apoprotein, apo-B, which are positively associated with atherosclerosis and coronary artery disease [2—7], vary considerably among individuals [8] Plasma LDL levels in an individual are affected by a number of genetic and environmental factors that modulate synthetic and/or catabolic rates of apo-B

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[9, 10]. These metabolic rates for LDL apo-B are measured by determining the turnover of isotopically labeled LDL. The separation of lipoproteins for these studies is often achieved by time-consuming techniques such as sequential ultracentrifugation or column chromatography. Since more rapid separations with improved resolution of various species of lipoproteins have been achieved by high-performance liquid chromatography (HPLC) employing molecular exclusion columns [11—14], the present study was conducted to establish suitable conditions for using this technique for the measurement of plasma [^{125}I] LDL turnover The feasibility of further time saving through the use of an on-line gamma counter was explored

EXPERIMENTAL

Experimental animals

Female New Zealand white rabbits (3—4 kg) were used for these studies Animals were maintained in individual cages, where they had access to feed (rabbit chow, Ralston Purina, St Louis, MO, U.S A) and water ad libitum.

Isolation and labeling of LDL

To isolate LDL for labeling, fasted (12—14 h) rabbits were bled from the ear artery and the blood was collected into tubes containing EDTA (1 mg/ml) and pooled The plasma was separated by low-speed centrifugation. Solid potassium bromide was added to the plasma to adjust the density (d) at 1 019 g/ml and overlaid with d 1 019 g/ml potassium bromide solution The plasma was then ultracentrifuged in a Beckman ultracentrifuge (Beckman, Palo Alto, CA, U S A.) using a 50 Ti rotor at a speed of 42 000 rpm (100 000 g) at 7°C for 20 h The tubes were sliced and the top 4-ml layer containing very-low-density and intermediate-density (IDL) lipoproteins were aspirated The IDL bottom was washed by overlayering with d 1.019 g/ml potassium bromide solution and centrifuging under similar conditions The tubes were sliced and the top 4 ml discarded. The IDL bottoms were then reset at d 1 063 g/ml by adding solid potassium bromide overlaid with d 1 063 g/ml potassium bromide solution, and ultracentrifuged in a 50 Ti rotor at a speed of 42 000 rpm (100 000 g) for 22 h At the end of the ultracentrifugation, the top 4-ml layer containing LDL was sliced. The LDL thus obtained was washed by overlaying with d 1 063 g/ml potassium bromide solution in a 50.3 Ti rotor and ultracentrifuging at a speed of 42 000 rpm (100 000 g) for 22 h.

The LDL was then dialyzed against normal saline containing 0 001 M EDTA in order to remove the salt. The protein content of the LDL was determined by the method of Lowry et al [15] with a slight modification The turbidity was removed by extracting the fraction with chloroform before reading the absorbance A known amount of LDL protein (3—4 mg) was labeled by the iodine monochloride procedure of McFarlane [16] as modified for lipoproteins by Bilheimer et al. [17]. LDL was dialyzed against 0.5 M glycine—0 5 M sodium chloride buffer (pH 10 00) LDL (2—3 ml) containing 3- 4 mg protein was labeled with ^{125}I (ICN Radiochemicals, Irvine, CA, U S A) while kept cold with ice. Free iodide was removed by passing the labeled LDL through a 20-ml column of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ, U S A)

and afterwards dialyzing against normal saline containing 0 001 *M* EDTA with four to five changes of dialysis solution.

Characterization of labeled LDL

A small fraction (10 μl) of labeled LDL (added to unlabeled LDL) was used for determining the trichloroacetic acid (TCA) precipitable radioactivity using 20% TCA. Free iodide in the TCA-soluble supernatant was determined by converting it into molecular iodine by adding 40% potassium iodide and 30% hydrogen peroxide and extracting with chloroform. The lipid-incorporated radioactivity was determined by extracting the labeled lipoprotein with 20 vols of chloroform—methanol (2.1), washing the extract with 0 7% potassium chloride in 0.02 *M* hydrochloric acid and counting the extract in a gamma counter after evaporating to dryness.

Radioactivity in the major protein of LDL (apo-B) was determined by diluting a small amount of labeled LDL with non-labeled LDL and treating it with tetramethylurea (TMU) (1 1). This was then incubated at 37°C and passed through a glass wool filter fitted in a pasteur pipet [18]. The precipitate was washed with saline and the TMU-soluble fraction was counted. The lipid radioactivity in the TMU precipitate was determined by extracting it with chloroform—methanol (2·1). The radioactivity in apo-B was calculated by subtracting the lipid and TMU-soluble radioactivity from the total [18]

Rabbit LDL apo-B turnover procedure

Rabbits were fasted (12—14 h) prior to the beginning of the turnover study. Four of the rabbits were injected with $5 \cdot 10^6$ cpm [^{125}I] LDL The fifth rabbit was injected with $30 \cdot 10^6$ cpm [^{125}I] LDL Blood samples (6 ml) were drawn from the ear at 5 and 30 min and 1, 2, 4, 6, 8, 24 and 30 h An additional blood sample was drawn at 12 h in the fifth rabbit. The blood was collected in tubes containing EDTA (1 mg/ml) and plasma was separated by low-speed centrifugation.

Plasma samples (2.0 ml) from each time point were ultracentrifuged using quick-seal tubes in a 50 3 Ti rotor using an L5-50 Beckman ultracentrifuge. The plasma was adjusted to *d* 1.019 g/ml by adding solid potassium bromide and centrifuged under similar conditions for 22 h. LDL was obtained by pipetting the 2.0-ml top layer after slicing the tube.

The radioactivity in the whole plasma and lipoprotein fractions (200 and 100 μl, respectively) at each time point was determined using a gamma counter. To avoid quenching owing to high salt concentration, the lipoproteins of *d* 1.063 g/ml and LDL bottoms were diluted 1 10 with deionized water prior to counting

After dialysis, TMU-soluble and -insoluble radioactivity in the lipoprotein fractions was determined by the following procedure. A lipoprotein sample (100 μl) was diluted with a small amount (10 μl) of concentrated non-labelled LDL and counted in a gamma counter An equal volume of nitrogen-purged glass-distilled TMU was incubated with the sample at 37°C for 30 min The sample was then transferred to a centrifugal filter apparatus (Nylon-66 membrane filter, 0 45 μm, Rainin Instruments, Emeryville, CA, U.S A.) The incubation tube was washed with saline and was added to the filter apparatus

The samples were then centrifuged at 3000 rpm (2000 *g*) for 10 min The filtrate was counted for TMU-soluble radioactivity.

Lipid-incorporated radioactivity was determined by extracting the lipids contained in the TMU-insoluble material remaining on the filter with 0 5 ml chloroform–methanol (2:1) The samples were then respun and the filtrate counted after evaporating to dryness.

Processing of turnover samples by HPLC

A Waters (Waters Assoc., Milford, MA, U S.A) HPLC system was employed for this study. For single injections a Rheodyne (Rheodyne, Cotati, CA, U S A) Model 7161 injector with a 200-μl fixed loop was used For varying the size of injection a WISP automated injection system (Waters Assoc) maintained at 10°C within a chromatographic chamber (Powers, Hatboro, PA, U S A) was used. This permitted the injection of samples over a 24-h period.

A series of Toya-Soda TSK PW columns (Kratos, Westwood, NJ, U.S A) were used to separate the lipoproteins first a GPWPH pre-column (100 × 7 6 mm) followed by a 4000 PW (600 × 7 6 mm) and finally a 3000 PW (600 × 7 6 mm) column. These columns were preceded by an in-line filter (2 μm). The mobile phase was 0.2 *M* Tris—acetate, pH 7 0, containing 0 05% sodium azide. High-purity water was obtained from a Millipore Milli-Q System (Millipore, Bedford, MA, U.S A.) Columns were stored in 10% methanol in HPLC water after use. The buffer and solvents were filtered through 0 45-μm Millipore filters (HA or Durapore) and degassed to avoid bubble formation during use A Model 6000A pump (Waters Assoc.) was used as the solvent delivery system. A Model 721 system controller reduced the time required for each sample by flow-programming the eluent delivered from 0 5 to 1 0 ml in 80 min. A Model 440 UV detector monitored the absorbance of lipoproteins at 280 nm (pen 1 of a Model 730 data module). An on-line radioactivity detector Flo-One (Radiomatic Instruments, Tampa, FL, U S A) was used to monitor the ^{125}I signal (pen 2 of the data module) The effluent from the Flo-One was attached to a Foxy automatic fraction collector (ISCO, Lincoln, NE, U.S A) maintained at 10° C in the chromatography chamber Fractions of 2 min were collected and counted in a Model 1185 gamma counter (Nuclear-Chicago, Des Plaines, IL, U S A.)

TMU-soluble and lipid radioactivity was also determined on the HPLC-separated lipoprotein fractions as follows The LDL peak from the HPLC system was pooled and dialyzed against deionized water After dialysis, the samples were lyophilized. After adding a small amount of water to the freeze-dried samples, TMU-soluble and lipid-incorporated radioactivity was determined using the centrifugal filters as described previously

Data analysis

The decay curves for LDL apo-B were plotted by using the radioactivity per ml of plasma divided by the total injected radioactivity The residence times and fractional catabolic rates were calculated by using the area under each radioactivity curve The area under the radioactivity curve was calculated by using a program for area calculations and an HP-3000 computer

The values in the tables have been expressed as mean ± standard deviation and were compared using the paired *t*-test

RESULTS

Characterization of radioactivity in LDL

Most of the radioactivity in LDL used for turnover studies was precipitable with TCA (97 6% of total radioactivity). The radioactivity associated with lipids and TMU-soluble proteins was low, 6 and 9%, respectively, and therefore a major proportion of radioactivity was associated with apo-B

Recovery of LDL by HPLC

Using a combination of TSK 4000 PW and TSK 3000 PW columns, almost all (95%) of the radioactivity was recovered in lipoprotein fractions Most of this radioactivity was present in the LDL fraction throughout the experiment At the 5-min time point 90 81 ± 3 95% (mean ± S.D , n = 4) of total radioactivity was present in the LDL fraction. The sequential ultracentrifugation procedure had a lower recovery when compared with HPLC However, as in case of HPLC, most of the radioactivity was present in LDL throughout the experiment At the 5-min time point 84 09 ± 0 23% of total radioactivity was recovered in LDL Most of the radioactivity recovered in LDL (96.1 ± 1.11%) by both methods was present in apo-B It appears, therefore, that smaller peptides are transferred rapidly to other lipoproteins However, apo-B stays quantitatively in the LDL particle and no apo-B was detected in any other fraction.

LDL apo-B turnover

These studies were conducted in five animals, which were maintained on the chow diet and were injected with iodinated LDL isolated from pooled plasma from the same animals The LDL isolated by ultracentrifugation and HPLC

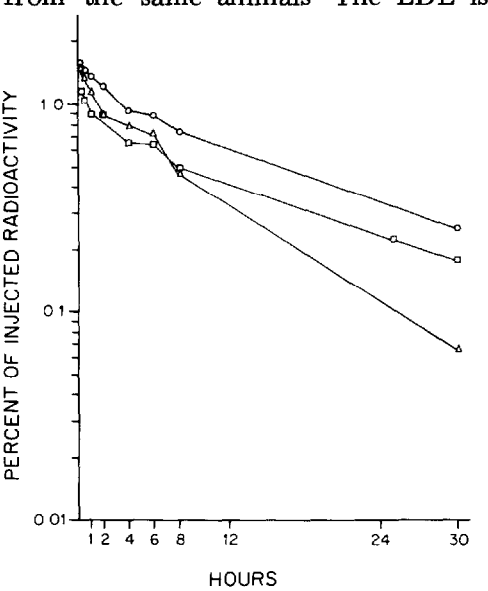

Fig 1 Decay of radioactivity in plasma LDL of one normal rabbit following injection of [^{125}I] LDL ($5 \cdot 10^6$ cpm) (□) Sequential ultracentrifugation method, (○) HPLC fractions counted in a gamma counter, (△) HPLC effluent counted by on-line detector (Flo-One) HPLC injections were 200 μl per sample Sequential ultracentrifuge samples were 2 0 ml

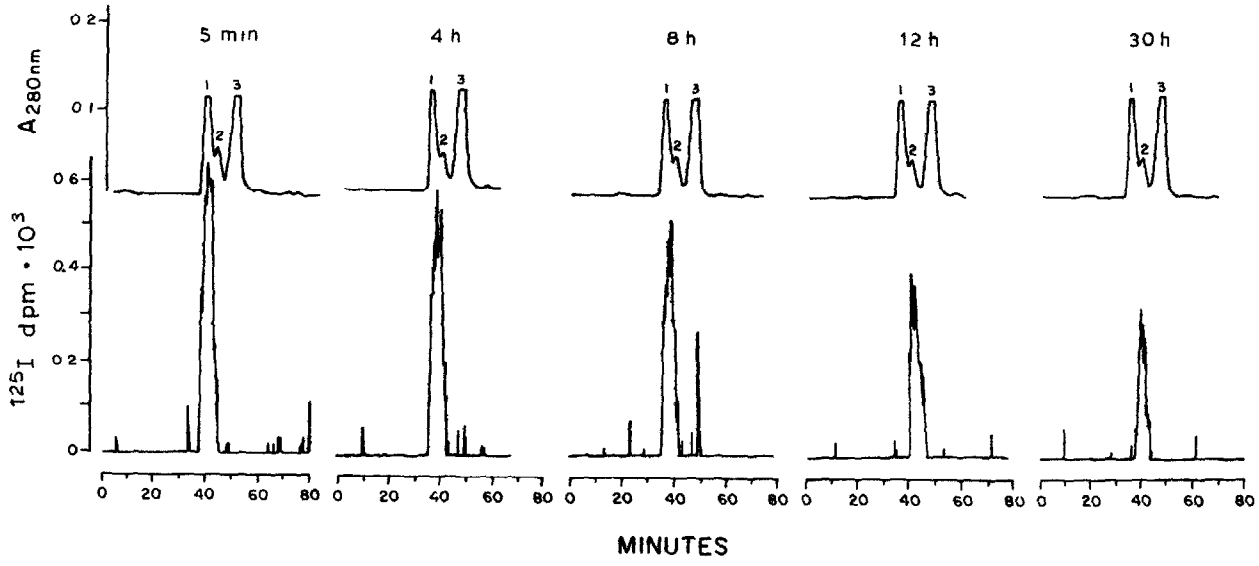

Fig 2 HPLC separation of ^{125}I-labeled LDL on gel molecular exclusion columns (4000 PW, 600 × 7 6 mm, 3000 PW, 600 × 7 6 mm) using 0 2 *M* Tris-acetate buffer, pH 7 0, with a flow-rate programmed from 0 5 to 1 0 ml/min in 80 min The upper pen is absorption at 280 nm, 0 5 a u f s Peaks 1 = LDL, 2 = high-density lipoprotein, 3 = albumin The lower pen is the on-line gamma counting, 10^3 cpm full scale, window setting 0-1000

was counted by a gamma counter At the same time radioactivity in LDL was measured by an on-line gamma counter. The decay of radioactivity in LDL apo-B calculated by these methods was plotted and fractional catabolic rates were calculated. The decay of radioactivity in a representative animal calculated by all three methods is given in Fig. 1

The radioactivity in LDL apo-B calculated by all three methods decayed biphasically in all animals. The decay of radioactivity calculated by sequential ultracentrifugation and HPLC (when fractions were counted by a gamma counter) were parallel and gave similar fractional catabolic rates (Table I). Residence times for LDL apo-B obtained by sequential ultracentrifugation and HPLC techniques were 10.38 ± 1.11 and 10 61 ± 1.56 h, respectively The radioactivity values calculated by the on-line gamma counter (Flo-One) were parallel to those for sequential ultracentrifugation up to 6—8 h. Later values were much lower and therefore resulted in lower fractional catabolic rates (Table I). The efficiency of counting by Flo-One varied with the amount of radioactivity in the samples. At the earlier time points, the efficiency of counting by Flo-One was 41% but decreased to 7% for the last sample At later time points the efficiency of counting was also variable and thus the later values were probably underestimated. It is possible therefore, that the fractional catabolic rates were lower when compared with sequential ultracentrifugation or HPLC without an on-line gamma counter The radioactivity detected by Flo-One was also mainly present in LDL (Fig 2).

Effect of varying the plasma volume applied to HPLC with a constant amount of radioactivity on the recovery of radioactivity by Flo-One

Since the efficiency of Flo-One was much more variable at later time points because of lower radioactivity in samples, an additional experiment was conducted using the plasma samples from the fifth animal In this experiment the amount of plasma sample applied to HPLC was varied to keep the amount of radioactivity constant. The radioactivity in LDL apo-B per ml plasma was calculated and plotted as percentage of the injected dose Fig 3 shows the recovery of radioactivity at five time points Most of the radioactivity was present in LDL and there was no variation in the recovery of total radioactivity

TABLE I

FRACTIONAL CATABOLIC RATES AND RESIDENCE TIMES OF LDL APO B

Normal rabbits were injected with variable amounts of [^{131}I]LDL (rabbit Nos 1—4) or with a fixed amount of [^{125}I]LDL (rabbit No 5) The fractional catabolic rates (FCR) and residence times (RT) were determined by sequential ultracentrifugation (A), HPLC with regular gamma counting (B) and HPLC with on line gamma counting (C)

Rabbit No	RT (h)			FCR (h $^{-1}$)		
	A	B	C	A	B	C
1	10 85	11 50	9 00	0 0922	0 0870	0 110
2	11 05	12 14	8 16	0 0905	0 0824	0 1230
3	10 88	10 16	6 87	0 0919	0 0984	0 1456
4	8 72	8 62	4 79	0 1147	0 1160	0 2090
Mean ± S D	10 38 ± 1 11	10 61 ± 1 56	7 21 ± 1 83*	0 0974 ± 0 0116	0 0985 ± 0 0137	0 1469 ± 0 0439**
5		15 71	16 39		0 0637	0 0600

*Mean RT value is significantly different ($P > 0.05$) from the values obtained by ultracentrifugation and HPLC with regular gamma counting

**Mean FCR value is not significantly different from the values obtained by ultracentrifugation and HPLC with regular gamma counting

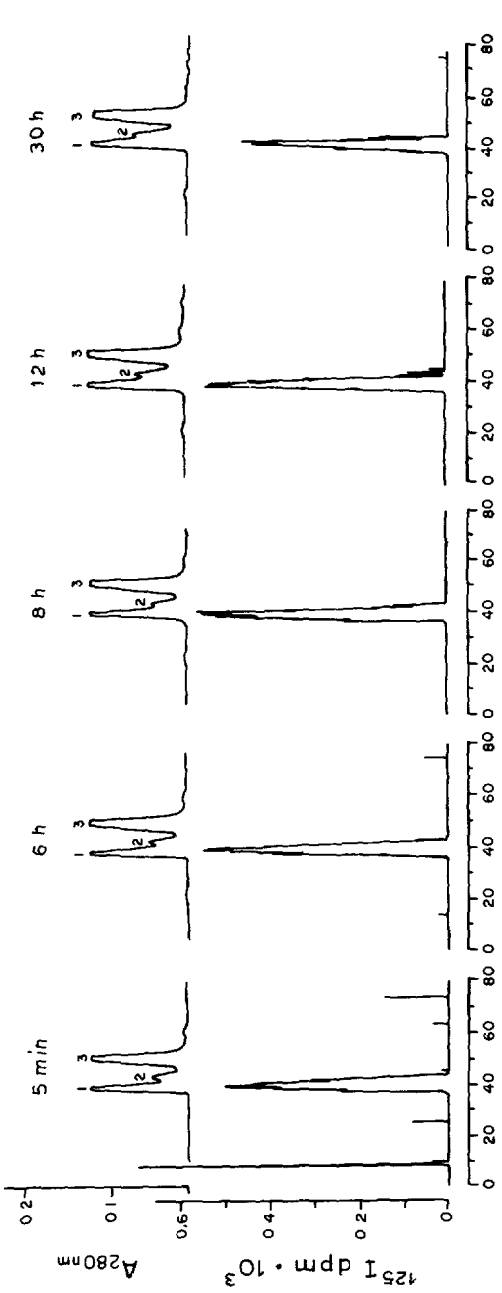

Fig 3 HPLC separation of ^{125}I-labeled LDL using various injection sizes (for uniform amount of radioactivity) under the same conditions as in Fig 2

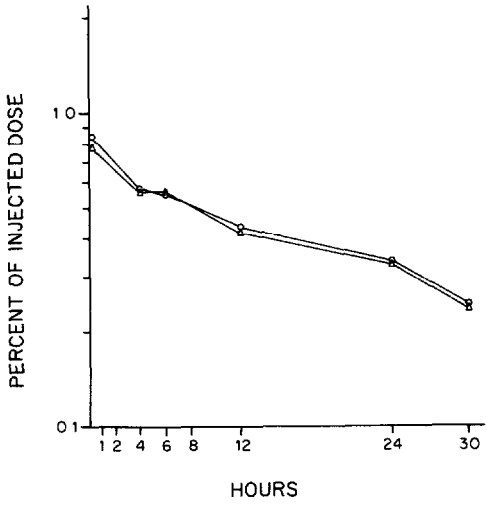

Fig 4 Decay of radioactivity in plasma LDL of a normal rabbit following injection of ^{125}I-labeled LDL (30 • 10^6 dpm) (○) HPLC fractions counted in a gamma counter, (△) HPLC with on-line detection (Flo-One) with variable-size injection and uniform amount of radioactivity

The radioactivity in LDL apo-B calculated by using Flo-One was compared with that counted by a gamma counter by pooling the fractions. The decay of radioactivity was similar, as shown in Fig 4 The fractional catabolic rates calculated by these two methods were similar (Table I). Thus, using a constant amount of radioactivity for separating lipoproteins by HPLC using a Flo-One gamma counter gave the best results and eliminated the need for pooling the radioactivity fraction and counting before determining apo-B radioactivity This method was very rapid compared to sequential ultracentrifugation

DISCUSSION

These studies demonstrate that HPLC with or without an on-line gamma counting can be used for the determination of LDL apo-B turnover without using the ultracentrifugation for separating plasma lipoproteins. This method is much more rapid than the sequential ultracentrifugation method [19] used widely for the measurement of LDL apo-B turnover [20—22]. When an automatic sample injector with automatic fraction collector and on-line gamma counter were used, all plasma samples for one animal were processed in 24 h In these studies we used the Flo-One as on-line gamma counter and the best results were obtained by keeping the injected radioactivity constant The recovery of radioactivity by HPLC was considerably higher than that obtained by sequential ultracentrifugation. At the same time the plasma volume required per time point was much smaller than that required for sequential ultracentrifugation Thus, this technique can be used effectively for smaller animals where a large volume of blood cannot be drawn without seriously endangering the life of the animal

Fractional catabolic rates for LDL apo-B measured by the sequential ultracentrifugation and HPLC method, where the fractions were counted by a

gamma counter, were very similar. These observations suggest that the two methods are comparable even though the recovery of radioactivity differed considerably by these methods. Since in the ultracentrifugation method less radioactivity was recovered without affecting the fractional catabolic rate, it appears that these losses were uniform probably during slicing of the lipoproteins

The LDL apo-B turnover values obtained by an on-line gamma counter were lower than those obtained either by the sequential ultracentrifugation or by the HPLC method without using an on-line gamma counter The radioactivity counts obtained at later time points were also lower than those obtained by the two other methods. Thus it appeared that the lower counts obtained at later points decreased the fractional catabolic rate and were related to the efficiency of counting The efficiency of counting by Flo-One varied considerably At earlier time points, when the plasma samples had high amounts of radioactivity, the efficiency was 41% This efficiency was reduced to 7% at the last time point The counts obtained at this point were much more variable Thus, the differences in fractional catabolic rates by Flo-One and direct counting were due to the lower efficiency of Flo-One at very low counts during later time points.

Since the efficiency of an on-line gamma counter varied with varying levels or radioactivity in the samples injected, another experiment was conducted. In this experiment the efficiency of Flo-One was kept constant by injecting a similar amount of radioactivity by varying the plasma volume. The fractional catabolic rates calculated by an on-line gamma counter (Flo-One) and a regular gamma counter were very similar. Thus, the use of a constant amount of radioactivity and an on-line gamma counter (Flo-One) eliminated the counting of lipoprotein fractions by a regular gamma counter Fractional catabolic rates for apo-B could be calculated after correcting these values for apo-B radioactivity. Thus, metabolic rates for LDL and LDL apo-B can be determined by HPLC with a flow-through gamma counter in considerably less time when compared with the ultracentrifugation procedure.

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