

The quantitative assay of 3-hydroxy-3-methylglutaryl coenzyme A reductase: comparison of a thin-layer chromatographic assay with a rapid chloroform extraction assay

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Summary Two assays for the quantitative measurement of HMG-CoA reductase, the major regulatory enzyme in hepatic cholesterol biosynthesis, are described. The first of these procedures employs thin-layer chromatography (TLC) of the reaction product on plastic-backed silica gel G strips impregnated with ammonium carbonate. The TLC strip is then cut into 14 segments and each segment is assayed for radioactivity. Extraction efficiency and exact chromatographic mobility are monitored by the use of authentic [4-³H]mevalonolactone as an internal reference. A wide and complete separation is achieved between HMG and mevalonolactone. Also the radiochemical purity of biosynthetic [3-¹⁴C]mevalonolactone can be assessed by measurement of the ³H/¹⁴C ratio across the mevalonolactone peak.

While the TLC assay is accurate and sensitive, it is laborious. Therefore a second assay procedure was developed using chloroform extraction of an incubation mixture saturated with solid equal molar KH₂PO₄-K₂HPO₄ at pH 6.8. Extraction efficiency was monitored by the addition of authentic [4-³H]mevalonolactone as an internal reference. The chloroform assay procedure was compared with the TLC procedure over a wide range of enzyme activities, both for rat liver microsomal HMG-CoA reductase and for solubilized enzyme. Excellent correspondence ($r = 0.999$) between the two assays was observed. TLC performed on the chloroform extract demonstrated that biosynthetic [3-¹⁴C]mevalonolactone was the only ¹⁴C-labeled compound present in the extract. The chloroform extraction assay is rapid; 20–30 samples can be processed in 2–3 hr. This procedure should facilitate studies concerning the nature and regulation of HMG-CoA reductase.

Supplementary key words HMG-CoA reductase · cholesterol biosynthesis

3-Hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) has been well established as the major regulatory enzyme in hepatic cholesterol biosynthesis (1–11). This enzyme catalyzes the reduction of HMG-CoA to mevalonic acid and is found in liver microsomal particles (4). Most assay methods for this

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLC, thin-layer chromatography; DTE, dithioerythritol; HMG, 3-hydroxy-3-methylglutaric acid.

enzyme involve the measurement of [¹⁴C]mevalonate formed during incubation of [¹⁴C]HMG-CoA with hepatic microsomes in the presence of NADPH or an NADPH-generating system. The product is extracted along with unreacted HMG-CoA and/or HMG and it is subsequently purified by column (12, 13), gas-liquid (14), or thin-layer chromatography (15, 16). At the present time the dual isotope, thin-layer chromatographic assay developed by Goldfarb and Pitot (16) is widely used. This method circumvents the need for quantitative extraction of [¹⁴C]mevalonate by using [³H]mevalonate as an internal reference to determine extraction efficiency.

The TLC assay described in the present article is similar to that independently developed by Goldfarb and Pitot (16); however, the present assay allows greater accuracy and sensitivity in the measurement of the amount of mevalonate synthesized, since it provides a means for the determination of the radiochemical purity of the product isolated.

Although the TLC assay is reliable, accurate, and sensitive, it is time consuming. Therefore, we developed a new and much more rapid assay which is essentially as accurate as the TLC assay. The assay is based on the fact that, in a slightly acidic (pH 6.7–6.9), saturated aqueous solution of an alkali metal salt, mevalonic acid is mainly present as mevalonolactone, whereas HMG is mainly present as the metal salt. The relatively nonpolar mevalonolactone can be extracted into an organic phase, while the polar HMG salt and very polar HMG-CoA remain in the aqueous phase. A preliminary report of these methods has appeared previously (17, 18).

Materials and Methods

Chemicals. All solvents and chemicals used were analytical reagent grade unless otherwise specified. NADPH, unlabeled HMG-CoA, and unlabeled HMG were purchased from Calbiochem (San Diego, CA), P-L Biochemicals Milwaukee, WI, and Schwarz/Mann Orangeburg, NJ, respectively. EDTA, unlabeled mevalonolactone, and DTE were obtained from Sigma Chemicals, St. Louis, MO. Plastic-backed TLC sheets, coated with Silica Gel G, were obtained from Eastman Kodak, Rochester, NY. Phase-separating filter papers (Whatman 1 PS) were from Arthur H. Thomas, Philadelphia, PA. [¹⁴C]Toluene and [³H]-toluene standards, [3-¹⁴C]HMG (7.37 μ Ci/ μ mol), [3-¹⁴C]HMG-CoA (8.96 μ Ci/ μ mol) and Aquasol were purchased from New England Nuclear, Boston, MA. [4-³H]Mevalonolactone was from Amersham/Searle, Arlington Heights, IL.

Measurement of radioactivity. In both of the assay procedures used, the ¹⁴C and ³H activities associated

with mevalonolactone were determined simultaneously using a three-channel liquid scintillation spectrometer (Packard 3375, Packard Instruments, Downers Grove, Il). The first two channels (red and green) were adjusted to give the following efficiencies: red channel, ^3H , 20.1% and ^{14}C , 4.9%; green channel, ^3H , 0.04% and ^{14}C , 54.7%. The third channel (blue) was adjusted to read the higher energy portion of the ^{14}C spectrum, so that quenching could be monitored by the ratio of the ^{14}C channels. However, absolute counting efficiencies were determined by the addition of [^{14}C]toluene and [^3H]toluene standards in all cases where a comparison was made between the TLC assay and the chloroform extraction assay.

In routine assay of the enzyme by the chloroform extraction method, it was found that the efficiencies remained constant from sample to sample. Therefore it was not necessary to standardize each sample routinely.

Preparation of ammonium carbonate-impregnated TLC strips. A plastic-backed Silica Gel G coated TLC sheet (20 × 20 cm) was activated by heating at 100°C for 30 min. The activated sheet was then developed in a sandwich type TLC apparatus using a saturated solution of ammonium carbonate in acetone. After drying in air, the sheet was cut into 2 × 9.5 cm strips. These strips were stored in a closed jar over solid ammonium carbonate until used.

Preparation of HMG-CoA reductase. Sprague-Dawley male rats (150–250 g) were maintained ad libitum on a diet of Wayne Lab-Blox and tap water. These animals were subjected to a 3 AM to 3 PM dark cycle for 10 days and they were killed at 9 AM local time, the peak of HMG-CoA reductase activity. Liver microsomes were prepared as previously described (18–20) in a buffer containing 0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate, 0.03 M EDTA, and 10 mM DTE at pH 7.2 (buffer A). HMG-CoA reductase was solubilized from the microsomal membranes as previously described (18–20).

Incubations. Microsomal suspensions were diluted to give the protein concentrations shown in Table 1. NADPH was added to give a final concentration of 4 mM. The samples were then preincubated at 37°C for 20 min. DL-[$^3\text{-}^{14}\text{C}$]HMG-CoA (66,500 dpm) was added to each sample to give a final concentration of 300 μM , and incubation was conducted for 20 min at 37°C. The total incubation volume was 500 μl .

When the chloroform extraction assay was used, a blank consisting of buffer A, NADPH, and DL-[$^3\text{-}^{14}\text{C}$]HMG-CoA (66,500 dpm) was incubated in the same manner as described above. This blank was then processed by the chloroform extraction assay as de-

scribed below. The blank value obtained in the ^{14}C channel was then subtracted from the value obtained in the ^{14}C channel for samples containing enzyme. Typical blank corrections were in the range of 30–45 cpm (55–82 dpm).

Chloroform extraction assay. The enzymatic reaction was stopped by the addition of 1 ml of 2 N HCl, followed by the addition of [^3H]mevalonolactone (83,550 dpm) in 20 μl of xylene; the [^3H]mevalonolactone served as an internal reference. The samples were allowed to stand for 30 min at room temperature to insure lactonization. Each sample was then transferred to a glass counting vial, and the small incubation test tube was rinsed with 2 ml of buffer A. Unlabeled mevalonolactone¹ (10 mg dissolved in 0.2 ml of ethanol–toluene 1:7) was added to each sample, followed by the addition of 2.3 g of an equal molar mixture of solid K_2HPO_4 – KH_2PO_4 . The samples were then incubated with shaking (2 oscillations per sec) in a Dubnoff incubator at 37°C for 30 min. Chloroform (6 ml) was added, followed by vigorous shaking of the mixture by hand for a few seconds, gently releasing pressure from the vial. Each sample was shaken vigorously again for 10 sec; the pressure was then released and the mixture was poured onto a fluted Whatman 1 PS phase separating filter (which was first wetted with chloroform). The chloroform extract filtered through the phase separating filter and was collected in a counting vial. The aqueous material was retained on the filter. The aqueous material was transferred with a Pasteur pipette back into the original extraction vial. More chloroform (6 ml) was added, followed by shaking and filtering as described above, gently jiggling the filter if necessary to facilitate the filtration. The chloroform filtrate was combined with the first chloroform filtrate. The aqueous retentate was discarded.

A saturated solution (1 ml) of equal molar KH_2PO_4 – K_2HPO_4 was added to the vial containing the chloroform filtrate. The vial was shaken vigorously and the contents were poured onto a fresh 1 PS phase separating fluted filter (prewetted with chloroform). The chloroform filtrate was collected in a counting vial. The filter was rinsed with chloroform, and the retentate was then discarded.

The chloroform filtrate was evaporated to dryness under a stream of nitrogen. For the purposes of

¹In the experiments shown in Tables 1 and 2, unlabeled carrier mevalonate was not added, so as to facilitate the TLC procedure. In experiments (not shown) in which carrier mevalonolactone was used and TLC was performed, correlation between the two assays was as good as that shown in Tables 1 and 2; however, the mevalonolactone peak was broader, due to the load of mevalonate applied to the TLC strip.

TABLE 1. Rat liver microsomal HMG-CoA reductase activity measured by TLC assay and chloroform extraction assay

Protein	TLC 1	TLC 2	TLC Avg.	CHCl ₃ Extraction Assay	Difference between Extraction Assay and TLC
mg/ml	nmol mevalonate formed/min. ml ⁻¹				%
15.00	2.44	2.31	2.38	2.46	3.4
7.50	1.30	1.25	1.28	1.41	10.2
3.75	0.64	0.66	0.65	0.65	0.0
1.50	0.24	0.26	0.25	0.26	4.0
0.75	0.12	0.13	0.13	0.13	0.0

Incubations and assays were conducted as described in Materials and Methods; the incubation time was 20 min. In the TLC assay, adjacent segments (TLC 1, and TLC 2) were compared across the mevalonolactone peak.

making a comparison between the chloroform extraction assay and the TLC assay, chloroform (6 ml) was added to each sample, and the sample was divided into two aliquots (3 ml each). One aliquot was assayed by the TLC method described below and the other aliquot was evaporated to dryness under a stream of nitrogen, and radioactivity was assayed using the same scintillation mixture (2 ml of 0.5 M acetic acid and 10 ml of Aquasol) as described below for the TLC assay (Tables 1 and 2).

When the chloroform extraction assay was used without comparisons to TLC, the following procedure was found to be more convenient. The entire chloroform filtrate was evaporated to dryness under a stream of nitrogen. Water (0.5 ml) was added, followed by the addition of 10 ml of Aquasol.² Radioactivity was then assayed as described above.

The chloroform extraction assay typically recovers 50–60% of the mevalonate present in the incubation mixture. This can be measured precisely for each sample, since the amount of [4-³H]mevalonolactone added and recovered as internal reference is known.

Thin-Layer chromatographic assay. An aliquot (one-half) of the chloroform filtrate described above was subjected to thin-layer chromatography (Tables 1 and 2). First the chloroform extract was transferred to a disposable glass test tube and the solvent was evaporated to dryness under a stream of nitrogen. Chloroform (20 μl) was added to each sample. The sample was then spotted onto an ammonium carbonate-impregnated TLC strip (2 × 9.5 cm). The strip was developed in a slide staining chamber using chloroform–methanol 25:1 as the solvent. After development the strip was cut at 0.5 cm intervals with scissors, placing each segment in a counting vial.

² An attempt to use a simpler scintillation mixture (0.3% diphenyloxazole in toluene) gave less satisfactory results, due to the lower solubility of mevalonolactone in toluene.

Acetic acid (0.5 M, 2 ml) was added to each vial, followed by brief shaking and then standing for 15 min. Aquasol (10 ml) was added, followed by vigorous shaking. After 10 min in the scintillation counter the samples were assayed for radioactivity.

Results

Table 1 shows the results of an experiment in which rat liver microsomal membranes were assayed for HMG-CoA reductase activity at five different protein concentrations, using both the TLC assay and the chloroform extraction assay. The results show excellent agreement between the two assays over a 20-fold range in protein concentration. A linear regression analysis between protein concentration and HMG-CoA reductase activity was performed. When subjected to this analysis, the data from the TLC assay showed a linear correlation coefficient equal to 0.998. The data from the chloroform extraction assay gave a linear correlation coefficient equal to 0.994. The correlation coefficient between the TLC assay and the chloroform extraction assay for this experiment (Table 1) equaled 0.999. This result strongly supports the conclusion that both assays are in fact measuring the same phenomenon, i.e., HMG-CoA reductase activity.

Table 2 shows the results of an experiment in which HMG-CoA reductase was solubilized from rat liver microsomes by the method of Heller and Gould (19, 20) and as previously described (18). The crude soluble extract was then diluted to six different protein concentrations by the addition of buffer A. HMG-CoA reductase activity was then measured, using both the TLC assay and the chloroform extraction assay. Again the results show excellent agreement between the two assays over a 33-fold range in protein concentration. A linear regression analysis between protein concentration and HMG-CoA reductase activity

TABLE 2. Soluble extract HMG-CoA reductase measured by TLC assay and chloroform extraction assay

Protein	TLC 1	TLC 2	TLC 3	TLC Avg.	CHCl ₃ Extraction Assay	Difference between Extraction Assay and TLC
mg/ml	nmole mevalonate formed/min. ml ⁻¹					%
0.67	1.33	1.23	1.33	1.30	1.44	10.8
0.33	0.74	0.73	0.77	0.75	0.75	0.0
0.17	0.40	0.37	0.37	0.38	0.37	2.6
0.08	0.16	0.17	0.17	0.17	0.18	5.9
0.04	0.07	0.07		0.07	0.08	12.5
0.02	0.03	0.03		0.03	0.03	0.0

Incubations and assays were conducted as described in Materials and Methods; the incubation time was 60 min. In the TLC assay, adjacent segments (TLC 1, TLC 2, and TLC 3) were compared across the mevalonolactone peak.

was performed. When subjected to this analysis, the data from the TLC assay showed a linear correlation coefficient equal to 0.991. The data from the chloroform extraction assay showed a linear correlation coefficient equal to 0.999. The correlation coefficient between the TLC assay and the chloroform extraction assay for this experiment (Table 2) equaled 0.998. Again this result strongly supports the conclusion that both assays were in fact measuring the same phenomenon, i.e., HMG-CoA reductase activity.

Fig. 1 shows the separation between [4-³H]mevalonolactone and [3-¹⁴C]HMG, obtained by subjecting a synthetic mixture of these two compounds to the TLC procedure. This figure demonstrates a wide and complete separation between [3-¹⁴C]HMG (segments 1, 2, and 3) and [4-³H]mevalonolactone (segments 10–13). A small amount of the salt form of [4-³H]mevalonic acid is seen at the origin (segments 1–3). This experiment demonstrates that this TLC assay can in fact separate the two compounds most likely to be present in an extract from an HMG-CoA reductase incubation, HMG and mevalonolactone. This is important since, when we attempted this separation using silica gel strips that were not impregnated with ammonium carbonate, HMG did not remain at the origin, but instead, streaked over much of the TLC strip.

The results of a TLC assay carried out on the chloroform extract from an enzyme incubation are shown in Fig. 2. This experiment demonstrates that no [3-¹⁴C]HMG is seen at the origin. Biosynthetic

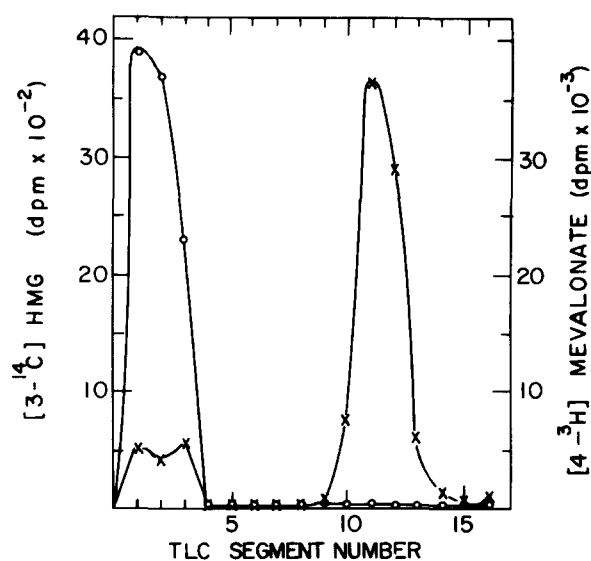


Fig. 1. Separation of [3-¹⁴C]HMG (○ — ○) from [4-³H]mevalonolactone (× — ×) on an ammonium carbonate-impregnated silica gel strip. DL-[3-¹⁴C]HMG (9400 dpm) and [4-³H]mevalonolactone (121,960 dpm) were mixed and subjected to thin-layer chromatography as described in Materials and Methods.

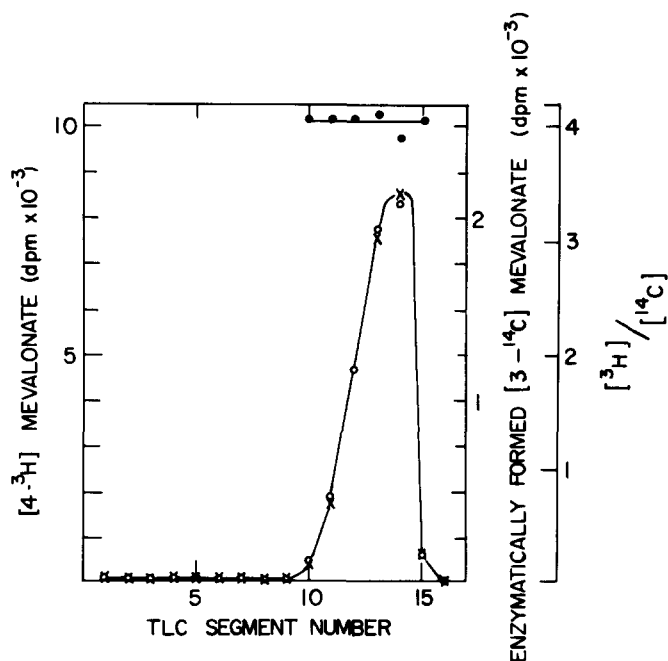


Fig. 2. Thin-layer chromatogram of a chloroform extract from an enzyme incubation (soluble extract, 0.67 mg/ml protein) with [3-¹⁴C]HMG-CoA as substrate; incubation time was 60 min; biosynthetic [3-¹⁴C]mevalonolactone (× — ×); authentic reference [4-³H]mevalonolactone (○ — ○); ³H/¹⁴C ratio (● — ●). Thin-layer chromatography was conducted as described in Materials and Methods.

[3-¹⁴C]mevalonolactone is virtually the only ¹⁴C-labeled compound (segments 10–14) seen in the chromatogram, and its chromatographic behavior correlates exactly with that of the authentic reference, [4-³H]mevalonolactone (segments 10–14). This coincidence is reflected in the relatively constant ³H/¹⁴C ratio across the mevalonolactone peak shown in Fig. 2.

This experiment demonstrates that the chloroform extract contains essentially pure mevalonolactone and that it is not contaminated with other ¹⁴C-labeled compounds from the incubation medium.

Discussion

The present article describes two techniques for the quantitative assay of HMG-CoA reductase. The TLC method described here uses ammonium carbonate-impregnated silica gel strips, and it achieves a wide and complete separation of HMG and mevalonolactone. This TLC method is also capable of measuring the radiochemical purity of enzymatically synthesized [3-¹⁴C]mevalonolactone, since the ¹⁴C radioactivity can be compared directly with authentic [4-³H]mevalonolactone by measuring the ³H/¹⁴C ratio in chromatographic segments across the mevalonolactone peak. These two points are advantages over previously published TLC methods for HMG-CoA reductase (15, 16).

The chloroform extraction assay described here correlates extremely well with the TLC assay (Tables 1 and 2). While the TLC procedure is inherently quite laborious, the chloroform extraction assay can be carried out quite rapidly. Using this technique, it is possible to process 20–30 enzyme incubations in a period of 2–3 hr, compared to several days if the TLC assay had been used. The cost per assay is significantly less with the chloroform extraction assay. Since a major objective of this laboratory has been the purification of HMG-CoA reductase (18), the chloroform extraction assay is ideally suited to the processing of the large numbers of enzyme assays that inevitably result from various purification procedures. The chloroform extraction assay procedure provides a rapid and accurate means for conducting these studies.

The chloroform extraction assay has been used thus far to assay HMG-CoA reductase activity in liver microsomes and in solubilized extracts from liver microsomes; however, there is no reason to believe a priori that the method would not work equally well for the assay of HMG-CoA reductase in other tissues or cells.

After the initial description of the chloroform extraction assay procedure (18), Goodwin and Margolis (21) described a benzene extraction procedure conducted in the presence of saturated sodium sulfite at pH 6.5. Good correlation with a TLC assay was achieved in a microsomal system possessing a specific activity of 0.098 nmol mevalonate formed/min per mg protein; however, correlations were not attempted over a wide range of HMG-CoA reductase activities.

Hulcher and Oleson (22) have described a colorimetric procedure using Ellman's reagent to measure released coenzyme A. Rodwell, Nordstrom, and Mitschelen (11) have noted that, while this method is rapid and convenient, it is less sensitive than the isotopic methods. Also the concentration of thiol reagents such as dithiothreitol (0.2 mM) must be maintained within narrow limits. This is a difficulty since dithiothreitol concentrations as high as 10 mM may be needed for maximal microsomal HMG-CoA reductase activities (19, 20). Huber, Latzin, and Hamprecht (13) and Avigan, Bhatena, and Schreiner (23) use small columns of an anion exchange resin for separation of mevalonolactone from other labeled incubation products, and Shapiro et al. (24) use small aqueous aliquots from incubations applied to TLC strips as an assay procedure.

The chloroform extraction procedure described in the present article allows the rapid assay of HMG-CoA reductase. The sensitivity of this assay is limited

only by the specific activity of the [3-¹⁴C]HMG-CoA used in the incubations. Since HMG-CoA reductase is the major regulatory enzyme in hepatic cholesterol biosynthesis, the chloroform extraction assay should significantly improve the ease with which studies can be conducted on the regulation of this interesting and important enzyme. ■

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