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Short Communication

High-performance liquid chromatographic determination of 3-hydroxy-3-methylglutaryl coenzyme A as diphenacyl ester of 3-hydroxy-3-methylglutarate

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Abstract

A method for derivatization of (*S*)-3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) by phenacylation in tetrahexylammonium hydrogensulphate as phase-transfer reagent and high-performance liquid chromatography of the HMG-diphenacyl ester is described. To avoid interference with buffers and other ions used to dissolve biological material, the alkaline hydrolysate of CoA esters has to be neutralized with phosphoric rather than hydrochloric acid. The latter reacts with phenacyl bromide. The pH during phenacylation has to be maintained in the range 6–7 to provide sufficient dissociation of HMG.

1. Introduction

In the terpenoid metabolism of microorganisms, plants and animals, (*S*)-3-hydro-3-methylglutaryl-CoA (HMG-CoA) is a prominent intermediate in the synthesis of isopentenyl diphosphate from acetyl-CoA [1]. A method hitherto routinely used for determination of HMG-CoA is the quantitation of HMG as acid-resistant residue after heating the biological material for several hours in 6 *M* HCl [2].

Based on the reaction of carboxylates with phenacylbromide [3–6], a method has been developed in our laboratory for quantitation of nanomolar amounts of HMG-CoA in the terpenoid metabolism of plants and other biological material during labeling studies.

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2. Experimental

2.1. Chemicals

Biochemicals were obtained from Sigma Chemie (Deisenhofen, Germany) and Boehringer Mannheim (Mannheim, Germany). All other chemicals were purchased from E. Merck (Darmstadt, Germany), Riedel de Haen (Seelze/Hannover, Germany), Baker Chemicals (Groß-Gerau, Germany) and Aldrich Chemie (Steinheim, Germany).

2.2. Assay of HMG-CoA in plant organelle suspensions

The organelle suspension (0.5 ml), containing up to 100 nmol of HMG-CoA, was deproteinized by adding 0.75 ml chloroform–methanol (1:2,

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v/v). After centrifugation for 15 min at 15 000 *g*, the upper (aqueous) phase was transferred into tubes, sealed with screw caps (Pyrex, Corning Glass Works, Corning, NY, USA) and subjected to hydrolysis of the CoA esters by adding 0.2 ml 2 *M* KOH and incubation for 2 h at *ca.* 20°C or 1 h at *ca.* 40°C.

A 1-ml volume of 50 m*M* tetrahexylammonium hydrogensulphate (adjusted with KOH to pH 12), used as phase-transfer reagent, and 3 ml of distilled water were then added, the pH of the mixture adjusted with 1 *M* phosphoric acid to pH 6–7 and 2 ml of 40 m*M* phenacylbromide in dichloromethane were added. The tightly screwed tubes were shaken in oblique position for *ca.* 14 h at 40°C. The dichloromethane phase, containing the phenacyl esters formed in the reaction, was washed with 3 ml of 0.2 *M* KOH and three times with 3 ml of distilled water each, and evaporated to dryness.

The residual phase containing the phenacyl esters was redissolved in 2 ml of dichloromethane, and the mixture was loaded onto a silica gel solid-phase (SPE-) 500-mg column (Baker Chemicals, Groß-Gerau, Germany) preconditioned with dichloromethane. The main portion of the non-converted phenacylbromide, phenacyl derivatives of monocarbonic acids and residual phase-transfer reagent were removed by washing three times with 2 ml of dichloromethane and once with 1 ml of dichloromethane–acetic acid ethylester (9:1, v/v) (solvent I). The HMG-diphenacyl ester (and some other phenacyl esters) were then eluted three times with 2 ml of solvent I each. The solution was evaporated to dryness and redissolved in dichloromethane. For further purification from contaminating phase-transfer reagent, the resuspended phenacylesters were loaded once again on a preconditioned SPE column and washed twice with 2 ml of dichloromethane each, and once with 3 ml of solvent I. The HMG-diphenacyl ester and some other esters were then eluted twice with 2 ml of solvent I, and the eluate was evaporated to dryness. The purified esters were stable for several weeks when stored in the dark at room temperature.

Quantitation of the HMG-diphenacyl ester was achieved by HPLC (Beckman 110B solvent delivery model and Beckman 163 variable wavelength detector) using a C_{18} column (Beckman Ultrasphere, ODS, 5 μm, 250 × 4.6 mm I.D. and a guard column containing the same packing). The mobile phase was acetonitrile–20 m*M* phosphoric acid, adjusted with triethylamine to pH 6.5 (1:1, v/v) (solvent II) at a flow-rate of 1.0 ml min^{-1}. The injection volume was 25 μl and the absorbance was recorded at 242 nm.

3. Results and discussion

The non-polar derivatizing reagent phenacylbromide was used in the nucleophilic substitution reaction with HMG (after hydrolysis of HMG-CoA). Both, the bromine anion as the leaving group and the carboxylate ion of HMG as nucleophilic group, are soluble in polar solvents. Thus, esterification of HMG with phenacylbromide in acetonitrile was unsuccessful. To avoid this problem, phase-transfer catalysis [7] was considered. In this case, the carboxylates have to be dissolved at a pH in the range providing sufficient dissociation (pH 6–7).

Of the phase-transfer reagents tested, only tetrahexylammonium as hydrogensulphate, which is also used in reactions of hydroxylated and branched-chain fatty acids [8,9], was effective but not the tetrabutylammonium cation.

Prerequisites for the determination of HMG-CoA in organelle suspensions and other biological material were (a) to maintain a pH value not exceeding pH 6–7 since hydroxyl ions compete with the phase-transfer reagent; and (b) to minimize the chloride concentration and to omit perchlorate. Apparently, both reacted preferentially with phenacylbromide. Only phosphoric acid could be used for acidification. Thus, increased concentrations of phase-transfer reagent and phenacylbromide were required (10 and 40 m*M*, respectively).

When HMG-CoA was used hydrolysis in 2 *M* KOH at 30°C required *ca.* 1 h (Fig. 1), and phenacylation of HMG *ca.* 8 h (Fig. 2).

The molar absorptivity at 242 nm of the phenacylation product of HMG was found to be

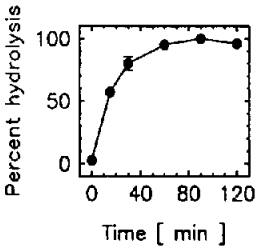

Fig. 1. Time course of hydrolytic cleavage of HMG-CoA. A 200-μl volume of 2 *M* KOH was added to aliquots of 50 μl 1 m*M* HMG-CoA (50 nmol) in 500 μl 50 m*M* 1,3-bis/tris-(hydroxymethyl)methylamino/propane (BTP) buffer (pH 8.2) and subjected to hydrolysis at 30°C for the indicated time. The reaction was stopped by addition of tetrahexylammonium hydrogensulphate, phosphoric acid and phenacylbromide, and the determination by HPLC of HMG formed by hydrolysis was performed as described in Experimental; 10 nmol from 50 nmol of the hydrolyzed HMG-CoA were injected. In the figure, the 100% value is equivalent to an absorbance of 0.19 A at 242 nm recorded by the HPLC detector system. Peaks were corrected to 1-cm base length. The data represent the mean values of two experiments.

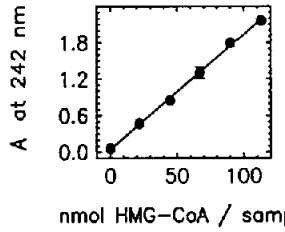

Fig. 3. Estimation of the molar absorptivity ϵ of HMG-diphenacyl ester and recovery of HMG from HMG-CoA of an incubation mixture. HMG-CoA at concentrations indicated in the figure was added to an incubation mixture containing heat-denaturated spinach chloroplasts (0.5 mg chlorophyll/ml) [10] in 50 m*M* BTP buffer (pH 8.2). Phenacylation and measurement by HPLC were carried out as described in Experimental. In the figure, 1 nmol HMG-CoA per aliquot is equal to 1 nmol HMG-diphenacyl ester after derivatization, HPLC, and absorbance measurement of the pooled eluate (1 ml). Under the assumption that no change of the molar absorptivity of the phenacyl moiety occurs during phenacylation of HMG, the molar absorptivity ϵ of the HMG-diphenacyl ester has been estimated at 18 920 l mol^{-1} cm^{-1} and a recovery of HMG of 99%.

$\epsilon = 18\,920$ l mol^{-1} cm^{-1} (Fig. 3), twice as high as that for phenacylbromide ($\epsilon = 9606$ l mol^{-1} cm^{-1}; Fig. 4), both determined in the HPLC buffer (solvent II). This is a strong indication of the formation of the diphenacyl ester of HMG.

The retention times of the phenacyl esters of biological interest listed in Table 1 show an effective separation of the HMG-diphenacyl ester from phenacyl esters of carboxylates involved in glycolysis, the citrate cycle and isoprenoid synthesis. The usefulness of the applied separation system is shown in an experiment with a chloroplast suspension to which 10 nmol HMG-CoA was added (Fig. 5).

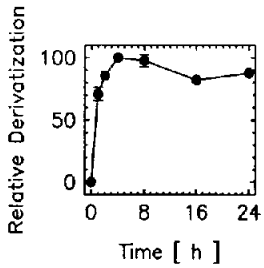

Fig. 2. Time course of the formation of HMG-diphenacyl ester in the presence of tetrahexylammonium hydrogensulphate as phase-transfer reagent. A 50-μl volume of 1 m*M* HMG in 500 μl 50 m*M* BTP buffer (pH 8.2) was subjected to phenacylation in the presence of the phase-transfer reagent as described in Experimental for the time indicated in the figure. For quantitation see Fig. 1. The data represent the mean values of two experiments.

4. Acknowledgements

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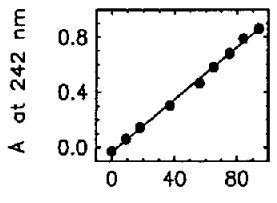

Fig. 4. Determination of the molar absorptivity ϵ of phenacylbromide in solvent II. The data in the figure represent mean values of two experiments recorded with a Kontron Uvikon 930 photometer. The molar absorptivity ϵ at 242 nm was calculated to be 9508 l mol^{-1} cm^{-1}.

Table 1
Retention time of phenacyl esters of some carboxylic acids in the applied HPLC system

Carboxylic acid moiety of the phenacylester	Relative retention time (phenacylbromide = 1.00)
D-Glucuronic acid	0.27
(−)-Shikimic acid	0.30
D-(+)-Glyceric acid	0.32
Pyruvic acid	0.40
Oxalacetic acid	not detectable
D,L-3-Hydroxybutyric acid	0.45
D,L-2-Hydroxybutyric acid	0.53
Formic acid	0.57
Acetic acid	0.63
2-Oxoglutaric acid	0.74
L-Malic acid	0.84
Acetoacetic acid	1.05
D,L-3-Hydroxy-3-methylglutaric acid	1.21
D,L-Isocitric acid	1.33
Malonic acid	1.40
Succinic acid	1.52
Fumaric acid	2.12
Citric acid	2.75

Phenacyl esters were prepared from 50 μl of 0.2 m*M* solutions of the respective acids (= 10 nmol) modified as described in Experimental. After derivatization of the acids, the phenacylesters formed were dissolved in 50 μl solvent II omitting the silica gel solid-phase steps. Aliquots (25-μl) equivalent to 5 nmol of the phenacylated carboxylic acids each were injected onto the HPLC system.

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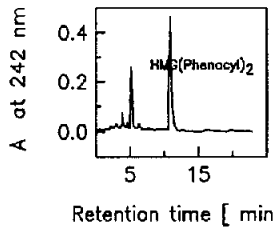

Fig. 5. HPLC analysis of a chloroplast suspension supplemented with HMG-CoA. A 50-μl volume of 0.2 m*M* HMG-CoA (10 nmol) were added to 0.5 ml of a reaction mixture containing spinach chloroplasts (0.5 mg chlorophyll/ml), 50 m*M* BTP buffer (pH 8.2), 1 m*M* NAD^+, 2 m*M* dithiothreitol, 1 m*M* diphosphothiamine, 0.5 m*M* CoA, 0.5 m*M* acetyl-CoA, 1 m*M* D,L-mevalonate, 1 m*M* pyruvate and 10 m*M* $MgCl_2$. The suspension was irradiated (830 W m^{-2} in the range between 400 and 900 nm; Osram Bellaphot spot) at 20°C for 30 min. After derivatization, half of the mixture (equivalent to 5 nmol HMG-CoA) was analyzed as described in Experimental.

5. References

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