Journal of Chromatography, 202 (1980) 439–445
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,257

DIRECT MEASUREMENT OF FREE COENZYME A IN BIOLOGICAL EXTRACTS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received June 27th, 1980; revised manuscript received August 18th, 1980)

SUMMARY

A high-performance liquid chromatographic system was developed with baseline separation of coenzyme A (CoASH) from dephospho-coenzyme A and acetyl-coenzyme A using isocratic elution. The chromatographic separation was achieved in a reversed-phase system with a high concentration (220 m*M*) of potassium phosphate buffer at pH 4.0 and appropriate amounts of methanol (*ca.* 12%). The eluate was monitored with a UV detector at 254 nm with the limit of detection at *ca.* 5 pmoles.

The system could be used without modification for the estimation of the content of CoASH in biological extracts, *e.g.* mitochondria.

INTRODUCTION

Different approaches have been used to measure coenzyme A (CoASH) in biological extracts. Indirect enzyme reactions are mostly used at present, either with spectroscopic monitoring (*e.g.* phosphotransacetylase, acetyl-CoA:orthophosphate acetyltransferase, E.C. 2.3.1.8) or with the CoASH-dependent incorporation of radioactive carnitine into palmitoyl carnitine[1–3]. Paper chromatography is employed, although this is a time-consuming procedure[4]. Thus, a quick, accurate and direct chemical method for estimation of CoASH in biological extracts is not available.

Recently, a high-performance liquid chromatographic (HPLC) method for determination of CoASH was introduced, using a microparticulate, strong anion-exchange resin[5]. This method was adequate for measurements of free CoASH after a controlled alkaline hydrolysis of the perchloride acid-insoluble materials from biological samples, thus giving an indication of the amount of long-chain acyl-coenzyme A (CoA) derivatives in the sample. However, the method is not optimal for direct measurements of the concentration of free CoASH in the neutralized perchloric extract of biological samples, *e.g.* mitochondria. An alternative system has therefore been sought, using other properties of the CoASH molecule as a basis for the chromatographic separation. This paper describes a reversed-phase system

0021-9673/80/0000–0000/$02.25

suitable for quantitation of CoASH in biological samples. After incubation with phosphotransacetylase and acetylphosphate at appropriate pH more than 95% of the UV-absorbing material of the CoA peak in the chromatogram disappeared, while a new peak with a retention time identical with that of acetyl-CoA was observed.

MATERIALS AND METHODS

Chemicals

CoASH was supplied by PL-Biochemicals (Milwaukee, WI, U.S.A.). Phosphotransacetylase (E.C. 2.3.1.8), acetyl-CoA, dephospho-CoA, β-mercaptoethanol, and thiodiglycol were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were of the highest purity commercially available.

Preparation of homogenate and mitochondrial fraction from rat livers

Rats of the Wistar strain were used, *ca.* 3 months old. The animals had free access to food and water before sacrifice. The livers were homogenized with a Potter-Elvehjem homogenizer (in five volumes) in 5 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) (pH 7.4 at 25°C) containing 0.25 *M* sucrose, 5 mM EDTA and 25 m*M* L-tartrate[6]. Cellular debris was removed by centrifugation at 2500 rpm for 10 min in the HB-4 rotor of a Sorvall centrifuge (type RC 5B). The supernatant was saved, and the mitochondrial fraction was obtained from this material by centrifugation for 10 min at 10,000 rpm in the HB-4 rotor in the Sorvall centrifuge. The pellet containing mitochondria was washed twice before resuspension in the homogenization buffer.

Preparation of extracts for analysis by HPLC

Appropriately diluted mitochondria (*ca.* 2–5 mg of mitochondrial protein per ml) were precipitated in 6% perchloric acid in the presence of 10 m*M* β-mercaptoethanol. After centrifugation in the Eppendorf centrifuge (Model 3200) for 4 min, the pH in the supernatant was adjusted to *ca.* 6 with KOH. After centrifugation in the Eppendorf centrifuge to remove precipitated potassium perchlorate, the supernatant was injected directly into the liquid chromatograph.

The content of CoASH was also measured in the "2500-rpm supernatant" obtained after removal of cellular debris (see above). To a sample (500 μl) of this material an equal volume of 12% perchloric acid in 20 m*M* β-mercaptoethanol was added, and then treated as described above for mitochondria. In the experiments with phosphotransacetylase, some material was adjusted further to pH 7.5 in the presence of 90 m*M* Tris buffer (pH 7.5). Acetyl phosphate was then added (10 m*M* final concentration) together with the enzyme.

Assay of CoASH by HPLC

An HPLC pump (Model 6000A, Waters Assoc., Milford, MA, U.S.A.) was used. The chromatographic separation was performed at room temperature using a microparticulate reversed-phase column (Spherisorb 5 ODS, 25 cm × 5 mm I.D.; HPLC Technology, Wilmslow, Great Britain). The particle size of this packing was 5 μm. A stainless-steel precolumn (10 × 2 mm I.D.) of pellicular silica (HC Pellosil-ODS from Whatman) was used to protect the column. The particle size of the packing

used in the precolumn was 37–44 μm. The mobile phase contained 220 m*M* potassium phosphate buffer pH 4.0, *ca.* 12% methanol and 0.05% (v/v) thiodiglycol, with a flow-rate of 1 ml/min. The pressure in the system under these conditions was 1700 p.s.i. The eluate was monitored with a Model 1203 UV detector at 254 nm (Laboratory Data Control, Riviera Beach, FL, U.S.A.), connected to a W+W recorder (Model 1107; W+W Electronic, Basel, Switzerland). The phosphate buffer was filtered through a Millipore filter type HA (0.45 μm). The potassium phosphate buffer and methanol were degassed for 5 min prior to the mixture of the two solvents and final adjustment of pH.

Protein determination

The protein was determined using the Folin method with bovine serum albumin as standard[7].

RESULTS

Relatively high ionic strengths are necessary to elute CoASH from an anion-exchange column[5]. In accordance with this, experiments with different ratios of methanol and water in the mobile phase resulted in broad elution profiles of the coenzyme, if eluted from the column at all (data not shown). However, when the ionic strength of the mobile phase was increased with phosphate buffer, CoASH was eluted as a distinct peak, completely separated from dephospho-CoA and acetyl-

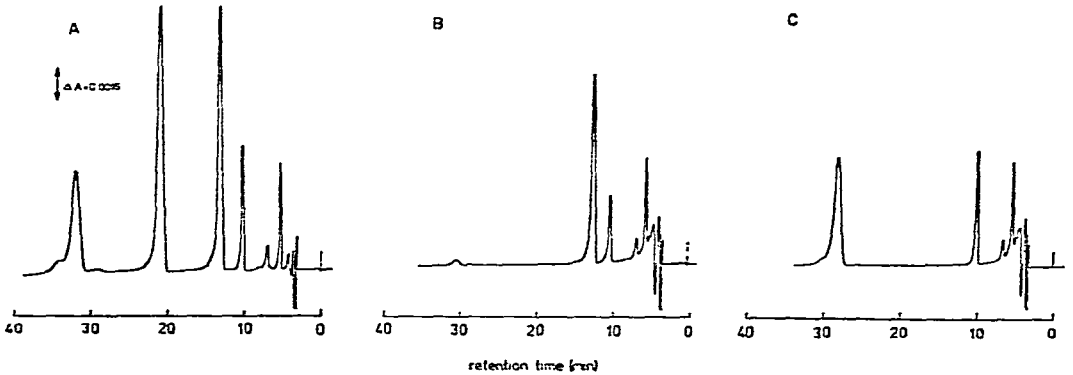

Fig. 1. (A) HPLC elution pattern of standards of β-mercaptoethanol (10 m*M*, t_R = 5.2 min and t_R = 10.2 min), CoASH (0.03 m*M*, t_R = 13 min), dephospho-CoASH (0.05 m*M*, t_R = 21 min), and acetyl-CoA (0.03 m*M*, t_R = 32 min). The solvent stock was 220 m*M* potassium phosphate, pH 4.0, and 12% methanol; 20 μl were injected into the chromatograph. Detection was at 254 nm. Full scale deflection represents 0.016 absorbance units. (B) HPLC elution pattern of CoASH (0.025 m*M*) in the presence of 10 m*M* acetyl phosphate, 10 m*M* β-mercaptoethanol and 90 m*M* Tris buffer, pH 7.5. The solvent stock was 220 m*M* potassium phosphate, pH 4.0, and 13% methanol. The solution of standards was treated with 6% perchloric acid and neutralized with KOH to pH 7.5 prior to the injection of 20 μl into the chromatograph. The retention time for CoASH was 12.5 min. (C) The standards from (B) were mixed with phosphotransacetylase (160 mU/ml) and incubated at 25°C for 10 min prior to injection of 20 μl of the assay mixture into the chromatograph. Full scale deflection represents 0.016 absorbance units in panels B and C. The same solvent system was used as shown in Fig. 1B. The retention time for acetyl-CoA was 28 min.

CoA. In the isocratic system adopted, 220 m*M* potassium phosphate buffer pH 4.0, *ca.* 12% methanol and 0.05% (v/v) thiodiglycol, the retention times were 13, 21, and 32 min, for CoASH, dephospho-CoA and acetyl-CoA, respectively (Fig. 1A). In order to avoid oxidation of CoA during analysis, 0.05% thiodiglycol was added to the solvent[5]. Adenine nucleotides like ATP and ADP are eluted in the solvent front in this system (data not shown).

CoASH is unstable, especially in alkaline solutions, in the absence of reducing agents such as β-mercaptoethanol[5]. A system applicable to the measurement of CoASH in biological extracts should be designed so that the CoASH peak is separated from β-mercaptoethanol. β-Mercaptoethanol is retained by the ODS column under the ionic strength and methanol concentrations selected. At pH 6, one of the peaks (see below) had the same retention time as CoASH. However, at pH 5 and below in the solvent, the retention time of CoASH was increased, and complete separation was obtained (Fig. 1A).

The injection of β-mercaptoethanol resulted in two peaks, with retention times 5.2 and 10.2 min (Fig. 1A). The first peak was reduced β-mercaptoethanol, and this is the dominant molecular species in the β-mercaptoethanol standard. Oxidation of β-mercaptoethanol to the disulfide resulted in a decrease in this peak with a concomitant increase in the second peak with retention time 10.2 min[8].

Control experiments with standards of CoASH and β-mercaptoethanol showed that the ratio of the two peaks derived from β-mercaptoethanol changed during storage and freezing and thawing of the solution. However, this did not influence the height of the CoASH peak in the concentration range used in this study (data not shown).

EDTA and tartrate did not influence the determination of CoASH (data not shown).

Definite proof of the composition of the CoASH peak in the chromatogram was obtained in experiments with the enzyme phosphotransacetylase and acetyl phosphate at pH 7.5. This enzyme catalyzes the reaction:

$$\text{acetyl-P}_i + \text{CoASH} \rightleftharpoons \text{acetyl-CoA} + \text{P}_i$$

and the equilibrium is far to the right. The reaction has thus been used to quantitate CoASH[2]. Fig. 1B shows that acetyl-P_i is eluted in the solvent front and thus does not influence the quantitation of CoASH. After addition of phosphotransacetylase and incubation at 25°C for 10 min, it is evident that more than 98% of the material in the CoASH peak was consumed, with a concomitant formation of acetyl-CoA (Fig. 1C).

The retention times of CoASH, dephospho-CoASH and acetyl-CoA are critically dependent on the methanol concentration at constant pH and ionic strength. Comparison of Fig. 1A with Figs. 1B and 1C shows that a change in the amount of methanol in the solvent from 12 to 13% resulted in small changes in the retention times, but had no significant effects on the separation and quantitation of CoASH and acetyl-CoA in biological samples (Figs. 1–3). Small differences in the batches of the solvent stocks probably explain the variations in the retention times in Figs. 2 and 3.

The reproducibility was good as the variations of the peak height in replicate runs were less than 1%.

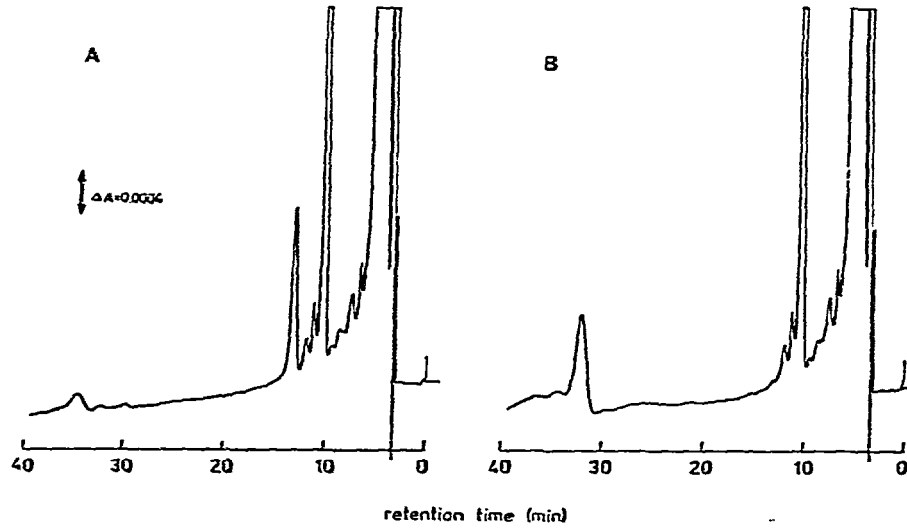

Fig. 2. (A) The HPLC chromatogram of an extract (20 μl) made from mitochondria of rat liver as described in Materials and methods. The solvent stock was 220 mM potassium phosphate, pH 4.0, and 12% methanol. The retention times were: for CoASH, 13.2 min; and for acetyl-CoA, 31.6 min. (B) The HPLC chromatogram of the extract shown in Fig. 2A after addition of acetyl phosphate (10 mM final concentration), and 160 mU/ml of phosphotransacetylase. The extract was analysed after incubation for 10 min at 25°C. Full scale deflection represents 0.004 absorbance units in panels A and B, and the retention times were the same as in panel A.

The method was applied to the measurement of the content of free CoASH in the mitochondrial fraction and homogenate from rat liver. The elution pattern of an extract (20 μl) made from the mitochondrial fraction is shown in Fig. 2A. At the high ionic strength of the solvent system used, most of the matrix compounds of low molecular weight and of ionic nature (*e.g.* ATP and ADP) were eluted in the solvent front, and the chromatogram is relatively "clear". Not much acetyl-CoA was present

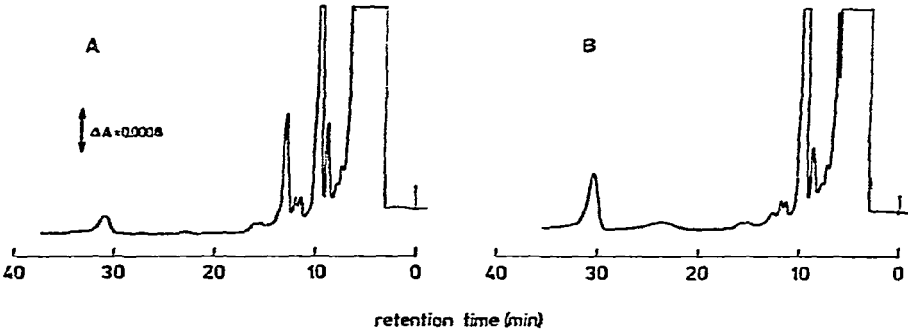

Fig. 3. (A) The HPLC chromatogram of an extract (20 μl) made from the "2500-rpm supernatant" of rat liver as described in Materials and methods. The solvent stock was 220 mM potassium phosphate, pH 4.0, 12% methanol. The retention times were: for CoASH, 12.8 min; and acetyl-CoA, 30.8 min. (B) The HPLC chromatogram of the extract shown in Fig. 3A after incubation at pH 7.5 with acetyl phosphate (10 mM final concentration) and 160 mU/ml of phosphotransacetylase for 10 min at 30°C. Full scale deflection represents 0.008 absorbance units in panels A and B. The retention times were the same as in panel A.

in the extract of the mitochondrial fraction. After incubation of the extract with phosphotransacetylase, more than 95% of the CoASH peak from the mitochondrial extract disappeared, and an increase in the acetyl-CoA content of the preparation was observed (Fig. 2B).

The elution pattern of an extract made from the "2500-rpm supernatant" of the rat liver is shown in Fig. 3A. Even after the dilution of the cytosol during the preparation procedure, the CoASH content was readily measured by this method. Also in this case, no interference with CoASH in the chromatogram was observed, as shown after incubation with phosphotransacetylase as described (Fig. 3B).

In mitochondria a free CoASH content of (mean) 1.95 nmol/mg protein, with standard deviation 0.06, $n = 4$, was found. These values were obtained by analysing four extracts made from the same stock of mitochondria. In the extracts from "2500-rpm supernatant" the standard deviation was larger, the data being (mean) 1.04 nmol/mg protein, with standard deviation 0.13, $n = 3$.

Standard curve for CoASH

A linear relationship was obtained between the injected amount of CoASH and the peak height. The limit of detection was *ca.* 5 pmoles of CoASH with a signal-to-noise ratio of 5 (data not shown).

DISCUSSION

The concentration of CoASH has been estimated directly without previous separation of possible interfering compounds by the UV-absorbing properties of the coenzyme, as well as by the reaction with N-ethylmaleimide[9,10]. These methods are not applicable to biological extracts. The determination of CoASH with phosphotransacetylase has been a standard procedure[2]. The end-point method[11] is based on the observation that acetyl-CoA absorbs more strongly than CoASH at 233 nm, and the increase in absorbance at 233 nm is thus measured in the sample; however, several compounds interfere with the assay[2]. The catalytic assay[12] with phosphotransacetylase has therefore been the method of choice for the determination of CoASH[2]. It should be noted that the present HPLC method is not influenced by the molecular composition of the sample. This is of some importance, since the overall composition of biological extracts in experimental studies may be dramatically changed, and thus possibly influence the activity of the enzyme(s) used to estimate CoASH by indirect methods.

In preliminary studies it was found that CoASH had low affinity for the alkyl-bonded packing if methanol–water was used as solvent. The capacity factors were low and the resolution poor. The addition of an appropriate ion-pair in the solvent usually increases the chromatographic performance in these situations[13,14]. However, it was found that an increase in phosphate buffer concentration in the solvent also had the same effect.

At constant pH and phosphate buffer concentration, an increase in the concentration of methanol in the solvent resulted in shorter retention times for CoASH, dephospho-CoA and acetyl-CoA. However, changes of 1–2% in the amount of methanol in the solvent did not seriously influence the chromatographic separation of CoASH from dephospho-CoA and acetyl-CoA. The content of CoASH in

mitochondria found by the HPLC method is in accord with data obtained by other authors[15].

The physicochemical principles underlying the increase in the chromatographic performance with high concentrations of phosphate in the solvent are not fully understood at present[16].

It has been reported that CoASH can be separated from acetyl-CoA and other thioesters in a reversed-phase system with ion-pair chromatography, using tetrabutylammonium ion as counter-ion[17]. However, the system was not evaluated for measurements of CoASH in biological extracts.

Considering the simple, isocratic system, and the rapid analysis of biological extracts, the HPLC procedure presented compares favourably with the enzyme methods in analysis time and precision. Furthermore, the system makes possible the assay of CoA, dephospho-CoA and acetyl-CoA in the same run.

ACKNOWLEDGEMENTS

The contribution of cand. mag. A. Bakken to this work is gratefully acknowledged.

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