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Separation and detection of tissue CoASH and long-chain acyl-CoA by reversed-phase high-performance liquid chromatography after precolumn derivatization with monobromobimane

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ABSTRACT

A method for the determination of tissue levels of free coenzyme A (CoASH) and long-chain acyl-CoA was developed using reversed-phase high-performance liquid chromatography and fluorescence detection. CoASH in acid-soluble and processed acid-insoluble liver homogenates was derivatized with the fluorescent agent monobromobimane, which selectively binds sulphydryl groups. The optimum requirements for sample preparation and conditions for derivatization with monobromobimane are discussed. The separation of the CoA-bimane adducts was achieved with a 3-μm Hypersil ODS C_{18} column (150 × 4.6 mm I.D.) using gradient elution with tetrabutylammonium hydroxide, acetic acid, phosphoric acid and acetonitrile. The detection limit was lower than 3 pmol. The method is more specific and more sensitive than the existing HPLC method with UV spectrophotometric detection. Furthermore, the method permits the detection and determination of other tissue thiols such as cysteine, cysteinylglycine and glutathione simultaneously.

INTRODUCI'ION

Tissue levels of acyl-coenzyme A (acyl-CoA) and particularly the ratios of long-chain acyl-CoA to free CoASH are known to act as intercellular regulators in several steps of intermediary metabolism [1–3]. For example, the levels of long-chain acyl-CoA and free CoASH in rat livers have been shown to increase after starvation and repeated administration of peroxisome proliferator compounds such as clofibrate and tiadenol [4–9]. A similar phenomenon was observed in our laboratory with peroxisome proliferating hypolipidaemic sulphur-substituted fatty acid analogues [10]. Furthermore, excess long-chain acyl-CoA levels in tissue have been implicated as potentially harmful amphiphiles with possible adverse effects on cellular metabolism through interaction with membrane structures [11]. It is of importance, therefore, to have

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reliable, sensitive, specific and reproducible means to determine these compounds.

The separation and detection of tissue levels of free CoA and long-chain acyl-CoA by high-performance liquid chromatography (HPLC) with UV spectrophotometryic detection, developed originally by Ingebretsen and Farstad [12] and subsequently modified by Berge et al. [13,14], is frequently used. The present paper describes a major modification of this procedure in terms of the reagent used to hydrolyse the thio-ester bond and the HPLC conditions for separation and detection of tissue levels of both free CoA and long-chain acyl-CoA. The principle of the detection of the CoASHs in this method is based on derivatization of the free sulphydryl groups with the fluorescent agent monobromobimane [15]. The method is superior in terms of sensitivity and specificity over the existing method. Moreover, other cellular protein-bound thiols such as glutathione, cysteine and cysteinylglycine can be determined in one run from the tissue precipitates.

EXPERIMENTAL

Materials

N-Ethylmalemide, N-ethylmorpholine, dithioerythritol (DTE), cysteine, cysteinylglycine, glutathione, sodium tetrahydroborate ($NaBH_4$) and coenzyme A (CoASH) were obtained from Sigma (St. Louis, MO, USA). 5-sulphosalicylic acid dihydrate, dimethyl sulphoxide (DMSO), hydrogen bromide (HBr), perchloric acid, acetic acid, phosphoric acid and methanol from Merck (Darmstadt, Germany), monobromobimane (mBrB) from Calbiochem, Behring Diagnostics (La Jolla, CA, USA), tetrabutylammonium hydroxide from Janssen Chimica (Beerse, Belgium) and Hypersil ODS (3 μm) from Shandon (Runcorn, UK). Columns for reversed-phase HPLC (150 × 4.6 mm I.D.) were packed with 3-μm Hypersil ODS at 9000 p.s.i. (1 p.s.i. = 6894.76 pa) using a Shandon column packer.

A mixed standard solution was prepared by dissolving 40 μM cysteine, 10 μM cysteinylglycine, 320 μM glutathione and 80 μM CoASH in a 5% solution of sulphosalicylic acid containing 50 μM DTE.

Principles of determination of long-chain acyl-CoA

The method was essentially developed first by using palmitoyl-CoA as a prototype of long-chain acyl-CoA. The thio-ester bond of palmitoyl-CoA was cleaved after incubation with sodium tetrahydroborate. Optimum assay conditions were developed by using different concentrations of sodium tetrahydrohorate, incubation temperatures and incubation times. Subsequently, the free sulphydryl group of the CoA moiety was derivatized with mBrB as described below. In acid precipitates of tissue, the method can also cleave the protein-bound thiols and hence can also be used to determine them simultaneously.

Tissue preparation for determination of long-chain acyl-CoA

Livers from male Wistar rats obtained from Møllegaard Breeding Laboratory (Ejby, Denmark) were used when they were *ca. 4* weeks old, weighing 170-180 g. The animals were killed under light halothane anaesthesia and the livers from individual rats were homogenized immediately in ice-cold 5% sulphosalicylic acid in 50 μM DTE to obtain 10% (w/v) liver homogenates. Aliquots of 500 μl of tissue homogenates were centrifuged at 600 g for 10 min. The resultant supematants were used to measure the acid-soluble CoASH, while the tissue precipitates were washed twice with 5% sulphosalicylic acid and once with distilled water and processed further.

The tissue precipitates were incubated with 25 μl of octanol (to prevent foaming) and 250 μl of either 1.4 or 2.0 M $NaBH_4$ for 30 min at 37°C in a water-bath. After incubation the samples were left on ice for 10 min and then 250 μl of 5% sulphosalicylic acid were added to neutralise the excess of $NaBH_4$. Subsequently, the samples were centrifuged at 600 g for 10 min and the supematants were used to determine the hydrolysed CoASH and other thiols.

Precolumn derivatization

The principle of the derivatization of free sulphydryl groups with mBrB described below was a further development of a previously pub-

lished method for the determination of various thiols in red blood cells [15] and plasma [16]. To 60 μl of either pretreated palmitoyl-CoA, untreated CoASH or tissue homogenates prepared as described above were added 30 μl of 20% sulphosalicylic acid, 130 μl of 140 m*M* HBr in 65% DMSO, 50 μl of 1.0 M N-ethylmorpholine and 10 μl of 20 m*M* mBrB in 100% acetonitrile in that order. After 10 min of incubation at ambient temperature in the dark, 20 μl of 10% perchloric acid were added to stop further reaction.

Chromatography

Volumes of 10 μl of derivatized samples were injected into a 150 x *4.6* mm I.D. column packed with 3-μm particles of Hypersil ODS (C,,), equipped with a guard column packed with Pelliguard LC-18. Elution solvent A consisted of 10 mmol of tetrabutylammonium phosphate, 2.5 ml of glacial acetic acid and 0.675 ml of orthophosphoric acid diluted to 1 1 with distilled water and adjusted to pH 3.4 with 2.0 *M* NaOH, solvent B was 200 ml of acetonitrile, 10 mmol of tetrabutylammonium phosphate, 2.5 ml of glacial acetic acid and 0.675 ml of orthophosphoric acid diluted with to 1 1 distilled water and the pH adjusted to 3.25 with 2.0 *M* NaOH and solvent C was acetonitrile-water (75:25). Elution was carried out at ambient temperature at a flow-rate of 1.5 ml/min. The profile of the elution with a mixture of solvents A and B was as follows: O-13 min, 4-22% B; 13.1-23 min, isocratic, 40% B; 23.1-30 min, 40–60% B; and 30.1-41.5 min, isocratic, 95% B. After each run the column was washed for 5 min with solvent C to remove late-eluting fluorescent material.

Instrumentation

A Spectra-Physics SP 8800 solvent-delivery system coupled to a Gilson 232-401 automatic sample processor was used. The detector was a Shimadzu RF 535 spectrofluorimeter equipped with concave diffraction grating excitation and emission monochromators operating at an excitation wavelength of 400 nm (13 nm bandpass) and an emission wavelength of 475 nm (15 nm bandpass). The sensitivity of the detector was enhanced about three-fold with a Hamamatsu R 982:08 photomultiplier. The integrator was a Spectra-Physics Chromjet SP 4400.

RESULTS AND DISCUSSION

Fig. 1A shows a typical HPLC trace of a standard mixture containing CoASH, cysteine, cysteinylglycine and glutathione that had been treated with $NaBH_4$ before derivatization with mBrB. With the gradient system adopted, a baseline separation of the CoA-bimane adduct

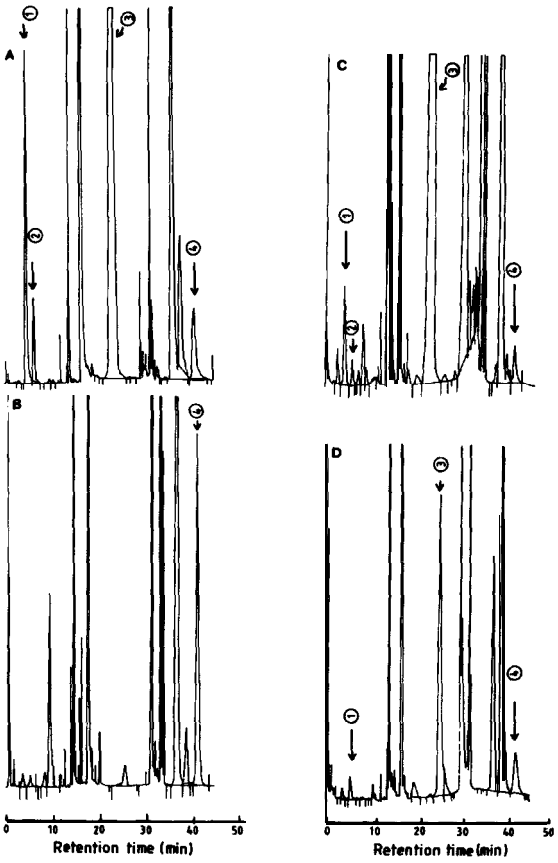

Fig. 1. Chromatograms obtained from monobromobimane-derivatized samples of (A) standard solution containing 80 μ*M* CoASH, 40 μ*M* cysteine, 10 μ*M* cysteinylglycine and 320 μ*M* ghrtathione dissolved in a 5% solution of sulphosalicylic acid containing 50 μ*M* DTE; (B) standard solution of palmitoyl-CoA (150 μ*M*) treated with 2.0 M $NaBH_4$; (C) acid-soluble liver extract obtained from 10% liver homogenates in 5% sulphosalicylic acid containing 50 μ*M* DTE; and (D) acid-insoluble liver precipitates incubated with 2.0 M $NaBH_4$. Peaks: 1 = cysteine; 2 = cysteinylglycine; 3 = glutathione; 4 = CoASH.

and the other thiol-bimane adducts from the reagent peaks was achieved. The retention times for the bimane derivative of CoASH was 40-41 min and for those of cysteine, cysteinylglycine and glutathione 7.0, 8.0 and 29 min, respectively. In a subsequent experiment in which a standard sample of palmitoyl-CoA was treated with $NaBH_4$, a CoA-bimane adduct with a retention time of 40-41 min was also observed (Fig. 1B).

The method was further developed to determine CoASH and long-chain acyl-CoA in biological samples. A CoA-bimane adduct with a similar retention time was also observed in chromatograms from both acid-soluble (Fig. 1C) and acid-insoluble (Fig. 1D) liver extracts treated in the same way as the palmitoyl-CoA standards. Thus, this method can be used without any modification for the determination of CoASH (in the acid-soluble extract) and long-chain acyl-CoA (in the acid-insoluble extract) in tissues. The detection limit for both free CoASH and long-chain acyl-CoA was about 3 pmol per injection with a signal-to-noise ratio of 3.

In another experiment, we investigated whether any materials co-eluted with the CoA–bimane adduct. A liver extract was spiked with a standard solution containing both CoASH and palmitoyl-CoA, treated with $NaBH_4$ before derivatization. The standards co-eluted exactly with the peaks from the tissue extracts (data not shown).

Calibration graphs obtained with different concentrations of pure CoASH and palmitoyl-CoA are shown in Fig. 2A and B, respectively. The correlation coefficent (r) between the areas of peaks measured at 475 nm and the concentrations of CoASH and palmitoyl-CoA used was 0.988. It is worth noting, however, that the addition of 50 μl of DTE was neccessary in order to obtain a linear calibration graph, especially at lower concentrations. Furthermore, as shown in Fig. 2A, the yield of the CoA-bimane adduct was higher following treatment of the standard CoASH solution with $NaBH_4$ than without. This may be explained by the fact that $NaBH_4$ acts as a reductant, thereby minimizing the possible oxidative degradation of the CoASH molecule during processing.

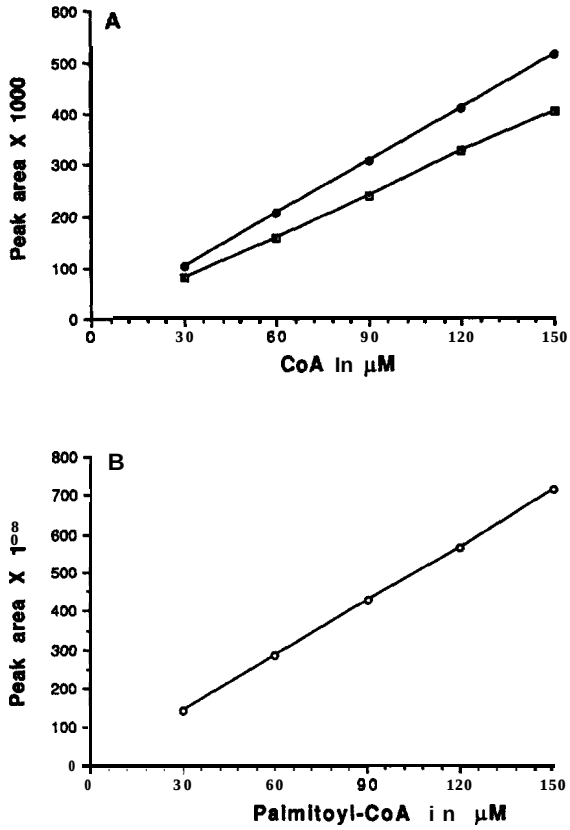

Fig. 2. Calibration graphs obtained from different concentrations of (A) free CoASH dissolved in 5% sulphosalicylic acid containing 50 μM DTE, (●) treated with $NaBH_4$ and (Cl) untreated, and (B) palmitoyl-CoA after incubation with 2.0 M $NaBH_4$ at 37°C for 30 min.

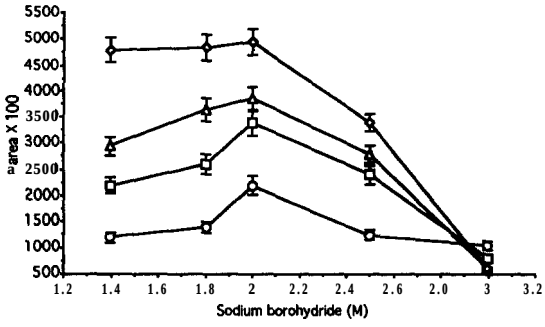

Fig. 3. Yield of CoA-bimane adduct from a solution of 150 μM palmitoyl-CoA incubated with 1.4, 1.8, 2.0, 2.5 and 3.0 M $NaBH_4$. Incubation time at 37°C: 0 = 5; •1 = 10; A = 20; 0 = 30 min. The values represent means ± standard deviations for four rat livers.

The optimum concentration of $NaBH_4$ required to cleave completely the thio-ester bond between the CoA and acyl moiety was studied by determining the yield of the CoA-bimane adduct obtained after incubating palmitoyl-CoA in different concentrations of $NaBH_4$ for different times (Fig. 3). The results indicated that $NaBH_4$ at a concentration of 2.0 *M* and incubation for 30 min were the optimum assay conditions. At concentration below 2.0 *M* the recovery of the CoASH from the long-chain acyl-CoA was low, mainly owing to incomplete bond cleavage. Similarly, a low recovery was obtained at concentrations higher than 2.0 *M*, mainly owing to the interference of the $NaBH_4$ with either the formation or the stability of the CoA-bimane adduct.

As shown in Fig. 4, a parallel experiment in which palmitoyl-CoA was incubated with 2.0 *M* $NaBH_4$ at different temperatures for various times showed a maximum yield of the adducts at 37°C with incubation for 30 min. Higher temperatures increased the effervescence of $NaBH_4$ and were therefore impractical and the yield at lower temperatures was erratic and low. Table I shows the recoveries obtained for free CoASH and palmitoyl CoA after incubation with 1.4 or 2.0 *M* $NaBH_4$ at 37" for 30 min, confirming the above findings.

The determination of long-chain acyl-CoA in 10% liver homogenates prepared in 5% sulphosalicylic acid containing 50 μM DTE is shown in Table II. In agreement with the experiment mentioned above, higher yields were obtained with tissue incubated with 2.0 *M* than with 1.4 *M* $NaBH_4$. Furthermore, larger amounts of free CoASH and long-chain acyl-CoA were obtained from liver homogenates with the present method than with the older UV method.

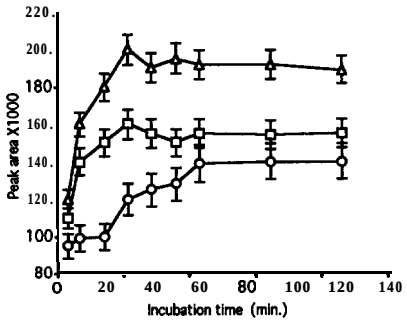

Fig. 4. Yield of CoA-bimane adducts from a 150 μM solution of palmitoyl-CoA in 5% sulphosalicylic acid containing 50 μM DTE at different incubation temperatures and times. 0 = 20°C; □ = 30°C; A = 37°C.

TABLE I

RECOVERIES OF FREE CoASH AND PALMITOYL-CoA FOLLOWING TREATMENT WITH (A) 1.4 *M* OR (B) 2.0 M $NaBH_4$ AT 37°C FOR 30 min

Type of compound	Treatment	Calculated concentration (m*M*)	Recovery (%)	
			25°C	37°C
Free CoASH	A	1.0	98	98
	B	1.0	98	98
Palmitoyl-CoA	A	1.0	68	85
	B	1.0	87	95

[a] Mean (n = 6).

TABLE II

LEVELS OF ACID-SOLUBLE CoASH AND ACID-INSOLUBLE LONG-CHAIN ACYL-CoA in LIVERS OBTAINED AFTER INCUBATION WITH (A) 1.4 M OR (B) 2.0 *M* $NaBH_4$ AT 37°C FOR 30 min, AND (C) LEVELS OBTAINED WITH THE PREVIOUS UV METHOD [13]

Type of compound	Concentration (nmol/g)		
	A	B	C
Acid-soluble CoASH	156.7 ± 10.2	159.2 ± 14.2	107 ± 23
Long-chain acyl-CoA	81.7 ± 6.5	98.8 ± 9.0	88 ± 10.2

[a] Means ± standard deviations (n = 4).

To test the reproducibility of method, we prepared three aliquots of a liver tissue homogenate as described above and derivatized the samples on three different days. The variations in the levels of both acid-soluble CoASH and acid-insoluble CoA between the samples were found to be less than 10% (data not shown).

In conclusion, the present method is more sensitive and more specific than the previous UV method for the detection and determination of tissue levels of CoASH. In addition, it can be used to measure protein- and non-protein bound

thiols such as cysteine, cysteinylglycine and gluthatione in biological specimens simultaneously.

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