High performance liquid chromatography of long-chain acylcarnitine and phospholipids in fatty acid turnover studies

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Summary In this paper we describe a rapid, isocratic high performance liquid chromatography (HPLC) method for the study of radioactive fatty acid incorporation into complex lipids of human erythrocytes, which allows the simultaneous separation of the major phospholipid classes and long-chain acylcarnitines. The lipid extract of erythrocytes pulsed with radioactive fatty acids was injected into an HPLC system equipped with a silica column. The individual components eluted were monitored by ultraviolet absorption and radioactive emission. With respect to the UV profile, the radioactive profile showed an additional peak between phosphatidylcholine and phosphatidylethanolamine, which was identified as long-chain acylcarnitine by different experimental approaches. The radioactivity recovered in the long-chain acylcarnitines contains essential information enabling definition of acyl trafficking in red cells.–**Arduini, A., A. Peschechera, S. Dottori, A. F. Sciarroni, F. Serafini, and M. Calvani.** High performance liquid chromatography of long-chain acylcarnitine and phospholipids in fatty acid turnover studies. *J. Lipid Res.* 1996. **37:** 684–689.

Supplementary key words erythrocytes • carnitine palmitoyltransferase • HPLC • long-chain acylcarnitines • phospholipids • reacylation–deacylation cycle

The membrane phospholipid fatty acid turnover of circulating human erythrocytes is accomplished mainly through the combined action of phospholipase A_2 and lysophospholipid acyl-CoA transferase activities (1, 2). We have recently shown, however, that the activity of carnitine palmitoyltransferase (CPT), an enzyme predominantly known for its role in mitochondrial fatty acid oxidation (3), is essential for the physiological expression of the deacylation–reacylation process of membrane phospholipids (4, 5). In addition, CPT activity has been demonstrated to be involved in neuronal triglyceride and phospholipid fatty acid turnover (6).

The involvement of CPT in membrane phospholipid fatty acid turnover has been verified by several studies. When a red blood cell suspension is incubated in the presence of radioactive long-chain fatty acid, a consistent amount of radioactive long-chain acylcarnitine is produced (5). The identification and separation of radioactive long-chain acylcarnitines and other radioactive components present in the red cell lipid extracts is usually carried out by two-dimensional thin-layer chromatography (TLC) (2, 5), a time-consuming methodology. A large number of studies using HPLC for membrane phospholipid analysis have been reported, though most of them still suffer from long elution time (7–9). More importantly, no information is available about the possibility of separating long-chain acylcarnitines and other long-chain acyl acceptors such as membrane phospholipids.

In this study, we report a fast, isocratic HPLC procedure which consistently reduces the elution time, still providing a good resolution and separation of radiolabeled long-chain acylcarnitines and major phospholipid classes obtained in the course of membrane complex lipid fatty acid turnover studies.

MATERIALS AND METHODS

Chemicals

Fatty acid-free bovine serum albumin (BSA), bovine liver phosphatidylinositol (PI), bovine brain phosphatidylserine (PS), bovine liver phosphatidylethanolamine (PE), egg yolk phosphatidylcholine (PC), bovine brain sphingomyelin (SM), 2-deoxy-D-glucose (DG), microcrystalline cellulose, and α-cellulose were obtained from Sigma Chemicals (St. Louis, MO). [1-^{14}C]palmitic acid (58 Ci/mol), [9,10-^{3}H]oleic acid (7 Ci/mmol), and [1-^{14}C]linoleic acid (53 Ci/mol) were obtained from New England Nuclear Corporation (Boston, MA). Thin-layer plates, Whatman LK6 (silica gel) (20 × 20 cm) with a pre-absorbent layer were obtained from Carlo Erba (Milan, Italy). Palmitoyl-L-carnitine hydrochloride (PCn) was given by Sigma Tau Pharmaceuticals (Pomezia, Italy). 2-Tetradecylglycidic acid (TDGA) was given by McNeil Pharmaceuticals (Spring House, PA). Mobile phase components of HPLC grade were obtained from Merck (Darmstadt, Germany). All other compounds used were reagent grade.

Preparation and incubation conditions of human erythrocytes

Heparinized blood was collected daily from normal human volunteers. White blood cells and platelets were removed with a column containing a mixture of microcrystalline cellulose and α-cellulose (1:1, w/w), and filtered blood cells were washed three times with 4 vol of

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; CPT, carnitine palmitoyltransferase; PCn, palmitoyl-L-carnitine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; TDGA, 2-tetradecylglycidic acid; DG, 2-deoxy-D-glucose; BSA, bovine serum albumin.

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cold 0.9% NaCl (10). Isolated erythrocytes were then washed with incubation buffer (NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1 mM, NaH_2PO_4 1 mM, saccharose 40 mM, 5 mM glucose, Tris-HCl 10 mM, at pH 7.4) and resuspended at a final hematocrit of 5%. A "Rotabath" shaking bath at 37°C was used for the incubations. Red cells were labeled with radioactive fatty acids by incubating them for 60 min with either palmitate, oleate, linoleate, or arachidonate (10 μM), complexed to fatty acid-free BSA (1.65 mg/ml). Incubations were terminated by washing cells once with cold incubation buffer, three times with fatty acid-free BSA 1% in incubation buffer, and finally once again with incubation buffer. In the course of DG studies, aliquots of red cell samples were used for ATP determination (5).

Lipid extraction and chromatographic procedures

Erythrocyte lipids were immediately extracted from intact cells according to Rose and Oklander (11). The lipid extracts were separated by two-dimensional TLC (5). Aliquots of the lipid extract were used for determination of lipid phosphorus content (12). Radioactive emissions from TLC plates were analyzed with a System 200 Imaging Scanner, Bioscan, Inc. (Edmonds, WA). Counting efficiency was evaluated by an external standard. Calculations are based on the specific activity of radioactive fatty acid.

The HPLC separation of long-chain acylcarnitines and phospholipids was carried out using a stainless column (250 × 4 mm I.D.) packed with Nucleosil 100-7.

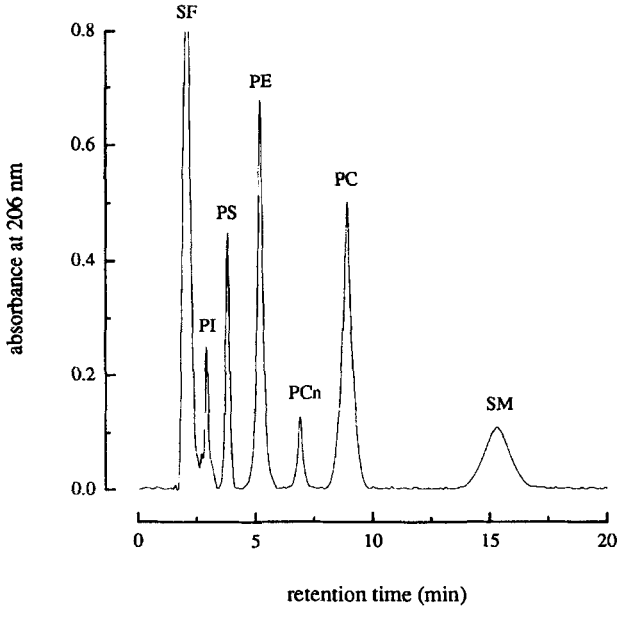

Fig. 1. Elution profile of a standard mixture of phospholipids and PCn detected by UV monitor at 206 nm. The amount injected of each standard component was 10 μg; SF, solvent front.

The mobile phase consisted of acetonitrile–hexane–methanol–phosphoric acid (918:30:30:17.5). The HPLC instrument used was a Waters (Milford, MA) apparatus consisting of a 510 solvent-delivery system, a 715 Ultraswip automatic injector, and a 486 variable-wavelength UV detector unit set at 206 nm equipped with a 7 μl flow cell. The flow rate was 1.5 ml/min. Radioactive emission was monitored by mixing the eluent with three volumes of Flow-Scint II Packard (Meriden, CT), and then passing it through a 500 μl flow cell installed in a Packard Radiomatic Flow-One A525 (Meriden, CT). The UV detector output was connected to the analog input channel of the radioactive detector, and both UV absorption and radioactive emission were processed by a Packard Radio-HPLC software package. The final output of the software package showed the combined UV and radioactive emission profiles, and allowed the computation of the respective peak areas. Static counting efficiency of the radiodetector was calculated using a known amount of radioactivity present in a radiolabeled fatty acid standard. Calculations are based on the specific activity of radioactive fatty acid. Results are given as pmol of radioactive fatty acid/μg of total lipid phosphorus.

RESULTS AND DISCUSSION

The utilization of radioactive tracers in membrane phospholipid fatty acid turnover studies represents the only experimental tool available to define the fate and to quantitate the apparent rate of membrane phospholipid reacylation. Previous work from our laboratory has shown that the radioactive fatty acid turnover in red cell membrane phospholipid was characterized by the formation of a significant amount of an additional radioactive acyl-acceptor: acylcarnitine (5). This carnitine derivative, an essential intermediate of mitochondrial β-oxidation of fatty acids, results from the reaction catalyzed by the erythrocyte CPT, an acyltransferase capable of reversibly transferring the acyl residue from CoA to L-carnitine (3). We have proposed that erythrocyte CPT is a component of the membrane phospholipid reacylation process, where its activity allows a fine tuning of the acyl-CoA/free CoA pool lying between the acyl-CoA synthesis and its utilization in the reacylation process. This interpretation also implies that long-chain acylcarnitines are an important reservoir of acyl-units, which may be utilized for the reacylation process at no ATP cost (5, 13).

Given these concepts, one of the most critical steps in membrane phospholipid reacylation experiments is the chromatographic separation of all radioactive components present in the lipid extract. Radioactive emissions

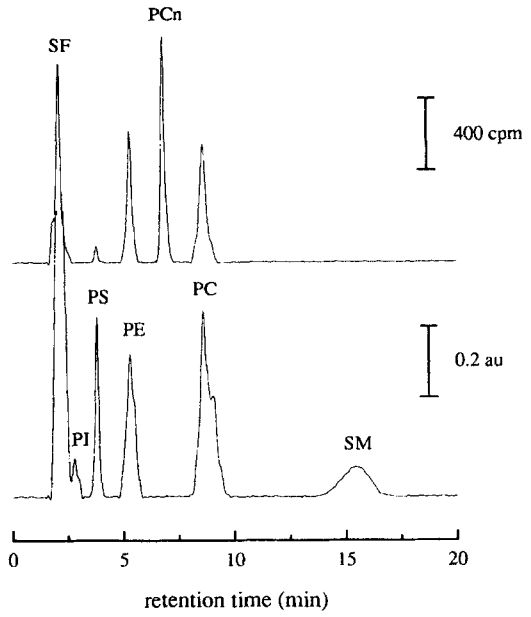

Fig. 2. Radioactive and UV elution profiles of a lipid extract of red cell incubated with radioactive palmitate. After 60 min of incubation at 37°C, red cells were washed, extracted, and chromatographed as described in the Materials and Methods section; SF, solvent front.

of a two-dimensional TLC plate from a lipid extract of pulsed red cells showed a complete resolution of the major phospholipid classes together with PCn (5). Interestingly, despite the large amount of radioactivity recovered into PCn, endogenous PCn was not visualized by iodine. In addition, we have demonstrated that one-dimensional TLC analysis, often utilized in red cell membrane phospholipid fatty acid turnover studies, failed to resolve PCn from PC (14). As PCn contains a high amount of radioactivity, most of the previous work on the reacylation rate of red cell membrane complex lipids either overestimated PC radioactivity or failed to show radioactive PCn.

This prompted us to develop a chromatographic method that was less time-consuming than two-dimensional TLC in separating phospholipids and long-chain acylcarnitine. We developed an HPLC procedure under isocratic conditions. Several HPLC protocols have appeared in the literature for the separation of membrane phospholipids (7–9, 15, 16). However, given the fact that the analysis of membrane lipid components often requires specific methodological intervention to resolve or to improve the chromatographic resolution of different lipid complexes (16), it is not easy to adopt a specific chromatographic procedure that answers a given analytical problem. Allen and Manning (17), in a study on the abnormal phospholipid metabolism in spur cell anemia, have reported an HPLC procedure for the separation of radioactively labeled phospholipids and long-chain acylcarnitines, though a complete analysis of the red cell lipid extract required a long elution time (more than 40 min) and a gradient approach. Isocratic separation of genuine phospholipids (PI, PS, PC, and PE) and PCn was achieved with a mobile phase composition of acetonitrile–hexane–methanol–phosphoric acid (918:30:30:17.5, v:v:v:v), which provided the greatest separation selectivity, overcoming the problem of long-chain acylcarnitine and PC co-elution (**Fig. 1**). Elution time for all phospholipid classes was completed within 18 min maintaining good resolution and reproducibility. A less polar mobile phase composition (i.e., reduction of phosphoric acid or methanol) or replacement of phosphoric acid with sulfuric acid, as recently described (16), either increased significantly the elution time, or did not allow a good separation of PCn and PC (data not shown).

Figure 2 shows the UV and radioactive profiles of a lipid extract from a red cell suspension pulsed with radioactive palmitate complexed to defatted BSA. The largest fraction of the radiolabeled fatty acid was recovered into PCn, though radioactivity was also recovered

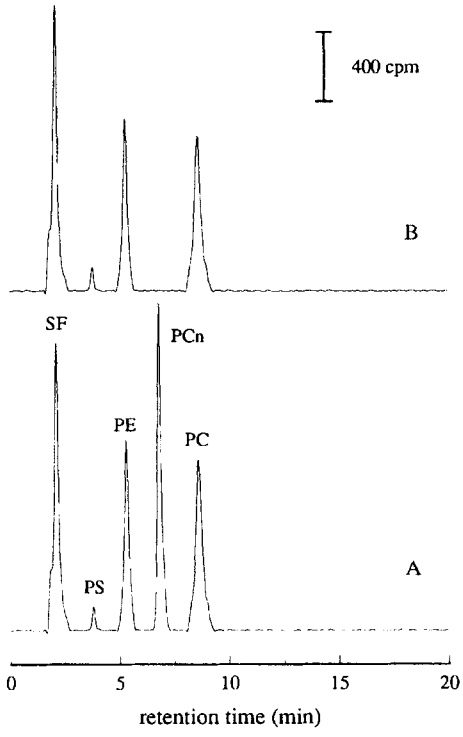

Fig. 3. Radioactive elution profiles of a lipid extract of either TDGA-treated or untreated red cells. (A) Control cells were processed as described in Fig. 2. (B) Red cells were pretreated with 15 μM TDGA for 60 min at 37°C. TDGA-treated cells were washed with defatted BSA twice, and reincubated with radioactive palmitate complexed to defatted BSA. After 60 min of incubation at 37°C, red cells were washed, extracted, and chromatographed as described in the Materials and Methods section; SF, solvent front.

into PC and PE. As expected, the UV profile shows only the major phospholipid classes, but not that of long-chain acylcarnitine. The apparent incorporation rates of the radioactive palmitate into PC, PE, and PCn calculated from the HPLC radioactive profile (3.5, 2.2, 2.0 pmol/h per μg P, respectively) were in good agreement with those calculated through the conventional TLC analysis of the same lipid extract (3.7, 2.1, 1.9 pmol/h per μg P, respectively). It should be noted that the lack of radioactivity in SM is in agreement with the concept that mature red cells do not reacylate such phospholipid (2). In other words, the radioactive profile may be considered complete within 10 min. Neutral lipids were eluted with the solvent front, where the majority of radioactivity was found in the free fatty acid fraction (5). The addition of radiolabeled PCn in intact red cell samples, immediately before lipid extraction, showed that radioactive PCn co-eluted with that produced by red cells pulsed with radioactive palmitate (data not shown). An additional proof that the HPLC radioactive profile of red cells incubated with radioactive palmitate contained PCn was obtained by treating red cells with TDGA, a specific CPT inhibitor (18). Previous work from our laboratory showed that the pretreatment of red cells with 15 μM TDGA for 60 min at 37°C resulted in a complete loss of CPT activity (5). Consequently, TDGA-treated red cells incubated with radioactive fatty acid were not capable of generating the palmitate ester of L-carnitine. The HPLC radioactive profile of a lipid extract from TDGA-treated red cells pulsed with radioactive palmitate showed the absence of a radioactive peak corresponding to the elution time of PCn (**Fig. 3**). TDGA treatment did not affect the reacylation of membrane phospholipids, though a significant inhibition of fatty acid incorporation was observed with radioactive oleate (5). Since a major difference between palmitate and oleate is the reacylation rate (the former is incorporated into membrane phospholipids at lower rates than the latter), we have proposed that with oleate a feedback inhibition of lysophospholipid acyl-CoA transferase by free CoA was more pronounced in CPT-inhibited red cells (5). The elution time of different long-chain acylcarnitine species (oleyl- and linoleyl-carnitine), generated by incubating red cells with the respective radioactive fatty acids complexed to defatted BSA, was the same of that found for PCn (**Table 1**).

DG, a reversible inhibitor of the glycolytic pathway, is often utilized in ATP-depletion studies. This compound allowed us to show that long-chain acylcarnitine production can be affected by the ATP content of red cells (5). To improve the characterization of red cell acyl-traffic occurring in the course of ATP depletion, we have carried out a pulse-chase study in control and DG-treated red cells. **Figure 4** shows the radioactivity recovered in red cell membrane PC, PE, and PCn during the chasing period. The system was pulsed with radioactive palmitate and chased with BSA fraction V (this fraction is rich in long-chain fatty acids) in the presence or in the absence of DG. As a result of radiodilution, the radioactivity present in membrane PC and PE of control cells decreased during the time of chase. However, a much lower radiodilution effect was observed in the ATP-depleted red cells. The lesser radiodilution effect found in PC and PE of the ATP-depleted cells cannot be easily explained in terms of differences in fatty acid turnover (i.e., deacylation–reacylation cycle). For example, if the deacylation rate were lower than that of reacylation in ATP-depleted cells, one would expect differences in the turnover during the pulse phase. However, ATP-depleted red cells are able to reacylate membrane phospholipids at physiological rates even when the ATP content is 26% that of control (19). Another possible explanation might be that some other source(s) of radioactive fatty acid is counteracting the radiodiluting effect exerted during the chasing period by cold fatty acids complexed to BSA. We have shown that ATP depletion increased the incorporation of radioactive palmitate into membrane phospholipids, when the reacylating substrate was PCn radiolabeled in the palmitate moiety. This observation led us to suggest that the decreased availability of acyl-CoA, due to the reduced amount of ATP, may shift the equilibrium of the CPT reaction toward acyl-CoA production. According to our prediction, even an endogenous pool of radioactive PCn, generated during the pulse phase, may feed the reacylation process of acyl units during the chase phase, in which a decrease of ATP levels may be further increased. As shown in Fig. 4, a greater reduction of radioactivity recovered into PCn in ATP-depleted cells than control was found. In addition, we have shown that the acyl traffic between long-chain acylcarnitine and phospholipids could be abolished by inhibiting CPT activity (14).

TABLE 1. Long-chain acylcarnitines: retention time and apparent fatty acid incorporation rate into carnitine

	Retention Time	Incorporation Rate
	min	pmol/h/μg *P*
Palmitoylcarnitine	6.9 ± 0.2	3.5 ± 0.4
Oleylcarnitine	6.8 ± 0.3	2.5 ± 0.4
Linoleylcarnitine;	6.9 ± 0.2	2.2 ± 0.3

Red cells were incubated with either radioactive palmitate, oleate, or linoleate complexed to defatted BSA for 120 min at 37°C. At the end of the incubation, red cells were washed, extracted, and chromatographed as described in the Materials and Methods section. TDGA-treated red cells were unable to generate radioactive long-chain acylcarnitines. Data are presented as the average ± SD of three different experiments.

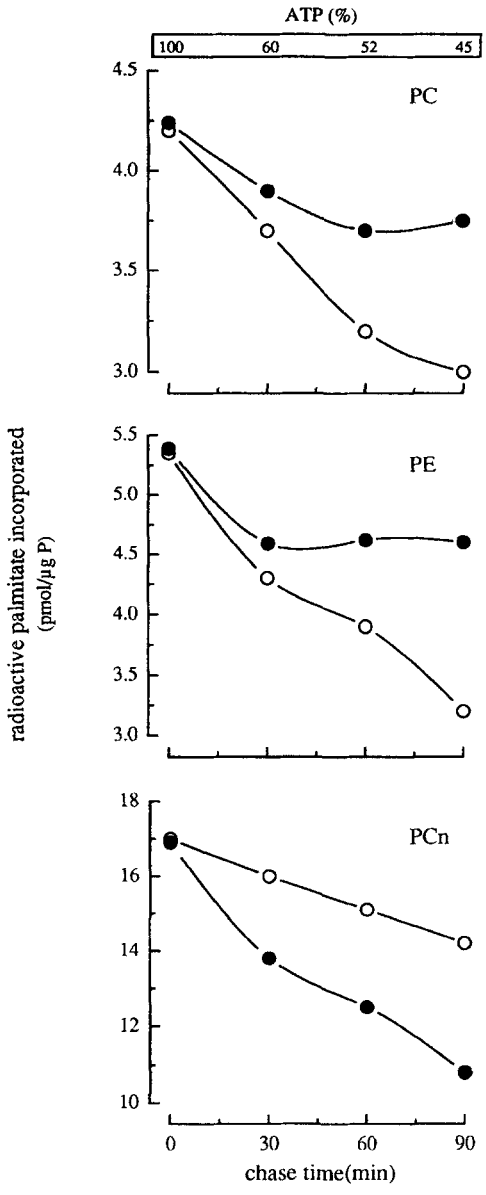

Fig. 4. Pulse-chase study in control and DG-treated red cells. Red cells were labeled (pulse phase) with radioactive fatty acids by incubating them for 4 h with radioactive palmitate complexed to defatted BSA. Incubations were ended by washing cells once with cold incubation buffer, three times with defatted BSA 1% (w/v) in incubation buffer, and finally once again with incubation buffer. Red cells were resuspended in the incubation buffer, and then reincubated (chase phase) with BSA fraction V (1.65 mg/ml) either in the presence or in absence of 1 mM DG. During the chasing period, glucose was present only in the control cell suspension. Aliquots were removed at fixed times for the measurement of radiolabeled fatty acid incorporation into membrane PC, PE, and PCn as described in Fig. 2. Residual ATP levels in DG-treated cells are expressed as percentage of control in the upper part of the figure. Open symbol, control cells; closed symbol, DG-treated cells. Values are the average of three experiments. The variation between experiments was not more than 8%.

The production of long-chain acylcarnitine in membrane phospholipid fatty acid turnover studies may be affected by various experimental conditions, which are used to mimic a particular pathophysiological state (5, 13). In this context, it is very important to resolve chromatographically the various radioactive components of a red cell lipid extract. Finally, the radioactivity recovered in the long-chain acylcarnitine contains essential information to define the acyl traffic scenario occurring in red cells.

We wish to thank Emma Mitchinson and Dr. Richard Porter for their comments and critical reading of the manuscript. This research was supported in part by Ministero dell'Universita e della Ricerca Scientifica e Tecnologica, and Sigma Tau S.p.A., Pomezia, Italy.

Manuscript received 28 September 1995 and in revised form 27 November 1995.

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