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Combined high-performance liquid chromatographic–continuous-flow fast atom bombardment mass spectrometric analysis of acylcoenzyme A compounds

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SUMMARY

A high-performance liquid chromatographic method for the analysis of coenzyme A thioesters which employs continuous-flow fast atom bombardment mass spectrometric detection is presented. The chromatographic system utilizes gradient elution with reversed-phase conditions using ammonium acetate–acetonitrile from both standard analytical (3.9 mm I.D.) and microbore (1 mm I.D.) columns. Applications to coenzyme A thioesters of various acyl group chain length (C_2–C_{18}) and functionality (-COOH, -OH, -C=C-) are described. The system is also applied to an in vitro enzyme reaction (crotonase) to directly follow the disappearance of substrate and appearance of product. The mass spectrometry of coenzyme A thioesters, their chromatographic behavior, system stability, and sensitivity of detection are discussed.

INTRODUCTION

The function of coenzyme A (Fig. 1) is to act as a carrier of acyl groups for enzymatic reactions in pathways of fatty acid and amino acid catabolism, fatty acid synthesis, pyruvate oxidation, and various biochemical acetylations [1]. A number of recognized metabolic diseases can be traced to specific enzyme deficiences in the catabolic pathways for fats and branched-chain amino acids, which result in the accumulation of toxic coenzyme A thioesters (acyl-CoA compounds) and other metabolites [2]. Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) [3], for example, results in the accumulation of me-

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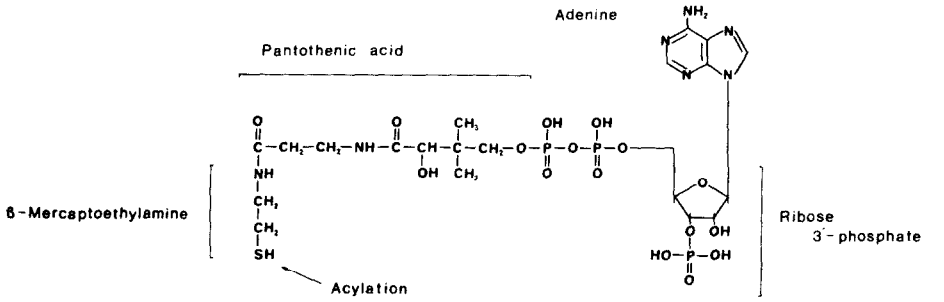


Fig. 1. Structure of coenzyme A and its thioester derivatives. Various subunits which make up the molecule are indicated.

dium-chain acyl-CoA compounds (hexanoyl- and octanoyl-CoA) which can produce symptoms of Reye's syndrome or result in sudden infant death syndrome (SIDS) [4].

In addition to their high polarity, acyl-CoA compounds are chemically and thermally unstable and are thus not amenable to analysis by techniques involving volatilization, such as gas chromatography or electron ionization mass spectrometry (MS). A number of methods for the separation and analysis of acyl-CoA compounds by high-performance liquid chromatography (HPLC) have been reported [5–13]. These, however, have employed UV detection which is relatively non-specific and thus cannot provide molecular mass or structural information for individual acyl-CoA compounds. Separation and analysis of several acyl-CoA compounds by combined HPLC–thermospray ionization mass spectrometry has been reported [14]. While structurally useful mass spectra were obtained for all compounds studied, no molecular mass information was observed owing to the relatively energetic nature of the thermospray process. In this study it was further reported that protonated molecular ions and structurally useful fragment ions were observed in the fast atom bombardment (FAB) mass spectra, obtained from direct insertion probe.

The recent introduction of the HPLC–continuous-flow (CF) FAB-MS interface [15,16] affords the opportunity for development of a detection system for acyl-CoA compounds with greatly enhanced specificity relative to UV.

This report describes the development and application of a HPLC–CF-FAB-MS system for acyl-CoA compounds of various chain length (C_2–C_{18}) and containing a variety of functional groups (–COOH, –OH, –C=C–). The system is evaluated with respect to chromatographic properties, stability, and sensitivity of detection.

EXPERIMENTAL

Reagents and materials

The acetonitrile for HPLC was UV grade (Burdick and Jackson, Muskegon, MI, U.S.A.). Aqueous mobile phases were composed of deionized, distilled

water, HPLC-grade ammonium acetate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and analytical-reagent-grade glycerol (Mallinckrodt, Paris, KY, U.S.A.). All mobile phase components were filtered through 0.45-μm Nylon 66 membranes (Alltech Assoc., Deerfield, IL, U.S.A.) and continuously sparged with helium throughout all HPLC experiments. Acyl-CoA compounds were obtained from Sigma (St. Louis, MO, U.S.A.) either as the free base or as the lithium salt. Crotonase (enoyl coenzyme A hydratase; EC 4.2.1.17) was obtained from Sigma as a crystallized and lyophilized powder from bovine liver.

Chromatographic system

For CF-FAB, two different instrumental configurations were employed with a standard pulse-damped HPLC solvent delivery system (Waters 600-MS; Waters Chromatography Division, Milford, MA, U.S.A.). The overall design is outlined in Fig. 2A. The first configuration incorporated an analytical column (Waters Nova-Pak C_{18}; 150 mm$\times$3.9 mm I.D., 4 μm particle size) with post-column flow splitting from 1.0 ml/min to approximately 10 μl/min, and the second a microbore column (Keystone Scientific Hypersil BDS; 250 mm$\times$1.0 mm I.D.; Bellefonte, PA, U.S.A.) with pre-column flow splitting from 1.0 ml/min to 50 μl/min and post-column splitting to 10 μl/min. Injections were from 1 to 20 μl on the analytical column with a Rheodyne 7125 (Cotati, CA, U.S.A) and 0.1 μl on the microbore column using a Valco CI4W (Houston, TX, U.S.A.).

The MS systems consisted of either a VG Trio-2 single quadrupole or VG Trio-3 triple quadrupole instrument (VG Masslab, Altrincham, U.K.) each of which incorporated a VG Masslab Dynamic–FAB probe. Each probe incorporated a fused-silica transfer line and conical stainless-steel tip (Fig. 2B).

HPLC–UV work using the Nova-Pak column was performed on a Waters high-pressure gradient system consisting of two Model 510 pumps, a Model 680 automated gradient controller, a Model 481 variable-wavelength UV detector (260 nm), and a Shimadzu C-R5A recording integrator (Kyoto, Japan).

Mobile phase conditions

Short- and medium-chain (C_2–C_8) acyl-CoA compounds were analyzed by both system configurations, each of which employed a dual solvent gradient (A: 0.2 *M* ammonium acetate–1.75% acetonitrile–2% glycerol; B: acetonitrile) with a two-step linear ramp (100% A; 2-min initial hold; to 90% A in 7 min; 1 min hold at 90% A; to 50% A in 9 min with a 10-min hold). Dicarboxylic acyl-CoA compounds were analyzed on the microbore system with the same solvents but a modified gradient (100% A; 2-min initial hold; to 90% A in 3 min; 1 min hold at 90% A; to 40% A in 4 min with a 5-min hold).

For the elution of the long-chain acyl-CoA compounds, solvent A was changed to 0.4 *M* ammonium acetate–2% glycerol. Analyses were performed on the

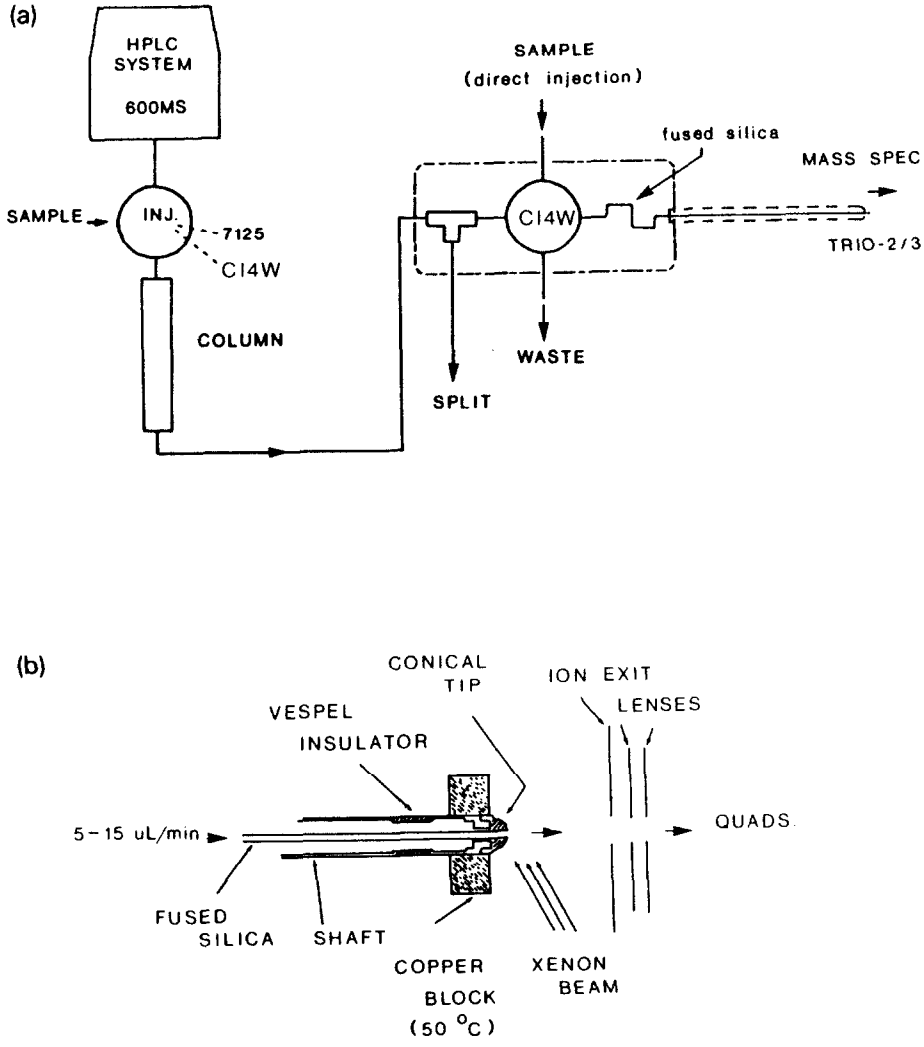


Fig. 2. (A) Schematic of the high-performance liquid chromatographic–continuous-flow fast atom bombardment (CF-FAB) mass spectrometric system. The CI4W injector mounted in the probe handle is optional and is by-passed during chromatography experiments. (B) Representation of the CF-FAB probe tip and ion source configuration.

Nova-Pak column with a three-step linear ramp (70% A; 5-min initial hold; to 60% A in 5 min; to 54% A in 9 min; to 38% A in 5 min with a 5-min hold).

Fast atom bombardment mass spectrometry

The FAB sources of both the Trio-2 and Trio-3 mass spectrometers incorporated saddle-field ion guns (Ion Tech, Teddington, U.K.) which generated

xenon atom beams of 5–6 keV kinetic energy at 1–2 mA current. In order to obtain a stable ion beam and good chromatographic peak shape, mobile phase flow to the probe tip was adjusted to obtain a source backing pressure of between 0.1 and 0.2 mbar (5–10 μl/min) at a source temperature of 50°C. The mass spectrometers were either scanned linearly over limited mass ranges (*m/z* 800–900 for the analysis of short- and medium-chain acyl-CoAs, see Fig. 3; *m/z* 100–900 for pure acetyl-CoA, see Fig. 4) or operated in the selected-ion monitoring (SIM) mode (see Figs. 5 and 7).

Enzymatic reaction

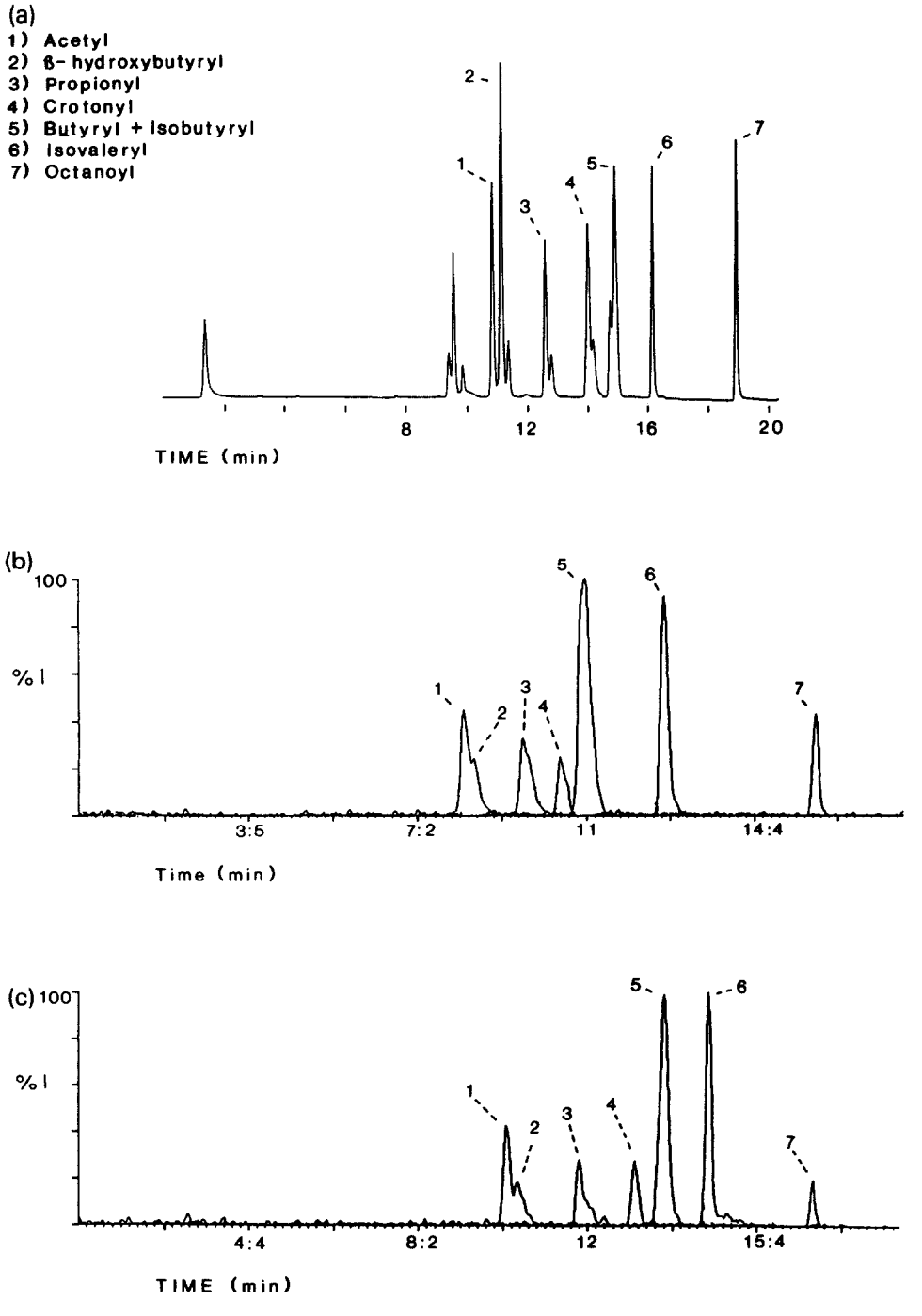
The reaction was started by dissolving 479 nmol crotonyl-CoA (*trans*-2-butenoyl coenzyme A) in 500 μl of 0.2 *M* ammonium acetate (pH 7.0), adding 50 μg of glycerol, then adding 0.048 U of crotonase. At selected time intervals, 10-μl aliquots of this reaction mixture were injected directly onto the microbore LC–MS system operating in isocratic mode [0.2 *M* ammonium acetate containing 1.75% acetonitrile and 2% glycerol–acetonitrile (60:40)]. The Trio-2 quadrupole mass spectrometer was operated in the SIM mode, alternating between *m/z* 836 and 854, the MH^+ ions of the substrate, and product of the enzyme reaction.

RESULTS AND DISCUSSION

Chromatographic and mass spectral performance

Fig. 3 compares the chromatographic analysis of a series of short- and medium-chain acyl-CoA compounds by HPLC–UV on the analytical column (a), HPLC–CF-FAB-MS on the analytical column (b) and HPLC–CF-FAB-MS on the microbore column (c). Chromatograms b and c are computer-reconstructed extracted ion current profiles of the corresponding protonated molecular ions (MH^+) for individual acyl-CoA compounds. Each chromatogram is normalized to the most intense MH^+ signal observed. The chromatographic performance of the two HPLC–MS systems is comparable to HPLC–UV and there is no evidence of excessive peak broadening or tailing due to effects at the CF-FAB probe tip or from the presence of glycerol in the mobile phase which acts as an ionization matrix in FAB [17,18].

Molecular mass information and structurally useful fragmentation were observed for each acyl-CoA compound. For example, the complete CF-FAB mass spectrum for acetyl-CoA derived from a similar HPLC–MS experiment is shown in Fig. 4. This spectrum was derived by computer averaging across the LC peak and then subtracting an averaged background spectrum to remove signals from glycerol and other mobile phase cluster ions which are typically observed in FAB [17,18]. Fig. 4 also indicates the characteristic fragmentation processes observed in the CF-FAB spectrum of acetyl-CoA. Cleavages at the phosphorus–oxygen bonds producing ions at *m/z* 303, 330, 428, and 508 are significant.


(a)
1) Acetyl
2) ß-hydroxybutyryl
3) Propionyl
4) Crotonyl
5) Butyryl + Isobutyryl
6) Isovaleryl
7) Octanoyl
8
12
16
20
TIME (min)
(b)
100
%I
3:5
7:2
11
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(c) 100
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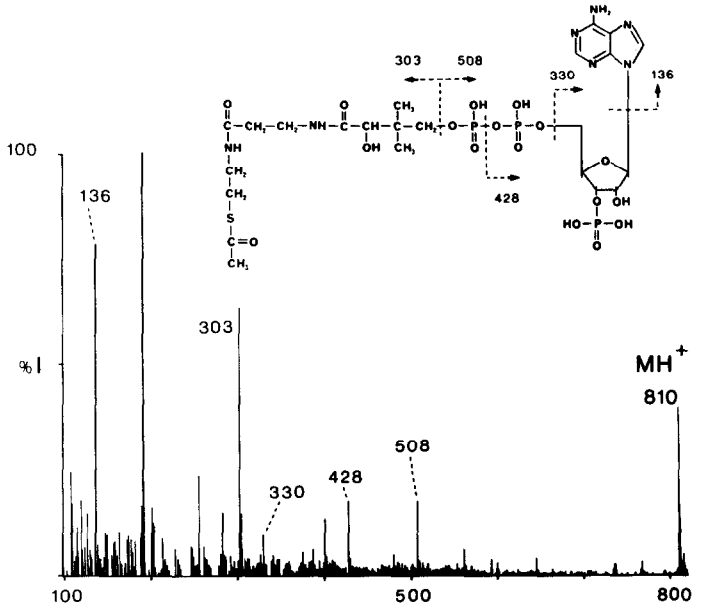

Fig. 4. CF-FAB mass spectrum of acetyl-CoA with likely origin of major fragment ions.

These processes were observed for all acyl-CoA compounds studied. The *m/z* 303 ion is of particular significance because it preserves the identity of the acyl group and therefore shifts in mass as the acyl group changes. Cleavage at the adenine–ribose bond produces a very stable positive ion at *m/z* 136 and is a dominant process observed for all acyl-CoA compounds. The spectra are comparable to those reported previously for acyl-CoA compounds obtained by direct insertion probe FAB on a double-focusing magnetic sector mass spectrometer [14].

The HPLC–CF-FAB-MS analyses of a mixture of long-chain acyl-CoA compounds is shown in Fig. 5A and of dicarboxylic acyl-CoA compounds in Fig. 5B. Each of these analyses was accomplished with the mass spectrometer operated in SIM mode on the appropriate MH^+ ions. The chromatographic traces represent summed and normalized total ion current profiles. Elution of the long-chain acyl-CoA compounds required a significant increase in mobile phase ionic strength relative to the short- and medium-chain species. However, even

Fig. 3. Chromatograms resulting from the analysis of a series of short- and medium-chain acyl-CoA compounds by: (a) HPLC-UV on a standard analytical (3.9 mm I.D.) column (260 nm; 30 nmol of each acyl-CoA injected); (b) HPLC–CF-FAB-MS on the same column (30 nmol of each injected); and (c) HPLC–CF-FAB-MS on a microbore (1 mm I.D.) column (0.5 nmol of each injected). The HPLC–MS traces are summed extracted ion current profiles (MH^+ ions) derived from scanned data. (MH^+: 1, 810; 2, 854; 3, 824; 4, 836; 5, 838; 6, 852; 7, 894.)

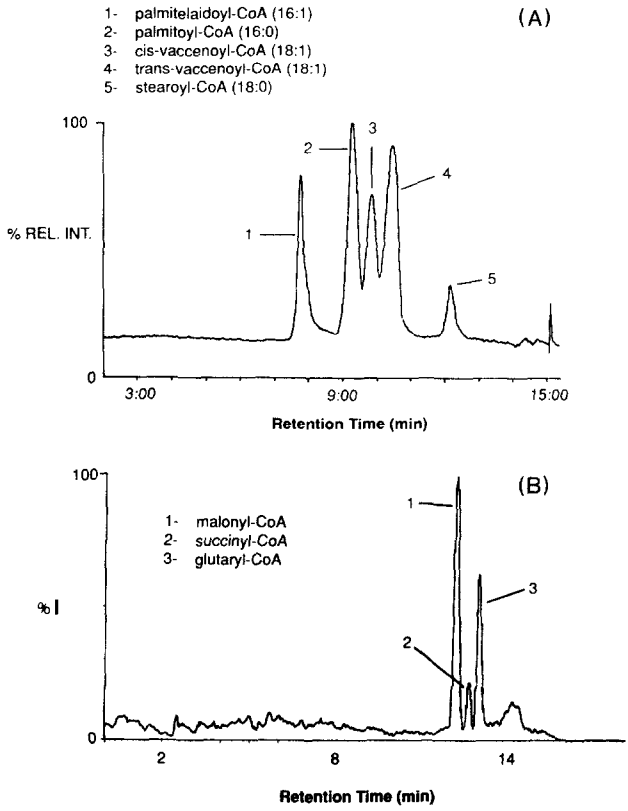

Fig. 5. (A) Summed ion chromatogram (MH^+ ions) resulting from the HPLC-CF-FAB-MS analysis by SIM of a series of long-chain acyl-CoA compounds (6–10 nmol of each injected onto the analytical column). (MH^+: 1, 1004; 2, 1006; 3, 1032; 4, 1032; 5, 1034.) (B) Summed ion chromatogram (MH^+ ions) resulting from the HPLC-CF-FAB-MS analysis by SIM of a series of dicarboxylic acyl-CoA compounds (6–10 nmol of each injected onto the microbore column). (MH^+: 1, 854; 2, 868; 3, 882.)

with these more optimum mobile phase conditions, relatively broad chromatographic peaks were observed. It is significant, however, that separation and identification of geometric isomers (*cis*- and *trans*-vaccenoyl-CoA) was possible.

Detection limits

For any MS analysis the detection limit for a given analyte depends on the criteria established for detection and on the particular acquisition mode employed. Fig. 6 shows a typical example for the detection of acetyl- and octanoyl-CoA from the microbore system. These are so-called "profile" spectra obtained by summing 10–20 scans across the MH^+ region of the eluting LC peak (similar to multi-channel analyzer acquisition). Using this particular acquisition

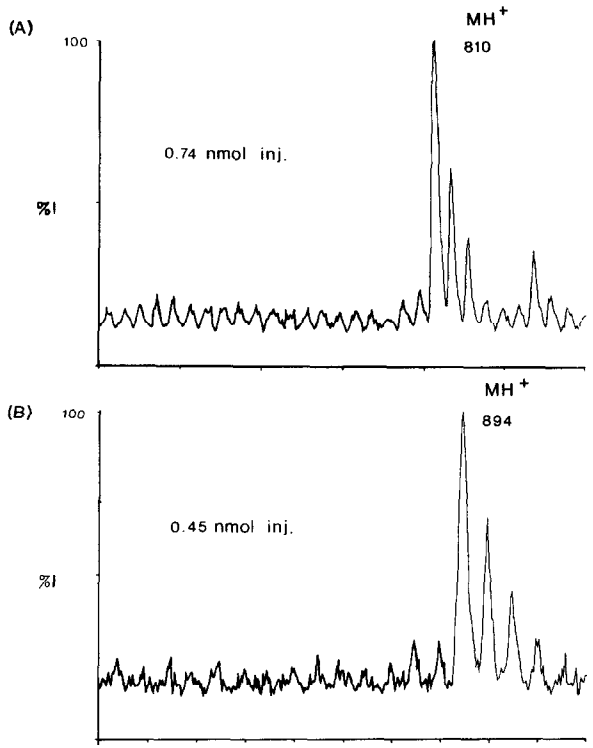

Fig. 6. Profile spectra (MH^+ region) of (A) acetyl-CoA and (B) octanoyl-CoA obtained by summing 10–20 scans across the eluting LC peak. Quantities listed refer to amounts injected onto the Hypersil BDS column

mode with unit mass resolution the detection of sub-nanomole quantities of material injected onto the column was achieved. Accounting for the post-column stream-splitting, the actual quantity reaching the ion-source was in the range 50–100 pmol. This is already within the range of the anticipated biological applications of the technique. Detection limits could be further reduced as required by using SIM of either the MH^+ or more abundant fragments ions (*m/z* 387 for octanoyl-CoA, for example). The use of packed microcapillary [19] or open-tubular [20] fused-silica columns which, because of their low operating flow-rates, do not require effluent splitting into the mass spectrometer would improve detection limits still further, if necessary.

System stability and robustness

Instability in this context refers specifically to ion beam instability caused by pressure fluctuations in the CF-FAB source and/or xenon atom beam instability. These phenomena most often result from imbalances in the flow of mobile phase to the CF-FAB probe tip, in the spreading of eluent across the

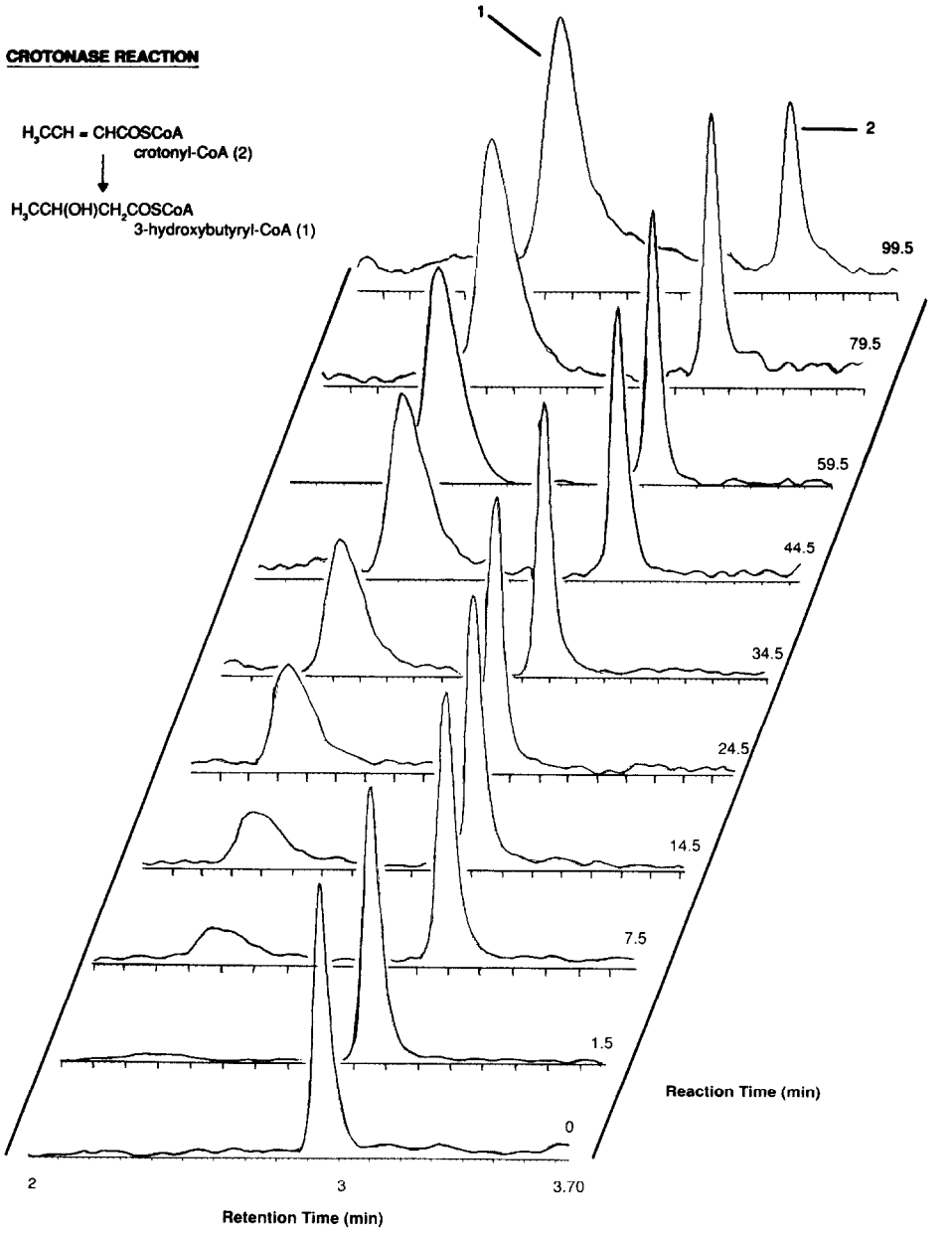

Fig. 7. Combined SIM chromatograms (MH^+ ions) obtained at different time points during the crotonase-catalyzed hydration of crotonyl-CoA to produce 3-hydroxybutyryl-CoA.

conical tip surface, and in the volatilization/ionization process from the tip. The parameters affecting these steady-state conditions are flow-rate, mobile phase composition, source temperature, and FAB ion gun conditions. The duration of stable system operation, or robustness, is further affected by the accumulation of involatile mobile phase and sample components at the probe tip and unstable flow in the fused-silica transfer line (Fig. 2).

Observations regarding stability and robustness may be summarized as follows:

1. Flow-rates to the probe tip which produced source backing pressures of greater than 0.2 mbar resulted in significant instability.
2. Loss of pressure stability was observed as the mobile phase composition changed to greater than 60% acetonitrile, which reduced the glycerol at the probe tip to 0.5–1%.
3. Source temperatures below 45 °C resulted in pressure instability and chromatographic peak broadening.
4. Operation of the saddle-field ion gun at lower voltage (5–6 keV) than is typical for static FAB ($\sim$8 keV) improved stability.
5. Approximately 4–6 h of stable operation was achieved routinely with this system, even when high concentrations of buffer (0.4 *M* ammonium acetate) and other involatile components (0.05 *M* K_2HPO_4 in some preliminary experiments) were added to the mobile phase.
6. No irregularities or interruptions of flow along the fused-silica transfer line were observed.

Application: in vitro enzyme reaction

Fig. 7 shows the progress of the crotonase reaction, in which the double bond of the acyl group in crotonyl-CoA is hydrated to form 3-hydroxybutyryl-CoA, as followed by HPLC–CF-FAB-MS. Each chromatogram was taken at a specific time point during the reaction and represents the sum of the ion current from *m/z* 836 (MH^+ crotonyl-CoA) and *m/z* 854 (MH^+ 3-hydroxybutyryl-CoA). The system was stable throughout the course of the experiment with each analysis requiring less than 4 min. Injection of macromolecular material (i.e. crotonase) had no effect on system stability.

This experiment serves to establish the principle of following enzyme catalyzed reactions of acyl-CoA compounds by monitoring changes in substrate and product concentration with high specificity. Kinetic parameters could be obtained by quantifying individual species using appropriate internal standards to calibrate instrument response, essentially as described by Caprioli and Smith [21,22] in their studies on proteolytic enzymes.

CONCLUSIONS

This research has demonstrated that the HPLC–CF-FAB-MS technique for the analysis of acyl-CoA compounds is versatile, stable, and robust, and has

the advantage of compound specificity relative to conventional HPLC–UV methods. Acyl-CoA compounds of various chain length (C_2–C_{18}) and containing a variety of groups could be separated and detected in the picomole range. In all cases, molecular mass information as well as structurally useful fragmentation were observed. The system was tolerant of solvent gradients, high-ionic-strength mobile phases, and involatile mobile phase components. The presence of glycerol in the mobile phase had no observable effect on chromatographic performance.

Application of the HPLC–CF-FAB-MS technique is indicated for studies of enzymes, identification of acyl-CoA compounds from biological matrices, and characterization of novel synthetic acyl-CoA compounds.

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