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Determination of tricarboxylic acid cycle acids and other related substances in cultured mammalian cells by gradient ion-exchange chromatography with suppressed conductivity detection

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Abstract

An ion-exchange chromatography method was established for simultaneously analyzing the tricarboxylic acid (TCA) cycle acids and other related substances in cultured mammalian cells, including citrate, *cis*-aconitate, isocitrate, α-ketoglutarate, succinate, malate, fumarate, oxaloacetate, *trans*-aconitate, phosphate, lactate and pyruvate. A Dionex 600 ion chromatograph with an ion suppressor and a conductivity detector, and an IonPac AS11-HC analytical column were employed. An NaOH gradient elution containing 13.5% methanol contributed to sufficient separation of target substances. The stability of carboxylic acids was investigated and oxaloacetate was found to be extremely unstable especially at pH 3. TCA cycle acids and other related substances in Chinese hamster ovary (CHO) cells were separated completely, and lactate, malate, phosphate, citrate and *cis*-aconitate were quantified due to their higher concentrations. In the quantification of the five substances, detection limits ($S/N=3$) ranged from 0.12 to 0.48 μ*M*, the correlation coefficients from 0.9982 to 1.0000 in their linear ranges of concentration, and the recoveries from 87 to 95%. The metabolic status of CHO cells was analyzed on the basis of the intracellular concentrations of TCA cycle acids.

Keywords: Tricarboxylic acid cycle; Cells; Carboxylic acids

1. Introduction

Recently mammalian cells, such as Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells and hybridomas, have been widely used in the biotechnological production of vaccines, diagnostics and therapeutics. The process optimization of mammalian cell culture extensively relies on the knowledge on the metabolism of cultured mammalian cells [1].

The tricarboxylic acid (TCA) cycle is the most important metabolic pathway for energy production that includes a series of oxidation–reduction reactions from pyruvate to carbon dioxide and water. In the TCA cycle, some organic acids such as citrate, *cis*-aconitate, isocitrate, α-ketoglutarate, succinate, malate, fumarate, and oxaloacetate are successively involved. Lactate, pyruvate and phosphate are the other important substances in the metabolism of mammalian cells. Information of the physiological status or metabolic flux shift under different nutrient

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conditions can be obtained by determining these substance concentrations in cultured mammalian cells.

However, some limitations in the available methods bring out an obstacle to the determination of these substances and the analysis of metabolic pathway in mammalian cells. Organic acids in mammalian cells are commonly determined by enzymatic analysis [2]. Each organic acid must be determined with an individual enzyme-catalyzed reaction system, which makes it impossible to analyze these organic acids simultaneously. Furthermore, this method is limited by strong interference because enzymes are easily affected by temperature, pH value of sample, and sample constituents. The most serious problem is that some adverse enzyme-catalyzed reactions exist when one organic acid is determined with a special enzyme reaction system, which is often unavoidable and interfering. Although isotope tracing can be used for the TCA cycle flux estimates by the analysis of isotope-marked glucose, glutamine or carbon dioxide, the concentrations of organic acids are still undetectable even with this method [3,4]. A nuclear magnetic resonance (NMR) method [5] has been adopted for the accurate quantification of phosphate in cells, instead of the colorimetric method [6]. Therefore, it is still quite difficult and time-consuming work to determine TCA cycle acids and other related substances in mammalian cells by the above methods.

Chromatography techniques have been applied to the analysis of organic acids in different types of samples [7–9,11,15–17]. Some organic acids in wine [7] or fruit juice [8] can be separated by reversed-phase high-performance liquid chromatography (HPLC) with either ultraviolet (UV) detection or refractive index (RI) detection, but these detection techniques lack specificity for carboxylic acids and are sensitive to many interfering compounds such as sugars and phenolic compounds. Gas chromatography–mass spectrometry (GC–MS) has been also applied to the separation of organic acids in human urine [9], but derivatization of the acids to either their trimethylsilyl or oxime-trimethylsilyl derivatives makes the keto acids unstable [10], preventing the simultaneous analysis of TCA cycle acids. Ion-exclusion chromatography by isocratic elution with UV detection has been applied to simultaneously separate TCA cycle acids in insect hemolymph [11], but it cannot provide a complete chromatogram of the homologous series of weak organic acids and inorganic anions [12]. Additionally, compared with samples of fruit juice, wine, human urine, or animal tissues, the concentrations of organic acids and inorganic anions in mammalian cells are lower. So the development of a more effective and sensitive chromatography technique is imperative.

Recently ion chromatography (IC) has made great progress, especially in the suppressed conductivity detection mode, which provides greater sensitivity than UV or RI detection [13,14]. Gradient ion-exchange chromatography has been considered a good substitute for ion-exclusion chromatography because of an excellent separation of a wide variety organic and inorganic acids [12]. So, gradient ion-exchange chromatography with suppressed conductivity detection has become an effective chromatography technique for sufficient separation of a wide variety of organic and inorganic substances, and it has been successfully applied to the separation of organic acids in some samples [15–17]. However, to our knowledge there has been no literature on the application of ion-exchange chromatography with suppressed conductivity detection for the determination of TCA cycle acids and other related substances in cultured mammalian cells. A new method has been established to simultaneously separate and determine these substances in this work.

2. Experimental

2.1. Instrumentation

A Dionex 600 IC system (Dionex, Sunnyvale, CA, USA) was used comprising a GP 50 gradient pump, an ED 50 conductivity detector and an LC20 chromatography enclosure. A PeakNet 6.4 chromatography workstation was used for instrument control, data collection, and data analysis. A Dionex ATC-3 trap column (24 mm×9 mm) was located between the pump and the injection valve to remove trace amounts of dissolved carbon dioxide and anionic contaminants in the eluents. A Dionex ASRS-ULTRA anion self-regenerating suppressor was operated in the auto-suppression external water mode

with 30 m*M* sulfuric acid solution and 5~8 ml/min flow-rate. Separation was carried out on an IonPac AS11-HC analytical column and an AG11-HC guard column. A sample loop of 25 μl was used for analysis.

2.2. Eluents and gradient conditions

Deionized water with a specific resistance of 18.2 MΩ cm from a Milli-Q Plus reverse osmosis water system (Millipore, Bedford Park, MA, USA) was used to prepare all the solutions. Sodium hydroxide solution (50%, GR for analysis), sulfuric acid (95~97%, GR for analysis) and methanol (gradient grade) were obtained from Merck (Darmstadt, Germany). Gradient elution with a 1.4 ml/min flow-rate was used with the following mobile phases: eluent A: deionized water; eluent B: 5.0 m*M* NaOH; eluent C: 100 m*M* NaOH; eluent D: 90% aqueous methanol. Good care must be taken to avoid the introduction of carbon dioxide from air into the aliquot of the 50% NaOH bottle or the deionized water for making the eluents. The gradient conditions are given in Table 1.

2.3. Cell line, cell culture and sample preparation

A recombinant CHO cell line that produces erythropoietin (EPO) was provided by Shandong Donge Ejiao. CHO cells were routinely cultivated in 25 cm^2 T-flasks with Dulbecco's Modified Eagle's medium (DMEM) containing high glucose and glutamine (GibcoBRL, Grand Island, NY, USA) and 5% fetal bovine serum (FBS, GibcoBRL) in a 37 °C incubator equilibrated with 5% CO_2. For determination of intracellular substances, CHO cells at mid-exponential growth phase were harvested and then inoculated in 120 cm^2 T-flasks in 30 ml DMEM without glucose or glutamine (Sigma, St. Louis, MO, USA) supplemented with 4 m*M* glutamine (Acros, NJ, USA), 10 m*M* glucose (GibcoBRL) and 5% FBS at an initial viable density of $2.5 \cdot 10^5$ cells/ml.

After CHO cells were cultivated for 24 h, medium was removed and cells were washed by 0.9% sodium chloride twice. About $1 \cdot 10^8$ viable cells were collected by trypsinization with 0.025% (w/v) trypsin (GibcoBRL) in phosphate buffer solution and centrifuged at 1200 *g* for 10 min. After decanting the supernatant, 10 ml of 0.9% sodium chloride was added and cells were gently resuspended with a pipette and then collected by centrifugation at 1200 *g* for 10 min. Subsequent removal of supernatant was followed by addition of 1 ml of 0.3 m*M* perchloric acid as extractant, the reaction mixture was kept on ice for 10 min. After centrifugation at 12 000 *g* for

Table 1
Gradient conditions

Time (min)	A (%)	B (%)	C (%)	D (%)	Curve	Comments
Equilibration						
0	35	50	0	15	5[a]	Start reading
7.0	35	50	0	15	5	Sample injection
Analysis						
7.1	35	50	0	15	5	
15.0	35	45	5	15	5	
25.0	30	40	15	15	5	
35.0	20	35	30	15	5	
45.0	5	35	45	15	5	
55.0	5	35	45	15	5	End reading
Column washing						
55.1	5	35	45	15	5	
65.0	20	35	45	0	5	
70.0	50	50	0	0	5	
75.0	35	50	0	15	5	

[a] The elution gradient follows a linear change between every two time points when ''curve 5'' is used.

10 min, supernatant was collected and neutralized by 0.6 m*M* KOH for 5 min. After centrifugation at 12 000 *g* for 10 min again, supernatant was collected and frozen at −20 °C, and the determination by IC was carried out within 24 h. It is critical that all reagents were kept on ice (below 6 °C) at all times and all containers including centrifuge tubes, pipettes and tips were thoroughly cleaned and presoaked in deionized water for at least 24 h.

2.4. Cell counting and concentration calculation of intracellular TCA cycle acids and other related substances

Cells were counted with a hemocytometer. The average diameter of an individual CHO cell is taken to 14 μm, which gives a single cell volume of $1.44 \cdot 10^{-9}$ ml [18]. Intracellular substance concentrations were calculated by the harvested cell number and CHO cell volume.

2.5. Standard solution

All organic acids for standard solution were purchased from Sigma. A standard solution, containing 0.1 m*M* lactate, 0.1 m*M* pyruvate, 0.13 m*M* oxaloacetate, 0.1 m*M* succinate, 0.1 m*M* malate, 0.1 m*M* α-ketoglutarate, 0.1 m*M* fumarate, 0.1 m*M* citrate, 0.1 m*M* isocitrate, 0.1 m*M* *cis*-aconitate and 0.04 m*M* phosphate, was used to measure retention time and identify the substances in samples. Another standard solution, containing 0.1 m*M* lactate, 0.05 m*M* malate, 1.0 m*M* phosphate, 0.05 m*M* citrate, 0.05 m*M* *cis*-aconitate, was used for the preparation of calibration standard solutions by diluting to the expected concentration range of target substances. All the standard solutions were prepared freshly each time and adjusted to pH 7 with 0.2 m*M* NaOH because of the instability of some acids, especially oxaloacetate.

3. Results and discussion

3.1. Chromatographic procedure

Fig. 1 shows the chromatogram of standard solution using an NaOH gradient elution. *trans*-Aconitate is identified because its peak was also detected when *cis*-aconitate was injected alone and its relative position to *cis*-aconitate has been reported [12,19]. Table 2 shows the retention time and reproducibility of TCA cycle acids and other related substances. In the investigation of reproducibility, a fivefold dilu-

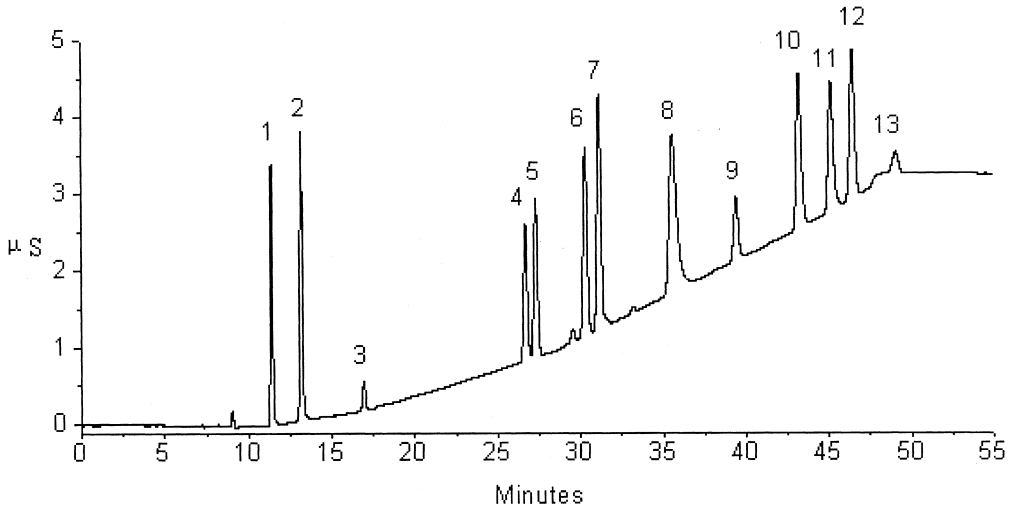

Fig. 1. The chromatogram of standard solution using an NaOH gradient elution. Peaks and concentrations: 1=lactate (0.1 m*M*), 2=pyruvate (0.1 m*M*), 3=chloride, 4=succinate (0.1 m*M*), 5=malate (0.1 m*M*), 6=α-ketoglutarate (0.1 m*M*), 7=fumarate (0.1 m*M*), 8=oxaloacetate (0.13 m*M*), 9=phosphate (0.04 m*M*), 10=citrate (0.1 m*M*), 11=isocitrate (0.1 m*M*), 12=*cis*-aconitate (0.1 m*M*), 13=*trans*-aconitate.

Table 2
Retention time and reproducibility of TCA cycle acids and other related substances

Analyte	Average retention time (min)	RSD (%) (n=6)
Lactate	11.37	0.34
Pyruvate	13.21	0.32
Succinate	26.68	0.45
Malate	27.29	0.36
α-Ketoglutarate	30.29	0.27
Fumarate	31.14	0.26
Oxaloacetate	35.62	0.57
Phosphate	39.41	0.33
Citrate	43.19	0.32
Isocitrate	45.11	0.30
cis-Aconitate	46.41	0.28
trans-Aconitate	49.04	0.26

tion of the standard solution was determined six times consecutively. The reproducibility, expressed as relative standard deviation (RSD), ranged from 0.26 to 0.57%. Some modifications have been made for the successful separation of target substances using an NaOH gradient elution. Firstly, the addition of methanol to the eluents contributes to a sufficiently good separation of succinate and malate because succinate will co-elute with malate when eluents contain no organic solvents. Methanol can mediate ion-exchange selectivity by changing the solvation of ion, the ability of the ion to shed the surrounding solvent molecules and the relative affinities of the analyte and eluent ions for the ion-exchange sites, which results in a longer retention time and better resolution of closely eluting ions [20,21]. After several tests with different methanol concentrations, a concentration of 13.5% was selected, where good resolution could be obtained and target substances suffered no interference from unknown substances due to the retention time extension. Secondly, perchlonic acid was selected as a satisfactory extractant of organic acids because perchlonic acid elutes last at about 58 min owing to its strong affinities for ion-exchange sites and avoids interfering with all the other peaks. Column washing was carried out for 20 min under the conditions in Table 1. In order to elute perchlonic acid completely and avoid its interference with the next analysis, the concentration of NaOH was kept at 46.75 m*M* from 55 to 65 min. The concentration of methanol was decreased to zero from 55 to 65 min and then kept at zero for 5 min, which minimized the deleterious impact on suppression by the electrolytic oxidization of methanol [20]. Then the elution was gradually returned to the initial status from 70 to 75 min.

3.2. Stability of TCA cycle acids and other related acids

It is known that some carboxylic acids are not stable in aqueous solution, so the stability of target acids is of great importance for analyte quantification and sample preparation. Table 3 shows the measured concentrations of target acids at different pH values and time when kept at 4 °C. The different acid solutions were prepared separately, adjusted to different pH values, and then stored at 4 °C for analysis.

Table 3
The measured concentrations of acids at different pH values and time when kept at 4 °C (RSD[a]<2%)

Analyte (m*M*)	0 h	pH 3		pH 7		pH 10	
		48 h	96 h	48 h	96 h	48 h	96 h
Lactate	0.600	0.598	0.586	0.595	0.581	0.587	0.583
Pyruvate	0.600	0.588	0.581	0.575	0.583	0.583	0.575
Succinate	0.600	0.595	0.590	0.594	0.584	0.595	0.587
Malate	0.600	0.595	0.571	0.595	0.575	0.601	0.583
α-Ketoglutarate	0.600	0.574	0.565	0.577	0.557	0.541	0.510
Fumarate	0.600	0.595	0.574	0.594	0.571	0.601	0.583
Oxaloacetate	0.600	0.078	0	0.390	0.276	0.384	0.258
Citrate	0.600	0.584	0.591	0.594	0.602	0.597	0.584
Isocitrate	0.600	0.596	0.601	0.596	0.585	0.602	0.583
cis-Aconitate	0.600	0.583	0.547	0.585	0.565	0.575	0.540

[a] The RSDs were calculated from six consecutive determinations of a standard solution.

The concentrations of lactate, pyruvate, succinate, malate, fumarate, citrate and isocitrate changed little in 96 h at different pH values when kept at 4 °C. The concentration of α-ketoglutarate decreased by 15% in 96 h at pH 10, but changed little at pH 3 or pH 7. The concentration of *cis*-aconitate decreased a lot owing to existing mutual transformation between *cis*-aconitate and *trans*-aconitate. Oxaloacetate is extremely unstable especially at pH 3 because it is easily transformed to pyruvate through a keto enol shift in aqueous solution [11]. In view of the instability of these acids, the standard solution must be prepared freshly each time and adjusted to pH 7, and the sample must be also adjusted to pH 7 finally and determined by IC within 24 h when kept at −20 °C.

3.3. Analysis of TCA cycle acids and other related substances in CHO cells

Fig. 2 shows the chromatogram of TCA cycle acids and other related substances in CHO cells using an NaOH gradient elution. The target substances were identified by both their retention times and standard addition of the respective components. Although there existed some unknown substances corresponding with unidentified peaks, the sufficiently long elution time contributed to good separation of target substances in CHO cells and prevented interference by these unknown substances. Lactate, malate, citrate, phosphate and *cis*-aconitate had higher concentrations than the other substances in mammalian cells and can be quantified accurately. The linear range, correlation coefficients of calibration curve, detection limits, recoveries and intracellular concentrations are shown in Table 4. The calibration curve of each substance was linear and the correlation coefficients ranged from 0.9982 to 1.0000 in the linear ranges of concentration listed in Table 4. When the recovery was investigated, sufficient CHO cells were collected and then divided into six equal cell amounts in six tubes. Three tubes were used to determine the concentrations of substances in cells, which are the averages of the three determinations. Then the solution containing lactate, malate, citrate, phosphate and *cis*-aconitate, whose concentrations were similar to determined amounts in CHO cells, was added to the collected cells in the other three tubes. After the cells were treated as for sample preparation, the cell extracts were determined by IC. The recovery of each substance was calculated from the determined amount minus the amount in CHO cells divided by the added amount and multiplying by 100%.

These results provided some valuable information about the metabolism in CHO cells. Lactate, with a

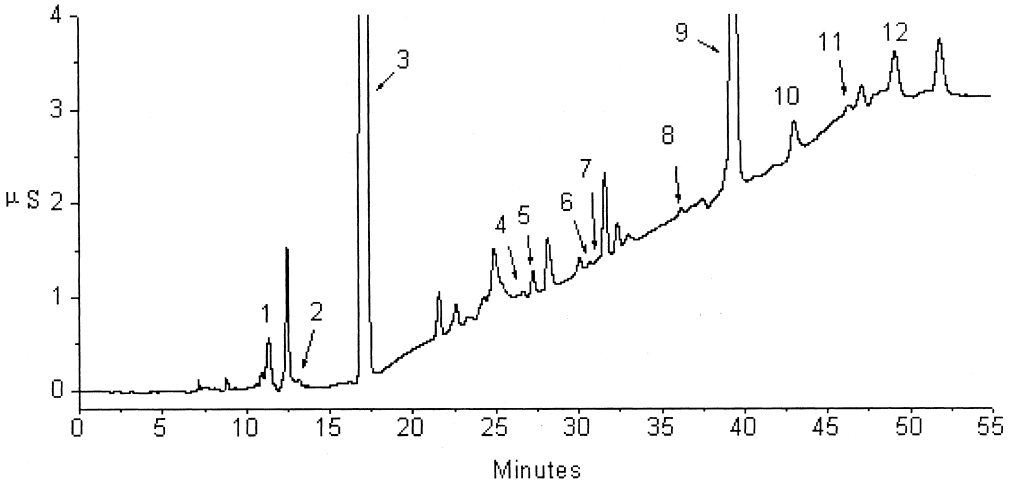

Fig. 2. The chromatogram of TCA cycle acids and other related substances in CHO cells using an NaOH gradient elution. Peaks: 1=lactate, 2=pyruvate, 3=chloride, 4=succinate, 5=malate, 6=α-ketoglutarate, 7=fumarate, 8=oxaloacetate, 9=phosphate, 10=citrate, 11=*cis*-aconitate, 12=*trans*-aconitate.

Table 4
Linear ranges, correlation coefficients of calibration curve, detection limits, recoveries and intracellular concentrations of some TCA cycle acids and other related substances

Analyte	Linear range (μM)	r^2 of calibration curve	Detection limit ($S/N=3$) (μM)	Recovery (%) ±RSD (%)	Intracellular concentration (mM) (χ^a ±SD)
Lactate	2~100	0.9998	0.29	90±6.2	0.52±0.08
Malate	4~50	1.0000	0.40	87±7.8	0.35±0.05
Phosphate	20~1000	0.9999	0.12	95±4.7	16.3±1.10
Citrate	4~50	0.9982	0.48	94±7.5	0.23±0.04
cis-Aconitate	2~50	0.9997	0.34	91±6.5	0.11±0.02

[a] χ represents the intracellular concentration which is calculated from the determined amount divided by the total cell volume.

high intracellular concentration, is the main byproduct of glucose metabolism in mammalian cell cultures [22]. Glutaminase is the key enzyme of glutamine metabolism in mammalian cells, and phosphate is a regulator of phosphate-activated glutaminase. The accurate determination of phosphate by IC will be a promising alternative method to NMR determination [5] in the study of the effect of phosphate on mammalian cell metabolism. The concentrations of malate, citrate and *cis*-aconitate are distinctively higher than the other acids in the TCA cycle, which shows the large accumulation of these acids in CHO cells. Furthermore, it is strong direct evidence of the hypotheses that a blockage exists in the TCA cycle of abnormal mammalian cells [23,24]. No detected peak of isocitrate indicates that there is lower concentration of isocitrate or no isocitrate in CHO cells, which further supports the same hypotheses that the TCA cycle is blocked at the point of citrate.

4. Conclusions

The determination of TCA cycle acids and other related substances in cultured mammalian cells by ion-exchange chromatography with suppressed conductivity detection is a new challenging work. The target substances in CHO cells can be completely separated and the interference by other unknown substances is ruled out with this method. Further efforts need to be made to quantify all the organic acids and other substances in mammalian cells accurately. In this paper, five substances with relatively high concentrations were quantified including lactate, malate, citrate, phosphate and *cis*-aconitate, which provides significant information on the metabolism of TCA cycle in CHO cells. The results indicate that a blockage exists in the TCA cycle of CHO cells and that citrate is one of the blocked points.

Acknowledgements

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