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Acta Chromatographica

33 (2021) 4, 322–332

DOI:

10.1556/1326.2020.00838

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ORIGINAL RESEARCH PAPER

New conditions of HPLC analysis for separation and quantification of simple organic acids of tricarboxylic acid cycle in psoriasis

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Received: August 18, 2020 • Accepted: August 22, 2020

Published online: October 3, 2020

ABSTRACT

Tricarboxylic acid cycle is an important pathway and the metabolites of tricarboxylic acid cycle, simple organic acids, play important roles in many physiological processes. The new conditions of high performance liquid chromatography (HPLC) with improved separation selectivity were developed and optimized by choosing suitable store solvent, methanol, which can catalyze the reaction, aldol condensation, and facilitate the formation of new compound to improve simultaneous detection of nine metabolites of tricarboxylic acid cycle, including lactic acid, pyruvic acid, citric acid, alpha-ketoglutaric acid, malic acid, succinic acid, *cis*-aconitic acid, itaconic acid, and fumaric acid, and be applied in analysis of the nine metabolites in psoriasis mice skin. This study shows that in process of developing methods, expect conventional chromatographic condition, solvent should also be considered carefully.

KEYWORDS

storage solvent, separation, small molecular organic acids, tricarboxylic acid cycle

INTRODUCTION

The TCA (tricarboxylic acid) cycle is an important pathway for energy metabolism of animals and the metabolites of the TCA cycle, simple organic acids, are involved in the synthesis of biological macromolecules, which provides materials for physiological processes such as post-transfer modification and epigenetic changes [1–13]. In recent study, itaconic acid showed the potential therapeutic value and it can also be formed by decarboxylation of aconitic acid, indicating the importance of TCA metabolites in physiological processes [14, 15]. However, the studies of metabolites in TCA cycle usually were implemented by GC-MS or LC-MS [16, 17], which often need complex pretreatment or expensive instrument.

To avoid above shortcomings, our work aimed to develop a new simple high performance liquid chromatography (HPLC) conditions to improve separation and selectivity for determination of these nine metabolites in TCA cycle, including lactic acid (Lac), pyruvic acid (Pyr), citric acid (Cit), alpha-ketoglutaric acid (Alp), malic acid (Mal), succinic acid (Suc), *cis*-aconitic acid (Cis), fumaric acid (Fum), and itaconic acid (Ita). In studies of small polar compounds, hydrophilic interaction chromatography (HILIC) has recently become one of the favorite chromatographic methods and been applied widely [18–20]. Similarly, in a study of organic acids, a polar embedded reverse phase column was successfully applied [21]. In our work, two columns, hydrophilic column and polar embedded reverse phase column, were compared and the polar embedded reverse phase column was chosen to detect the nine small molecular acids. For HPLC analysis, solvent is an important factor of affecting separation. There were several reports about effect of sample solvent on analytes dispersion in chromatographic columns, peak shape and analysis time [22–24]. However, to our best knowledge, the effect of solvent on determination in HPLC was rarely discussed. In this study, one of targeted analytes, pyruvic acid stored in methanol, can be catalyzed to the reaction, aldol condensation, which will from pyruvic

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acid to a new compound. By a simple pretreatment, pyruvic acid can be transformed to the new compound, which is beneficial for determination of 9 acids in HPLC. According to this result, the new HPLC method conditions with a simple sample pretreatment were developed.

Psoriasis is a serious, heterogeneous, systemic immune disease with obvious genetic predisposition and autoimmune characteristics [25, 26]. Psoriasis patients, in addition to suffering from diseases, often also bear a great psychological burden and are prone to anxiety, inferiority, self-injury, and even depression, which affects quality of their life seriously [27]. The etiology of psoriasis involves many factors, including environment, genes, infection and individual lifestyle [28–30]. In this work, the new method conditions were applied in the analysis of the nine metabolites of TCA cycle in psoriasis mice skin. The result showed that there is obvious difference in metabolites of TCA cycle between psoriasis and normal mice, which may provide potential therapeutic strategy of psoriasis.

MATERIALS AND METHODS

Chemicals and materials

Lactic acid (98% purity), pyruvic acid (99% purity), *cis*-aconitic acid (99% purity), itaconic acid (98% purity), and citric acid (99% purity) were purchased from Aladdin company. Succinic acid (99% purity) was purchased from Sigma-Aldrich company. Fumaric acid (98% purity) and malic acid (98% purity) were purchased from Fluka company. Alpha-ketoglutaric acid (98% purity) and acetaminophen (98% purity) (internal standard, IS) were purchased from Sinopharm Chemical Reagent Co., Ltd. HPLC-grade acetonitrile and methanol were purchased from Beijing Dikma Technology Co., Ltd. Water was purchased from Wahaha company.

Instrumentation and chromatographic conditions

The chromatographic separation of analytes was implemented with an HPLC system (HITACHI corporation, L-2000, Japan) equipped with a L-2130 HPLC pump, a L-2300 column oven and a L-2400 UV detector at 210 nm.

A polar embedded reverse phase column (250 × 4.6 mm I.D., YMC-Pack ODS-AQ/S-5 μ m/12 nm, YMC CO., LTD) and a HILIC column (5 μ m, 4.6 × 250 mm, Agela) were selected and compared with each other. The polar embedded reverse phase column was chosen to be applied in the analysis of the 9 acids in mice skin.

The identification of aldol condensation reaction of pyruvic acid was implemented on an Agilent 1100 LC-MS high performance liquid chromatography mass spectrometer. The instrument consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degassing pump, and a 1100 mass spectrometer. The mobile phase was pure water and the flow rate was 0.5 mL/min. The drying gas flow and temperature was set at 12 L/min and 350 °C, respectively. The capillary voltage (negative) was 3,000 V and scan range was *m/z* 50–500.

The optimized HPLC condition, which was applied on the polar embedded reverse phase column, was: A phase: 5 mM $\text{NH}_4\text{H}_2\text{PO}_4$ aqueous phase (pH modulated by phosphoric acid to 2.2), B phase: acetonitrile; 0 → 2 min, phase A: 100%; 2 → 22 min, B phase: 0 → 5%; 22 → 30 min, phase A: 100%. The flow rate was 1 mL/min and the temperature was 35 °C.

Preparation of standard solution

Individual stock standard solutions of each acid and IS were prepared in HPLC-grade methanol at concentration of 10 mg/mL. Working standards containing 9 acids and IS were prepared in HPLC-grade methanol by mixing stock solution and series dilution.

Working standard solutions were heated at 40 °C for 20 min, and then was evaporated to dry under nitrogen flow and reconstituted in water, as the methanol solution will lead to poor retention if it is injected directly.

The storage solutions were stored at –20 °C, while working solutions were stored at –4 °C in the dark.

Preparation of mice samples

Psoriasis on mice model. Female C57BL/12 mice of six to eight weeks old were purchased from Shanghai Lingchang biotechnology Co., Ltd. All use and care of experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC), Roche R&D Center (Shanghai, China).

Skin lesions were induced in model groups of mice by applying 62.5 mg/d imiquimod ointment on back skin of the model mice for 5 days after their back hair were removed. Mice in the control group received no other treatment after back hair removal (one mouse in control group was dead during the experimental process). After 5 days, mice treated with imiquimod ointment showed similar symptoms of psoriasis on their backs. The control group and the model group were euthanized and the back skin of the mice was acquired and stored at –80 °C.

Mice sample pretreatment. About 45 mg of mice skin was cut into pieces and mixed with 800 μ L of methanol in a 1.5 mL tube, then it was spiked with IS (10 μ L 100 μ g/mL). After homogenization at 50 Hz for 180 s followed by ultrasonication for 15 min, sample was centrifuged under 12,000 rpm for 10 min and supernatant was collected and filtered through organic nylon membrane. The filtrate was heated at 40 °C for 20 min. Then, the filtrate was evaporated to dry under nitrogen flow and then reconstituted in water.

Method validation

The optimized HPLC method conditions and the simple pretreatment, which were mentioned in “Preparation of standard solution” and “Preparation of mice samples” respectively, were validated by conventional validation procedures, including the calibration curve linearity, limit of detection (LOD), limit of quantification (LOQ), precision

Fig. 1. Chromatogram of 9 acids on polar embedded reversed phase column (a) and HILIC liquid column (b). 1, pyruvic acid; 2, malic acid; 3, lactic acid; 4, alpha-ketoglutaric acid; 5, citric acid; 6, succinic acid; 7, fumaric acid; 8, *cis*-aconitic acid; 9, itaconic acid

and accuracy, and recovery. The area response ratio of analyte/IS was used for regression analysis. Calibration curve linearity was assessed by three sets of calibration curves for each acid (each set had eight data points) and was studied over a wide range to cover content levels of 9 acids in skin of psoriasis mice. The relative standard deviation (%RSD) of the slopes of the three curves was determined. The regression curve was calculated as “ $y = mx + b$ ”, where “ x ” was the content of each analyte in mice skin, “ m ” was the slope, and “ y ” was the rate of detector responses of analytes to the internal standard. The detection limit (LOD) was based on the signal-to-noise ratio above 3 and the lowest standard on the calibration curve was accepted as the quantitative limit (LOQ). Precision and accuracy were determined by analyzing five replicates of QC samples at HQC, MQC, and LQC three levels on the same day, while the inter-batch accuracy and precision were assessed by analyzing three batches on three consecutive days. As there are the nine analytes in mice skin, the recovery was calculated with following formula:

$$\text{Recovery} = (A_1 - A_2)/A_c$$

A_1 = concentration of analytes of mice skin with adding standard sample

A_2 = concentration of analytes of mice skin without adding standard sample

A_c = concentration of standard sample

RESULT AND DISCUSSION

Development and optimization of chromatographic condition

As HILIC column was often applied in studies of polar compounds to achieve suitable retention and polar embedded reversed phase column can applied successfully in a study of organic acids, in this study, the 9 acids were analyzed by HILIC column and polar embedded reversed phase column, respectively, under the same condition of 20 mM $\text{NH}_4\text{H}_2\text{PO}_4$ 100% aqueous phase (pH modulated by phosphoric acid to 2.4 and temperature of column set at 35 °C). The result showed that a better separation was

achieved in polar embedded reversed phase column (Fig. 1a) and the 9 acids cannot be separated entirely on the HILIC column, in Fig. 1b.

To optimize the method, ammonium formate, ammonium acetate and ammonium dihydrogen phosphate were added in 100% aqueous phase, respectively. Consideration of acidity and cutoff wavelength, 5 mM ammonium dihydrogen phosphate was used as salt solution in mobile phase.

To further optimize the separation, peak shape and speed of analysis, gradient elution was adopted and dosage of acetonitrile, pH, temperature and flow rate were investigated. The final optimized HPLC conditions were described in “Instrumentation and chromatographic conditions”.

Improvement of separation by utilizing Aldol condensation

Water and methanol are generally used as solvent for polar compounds. However, the chemical reaction of compounds may be different in different solvents. Usually solvent is chosen to minimize the changes in chemical compounds, but in this study, methanol which catalyze the reaction of stored compounds, can improve the determination in HPLC. In this study, water and methanol were used as store solvents respectively to investigate the effect of solvent on compounds. The standard sample stored in water and methanol were blown to dry respectively, then reconstituted by water and analyzed by HPLC under the same condition. It is interesting that when water was used as store solvent, the retention time of pyruvic acid and malic acid were both at 4.1 min and overlapped in chromatogram (Fig. 2a). While methanol was used as store solvent, there were two peaks in chromatogram of pyruvic acid and the chromatogram is shown in Fig. 2b. The first peak was at the original position at 4.1 min and the second peak (new peak) was around at 14 min, indicating that pyruvic acid might have a reaction in methanol and form another compound (new peak). As the pyruvic acid in methanol could be further transformed by being heated, the heating time (10, 20, 30 min) and temperature (20, 40, 60) were investigated. The result showed that pyruvic acid could be transformed to another compound by being heated at 40 °C for 20 min (Fig. 2c), which suggested that pyruvic acid and malic acid could be separated by a simple pretreatment.

Fig. 2. Chromatogram of pyruvic acid with water and methanol as store solvent respectively: (a) pyruvic acid in water was injected directly; (b) pyruvic acid stored in methanol was blown to dry and reconstituted in water; (c) pyruvic acid stored in methanol was heated at 40 °C for 20 min, then blown to dry and reconstituted in water

For this phenomenon, we compared the structure of pyruvic acid with structures of other acids. The structure of pyruvic acid (Fig. 3a) shows that alpha position of its carboxyl group has a carbon-oxygen double bond, which is prone to aldol condensation; while among other acids, only alpha-ketoglutaric acid (Fig. 3b) has a similar structure. However, alpha-ketoglutaric acid has a long carbon chain, which restricts aldol condensation reaction.

The UV absorption spectrometry and mass spectrometry of the new compound were implemented and the result (Fig. 4) showed that the UV spectrograms of pyruvic acid in water and methanol were obviously different. The

UV absorption decreased with wave length, when water was used as store solvent (Fig. 4a). When methanol was used as store solvent, in the second peak (new peak), the maximum absorption peak was observed at 238 nm (Fig. 4c); in the first peak (old peak), UV absorption spectrometry (Fig. 4b) of the pyruvic acid (methanol used as store solvent) was consistent with UV absorption spectrometry of the pyruvic when water used as store solvent, suggesting that there is no difference in influence of methanol and water on the UV spectrogram. This result suggested that a new compound with conjugated structure might be formed. The result of mass spectrometry (Fig. 5)

Fig. 3. Structures of pyruvic acid (a) and alpha-ketoglutaric acid (b)

Fig. 4. UV spectrogram of pyruvic acid when water and methanol as storage solvent respectively: (a) UV spectrogram of pyruvic acid when water used as storage solvent; (b) UV spectrogram of the first peak of pyruvic acid when methanol used as storage solvent; (c) UV spectrogram of the second peak of pyruvic acid when methanol used as storage solvent

showed that the m/z of the new compound is 157 after being heated in methanol, which could be inferred as the product of condensation of two pyruvic acid molecules. There are some studies about the chemical change of pyruvic acid [31–34], and there are two most possible products formed from pyruvic acid by aldol condensation, 2-methyl-4-oxopent-2-endioic acid (OMPD), and zymonic

acid, which are structural isomers (Fig. 6). The structures of both compounds have conjugated structure and under ESI (negative mode) conditions the m/z of $[M-H]^-$ of both compounds was 157. However, the study from Duwel S. showed that the most abundant peak in the mass spectrometry of zymonic acid was m/z 315 which could be attributed to $[2M-H]^-$, while the mass spectrometry of

Fig. 5. Mass spectrum of pyruvic acid when water and methanol as storage solvent respectively: (a) Mass spectrum of pyruvic acid when water used as storage solvent; (b) Mass spectrum of pyruvic acid when methanol used as the storage solvent, which was heated for 20 min at 40 °C, blown to dry and reconstituted in water

Fig. 6. Structures of OMPD (a) and zymonic acid (b)

OMPD from the most recent study [34] shared a similar pattern to our study as shown in Fig. 5b, in which the most significant peaks were both m/z 157 from ion $[M-H]^-$.

The synthesis condition of zymonic acid is vacuum distillation of pyruvic acid, while synthesis condition of OMPD is that methyl pyruvate ester is heated in methanol, which is more similar with experimental situation of this study [31, 34].

In addition, as reported by Rios zymonic acid might be transformed to OMPD under wide range of pH value when heated. Thus it seems to be important for evaluation of chemical properties of these isomers [34]. Thus, the new

Fig. 8. Chromatogram of 9 acids: 1, malic acid; 2, lactic acid; 3, alpha-ketoglutaric acid; 4, citric acid; 5, succinic acid; 6, fumaric acid; 7, cis-aconitic acid; 8, pyruvic condensation acid, the new compound transformed from pyruvic acid; 9, itaconic acid; 10, internal standard

compound is more likely to be OMPD, and the speculation of mechanism of aldol condensation in pyruvic acid is shown in Fig. 7. In this case, methanol may promote the

Fig. 7. Hypothesis of mechanism of pyruvic acid reaction in methanol

aldol condensation of pyruvic acid, but it is also possible that methanol reacts with pyruvate to form methyl pyruvate which forms into OMPD [34].

Therefore, the more investigation needs to be done to confirm the mechanism of forming the new compound and its structure. In this study, the new compound from pyruvic

acid was named as pyruvic condensation acid which was abbreviated as pyrs.

By the simple pretreatment, the 9 acids were detected successfully by the new method conditions (HPLC conditions were in “Instrumentation and chromatographic conditions”) and the result showed in Fig. 8.

Table 1. Regression (calibration) equations and coefficients (R^2)

Acids	Range ($\mu\text{g/mL}$)	Regression equation	%RSD (slope)	R^2
Lac	234.4–30,000	$y = 0.0011x - 0.1828$	3.19	0.9997
Pyrs	7.8–1,000	$y = 0.0018x + 0.0033$	1.61	0.9995
Cit	7.8–1,000	$y = 0.0023x + 0.0029$	3.9	0.9992
Alp	7.8–1,000	$y = 0.0152x - 0.0114$	7.94	0.9992
Mal	15.6–2000	$y = 0.0015x + 0.0037$	1.5	0.9997
Suc	7.8–1,000	$y = 0.0013x - 0.0002$	5.32	0.9997
Cis	1.6–200	$y = 0.0866x + 0.0485$	0.49	0.9999
Ita	0.8–100	$y = 0.1084x - 0.0155$	3.71	0.9997
Fum	0.3–40	$y = 0.2527x + 0.0133$	4.89	0.9997

Table 2. LOD and LOQ for 9 acids ($\mu\text{g/mL}$)

	Lac	Pyrs	Cit	Alp	Mal	Suc	Cis	Ita	Fum
LOD	5.0	3.0	1.5	1.0	2.0	3.0	3.0E–02	4.0E–02	2.0E–02
LOQ	2.3E+02	7.8	7.8	7.8	1.6E+01	7.8	1.6	8.0E–01	3.0E–01

Table 3. Intra-day and inter-day precision (area/area)

TCA acids		Intra-day precision ($n = 5$)						Inter-day precision	
		Day 1		Day 2		Day 3		A/A	RSD%
		A/A	RSD%	A/A	RSD%	A/A	RSD%		
Lac	LQC	0.08	11.9	0.09	9.6	0.10	12.9	0.09	13.1
	MQC	15.24	2.9	15.90	1.3	16.66	4.7	15.93	4.5
	HQC	25.82	2.9	27.24	2.8	28.09	1.9	27.05	4.3
Pyrs	LQC	0.02	8.2	0.02	7.4	0.02	8.8	0.02	13.4
	MQC	0.91	3.4	0.90	0.7	0.92	0.4	0.91	1.0
	HQC	1.48	4.0	1.57	1.2	1.60	0.7	1.55	4.1
Cit	LQC	0.02	4.8	0.02	4.7	0.02	6.4	0.02	9.1
	MQC	1.21	1.7	1.19	0.9	1.17	0.9	1.19	1.7
	HQC	1.55	1.9	1.60	1.7	1.64	1.4	1.60	3.0
Alp	LQC	0.08	1.7	0.09	3.5	0.09	2.1	0.09	5.8
	MQC	7.64	1.8	7.83	1.0	8.04	2.6	7.84	2.5
	HQC	10.64	1.4	11.03	2.7	11.44	0.8	11.04	3.6
Mal	LQC	0.02	2.6	0.02	10.6	0.03	3.2	0.02	13.1
	MQC	1.54	4.4	1.61	2.1	1.69	2.4	1.62	4.4
	HQC	2.52	1.7	2.59	1.7	2.64	0.6	2.58	2.3
Suc	LQC	0.01	7.2	0.01	3.4	0.01	6.5	0.01	8.0
	MQC	0.63	2.2	0.65	1.0	0.67	3.0	0.65	2.7
	HQC	1.07	3.2	1.12	0.9	1.14	0.4	1.11	3.0
Cis	LQC	0.16	2.6	0.17	4.3	0.19	3.3	0.17	7.1
	MQC	8.54	2.9	8.83	1.2	8.99	0.5	8.79	2.6
	HQC	13.82	2.2	14.18	1.1	14.38	0.6	14.13	2.0
Ita	LQC	0.07	2.7	0.08	2.9	0.08	6.3	0.08	6.1
	MQC	5.31	0.8	5.39	0.9	5.50	2.6	5.40	1.7
	HQC	8.11	0.3	8.01	0.8	7.90	0.8	8.00	1.3
Fum	LQC	0.08	7.9	0.09	10.7	0.11	7.9	0.09	15.4
	MQC	5.16	3.1	4.98	0.7	4.92	0.7	5.02	2.5
	HQC	7.05	1.1	7.20	1.3	7.29	0.5	7.18	1.7

Table 4. Intra-day and inter-day accuracy ($\mu\text{g/mL}$)

		Intra-day accuracy ($n = 5$)							Inter-day accuracy ($n = 15$)	
TCA acids		Nominal concentration	Day 1		Day 2		Day 3			
			Calculated concentration	%	Calculated concentration	%	Calculated concentration	%	Calculated concentration	%
Lac	L	2.3E+02	2.5E+02	106.9	2.5E+02	106.7	2.4E+02	103.8	2.5E+02	105.8
	M	1.5E+04	1.4E+04	96.2	1.5E+04	98.2	1.5E+04	98.5	1.5E+04	97.7
	H	2.3E+04	2.5E+04	109.7	2.5E+04	109.9	2.5E+04	110.4	2.5E+04	110.0
Pyr	L	7.8	9.3	118.9	9.4	119.9	8.7	111.4	9.1	116.7
	M	5.0E+02	5.0E+02	100.1	5.0E+02	99.9	5.1E+02	101.3	5.0E+02	100.4
	H	7.5E+02	8.6E+02	114.3	8.6E+02	114.8	8.6E+02	114.4	8.6E+02	114.5
Cit	L	7.8	7.1	91.0	7.1	90.3	7.0	90.0	7.1	90.4
	M	5.0E+02	5.2E+02	103.7	5.2E+02	103.1	5.2E+02	103.1	5.2E+02	103.3
	H	7.5E+02	6.9E+02	92.2	6.9E+02	92.4	6.9E+02	92.4	6.9E+02	92.3
Alp	L	7.8	6.6	84.1	6.6	84.7	6.6	84.9	6.6	84.6
	M	5.0E+02	5.1E+02	102.6	5.1E+02	102.9	5.2E+02	104.3	5.2E+02	103.3
	H	7.5E+02	7.3E+02	96.8	7.3E+02	96.8	7.3E+02	97.1	7.3E+02	96.9
Mal	L	15.6	13.6	86.8	13.7	88.0	14.1	90.5	13.8	88.4
	M	1.0E+03	1.1E+03	106.0	1.1E+03	108.1	1.1E+03	108.1	1.1E+03	107.4
	H	1.5E+03	1.7E+03	114.2	1.7E+03	114.9	1.7E+03	115.0	1.7E+03	114.7
Suc	L	7.8	7.9	100.6	8.0	102.1	8.1	103.1	8.0	101.9
	M	5.0E+02	5.0E+02	99.1	5.0E+02	99.4	5.0E+02	100.9	5.0E+02	99.8
	H	7.5E+02	8.6E+02	114.7	8.5E+02	113.6	8.5E+02	113.5	8.5E+02	113.9
Cis	L	1.6	1.3	86.1	1.4	86.4	1.3	86.3	1.3	86.3
	M	1.0E+02	1.0E+02	104.2	1.0E+02	104.0	1.0E+02	103.9	1.0E+02	104.0
	H	1.5E+02	1.6E+02	107.7	1.6E+02	108.4	1.6E+02	108.8	1.6E+02	108.3
Ita	L	0.8	0.9	110.2	0.9	110.8	0.9	112.5	0.9	111.1
	M	5.0E+01	5.0E+01	99.6	5.0E+01	99.7	5.0E+01	100.6	5.0E+01	100.0
	H	7.5E+01	7.4E+01	98.6	7.4E+01	98.6	7.4E+01	98.7	7.4E+01	98.6
Fum	L	0.3	0.3	99.6	0.3	99.0	0.3	98.3	0.3	99.0
	M	2.0E+01	2.0E+01	99.9	2.0E+01	98.7	2.0E+01	98.5	2.0E+01	99.0
	H	3.0E+01	2.8E+01	94.7	2.8E+01	94.6	2.8E+01	94.4	2.8E+01	94.6

Method validation

Linearity, LOD and LOQ. Linear range, calibration slope RSD%, regression coefficient (R^2), and calibration equation are shown in Table 1. The detector response (peak area) of all acids has a linear relationship with the known content ($R^2 = 0.9992\text{--}0.9999$), which showed a good linearity. The results of LOD and LOQ were shown in Table 2.

Precision and accuracy. The precision and accuracy of intra-day and inter-day each concentration level were shown in Tables 3 and 4.

The result showed that the precision and accuracy of HQC and MQC from the nominal concentration were within $\pm 15\%$, while the precision and accuracy of LQC from the nominal concentration were no greater than $\pm 20\%$. This indicated that the optimized method is reliable for the determination of nine metabolites.

Recovery. The recovery of this method was evaluated as described in “Method validation” in three independent times. The result, which was showed in Table 5, inducted that no significant signal enhancement or signal suppression were observed.

Analysis of metabolites in normal and psoriasis mice skin

The new method with the pretreatment was applied in the analysis of the 9 acids in mice skin and the result was showed in Table 6.

The result showed that compared control mice, lactic acid, pyruvic acid, malic acid, and alpha-ketoneglutaric acid in psoriasis mice skin had an obvious increase, while other acids were observed an opposite trend.

To further compare the difference of metabolites in TCA cycle between skins of psoriasis mice and control mice, the website “MetaboAnalyst” was used to analyze the concentrations of 9 metabolites (m/m) in mice skin of the model group and the control group. Partial least square discriminant analysis (PLS-DA) model and principal component analysis (PCA) model were able to completely distinguish the samples of the two groups (Fig. 9), indicating that there is remarkable difference in metabolites of TCA cycle between psoriasis and normal mice.

According to the T -test ($P < 0.01$) and the ratio analysis (FC, $FC > 2$), five metabolites, lactic acid, alpha-ketoglutaric acid, pyruvic acid, succinic acid and itaconic acid, may play important roles in the pathogenesis of psoriasis. During

Table 5. Recovery of 9 acids (µg/mL)

TCA acids	Calculated concentration					Added concentration	Recovery %
	1	2	3	Mean	%RSD		
Lac	5.0E+03	4.9E+03	4.9E+03	4.9E+03	0.5	5.0E+03	98.6
Pyrs	1.1E+03	1.0E+03	1.0E+03	1.0E+03	2.3	1.0E+03	103.8
Cit	2.0E+03	2.0E+03	2.0E+03	2.0E+03	0.5	2.0E+03	100.7
Alp	5.4E+02	5.4E+02	5.6E+02	5.5E+02	2.0	5.0E+02	109.1
Mal	4.1E+03	4.8E+03	4.8E+03	4.6E+03	8.8	4.0E+03	114.7
Suc	4.3E+03	4.3E+03	4.2E+03	4.3E+03	0.4	5.0E+03	85.3
Cis	5.0E+01	5.0E+01	5.1E+01	5.0E+01	1.8	6.0E+01	84.0
Ita	2.0E+01	2.0E+01	2.0E+01	2.0E+01	1.3	2.0E+01	100.0
Fum	5.1E+01	5.1E+01	5.2E+01	5.1E+01	0.7	5.0E+01	102.3

Table 6. Content of 9 acids in skins of model and control mice (m/m, %)

TCA acids	Model						Control				
	1	2	3	4	5	6	1	2	3	4	5
Lac	5.7E-01	7.6E-01	6.6E-01	6.7E-01	4.8E-01	6.2E-01	3.1E-01	2.7E-01	2.7E-01	2.3E-01	2.4E-01
Pyrs	5.3E-02	4.8E-02	3.9E-02	3.7E-02	4.4E-02	4.0E-02	2.6E-02	6.7E-03	1.2E-02	1.7E-02	1.4E-02
Cit	6.2E-02	7.2E-02	1.1E-01	9.5E-02	7.5E-02	7.7E-02	1.5E-01	1.7E-01	1.8E-01	1.2E-01	1.1E-01
Alp	1.8E-02	1.7E-02	1.1E-02	1.9E-02	1.5E-02	1.8E-02	9.9E-03	7.3E-03	7.4E-03	5.8E-03	7.3E-03
Mal	1.2E-01	1.2E-01	1.0E-01	9.2E-02	1.1E-01	1.0E-01	5.0E-02	3.5E-02	3.7E-02	1.6E-01	2.1E-01
Suc	1.9E-01	2.6E-01	3.7E-01	2.0E-01	1.7E-01	2.4E-01	7.6E-01	6.5E-01	7.4E-01	7.4E-01	4.8E-01
Cis	1.7E-03	1.7E-03	1.6E-03	1.3E-03	1.2E-03	1.2E-03	5.6E-01	3.0E-01	3.8E-01	3.3E-01	3.5E-01
Ita	7.0E-04	7.1E-04	6.5E-04	5.6E-04	5.5E-04	6.5E-04	5.0E-03	4.1E-03	6.0E-03	1.0E-02	4.2E-03
Fum	1.5E-03	1.3E-03	1.3E-03	1.3E-03	1.4E-03	1.6E-03	2.0E-03	1.6E-03	1.7E-03	2.0E-03	1.9E-03

Fig. 9. 2D and 3D schematic diagram of the control group and model group under PCA and PLS-DA models: (a) 3D schematic diagram of PCA model; (b) 3D schematic diagram of PLS-DA model; (c) 2D schematic diagram of PCA model; (d) 2D schematic diagram of PLS-DA model

immune processes, lactic acid can not only promote anti-inflammatory factor interleukin 10 (IL-10) and reduce pro-inflammatory factor IL-12, but also can down-regulate TNF, NF- κ B and PTX-3 [35, 36]. It also can induce vascular endothelial growth factor and collagen, which accelerates the process of injury repairing [37, 38]. Alpha-ketoglutaric acid can be transformed into ornithine alpha-ketoglutaric acid (OKG) by combining with ornithine, while OKG can enhance macrophage reactivity and toxicity under stress [39–44]. Pyruvic acid can have anti-inflammatory function by reducing Ca^{+} mismanagement and oxidative stress [45, 46], while succinic acid can act as a pro-inflammatory signal during the immune process [47, 48]. In skins of psoriasis mice, the increase of lactic acid, alpha-ketoglutaric acid and pyruvic acid and decrease of succinic acid may be related to anti-inflammatory function and immune enhancement. However, itaconic acid has also anti-inflammatory effects in activated immune cells, but it showed descending trend in skins of psoriasis mice, which needs be further investigated. Although this result showed that there is important difference in metabolites of TCA cycle between skins of psoriasis and normal mice, the study was implemented on a small group and just preliminary for the further research of exact mechanism, so the result need to be verified in future.

CONCLUSION

In this study, the new simple conditions of HPLC method with improved separation selectivity were developed for simultaneous detection of nine simple organic acids in the skin of mice. It is notable that certain quick conversion of analytes prior to analysis could be considered as a complementary approach to improve chromatographic determination. In this work, aldol condensation was utilized to from pyruvic acid to a new compound for simultaneous detection of 9 acids, which was simply achieved by mild heating and reconstitution. The result showed that during the pathogenesis of psoriasis, the metabolites of TCA cycle play important roles.

Conflict of interest: There is no conflict of interest in this article.

ACKNOWLEDGMENT

This study was funded by The National Natural Science Foundation of China (81673056, 81773325).

ABBREVIATIONS

TCA	tricarboxylic acid
Lac	lactic acid
Pyr	pyruvic acid
PyrS	pyruvic condensation acid
Cit	citric acid

Alp	alpha-ketoglutaric acid
Mal	malic acid
Suc	succinic acid
Cis	cis-aconitic acid
Fum	fumaric acid
Ita	itaconic acid
IS	internal standard

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