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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF SELECTED BLOOD CITRIC ACID CYCLE INTERMEDIATES

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SUMMARY

We describe a high-performance liquid chromatographic (HPLC) method for analysis of the intermediates of the citric acid cycle. Using two Aminex HPX-87H columns in series at 36°C, the early eluting compounds *cis*-aconitate, oxaloacetate, α-ketoglutarate and citrate–isocitrate can be resolved. Acetonitrile is used for extraction of citric acid cycle intermediates from blood as interfering ultraviolet absorbing peaks are present with perchloric acid or trichloroacetic acid extraction. Acetonitrile extraction is compared with perchloric acid extraction of citric acid cycle intermediates from plasma. Low recovery of some organic acids from blood seems not to be due to enzymatic degradation. Storage of acetonitrile extracts in liquid nitrogen led to a small but significant decrease in pyruvate levels in human blood. However, significant changes in other organic acids were not seen. HPLC methodology allows study of the citric acid cycle in tissue samples as well as blood and promises to facilitate the investigation of human disorders of energy metabolism.

INTRODUCTION

Many human metabolic diseases have been described which arise from disorders of energy metabolism. Patients with defects of glycolysis [1], defects in enzymes immediately concerned with pyruvate metabolism [2] and cytochrome abnormalities have all been described [3]. Recently, the first human defect in the citric acid cycle was described [4]. This patient with fumarase deficiency died in infancy. Most patients with lactic acidemia suffer degenerative neurological disease, often with a fatal outcome, but have no identifiable molecular biochemical defect. The citric acid cycle is a dynamic and critical component of cellular oxidative metabolism. Altered flux and absolute levels of citric acid cycle intermediates might be expected with defects of energy metabolism changing substrate inflow, product outflow and the cellular redox state. There is some evidence

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that this is the case. In a renal model of acidosis [5], levels of succinate and α-ketoglutarate decrease. The patient recently described with fumarase deficiency [4] had high levels of fumarate in the urine — an important clue in identifying the defective enzyme.

High-performance liquid chromatographic (HPLC) technology has been described for measurement of many of the components of the citric acid cycle [6–12]. These methods are routinely used in the wine industry [13,14]. However, in only one paper has an adequate separation of sample extracts containing pyruvate, α-ketoglutarate and malate been reported [11]. We were not able to replicate separation of these compounds using a single Aminex HPX-87H column. We found that two Aminex HPX-87H columns in series, however, reproducibly allow separation of the early eluting citric acid cycle intermediates.

In this paper acetonitrile is used to denature the protein in blood samples. Losses due to this extraction procedure are quantified allowing calculation of the true blood content of each organic acid of interest. Recovery studies included a comparison of acetonitrile and perchloric acid extraction of plasma, the effect of enzyme inhibitors on organic acid levels in human blood, as well as effects of storage in liquid nitrogen.

EXPERIMENTAL

Chromatography

The isocratic HPLC system consisted of two (strong cation-exchange resin) columns in series (Aminex HPX-87H; Bio-Rad Labs., Richmond, CA, U.S.A.) protected by a Bio-Rad Aminex micro-guard cartridge. Column temperature was maintained at 36 °C by means of two water jackets (Alltech Assoc., Deerfield, IL, U.S.A.). Some studies were performed with both columns at 65 °C allowing separation of succinate from an unidentified blood peak. The system was equipped with a dual-piston pump (Model 1330, Bio-Rad Labs.) and a refrigerated automatic sampler (Model AS-48, Bio-Rad Labs.). The mobile phase was 0.002 *M* sulfuric acid, delivered at 0.7 ml/min. Column effluents were monitored at 205 nm with a spectrophotometer (Model 2600 XY, Gilford, Oberlin, OH, U.S.A.) using an 8-μl quartz flow cell (Model 178.32 QS, Hellma Cells, Jamaica, NY, U.S.A.). Plotting of the chromatogram and integration of peak areas were performed by an integrator (Model 7225B, Hewlett-Packard, Avondale, PA, U.S.A.). The whole system was controlled by an Apple IIe computer, using the Bio-Rad GPS software. Data were stored on a hard disk (Apple Profile, Apple Computers, Cupertino, CA, U.S.A.). All samples were filtered with an 0.2-μm filter (Acro LC13, Gelman Sciences, Ann Arbor, MI, U.S.A.); 100 μl of each sample were injected.

Standards

Standards of citric acid cycle intermediates as well as pyruvate, lactate, β-hydroxybutyrate and acetoacetate were prepared individually in 0.025 *M* sulfuric acid and chromatographed singly in order to determine the retention time for each organic acid. Once the individual retention times were established, the acids

were chromatographed as a mixture. A standard solution of ten different organic acids in 0.025 *M* sulfuric acid was prepared, containing 2 *μM cis*-aconitate, 20 *μM* oxaloacetate, 10 *μM* α-ketoglutarate, 40 *μM* citrate, 10 *μM* pyruvate, 120 *μM* malate, 220 *μM* succinate, 240 *μM* lactate, 240 *μM* β-hydroxybutyrate, 0.05 *μM* fumarate and 432 *μM* isobutyrate. Reference carboxylic acids were purchased from Sigma (St. Louis, MO, U.S.A.). Water was purified using a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.). Standards were stored in liquid nitrogen until use.

Different concentrations of the standard mixture, varying between 0.1 and 10 times the concentration described above, were used to obtain calibration curves for each organic acid. Standard curves were also obtained with standards carried through an extraction procedure with HPLC-grade acetonitrile (Aldrich, Milwaukee, WI, U.S.A.).

Recovery studies

In order to study the recovery of organic acids in human blood and to obtain calibration curves for further analysis, different concentrations of the standard solution varying over a concentration range of 0.1–100 times the standard solution mixture were added to freshly drawn blood. These were immediately extracted with acetonitrile and analyzed. Recoveries were calculated by comparing the slopes of the regression lines obtained from these studies.

Extraction procedures

Two extraction techniques were tested with respect to their influence on recovery. All extractions were carried out at 4 °C.

Acetonitrile extraction. The sample (0.25 ml blood or plasma) was added to 0.25 ml of 0.025 *M* sulfuric acid and 1.5 ml acetonitrile, vortexed and centrifuged (1000 *g*). Acetonitrile was removed by drying the sample with an air jet to an approximate volume of 0.2 ml. The volume was adjusted to 0.25 ml with water and the sample filtered (0.2 μm); 100 μl were then injected on the column.

Perchloric acid extraction. The sample (0.5 ml plasma) was added to 1.0 ml perchloric acid (8%), vortexed and centrifuged (1000 *g*). To remove excess perchloric acid, 1.0 ml of the supernatant was added to 0.16 ml potassium hydroxide, vortexed and centrifuged (1000 *g*). Then, 0.8 ml supernatant was taken, adjusted to a pH of approximately 3–4 with 0.01 ml of 0.5 *M* sulfuric acid, filtered, and 100 μl were then injected.

Enzyme inhibitors

In an attempt to investigate whether the citric acid cycle intermediate losses observed in blood and plasma with both perchloric acid and acetonitrile extraction could be due to residual enzyme activity, we studied the effect of enzyme inhibitors on recovery. Transaminases were inhibited by aminooxyacetic acid and aconitase by fluorocitrate. Tartronate (an inhibitor of malic enzyme) could not be used because it produces large UV peaks in the early part of the chromatogram. For this study 0.25 ml blood was extracted using a mixture of 0.25 ml of 0.025 *M* sulfuric acid and 1.5 ml acetonitrile with and without inhibitors (0.15

m*M* aminooxyacetic acid and 0.0015 m*M* fluorocitrate). A second experiment was carried out with the standard organic acid solution added to the blood extraction mixture. All four samples were then carried through the drying procedure described above and analyzed.

Storage

The effect of storage in liquid nitrogen on levels of organic acids in human blood was investigated. Freshly drawn blood was added to 0.025 *M* sulfuric acid and acetonitrile with and without added standards, immediately vortexed and centrifuged. A portion of the supernatant was taken, dried and analyzed. The rest was divided into five portions, stored in liquid nitrogen; portions were analyzed, 7, 14, 29 and 38 days after sampling.

Data analysis

The chromatographic data were integrated on-line for calculation of areas under each peak with raw data storage on an Apple Profile 5-Mb hard disk. When necessary, the data were re-integrated to assure accurate calculation of peak areas. Retention times (t_R) and peak areas were statistically analyzed by means of the BMDP statistical software (BMDP, University of California, Los Angeles, CA, U.S.A.) on a VAX/VMS main frame computer (Digital Equipment, Nashua, NH, U.S.A.). Characteristics of the calibration curves and effects of storage in liquid nitrogen were obtained by regression analysis. The minimal detection limit for each organic acid was estimated by extrapolating the calibration curve (area versus concentration) to an area of zero. The 99% confidence interval of the corresponding concentration gives the minimal detection limit. Response factors were calculated from the slopes of the calibration curves. The effects of different extraction procedures were verified by two-way analysis of variance. Data are shown as mean ± S.D. A p value of 0.05 was considered to be significant, except for testing the zero hypothesis, that storage in liquid nitrogen does not affect the levels of organic acids in human blood. In this case, to avoid falsely accepting the zero hypothesis, a p value of 0.1 was chosen.

RESULTS

Use of two Aminex HPX-87H columns in series and isocratic elution with 0.002 *M* sulfuric acid allows the reproducible separation displayed in Fig. 1. Citrate and isocitrate as well as acetoacetic acid and fumarate could not be resolved independently. Retention times, response factors and correlation coefficients of the calibration curves of citric acid cycle intermediates are shown in Table I. The very high correlation coefficients of 0.96–0.99 show the linearity of the system over a wide range, e.g., lactate concentration varied between 24 and 2400 μM. Table I also shows the low minimal detection limits of the analytical system detecting injected amounts below 26 μM. Fumarate and *cis*-aconitate are strong UV absorbers with very low detection limits of $3.3 \cdot 10^{-3}$ and $1.3 \cdot 10^{-1}$ μM, respectively.

In Fig. 2, a typical chromatogram of a blood sample extracted with acetonitrile is displayed. Most of the organic acids of interest can be resolved. Glucose, uric

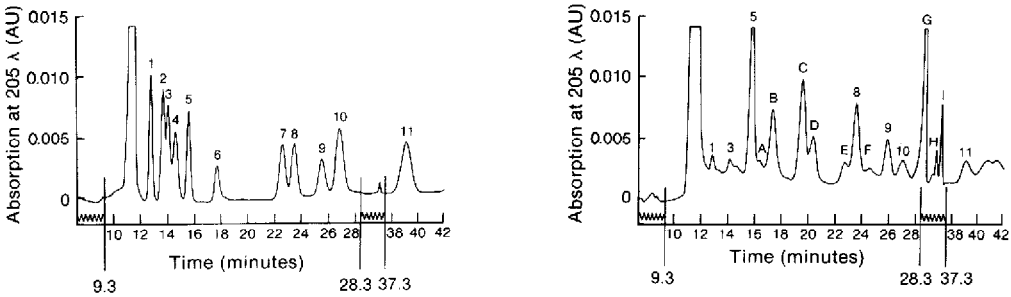

Fig. 1. Chromatogram of a standard solution of the following organic acids (concentrations in 100-μl injection volumes are as indicated): (1) *cis*-aconitate, 2 μM; (2) oxaloacetate, 20 μM; (3) α-ketoglutarate, 10 μM; (4) citrate, 40 μM; (5) pyruvate, 10 μM; (6) malate, 120 μM; (7) succinate, 220 μM; (8) lactate, 240 μM; (9) β-hydroxybutyrate, 240 μM; (10) fumarate, 0.05 μM; (11) isobutyrate, 432 μM. Tic marks signify chart speed changes and zig zag line indicates slow chart speed.

Fig. 2. Typical chromatogram of a blood sample from a healthy control, extracted with acetonitrile. Blood concentrations are as indicated. Identified peaks: (1) *cis*-aconitate, 1.29 μM; (3) α-ketoglutarate, 4.60 μM; (5) pyruvate, 24.2 μM; (8) lactate, 580 μM; (9) β-hydroxybutyrate, 711 μM; (10) fumarate, 0.04 μM; (11) isobutyrate, 740 μM. A (t_R = 16.2 min) is glucose; G (t_R = 28.97 min) is uric acid. Isobutyrate is an added standard. Unidentified peaks: B (t_R = 17.21 min); C (t_R = 19.35 min); D (t_R = 20.18 min); E (t_R = 22.59 min); F (t_R = 24.44 min); H (t_R = 33.77 min); I (t_R = 35.49 min). Tic marks signify chart speed changes and zig zag line indicates slow chart speed.

acid and several unknown UV-absorbing compounds are marked with letters. One such unidentified compound is coeluting with malate, producing a combined peak (B in Fig. 2) and unknown peak E has the same retention time as succinate

TABLE I

RETENTION TIMES (t_R), RESPONSE FACTORS (RF), CORRELATION COEFFICIENTS OF REGRESSION LINES (r) AND THEORETICAL MINIMAL DETECTION LIMITS OF STANDARDS AND OF WHOLE BLOOD EXTRACTS FOR TEN DIFFERENT ORGANIC ACIDS (100-μl INJECTION)

Substance	t_R (mean ± S.D.) (min)	RF (mean ± S.D.) (μmol/l per unit)	r	Minimal detection limit (μmol/l)	
				Standards (n = 10)	Acids in blood (n = 22)
cis-Aconitate	12.73 ± 0.04	$(3.40 \pm 0.03).10^{-6}$	0.99	$1.3 \cdot 10^{-1}$	$4.2 \cdot 10^{-1}$
Oxaloacetate	13.65 ± 0.04	$(2.67 \pm 0.07).10^{-5}$	0.99	3.9	28
α-Ketoglutarate	14.04 ± 0.05	$(1.37 \pm 0.06).10^{-5}$	0.99	3.2	8.6
Citrate	14.59 ± 0.04	$(5.33 \pm 0.52).10^{-5}$	0.96	22	16
Pyruvate	15.57 ± 0.06	$(1.39 \pm 0.06).10^{-5}$	0.99	3.2	2.8
Malate	17.68 ± 0.05	$(3.38 \pm 0.08).10^{-4}$	0.99	24	918
Succinate	22.50 ± 0.05	$(3.74 \pm 0.02).10^{-4}$	0.99	11	27
Lactate	23.36 ± 0.04	$(3.81 \pm 0.05).10^{-4}$	0.99	26	38
β-Hydroxybutyrate	25.54 ± 0.10	$(5.87 \pm 0.08).10^{-4}$	0.99	25	57
Fumarate	26.81 ± 0.14	$(6.76 \pm 0.05).10^{-8}$	0.99	$3.3 \cdot 10^{-3}$	$5.2 \cdot 10^{-3}$

TABLE II

SLOPES (m) AND CORRELATION COEFFICIENTS (r) OF REGRESSION LINES FOR CONCENTRATIONS PLOTTED AGAINST AREAS FOR ACETONITRILE-EXTRACTED STANDARD MIXTURE ALONE AND AFTER ADDITION OF BLOOD

Substance	Acetonitrile-extracted standards ($n=14$)			Acetonitrile-extracted standards + blood ($n=22$)		
	m (mean ± S.D.)	r	Recovery (mean ± S.D.) (%)	m (mean ± S.D.)	r	Recovery (mean ± S.D.) (%)
cis-Aconitate	$(2.35 \pm 0.08) \cdot 10^5$	0.99	80 ± 3	$(0.74 \pm 0.03) \cdot 10^{5*}$	0.98	31 ± 23
Oxaloacetate	$(2.5 \pm 0.09) \cdot 10^4$	0.99	67 ± 2	$(0.34 \pm 0.03) \cdot 10^{5*}$	0.91	14 ± 2
α-Ketoglutarate	$(5.42 \pm 0.14) \cdot 10^4$	0.99	75 ± 2	$(2.02 \pm 0.11) \cdot 10^{4*}$	0.96	37 ± 4
Citrate	$(9.64 \pm 0.37) \cdot 10^3$	0.99	55 ± 2	$(3.43 \pm 0.37) \cdot 10^{3*}$	0.87	36 ± 8
Pyruvate	$(6.11 \pm 0.23) \cdot 10^4$	0.99	87 ± 3	$(6.98 \pm 0.55) \cdot 10^4$	0.92	114 ± 18
Malate	$(4.02 \pm 0.32) \cdot 10^3$	0.97	134 ± 11	$(3.47 \pm 0.58) \cdot 10^3$	0.88	88 ± 29
Succinate	$(2.22 \pm 0.08) \cdot 10^3$	0.99	83 ± 3	$(0.92 \pm 0.05) \cdot 10^{3*}$	0.96	41 ± 5
Lactate	$(2.02 \pm 0.06) \cdot 10^3$	0.99	77 ± 2	$(1.36 \pm 0.1) \cdot 10^{3*}$	0.93	68 ± 5
β-Hydroxybutyrate	$(1.59 \pm 0.07) \cdot 10^3$	0.99	94 ± 4	$(0.69 \pm 0.04) \cdot 10^{3*}$	0.96	44 ± 5
Fumarate	$(1.22 \pm 0.03) \cdot 10^7$	0.99	82 ± 2	$(0.77 \pm 0.04) \cdot 10^{7*}$	0.97	63 ± 6

* Significant ($p < 0.05$) difference to acetonitrile-extracted standard mixture.

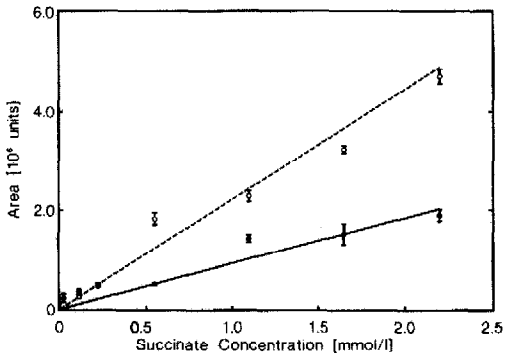

Fig. 3. Peak areas of different concentrations of succinate standards (open circles, $n=18$) and human blood with added succinate (closed circles, $n=32$). All points are means ± S.D. Both samples have been carried through acetonitrile extraction.

when analysed at 36°C. Glucose elutes at $t_R = 16.2$ min (peak A in Fig. 2) and uric acid at $t_R = 28.97$ min (peak G in Fig. 2).

Recovery

Instead of using the internal standard method for correction of losses during the extraction procedure, we analyzed the loss for each organic acid separately (Table II). The first part of this table gives the slope and correlation coefficient of the regression lines for each standard, carried through the extraction procedure with acetonitrile. The high correlation coefficients show that linearity is not af-

TABLE III

BLOOD VALUES OF ORGANIC ACIDS IN FOUR CONTROLS (ADULT, HEALTHY) AND IN ONE PATIENT WITH LACTIC ACIDEMIA

Substance	Controls (μM)	Patient (μM)
cis-Aconitate	1.69 ± 0.9	14.7
Oxaloacetate	–	–
α-Ketoglutarate	14.5 ± 5.4	65.0
Citrate	87 ± 36	180
Pyruvate	43 ± 10	138
Malate	–	–
Succinate	–	–
Lactate	465 ± 165	5672
β-Hydroxybutyrate	554 ± 288	1437
Fumarate	0.058 ± 0.018	0.266*

*Combined fumarate-acetoacetate peak.

TABLE IV

CORRELATION BETWEEN TIME AND CONCENTRATIONS OF ORGANIC ACIDS IN HUMAN BLOOD, STORED IN LIQUID NITROGEN ($n=7$)

Substance	Correlation coefficient	p Value
cis-Aconitate	0.27	0.60
α-Ketoglutarate	−0.20	0.73
Citrate	−0.15	0.79
Pyruvate	−0.79	0.06*
Succinate	−0.51	0.26
Lactate	−0.34	0.47
β-Hydroxybutyrate	−0.57	0.19
Fumarate	−0.16	0.76

*Statistically significant ($p<0.1$).

fected by the extraction, but recovery is, varying between 55 ± 2% for citrate and 134 ± 11% for malate. When standards are added to blood and then carried through the acetonitrile extraction recoveries are lower than with extracted standards alone, except for pyruvate (87 ± 3% versus 114 ± 18%). A typical result is shown in Fig. 3. Different concentrations of succinate with and without addition to blood are plotted against the resulting peak areas. Recoveries from blood range between 14 ± 2% for oxaloacetate and 114 ± 18% for pyruvate. The striking loss of oxaloacetate in particular leads to the inhibitor studies, results of which are detailed below.

Correlation coefficients between 0.88 and 0.98 for carboxylic acids in blood suggest linearity between peak area and concentration. Blood values for organic acids in four healthy controls and in a patient with lactic acidemia of unknown etiology are displayed in Table III. Peak areas have been converted to concentrations using factors derived from the regression lines of acetonitrile extracted standards in blood (Table II, column 4). *cis*-Aconitate and fumarate are detected

at very low concentrations as a result of their high UV absorption. A high detection limit was found for malate in blood: 918 μM compared to 24 μM, when only standards are injected. This is due to an unknown compound coeluting with malate ($t_R = 17.15$ min). Only malate concentrations higher than 918 μM give a separate peak. Large concentrations of pyruvate and lactate are seen in the patient's blood sample. Of interest, glucose and unknown peaks B, C, D, F and H are also highly elevated (data not shown).

Detection limits for blood organic acids whose normal values are reported following enzymatic assay [15, 16] are: lactate (3%), pyruvate (4%), α-ketoglutarate (50%) and citrate (60%). No succinate was detected in our control blood samples when chromatography at 65°C (see Experimental) was used to separate succinate from unknown peak E (Fig. 2) which at 36°C has the same retention time as succinate.

Enzyme inhibitors

Addition of the enzyme inhibitors aminooxyacetic acid and fluorocitrate did not improve recovery of citric acid cycle intermediates in blood. No change in oxaloacetate was observed and a large pyruvate loss occurred.

Extraction procedures

While the extraction of plasma as well as blood with acetonitrile gives good chromatograms, the extraction of blood with perchloric acid produces a UV-absorbing peak that contaminates the early region of the chromatogram in which *cis*-aconitate, oxaloacetate, α-ketoglutarate, citrate, pyruvate and malate elute. The same problem occurred with trichloroacetic acid. Therefore, only plasma was extracted with perchloric acid for comparison with the acetonitrile extraction technique. Perchloric acid extraction of plasma yields significantly higher levels for each organic acid than acetonitrile extraction. The difference between acetonitrile and perchloric acid extraction of plasma is lowest for lactate (ratio = 0.68) and highest for citrate (ratio = 0.41). In a small number of experiments perchloric acid extraction of organic acids from plasma yielded recoveries of close to 100% except for oxaloacetate and β-hydroxybutyrate where losses were considerable.

Storage

Table IV shows that only in the case of pyruvate did significant loss occur during liquid nitrogen storage of acetonitrile extracts. This loss (approximately 28%) occurred within the first two weeks. Succinate ($r = 0.51$) and β-hydroxybutyrate ($r = 0.57$) also show relatively high correlation coefficients. Although they are not statistically significant, a slight decrease during the first two weeks must be assumed for these compounds also.

DISCUSSION

A method for separation of citric acid cycle intermediates in insect haemolymph by HPLC has been described by Womersley et al. [11]. In our laboratory,

this separation could not be replicated with respect to oxaloacetate, α-ketoglutarate and citrate. We found, however, that using two heated (36°C) Aminex HPX-87H columns in series provides a satisfactory separation of all the citric acid cycle intermediates of interest as well as lactate and pyruvate.

Our experience with acetonitrile extraction is similar to data reported by Marsili et al. [7]. As shown in Table I, our system has linear characteristics over a wide concentration range. This linear relationship between peak area and concentration is confirmed not only for standard solutions but also for blood. The minimal detection limits of our technique are 17–400 times lower than values published by Turkelson and Richards [6], using an Aminex 50W-X4 cation-exchange resin and UV detection at 210 nm.

The results from our lactic acidemia patient (Table III) illustrate the point that greatly elevated levels of citric acid cycle intermediates may be seen in this patient population. Our detection limits are adequate for identification of elevated citric acid cycle intermediates with our limits ranging from 3 to 60% of published normal blood levels determined enzymatically [15, 16]. An exception is succinate which we were unable to detect in our control blood samples in spite of the report by Krebs [17] of normal plasma levels of 43 μmol/l. The reason for this discrepancy is not obvious as our detection limit for succinate is 50% of this value.

Marsili et al. [7] and Adams et al. [8] reported the use of acetonitrile for extraction of organic acids from dairy products. Recoveries of organic acids added to milk were better than 90% [7] for most of the citric acid cycle intermediates. We found recoveries ranging from 55 to 134% for standard solutions extracted with acetonitrile. Lower recoveries (14–114%) were found with standard solutions added to human blood. Using different correction factors for each organic acid appropriate measurements of blood levels may be obtained.

It is unlikely that the loss of organic acids following acetonitrile extraction of blood is due to a specific effect of acetonitrile, because concentrations of these compounds in plasma are very similar with either acetonitrile or perchloric acid extraction (Table V). Low recoveries of fumarate (66%) and lactate (25%) when added to culture medium and then extracted with diethyl ether have been also reported [18]. Moreover, Marsili et al. [7] found recoveries of 87–99% for standards added to milk using acetonitrile extraction.

Oxaloacetate is the most labile of the citric acid cycle intermediates; with either acetonitrile or perchloric acid extraction most of the added oxaloacetate is lost. It is unclear whether this is the result of absorption to protein, non-enzymatic or enzymatic degradation. The results of acetonitrile extraction of a mixed standard solution in 0.025 *M* sulfuric acid allows estimation of the loss due to the acetonitrile extraction procedure with drying in the absence of protein. Recovery of oxaloacetate with acetonitrile extraction of standards is $67 \pm 2\%$ (Table II). Thus, the extraction procedure itself cannot explain the much greater loss of oxaloacetate seen with blood and plasma extraction.

Because the addition of enzyme inhibitors could not improve the recovery of citric acid cycle intermediates in blood an enzymatic degradation seems a less likely explanation. However, this possibility cannot be completely excluded by

TABLE V

EFFECT OF TWO DIFFERENT EXTRACTION PROCEDURES ON BLOOD ORGANIC ACIDS (FOUR CONTROLS, DOUBLE MEASUREMENTS)

pl-acn = plasma extracted with acetonitrile; pl-pca = plasma extracted with perchloric acid.

Substance	pl-acn (μM)	pl-pca (μM)	acn/pca ratio
cis-Aconitate	0.60 ± 0.88	1.1 ± 0.2*	0.55
Oxaloacetate	–	–	
α-Ketoglutarate	2.99 ± 0.28	5.6 ± 1.6*	0.53
Citrate	21.0 ± 6.9	51.3 ± 22.1*	0.41
Pyruvate	44.6 ± 5.5	66.2 ± 8.4*	0.67
Malate	–	–	
Succinate	–	–	
Lactate	367 ± 135	543 ± 208*	0.68
β-Hydroxybutyrate	264 ± 59	530 ± 170*	0.49
Fumarate	0.026 ± 0.01	0.056 ± 0.011*	0.46

*Significant difference to pl-acn at $p < 0.01$.

our data because not all the enzymes involved have been inhibited. The fall of pyruvate observed after selective enzyme inhibition is unexplained. Some residual enzyme activity may remain after acetonitrile extraction although the pyruvate loss could be non-enzymatic.

Although perchloric acid extraction is generally used for deproteinization [15], the use of acetonitrile extraction allows study of whole blood samples which can be rapidly deproteinized immediately on collection.

Lowry and Passonneau [19] found that liquid nitrogen storage of non-extracted tissue produced little or no loss of the citric acid cycle intermediates studied. However, after extraction several organic acids become unstable. Pyruvate, in particular, is lost in perchlorate extractions stored in liquid nitrogen [19]. Our experience with acetonitrile extraction similarly showed loss of pyruvate during storage and also probably succinate and β-hydroxybutyrate. This lack of stability in extractions contrasts to the excellent stability of our standard citric acid cycle intermediates dissolved in 0.025 M sulfuric acid and stored in liquid nitrogen. These standards show no change over months of storage.

The nature of the unidentified peaks found in blood (Fig. 2) remains unclear. UV detection is a relatively non-specific detection method. Aminex HPX-87H strong cation-exchange column material separates alcohols and carbohydrates as well as organic acids. Most sugars and alcohols will not be detected at 205 nm unless present in high concentration (i.e., glucose, $t_R = 16.2$ min, Fig. 2), but many organic acids will be and thus most of our unidentified peaks are probably produced by organic acids unrelated to the citric acid cycle. Uric acid produces a large peak (G in Fig. 2, $t_R = 28.97$ min).

Daish and Leonard [10] report identification of the common organic acidurias by UV detection of plasma extracts following Aminex HPX-87H column chromatography. In our system methylmalonate, isovalerate and propionate have retention times of 19.25, 48.79 and 33.43 min, respectively. These organic acids are

not detected in our control blood samples, but in patients with organic aciduria caution is needed in the interpretation of blood chromatograms.

Our results highlight the limitations of a simple internal standard method for correction of recovery when several different compounds are to be quantified. As the recovery through our extraction is different for each organic acid, a separate correction factor calculated from the slope of a concentration versus peak area plot must be applied to each organic acid. Using such correction factors the original concentration of organic acids present in the sample can be estimated from the area measured on the chromatogram.

The ability to so easily measure the intermediates of the citric acid cycle in blood and other biological tissues will facilitate the investigation of defects in intermediary metabolism and has particular application for the study of human lactic acidemia.

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