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Note

Advantages and limitations of isotachophoretic determinations of metabolites in tissue extracts

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Isotachophoresis is a very useful technique for analyzing biochemical samples because of its high resolution power which allows analysis of small sample volume as well as of products in low concentration. Metabolite and nucleotide analysis, a very important aim in biological studies, has been resolved in part by isotachophoresis. Conventional techniques, *i.e.*, NMR spectroscopy, firefly luciferase method and enzymatic end-point assay, give good results but are time-consuming, require specific reactions for each substance and large sample volumes —conditions which are not always possible. Several authors have developed experimental methods for isotachophoretic metabolite and nucleotide separation in biological samples[1–6], generally by using UV detection. A calibration with commercial UV-absorbing substances is possible but cannot be directly applied in routine procedures for biological sample analysis because it is necessary to show that each nucleotide used in the isotachophoretic analysis is not mixed with other UV-absorbing products.

The aim of this study was the experimental determination of several metabolites and nucleotides in biological samples using conductivity detection.

EXPERIMENTAL

Tissue samples from muscle or liver were excised and frozen with liquid nitrogen, followed by an extraction with hydrochloric acid and a neutralization with $KHCO_3$[7,8]. The leading electrolyte was 5 m*M* HCl with β-alanine as a counter ion, adjusted to pH 3.89 and 0.5% hydroxypropylmethylcellulose (HPMC) as an additive to increase the viscosity and thus minimize the electroendosmosis. The terminating buffer was 5 m*M* *n*-caproic acid. Analysis were performed on a LKB 2127 Tachophor equipment with UV and CD detection systems connected to an Hewlett-Packard integrating printer plotter 3390 A. Analytical separations were carried out in a PTFE capillary tube (length 430 mm) thermostatted at 15°C. The average volume injected was 5 μl. Separation was started at 125 μA and manually reduced to 25 μA so that the effect never exceeded 2.5 W.

RESULTS

Each individual substance (or zone) has a defined resistance and thus a defined

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conductivity step height under these conditions —this is a characteristic parameter for each substance. Table I shows the relative step heights for eleven standard solutions of metabolites and nucleotides. The coefficient of variation in average is 2.5%. The results of the calibration are also shown. Calibration curve were constructed by using the zone length obtained from the differential signal of the conductivity detector. The correlation coefficient ranged from 0.997 to 0.999 with an average coefficient of variation of 4.0%.

TABLE I

CALIBRATION AND QUANTITATION BY CONDUCTIVITY DETECTION

PEP = Phosphoenolpyruvate; PYR = pyruvate; FDP = fructose diphosphate; P_i = inorganic phosphate; F6P = fructose-6-phosphate.

Compound	*Rel. step height*		*Coefficient of variation,* v_K *(%)*	*Correlation coefficient, r*	*Coefficient of variation* v_K*, (%) (means)*	*Range of concentration (nmol)*
	$\bar{x}$	$\pm s$				
Leading ion	0.0	–	–	–	–	–
PEP	0.113	0.002	1.8	0.999	3.8	0.5– 5.0
PYR	0.138	0.006	4.4	0.998	4.7	0.5–14.0
ATP	0.179	0.006	3.4	0.998	1.0	0.5– 5.0
FDP	0.183	0.005	2.7	0.999	5.1	0.5– 5.0
P_i	0.205	0.005	2.4	0.997	5.1	1.0–10.0
CP	0.270	0.009	3.3	0.999	5.5	0.3– 6.0
ADP	0.293	0.008	2.7	0.998	7.2	0.5– 5.0
Lactate	0.316	0.009	2.9	0.998	3.7	1.0–20.0
F6P	0.391	0.006	1.5	0.998	3.1	0.5– 5.0
cAMP	0.586	0.005	0.9	0.999	7.4	0.5– 5.0
AMP	0.675	0.018	2.7	0.998	4.0	0.5– 5.0
Terminating ion	1.0	–	–	–	–	–

Table II shows some results of recovery studies. The recoveries of ATP, creatine phosphate (CP) and lactate in a muscle extract ranged from 96 to 105%; the correlation coefficients are nearly the same as in aqueous solutions, Fig. 1. We can conclude that the analytical precision is very good in this concentration range even in tissue extracts.

The stability of the tissue extract was also studied. Fig. 2 shows that there are no remarkable differences within 3 h for ATP and CP if the sample is kept at 4°C.

TABLE II

RECOVERY STUDIES

	Recovery (%) (n = 3)		*Correlation coefficient, r (n = 10)*	
	Range	*Average*	*Aq. solution*	*Extract*
ATP	97–103	99.0	0.998	0.996
CP	97–105	99.5	0.999	0.998
Lactate	96–102	98.8	0.998	0.996

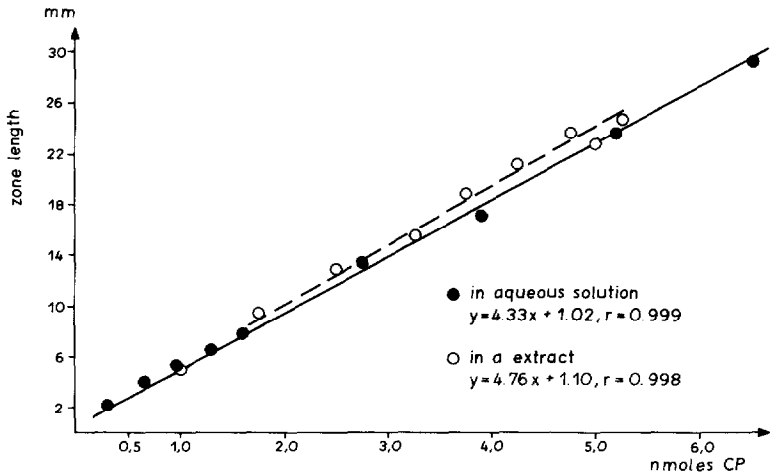

Fig. 1. Calibration curves for creatine phosphate.

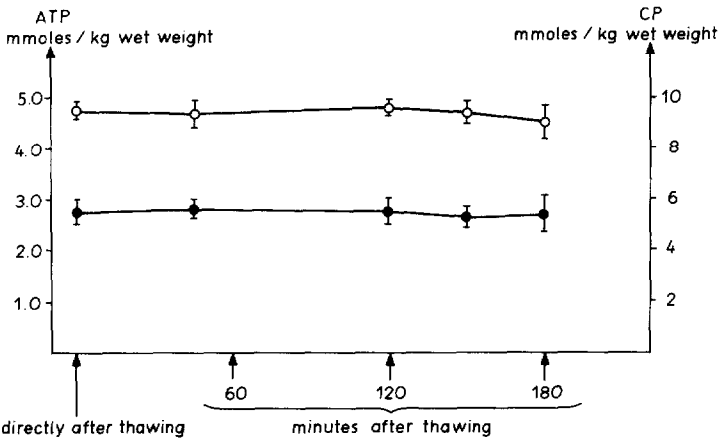

Fig. 2. Stability of ATP (●) and CP (○) in a tissue extract.

Fig. 3 compares isotachophoretic analysis with the enzymatic end-point assay. The tissue concentrations determined by the former method are throughout slightly lower than those of the enzymatic assay for ATP, CP and lactate. However, there is a good correlation between both methods the slopes of the plots in Fig. 3 ranging between 45 and 46.5°. Similar results have been reported by several authors, *e.g.*, Aomine *et al.*[9]. Surholt[6] obtained slightly higher values for ATP and ADP, and corresponding values for AMP by using the UV peak areas in isotachophoresis.

CONCLUSIONS

It can be concluded that conductivity detection is a viable possibility for the isotachophoretic analysis of various chemical compounds and has several advantages, *e.g.*, short separation time, high sensitivity, high resolution and good repro-

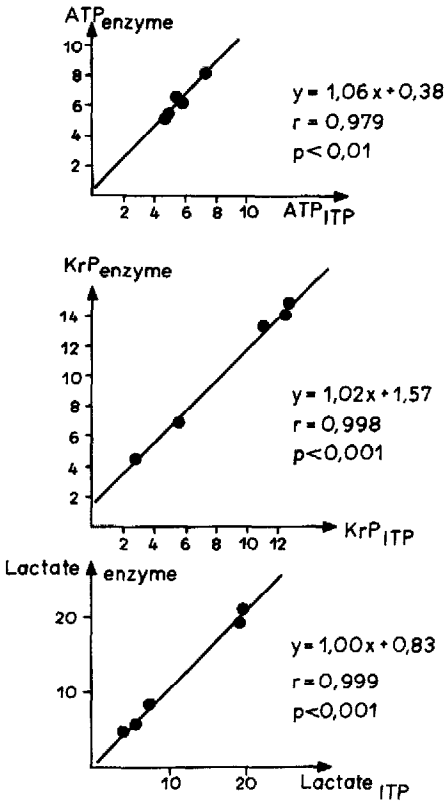

Fig. 3. Comparison of ITP data with enzymatic values (mmoles/kg wet weight).

ducibility. With this method, it is possible to detect a large number of substances during a single analysis. This approach has been used to analyze proteins, nucleotides, amino acids and metal ions. The chemicals used were of analytical grade. For analysis of biological extracts, *e.g.*, muscle or liver tissue, there are some limitations. For capillary injection, sample volumes of approximately 5 μl are useful because volumes over 10 μl resulted in poor zone separation and with less than 5 μl the quantitative analysis was less reliable. Another problem is that only part of the high information content of the detector signal is employed by a simple recorder. Concentration of the tissue extract by freeze-drying is one way of circumventing this problem. Also new developments, such as the steady-state mixed-zone technique, the coupled-columns system and the combination of both methods, may be used to solve the limitations. The application of computer analysis for UV-peak characterization seems also to be successful. The Hewlett-Packard printer plotter with its several integrating programs provides good results in this respect.

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