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Journal of Chromatography A, 706 (1995) 459–462

JOURNAL OF
CHROMATOGRAPHY A

Analysis of the acidic microenvironment in murine tumours by high-performance ion chromatography

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Abstract

High-performance ion chromatography (HPIC) has been utilised to probe the biochemistry associated with changes in tumour pH following total vascular occlusion. Samples from the tumour extracellular compartment were obtained by insertion of a microdialysis probe and analysed by HPIC with conductivity detection. Separations were carried out by ion-exclusion chromatography using an IonPac ICE AS1 weak-acid column. The eluent (0.5 m*M* octanesulphonic acid) was chemically suppressed with 5 m*M* tetrabutylammonium hydroxide through a micromembrane suppressor. After complete vascular occlusion induced by a clamp, lactate levels increased in the extracellular compartment.

1. Introduction

Early reports suggested that tumours preferentially metabolise glucose by anaerobic glycolysis to lactic acid even if oxygen were available [1]. Because of the poor vascularity of tumours, lactic acid accumulates within the tumour and has often been proposed as a determinant of tumour acidity. The pH of the tumour microenvironment may be an important factor affecting the cellular uptake, distribution and activity of anticancer drugs [2–4]. The intra- to extracellular pH gradient (pH_i/pH_e) is in the opposite direction to, and of a larger magnitude than in normal tissues [3,5], and may be used to accumulate drugs with weak acidic functions within the relatively higher pH of the intracellular compartment, thereby enhancing tumour cell kill [6–8]. In this study, tumour acidity was altered by vascular occlusion in a murine tumour model [9] or using vinblastine, a vasoactive drug, which reduces tumour blood flow [10,11].

Microdialysis has previously been widely used to monitor changes in tissue metabolites during ischaemic periods, e.g. in heart or skeletal muscle [12,13]. We have investigated the relationship between tumour lactate concentrations and extracellular acidity using this technique, combined with ion-exclusion chromatography.

2. Experimental

2.1. Animals

Mice (CBA/Gy f TO males) were transplanted subcutaneously on the dorsum with the syngeneic adenocarcinoma CaNT. When the

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SSDI 0021-9673(95)00016-X

tumours reached 7–9 mm in diameter, blood flow was occluded using a metal clamp and microdialysis was performed at various periods of time between 0 and 6 h of occlusion. Microdialysis was also performed up to 1 h after the clamp was removed. The cytotoxic agent vinblastine was administered intraperitoneally at 5 mg/kg (0.01 ml/g) body weight and microdialysis performed up to 6 h after injection. Immediately prior to the insertion of the microdialysis probe, animals were killed by cervical dislocation.

2.2. Sample preparation

Normal saline was pumped through the microdialysis probes (CMA/12, Biotech Instruments, Herts, UK) at 1 μl/min using a syringe infusion pump (Harvard Apparatus, MA, USA). The first 6 μl of the dialysate, which represent the dead volume of the probe and tubing, were discarded, and the next 16 μl collected directly into vials (limited-volume inserts, Waters, Watford, UK). The samples were then frozen at −20°C until analysis by HPIC.

2.3. Chromatography

Dialysates (5 μl) were injected using a WISP autosampler (Waters) onto a DX 100 system (Dionex, Camberley, UK). The column was a weak-acid IonPac ICE AS1 ion-exclusion column (Dionex), eluted with 0.5 m*M* octanesulphonic acid (Dionex) at a flow-rate of 1.2 ml/min. Detection was by conductivity following chemical suppression with 5 m*M* tetrabutylammonium hydroxide in counterflow through an AMMS ICE anion micromembrane suppressor at a flow-rate of 2 ml/min. Where spectrophotometric detection was used, a fixed-wavelength detector operating at 214 nm was inserted after the conductivity detector (Waters). Chromatograms were processed using an 840 data analysis system (Waters).

3. Results

Lactate recovery from stock solutions of lactate in saline was determined and found to be linear with respect to lactate concentrations up to 10 m*M* ($r > 0.999$). No significant differences were found between each probe, and the average recovery of 30% at 1 μl/min was used to calculate the tumour concentrations reported. Higher flow-rates resulted in a much reduced recovery (13% at 3 μl/min, 9% at 6 μl/min). The extent of carry-over from each consecutive sample was determined by allowing increasing volumes of dialysate to be discarded before collection of the next sample after transfer of the probe between saline and lactate solutions. If the first 6 μl of the dialysate were discarded, carry-over was minimal (Table 1). Intra-assay variability was assessed using replicate analyses of a control tumour dialysate ([lactate] = 1.95 ± 0.06 m*M*, mean ± S.D., $n = 5$). The intra-tumour heterogeneity was determined from the variation in lactate concentration obtained after inserting two probes into a number of tumours; the error was found to be 15.7 ± 12.3% (mean ± S.D., $n = 20$). Dialysates of standard lactate solutions were found to be stable; however, degradation of lactate was initially observed in dialysates obtained from some tumours if stored at room temperature overnight prior to analysis (Fig. 1). Also shown in Fig. 1 is the peak corresponding

Table 1
Carry-over of 45 m*M* lactate into saline during microdialysis at 1 μl/min

		[Lactate] (m*M*)
Lactate dialysed	Collect 15–30 min	13.9
Saline dialysed	Discard 0–6 min (6 μl), collect 6–18 min	0.18

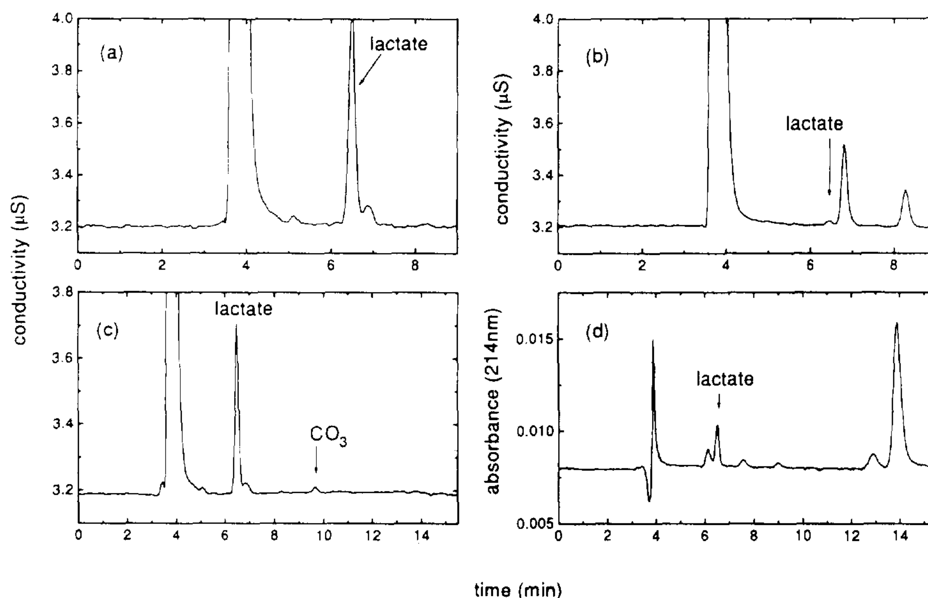

Fig. 1. HPLC profiles showing lactate in a tumour microdialysate analysed (a) immediately and (b) after 21 h at room temperature (sample diluted 1:3 with water); (c, d) extended analysis time showing carbonate (c) and unidentified UV-absorbing species (d). Chromatography was carried out on an IonPac-AS1 column, eluent 0.5 m*M* octanesulphonic acid.

to carbonate and a chromatogram showing the absorbance at 214 nm.

Lactate accumulation was determined in clamped or vinblastine-treated tumours as shown in Fig. 2. After vinblastine treatment there was no significant change in lactate concentration. In contrast, there was significant accumulation of lactate up to 6 h after vascular occlusion induced by clamping. It can be seen that the lactate accumulation appeared to correlate with the decrease in extracellular pH measured under identical treatment conditions. The open symbols indicate the reduction in tumour lactate concentration which occurs if the clamps were removed following a period of 6 h occlusion.

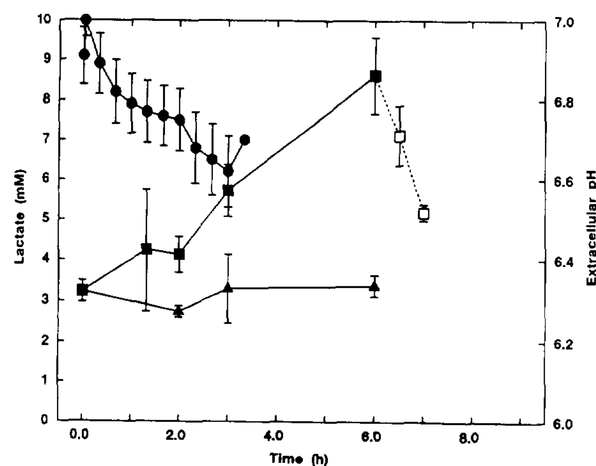

Fig. 2. Lactate accumulation and extracellular pH in CaNT tumours after clamping or vinblastine treatment. (▲) Lactate following vinblastine; (■) lactate following clamping; (□) lactate following clamp removal; (●) extracellular pH during clamp.

4. Discussion

The data presented here illustrates the use of ion-exclusion chromatography with conductimetric detection for analysis of lactate and other weak acids in the small volumes that are characteristically collected using microdialysis probes. The strong anions, particularly chloride which are present at high concentration in all biological samples, elute unretained under these conditions, allowing a rapid analysis. The peak shape

for lactate was good, and the error of replicate injections was ~3%. The sensitivity of the method is such that duplicate analyses can be made from as little as 16-μl collected volumes. Other anions such as carbonate were also detected although the quantification of this ion is complicated by its equilibration with atmospheric CO_2. The observation that lactate in tumour dialysates was sometimes degraded is unexplained, as dialysed lactate solutions are stable and degradative enzymes would not be expected to cross the dialysis membrane.

The increased capacity of tumour cells to metabolise glucose by glycolysis is a characteristic of solid tumours, and accumulated lactate has been shown using a variety of techniques, e.g. bioluminescence [2]. The present microdialysis data support these observations of high concentration in tumour relative to normal tissue (e.g. kidney, [lactate] = 0.1 m*M* [14]). The significant accumulation of lactate observed in response to ischaemia is not unexpected and not tissue-specific, although the magnitude in normal tissues are less than reported here in murine tumours. The reduction in tumour blood flow induced by the cytotoxic agent vinblastine is of the order of 50% at 6 h after the drug dose used here [10]. This partial vascular occlusion resulted in no significant accumulation of lactate over the time period studied, presumably because the residual blood flow was sufficient to remove any accumulated lactate. By comparison, the application of complete vascular occlusion, by clamping, resulted in a time-dependent increase in lactate. Because the pK_a of lactic acid is 3.7, the ion will be essentially fully dissociated under physiological conditions. The high extracellular proton concentration would be expected to result in the reduction in extracellular pH as shown, although the time course of the changes are slightly different. This may suggest that other factors, e.g. accumulation of other metabolites, may complicate the relationship. Preliminary observations were also made on the tumour lactate concentration during the period following clamp removal. The rapid decrease in tumour lactate observed suggests a reperfusion of the tumour allowing any accumulated lactate to be removed. Indeed, studies on tumour blood flow recovery after clamp removal would support this hypothesis [15].

In conclusion, we have used the combination of microdialysis with HPIC to investigate some of the biochemical changes occurring in the tumour microenvironment.

Acknowledgements

This work is supported by the Cancer Research Campaign. Sponsorship from Dionex is gratefully acknowledged.

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