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Note

High-performance liquid chromatographic determination of plasma lactate specific radioactivity

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The supply and utilisation of lactate in vivo can be studied by administration of radiolabelled lactate into the circulation and subsequent determination of the specific radioactivity of plasma L-(+)-lactate [1]. Several different approaches to this determination have been used, including ion-exchange chromatography [2, 3] and radioisotopic dilution analysis [4, 5]. However, methods involving the isolation of lactate from plasma by step gradient ion-exchange chromatography and subsequent enzymatic analysis of the isolated fraction for lactate and liquid scintillation counting of radioactivity content require at least 1.0 ml of plasma or blood and are of possibly variable specificity [2, 3, 6]. While radioisotopic dilution analysis with derivatisation of lactate is more specific, it requires a comparable volume of plasma, is more time-consuming and can involve the removal of a potentially labelled carbon atom, leading to errors in samples of unknown label distribution [5].

To study lactate metabolism in the sheep fetus [7], we attempted to develop a simple and rapid method for the determination of plasma lactate specific radioactivity, which would utilise smaller volumes of plasma than conventional methods, permitting repeated sampling of a limited blood volume. The application of high-performance liquid chromatography (HPLC) to the isolation of lactate seemed an obvious approach because separation of some organic acids by HPLC had already been achieved using octadecyl silica columns at low pH with acetonitrile—water gradients [8] or ion-exchange chromatography [9]. The first method was adopted because it had the adavantages of being inexpensive, simple and rapid. Detection of lactate is difficult, however, due to its lack of absorption in the ultraviolet—visible range. Although conversion of α-keto acids to their 2,4-dinitrophenylhydrazones, which absorb between 380 and 520 nm, has been used to overcome this problem, lactate cannot be

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derivatised in this fashion [10]. The feasibility of monitoring in the low ultraviolet region was therefore examined.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. L-(+)-lactate, Triton X-100, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPOP) and 2,5-diphenyloxazole (PPO) were obtained from Sigma (St. Louis, MO, U.S.A.). Trichloroacetic acid, orthophosphoric acid, diethyl ether and toluene were obtained from BDH (Port Fairy, Australia). Acetonitrile (HPLC grade) was obtained from Waters Assoc. (Sydney, Australia). Millipore Q reagent grade water was used throughout. L-[U-^{14}C]lactic acid, sodium salt (1.85—5.5 GBq/mmol) was obtained from Amersham Australia (Sydney, Australia).

Preparation of samples

Samples (5.0 ml) of heparinised blood were obtained from sheep fetuses with indwelling vascular catheters during late gestation [7]. Following centrifugation at 4°C and 2500 *g* for 15 min, plasma was removed and stored at −20°C until extraction.

Aliquots (0.1—0.5 ml) of plasma were deproteinised by the addition of 0.2 ml of 14% (w/v) trichloroacetic acid at 4°C. After vigorous mixing, samples were incubated at 4°C for 5 min, then centrifuged at 4°C and 2500 *g* for 20 min. The supernatants were poured into 1-ml disposable plastic syringes attached to pre-equilibrated C_{18} Sep-Pak cartridges (5 ml of acetonitrile followed by 15 ml of water) and washed through with water (see Fig. 1). Drops 11—55 were collected for further processing. The eluent was extracted three times with an equal volume of water-saturated diethyl ether in glass-stoppered tubes (10 ml) on a rotary mixer for 5 min. The water phase was then removed and freeze-dried. Lyophilised extracts were stored at −20°C until analysis.

Apparatus

A Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) was used for solvent delivery. Injections were made using a Waters U6K universal injector. The eluent was monitored at 190, 220 and 245 nm using a Waters variable UV/Vis detector, Model 440, and the signal recorded on an Omniscribe recorder (Houston Intruments).

Chromatography

Plasma extracts (reconstituted in 50 or 100 μl of 0.01 *M* phosphate buffer of the same pH as the mobile phase) and lactate standards (in mobile phase) were chromatographed on a 4.6 × 250 mm μBondapak octadecyl silica (10 μm) column (Waters) with a mobile phase of 0.01 *M* phosphate buffer at varying pH. Aliquots of 1—10 μl were injected. The mobile phase of 0.01 *M* phosphate buffer was prepared by diluting 1.0 ml of orthophosphoric acid to approximately 50 ml, adjusting the pH appropriately with 2 *M* KOH, then diluting to 1000 ml with water, with a final adjustment made to give the required pH. A flow-rate of 2.0 ml/min was used with a back-pressure of 1950 p.s.i.

Protection of the column with a guard column (C_{18}/Corasil, Waters) was necessary to minimise the hydrolytic action of the low-pH mobile phase. Storage of the column in water only and use of the guard column resulted in little change of retention time with extensive use.

Peak areas were calculated by multiplying peak height by peak width at half height. All results are given as means with standard errors.

Measurement of radioactivity content in chromatography eluent

Chromatography eluent was collected in scintillation vials as 5-sec fractions for 4 min from the time of injection. Scintillation fluid (4 g PPO, 0.1 g POPOP, 330 ml Triton X-100 made up to 1 litre with toluene) (10 ml) was then added to each fraction. Samples were left to stand for 12 h in the dark before measurement of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. A quench curve was constructed using the external standards channels ratio method, and corrections for quenching were made.

RESULTS

Sample preparation

To prepare an extract suitable for HPLC, plasma was deproteinised, then hydrophobic components that would have accumulated on the analytical column were removed by C_{18} Sep-Pak chromatography (Fig. 1). The deproteinising agent, trichloroacetic acid, was itself removed by extraction into diethyl ether as it would have rendered lyophilisation and reconstitution in a small volume difficult. It also interfered with the chromatographic analysis and attacked the analytical column packing, reducing column efficiency with use. Deproteinisation with perchloric acid and neutralisation with potassium hydroxide were not feasible due to interference in detection of lactate. Recovery of lactate in the final reconstituted extract (as estimated by extracting ten plasma samples to which 67 pmol, 0.37 kBq L-[U-^{14}C]lactate had been added) was 94 ± 1.4% (S.E.M.).

Chromatographic analysis

To optimise the chromatographic separation of lactate from other compounds present in the plasma extracts, the pH of the mobile phase was varied from 2.3 to 3.5 and the eluent monitored at 190 nm. Baseline separation of a peak with the same capacity factor (k') as pure lactate was achieved with a mobile phase of pH 2.7 (Fig. 2). Accordingly, this optimum pH was adopted in subsequent studies.

Chromatography of pure amino acid standards — alanine, glutamine and glutamate — under these conditions indicated that they eluted just after the void volume at approximately 1.4 min (k' 0.001—0.065) as did the carbohydrates glucose and fructose (k' 0.064—0.069), and could be collected for further analysis if required.

Some trichloroacetic acid was still present in plasma extracts as indicated by a large peak eluting with the characteristic retention time of 4.5 min (k' 2.46). Elution of all compounds present was complete within 6 min of injection, allowing analysis of at least 60 samples plus standards per day.

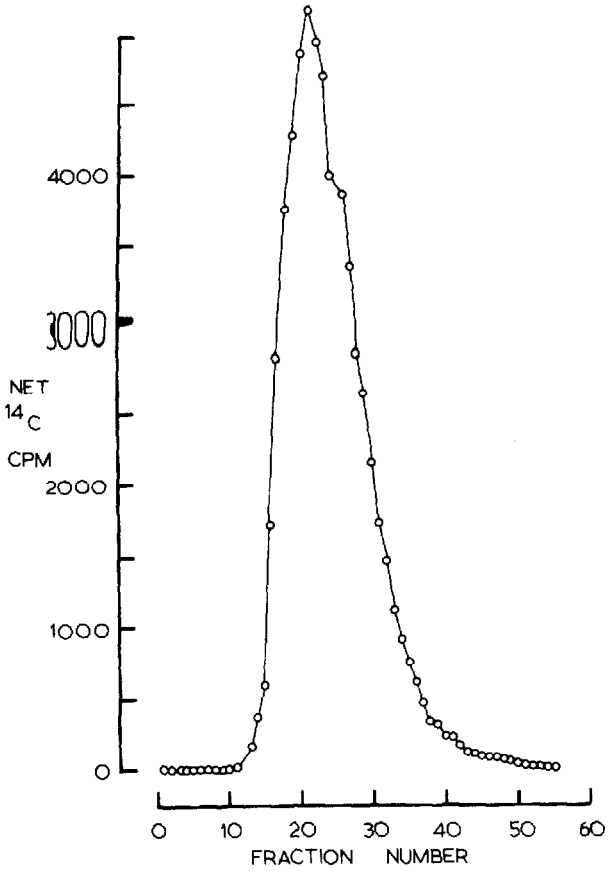

Fig. 1. Elution profile of L-[U-^{14}C]lactate in deproteinised plasma applied to a C_{18} Sep-Pak cartridge followed by washing with water. Fractions consisting of one drop were collected into scintillation vials and radioactivity content was measured as described for chromatography of eluent in text.

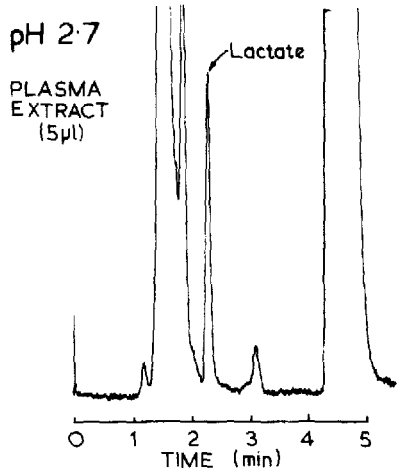

Fig. 2. HPLC of plasma extracts (reconstituted in 100 µl of appropriate mobile phase) at pH 2.7. Conditions: column, µBondapak C_{18} 4.6 × 250 mm; mobile phase, 0.01 *M* phosphate buffer of varying pH; flow-rate, 2.0 ml/min; detection wavelength, 190 nm; a.u.f.s., 0.04; temperature, 22° C.

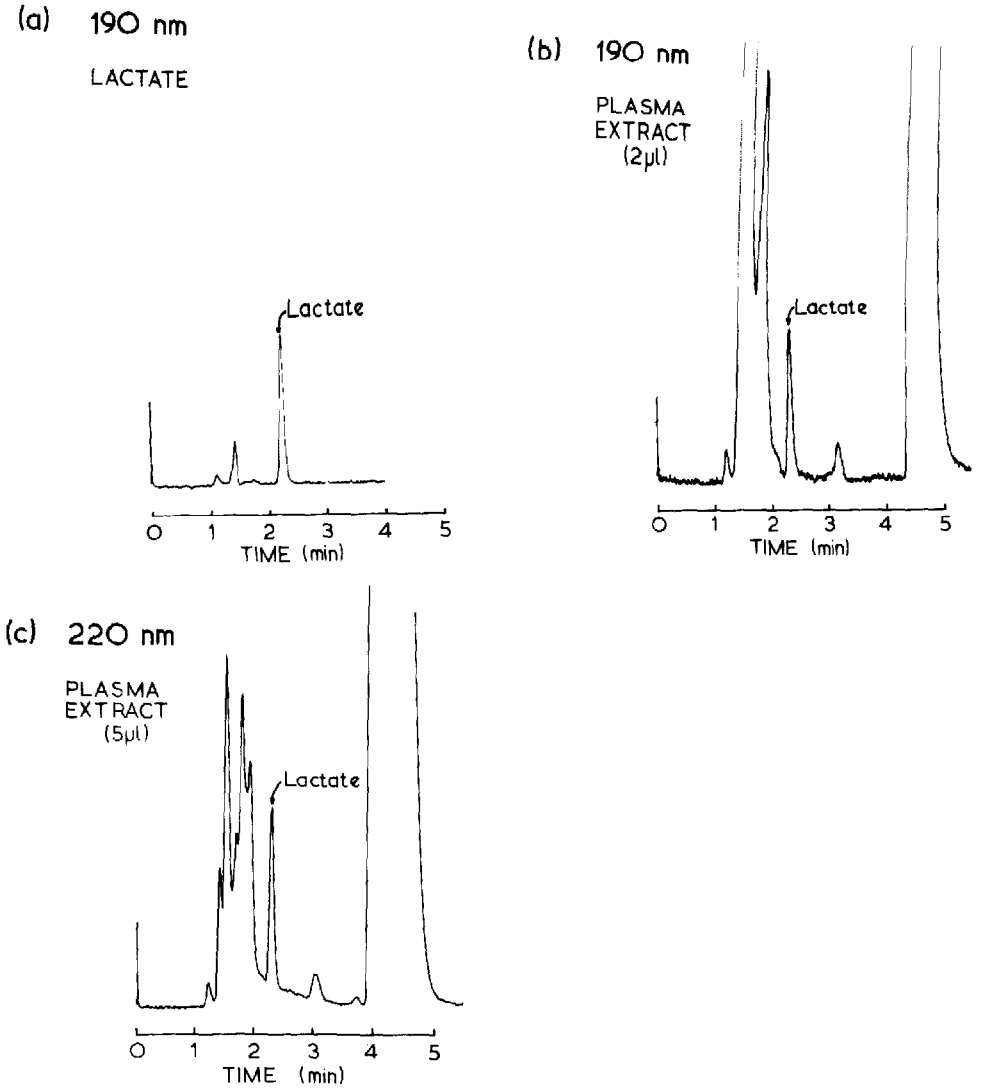

Fig. 3. HPLC of (a) lactate with detection at 190 nm, and of a plasma extract with detection at 190 nm (b) and 220 nm (c). Conditions: column, µBondapak C_{18}, 4.6 × 250 mm; mobile phase, 0.01 *M* phosphate buffer, pH 2.7; flow-rate, 2.0 ml/min; a.u.f.s., 0.04; temperature, 22°C.

The elution profiles of plasma extracts at different wavelengths were then examined (Fig. 3). As expected, significant absorbance by lactate in plasma extracts was seen only with the low-ultraviolet wavelengths, 190 and 220 nm (Fig. 3) and not with detection at 254 nm. The former was selected for use as it gave greater sensitivity and a more stable baseline (Fig. 3).

The specificity of the separation was examined by comparing retention times and peak area ratios (190 nm/220 nm) of lactate standards and the putative lactate peak in chromatographed plasma extracts (Table I). No significant differences in capacity factors or peak ratios were seen, indicating that lactate was in fact completely separated from other components in the extracts. Co-chromatography of pure lactate with plasma extract resulted in no change in retention time and capacity factor.

A linear response of peak area to lactate concentration was observed and the

TABLE I

IDENTIFICATION OF LACTATE IN PLASMA EXTRACTS FOLLOWING HPLC

Conditions were as described in Fig. 3. Results are expressed as mean ± S.D. (n = 5).

Sample	Capacity factor (k')	Peak area ratio (190 nm/220 nm)
Lactate	0.60 ± 0.02	2.35 ± 0.06
Plasma extract	0.59 ± 0.01	2.37 ± 0.10
Plasma extract plus lactate	0.59 ± 0.02	

limits of sensitivity under these conditions was 0.5 μg. The coefficients of variation of three determinations of the following lactate standards, 1, 4 and 10 μg, were 1.7, 0.9 and 0.8%, respectively.

Measurements of radioactivity content

The ^{14}C radioactivity content of lactate in chromatographed plasma extracts was estimated by summing all activity above background that eluted with the same retention time as the isolated lactate peak. For routine analysis, the peak was collected in bulk as one fraction. A quench curve was prepared using the external standards channels ratio method to correct for quenching. However, the efficiency of counting of eluent fractions only varied between 79.0 and 79.5%. The specific radioactivity of lactate in plasma to which 67 pmol, 0.37 kBq L-[U-^{14}C]lactate were added per 0.5 ml aliquot prior to extraction was 0.348 ± 0.028 kBq/μmol (n = 10), giving a coefficient of variation of 3.2% for the determination of plasma lactate specific radioactivity.

DISCUSSION

The determination of plasma lactate specific radioactivity by high-performance liquid chromatography on octadecyl silica is both rapid and precise. The technique requires only small volumes of plasma compared to conventional methods because of its high sensitivity in quantitation. Thus the minimum plasma volume extracted is usually limited only by the radioactivity content of lactate in the plasma sample. The addition of a suitable internal standard prior to extraction would also permit simultaneous determination of plasma lactate concentrations if required.

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REFERENCES

1 J. Katz, F. Okajma, M. Chenoweth and A. Dunn, Biochem. J., 194 (1981) 513.

2 M.W. Riley, Anal. Biochem., 22 (1968) 341.
3 D.G. Clark, R. Rognstad and J. Katz, J. Biol. Chem., 249 (1974) 2028.
4 E.F. Annison, D.B. Lindsay and R.R. White, Biochem. J., 88 (1963) 243.
5 C.L. Long, Y. Mashima and F. Gump, Anal. Biochem., 40 (1971) 386.
6 P.E.B. Reilly, Anal. Biochem., 64 (1975) 37.
7 J.S. Robinson, E.J. Kingston, C.T. Jones and G.D. Thorburn, J. Dev. Physiol., 1 (1979) 379.
8 I. Molnár and C. Horváth, J. Chromatogr., 143 (1977) 391.
9 C.D. Scott, D.D. Chilcote and N.E. Lee, Anal. Chem., 44 (1972) 85.
10 B.C. Hemming and C.J. Gubler, Anal. Biochem., 92 (1979) 31.