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ISOLATION AND QUANTITATION OF CARBOHYDRATES IN SHEEP PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The application of ultraviolet detection at 190 nm following chromatography on a Ca^{2+} cation-exchange column with a mobile phase of water enables the low amounts of carbohydrates present in plasma to be quantitated. The separation and quantitation of carbohydrates in maternal and fetal sheep plasma and amniotic fluid are described, as is the application of this method to the determination of specific radioactivities of glucose and fructose in plasma.

INTRODUCTION

The utilisation and metabolic fate of glucose in the fetus and placenta in vivo have been studied by infusion of [2-^{3}H]glucose and [U-^{14}C]glucose via indwelling catheters into the fetus and subsequent collection of samples from both the fetal and maternal circulations for determination of the specific radioactivity of glucose in plasma [1]. As a major metabolic substrate of the gravid uterus, glucose may be metabolised in several ways, including oxidation for energy production or utilisation for biosyntheses via glycolysis, the pentose phosphate pathway and hexose isomerisations. Because the latter pathways are more active in the fetus and/or placenta than in the adult [2], significant incorporation of ^{14}C-radioactivity from glucose into other carbohydrates including fructose may occur [3]. We required a rapid and sensitive method for the determination of the specific radioactivities of glucose and other carbohydrates, particularly fructose and polyols such as dulcitol and sorbitol, in plasma for such studies. A further constraint upon the method was the limited volume of fetal plasma available for analysis.

There are several other approaches to the determination of the specific radio-

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activities of different monosaccharides and their derivatives in plasma, following the administration of radioactive precursors in vivo. Most involve deproteinisation of plasma, removal of charged compounds by ion-exchange chromatography, then separation of the various carbohydrates present. The techniques most commonly used to achieve this separation are paper or thin-layer chromatography [4, 5], electrophoresis [6] or further ion-exchange chromatography of the carbohydrates as borate complexes [7]. In most instances, the carbohydrates must then be located and eluted from the support medium before radioactivity content and the amount present can be quantitated. These techniques are relatively time-consuming and not appropriate to large numbers of samples. In addition, sufficiently sensitive assays for the quantitation of many carbohydrates, particularly polyols, are not available.

In this paper, the successful application of high-performance liquid chromatography (HPLC) to the simultaneous separation and quantitation of carbohydrates in plasma from fetal and maternal sheep for the determination of their specific radioactivities is reported.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Carbohydrates, potassium phosphate, glucose oxidase, peroxidase, 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid), 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.). Barium hydroxide, zinc sulfate and toluene were obtained from BDH (Victoria, Australia). Millipore-Q reagent-grade water was usd throughout. D-[U-^{14}C]Glucose (10.47 GBq/mmol) and D-[2-^{3}H]glucose (444 GBq/mmol) were obtained from Amersham Australia (Sydney, Australia).

Cation and anion exchangers were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Preparation of samples

Samples of blood and amniotic fluid were obtained from chronically catheterised ewes and their fetuses during late gestation [8]. Following centrifugation at 2500 *g* for 15 min at 4°C, plasma was removed and stored at −20°C until extraction.

Aliquots (0.2—0.5 ml) of plasma or amniotic fluid were deproteinised by the addition of 1.0 ml of 0.16 *M* zinc sulfate. After centrifugation at 2500 *g* for 30 min at 4°C, the supernatant was removed and three aliquots (0.025 ml) reserved for enzymatic assay of glucose.

The remaining supernatant was then deionised by passage through tandem cation (1.0 ml, AG 50W-X8, H^+) and anion (1.0 ml, AG1-X8, CH_3COO^-) exchange columns, followed by two 2.0-ml washes of water. The combined eluent was then washed through C_{18} Sep-Pak (Waters Assoc., Milford, MA, U.S.A.), already equilibrated with water, and freeze-dried. Extracts were reconstituted in water (0.05 ml) for chromatography.

Apparatus

A Waters Model 6000A pump was used for solvent delivery. Injections were made using a Waters U6K universal injector. Eluent was monitored at 190 nm using a Waters Lambda Max Model 480 spectrophotometer and the detector signal plotted and integrated with a Model 730 data module. Eluent fractions were collected using a Gilson Model 202 fraction collector.

Chromatography

Extracts and standards were chromatographed on a Waters Sugar-Pak 1 column (Ca^{2+} cation exchange microparticulate gel) maintained at 65° C by a Waters column temperature controller. The mobile phase of water was filtered through an 0.45-μm HA Millipore filter and degassed under vacuum with sonication for 15 min. A flow-rate of 0.4 ml/min^{-1} was used with a back-pressure of 62 bars. In addition, a guard column containing C_{18} Corasil was placed in-line, preceding the analytical column.

Measurement of radioactivity content in chromatography eluent

Chromatography eluent was collected in 10-sec fractions into scintillation vials for 30 min from the time of injection. Of the scintillation fluid (4 g PPO, 0.1 g dimethyl POPOP, 330 ml Triton X-100 made up to 1 l with toluene) 10 ml were then added to each fraction. For routine determinations, each peak was collected as a single fraction. Samples were stored in the dark for 12 h before measurement of ^{14}C- and ^{3}H-radioactivity in an LKB liquid scintillation spectrometer. Quench curves were constructed using the external standard ratio method and corrections for quenching and cross-over made.

Enzymatic determination of plasma glucose concentration

The concentration of glucose in plasma was determined in triplicate by adding 1.25 ml glucose reagent [0.12 *M* phosphate buffer, pH 7.0; 1.5 U/ml peroxidase, 5.0 U/ml glucose oxidase, 0.92 m*M* 2,2′-azino-di(3-ethylbenz-thiazoline sulfonic acid)] to 0.025 ml deproteinised plasma, water and standards and incubating at 37° C for 60 min. Absorbance at 420 nm was then measured for each sample against water. Intra-assay and inter-assay coefficients of variation were 0.8% and 3%, respectively.

Statistical analysis

Coefficients of variation were calculated as standard deviations expressed as a percentage of mean values.

RESULTS

Preparation of samples

To render samples suitable for HPLC on an ion-modified resin column, plasma was deproteinised, then passed through tandem cation and anion exchangers to remove all charged compounds. Hydrophobic components were then removed by washing the neutral eluent through a C_{18} Sep-Pak before freeze-drying. Recoveries of plasma carbohydrates, based upon that of glucose in the final freeze-dried extract were 94% (S.E.M. 4, $n = 8$).

Separation and quantitation of carbohydrates

Ultraviolet (UV) detection of carbohydrates was carried out at 190 nm since most have an absorption maximum at approximately 188 nm and the slight decrease in absorption by comparison at 190 nm was more than compensated for by a reduction in baseline noise [9]. The offset of the detector at this wavelength typically ranged from 0.36 to 0.51 absorbance units full scale (a.u.f.s.). This was due largely to the temperature-dependent continuous elution of an unknown compound from the column, which limited its use to temperatures below 70°C.

The separation of various mixtures of carbohydrates under the conditions described in Materials and methods is shown in Fig. 1. Their retention times and those of other known neutral plasma components are presented in Table I, together with their response factors in peak area (arbitrary units) per nmol carbohydrate. Hexoses tended to elute before polyols while pentoses eluted with a wide range of retention times.

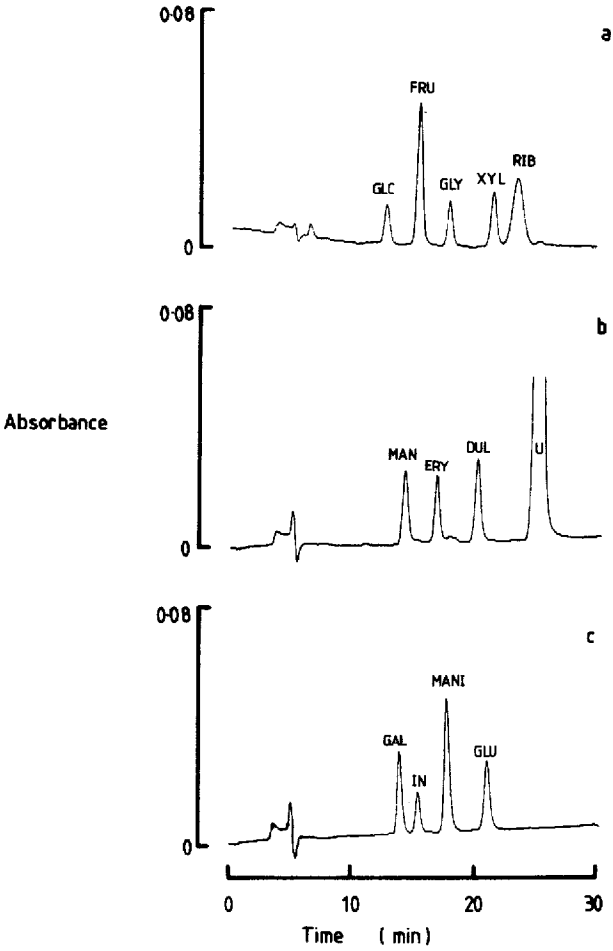

Fig. 1. Chromatographic analysis of carbohydrates. (a) Glucose (GLC), fructose (FRU), glycerol (GLY), xylitol (XYL), ribose (RIB). (b) Mannose (MAN), *i*-erythritol (ERY), dulcitol (DUL), urea (U). (c) Galactose (GAL), inositol (IN), mannitol (MANI), glucitol (GLU). Conditions were as described in Materials and methods.

TABLE I

RETENTION TIMES AND RESPONSE FACTORS OF MONOSACCHARIDES AND THEIR DERIVATIVES

Compound	Retention time (min)	Response factor		Detection limit (nmol)
		Area/nmol	Relative to glucose	
D-Glucose	12.05	20.89	1.0	5.5
L-Sorbose	13.25			
D-Galactose	13.40			3.7
D-Mannose	13.65	41.03	1.96	3.1
D-Fructose	14.70	68.96	3.30	1.6
meso-Inositol	14.90			3.7
i-Erythritol	16.20	23.84	1.14	3.25
Glycerol	17.10	11.28	0.54	
Mannitol	17.25			
Arabitol	17.85			
Dulcitol	19.55	48.07	2.30	1.95
Sorbitol	20.60			2.20
Xylitol	20.70			
d-Ribose	22.90	44.32	2.12	4.75
Urea	23.45	58.62	2.81	1.85

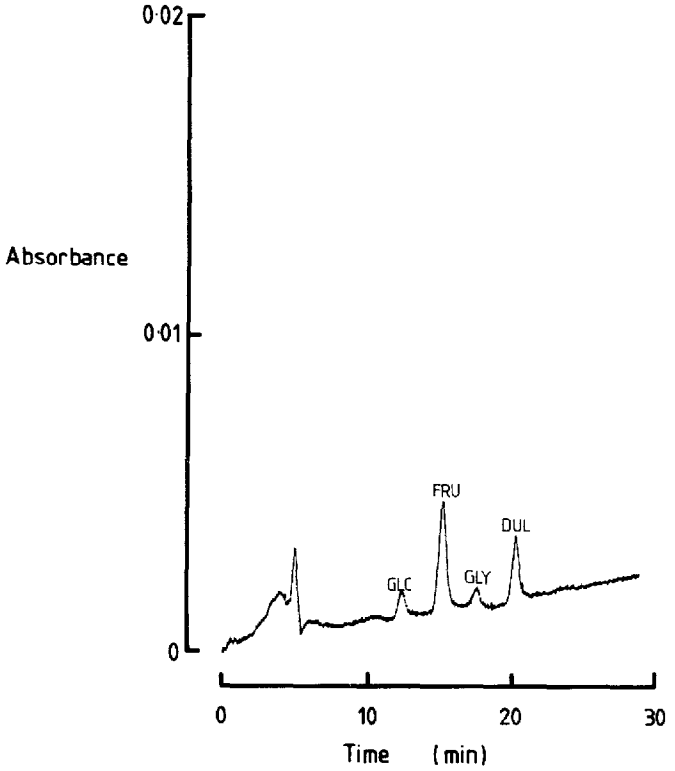

Fig. 2. Chromatographic analysis of 5 nmol each of glucose (GLC), fructose (FRU), glycerol (GLY) and dulcitol (DUL) injected in a 0.015-ml aliquot.

Most of the compounds studied had greater absorbance at 190 nm glucose with the exception of glycerol and *i*-erythritol. This is reflected in their detection limits which were determined as the amount injected in 0.005 ml which gave a peak-height-to-noise ratio of 10 (Table I).

The detection limit of 5.5 nmol for glucose indicates that UV detection under these conditions is at least twenty times more sensitive than detection by refractive index [9]. The chromatogram obtained when 5 nmol of glucose, fructose, glycerol and dulcitol were injected in a 15-μl volume is shown in Fig. 2.

Peak area showed a linear response to concentrations up to approximately 250 nmol for all compounds examined. Coefficients of variation at the 5-nmol level for glucose, fructose and dulcitol were 2.3%, 1.9% and 1.8% respectively.

Determination of specific radioactivities of carbohydrates in sheep plasma

Typical chromatograms obtained when extracts of fetal and maternal plasma were analysed are shown in Figs. 3 and 4. Upon chromatography of extracts of fetal plasma, peaks co-eluting with glucose, mannose, fructose, dulcitol,

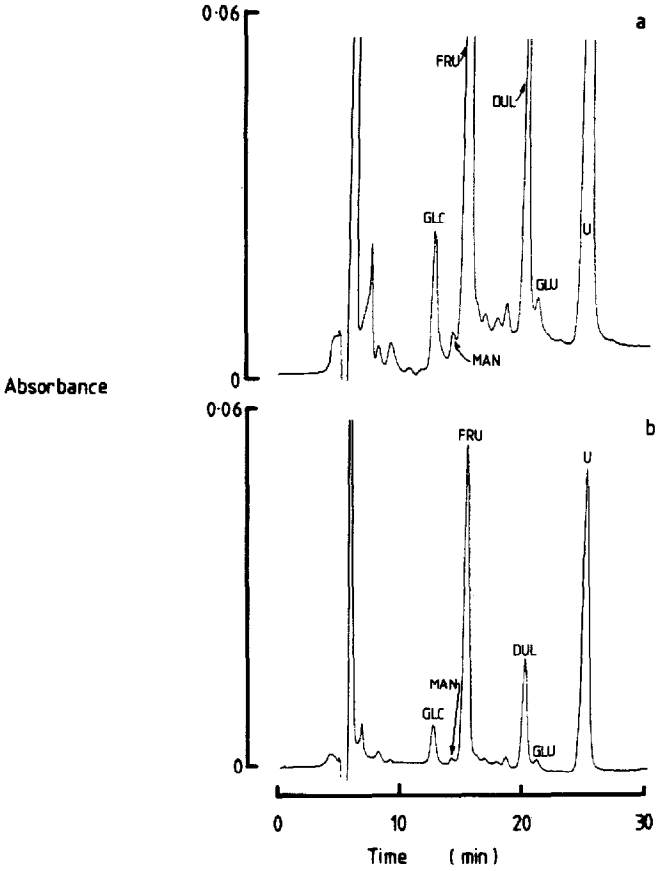

Fig. 3. Chromatographic analysis of fetal plasma: (a) 0.015 ml of fetal plasma extract reconstituted in 0.05 ml of water; (b) 0.005 ml of fetal plasma extract reconstituted in 0.05 ml of water. Conditions as described in Materials and methods. For peak identification, see legend to Fig. 1.

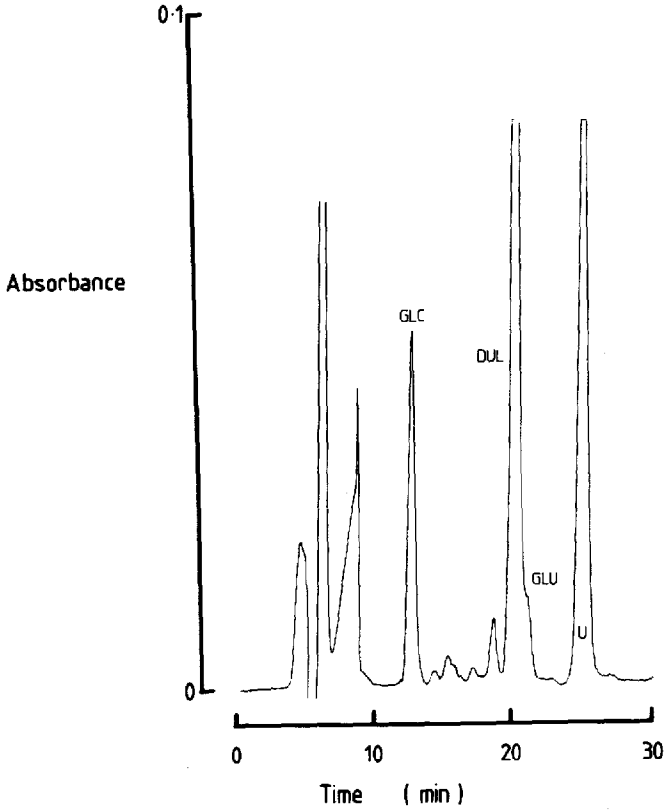

Fig. 4. Chromatographic analysis of maternal plasma (0.015 ml of maternal plasma extract reconstituted in 0.05 ml water). Conditions as described in Materials and methods. For peak identification, see legend to Fig. 1.

sorbitol and urea were observed (Fig. 3). Of these, glucose, dulcitol, sorbitol and urea were separated with baseline resolution. HPLC of maternal plasma extracts revealed a number of peaks, some eluting at the same time as the pure standards, glucose, galactose, mannose, inositol, dulcitol, sorbitol and urea (Fig. 4). All, with the exception of galactose and mannose were usually resolved to the baseline from neighbouring peaks.

The concentration of these compounds in plasma was determined by using glucose as an internal standard whose plasma concentration was measured by enzymatic assay. Where the latter is not required for the purposes of the study, addition of a suitable compound, as an internal standard, to plasma prior to extraction should be a practicable alternative.

The determination of specific radioactivity of glucose in plasma was assessed by adding 0.37 μBq of D-[U-^{14}C]glucose in 0.01 ml to each of ten aliquots of 1.5 ml deproteinised fetal plasma containing 0.60 μmol glucose as determined by enzymatic assay. The specific radioactivity of glucose in plasma was found to be 0.618 μBq/μmol (S.E.M. 0.002, n = 10) compared to the theoretical specific radioactivity of 0.617 μBq/μmol.

This method was then applied to the determination of the specific radioactivity of carbohydrates in fetal and maternal plasma sampled during a

constant infusion of D-[U-^{14}C]glucose and D-[2-^{3}H]glucose (37 μBq/min and 111 μBq/min, respectively) into the tarsal vein of a fetal sheep at 120 days gestation.

The chromatographic profiles of absorbance at 190 nm and of ^{14}C- and ^{3}H-radioactivity of extracted fetal plasma are shown in Fig. 5. In fetal plasma, most ^{3}H-radioactivity co-eluted with glucose while ^{14}C-radioactivity co-eluted with glucose, mannose and fructose. No other significant peaks of radioactivity were observed. The specific activities of glucose and fructose in plasma throughout the infusion, determined in this way, are shown in Fig. 6. The average

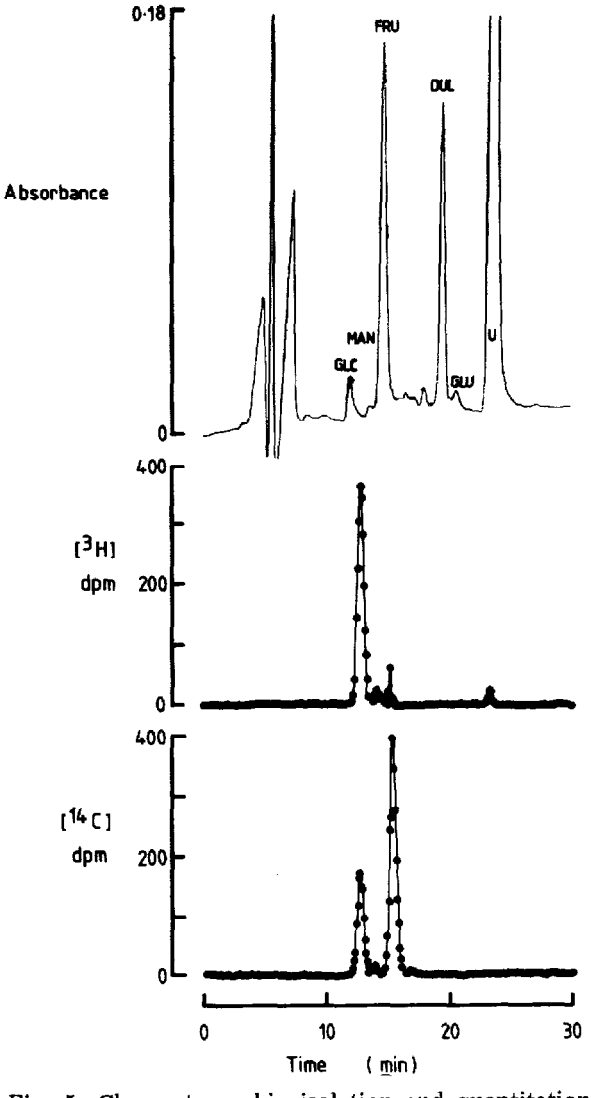

Fig. 5. Chromatographic isolation and quantitation of carbohydrates in plasma obtained from a fetal lamb during the continuous infusion of D-[U-^{14}C]glucose and D-[2-^{3}H]glucose into a chronically catheterised fetal lamb. Fractions (0.1 min) were collected continuously following injection of 0.015 ml of plasma extract reconstituted in 0.05 ml water and ^{14}C and ^{3}H content determined as described in Materials and methods. For peak identification, see legend to Fig. 1.

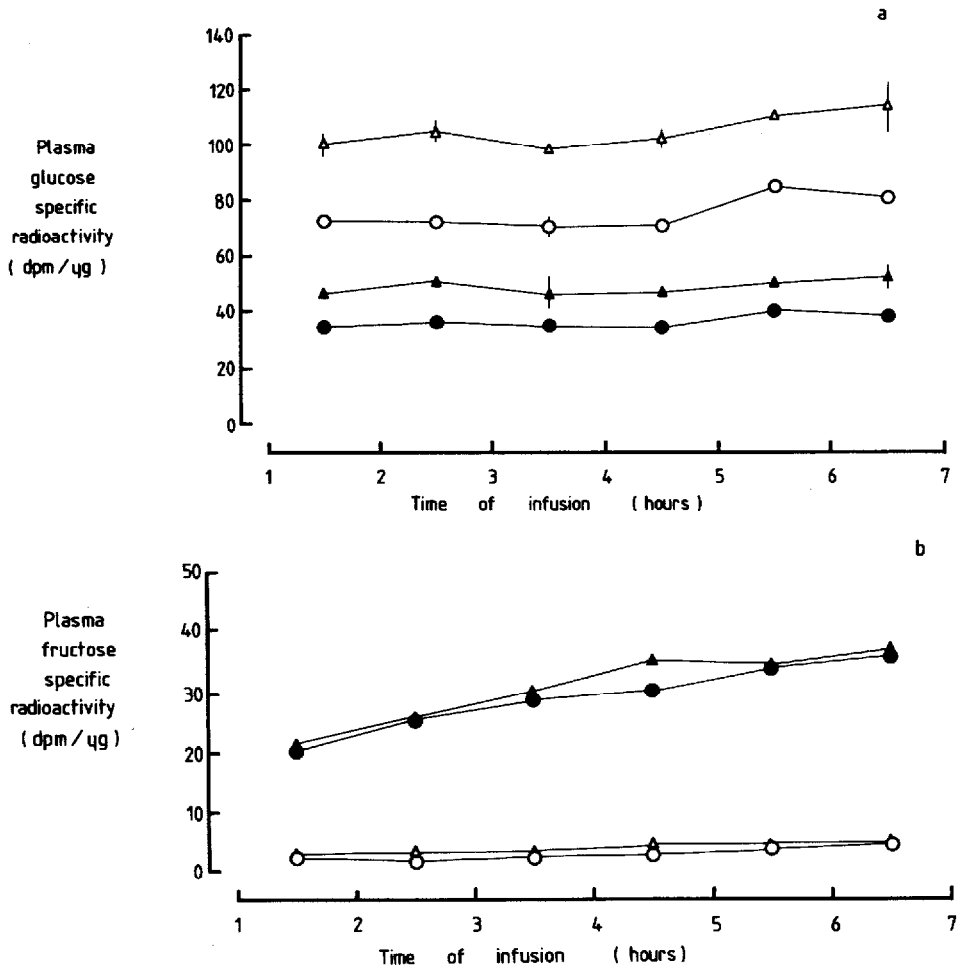

Fig. 6. The specific radioactivities in plasma of (a) glucose and (b) fructose in fetal lamb plasma obtained from the umbilical vein (○) and the femoral artery (△) during a constant intrafetal infusion of D-[U-^{14}C]glucose (●, ▲) and D-[2-^{3}H]glucose (○, △). Each point represents the mean of duplicate determinations with standard deviation shown by bars where sufficiently large.

coefficients of variation for duplicate determinations of specific radioactivity of glucose in fetal plasma were 1.6% for [^{14}C]glucose and 1.7% for [^{3}H]glucose. For duplicate measurements of specific radioactivities of fructose in fetal plasma the average coefficients of variation were 3.11% for [^{14}C]fructose and 4.64% for [^{3}H]fructose.

The chromatographic profiles of absorbance at 190 nm and of ^{14}C-and ^{3}H-radioactivity of extracted maternal plasma are shown in Fig. 7. Both ^{14}C- and ^{3}H-radioactivity co-eluted with glucose only. The specific activities of glucose in maternal plasma glucose determined at intervals throughout the infusion are shown in Fig. 8. The average coefficients of variation for specific radioactivities of [^{14}C]glucose and [^{3}H]glucose in maternal plasma were 1.95% and 2.95%, respectively.

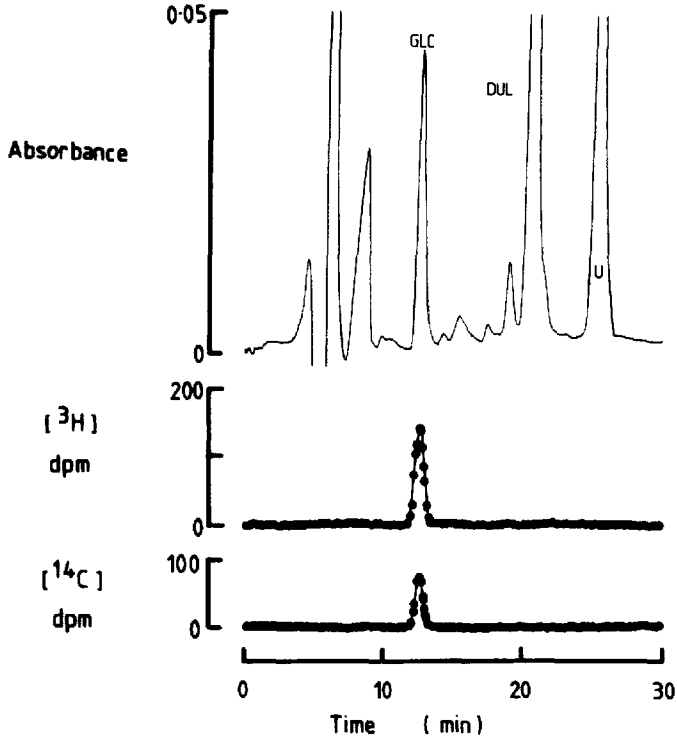

Fig. 7. Chromatographic isolation and quantitation of carbohydrates in plasma of maternal sheep obtained during a continuous infusion of D-[U-^{14}C]glucose and D-[2-^{3}H]glucose into a chronically catheterised fetal lamb. Fractions (0.1 min) were collected continuously following injection of 0.015 ml of plasma extract reconstituted in 0.05 ml of water and ^{14}C and ^{3}H content determined as described in Materials and methods. For peak identification, see legend to Fig. 1.

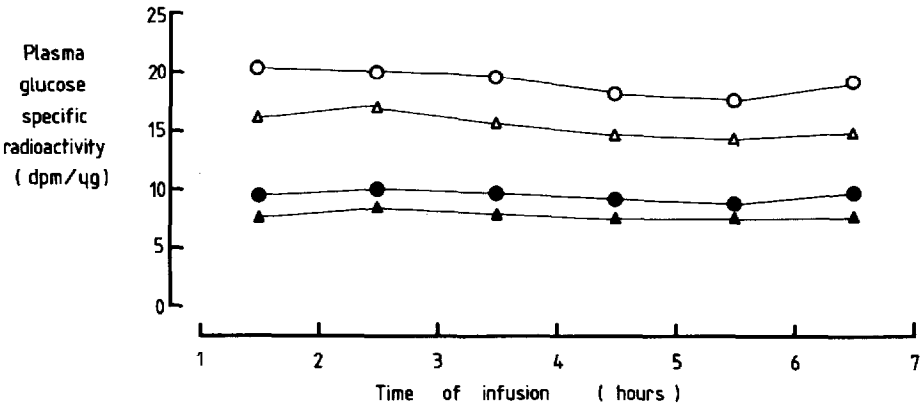

Fig. 8. Specific radioactivities of glucose in plasma of maternal sheep during a constant intrafetal infusion of D-[U-^{14}C]glucose (•, ▲) and D-[2-^{3}H]glucose (○, △). Each point represents the mean of duplicate determinations.

To assess their homogeneity, [^{14}C]glucose, [^{3}H]glucose, [^{14}C]fructose and [^{3}H]fructose peaks were collected and then lyophilised. Following reconstitution in water, an aliquot was chromatographed on an amine—silica column (Waters Assoc.) at 25°C, with a mobile phase of acetonitrile—water (85:15) at a flow-rate of 2 ml/min and detection at 190 nm (a.u.f.s. 0.1). An aliquot of the remainder was counted for ^{14}C- and ^{3}H-radioactivity. Continuous collection of 0.1-ml fractions and measurement of their ^{14}C- and ^{3}H-radioactivity showed recovery of 99% and 102% of ^{14}C- and ^{3}H-radioactivity, respectively, in a peak co-eluting with glucose following rechromatography. Similarly, recovery of ^{14}C-radioactivity in fructose was 99%.

DISCUSSION

Using an HPLC cation-exchange column and a mobile phase of water at a temperature lower than that generally recommended, 65°C, it was possible to quantitate carbohydrates by UV detection and still efficiently separate a wide range of hexoses, pentoses and polyols. In this system, quantitation at 190 nm resulted in detection limits which were lower than previously achieved with UV or refractive index detection [9] and comparable to those reported for post-column derivatisation techniques [10—12].

Other methods of HPLC of carbohydrates largely utilise partition chromatography on silica columns with either chemically or dynamically amine-coated silica stationary phases [13, 14] and detection by refractive index [15]. However, these approaches are in general applicable only to samples with high carbohydrate content due to the insensitivity of this type of detection. In addition, their limited ability to resolve different carbohydrates and polyols requires that relatively few compounds be present in the sample.

Some of these limitations have been overcome by the development of both pre- and post-column derivatisation methods which have in two instances been applied to the analysis of complex biological samples such as human serum [10, 16]. However, these methods are associated with other problems such as variable efficiency of derivatisation and formation of multiple derivatives. In addition, covalent modification of the sample precludes further analysis, in particular the determination of radioactivity content. They also require additional equipment such as pumps, mixing coils and fluorescence detectors as well as the use of highly corrosive reagents.

More recently, heavy-metal cation-exchange resin columns have been used for the separation of carbohydrates in food products and hydrolysates of plant cell walls with refractive index detection [17]. Because such columns use water as a mobile phase, UV detection of carbohydrates at 190 nm appeared feasible. Accordingly, the present method for the extraction of carbohydrates from sheep plasma and their separation and quantitation was developed.

The principal carbohydrates present in the plasma of fetal and maternal sheep were successfully separated and quantitated by this method, using as little as 0.2 ml plasma. In addition, the specific radioactivities of labeled glucose and fructose, present simultaneously in plasma, were accurately determined with good reproducibility.

This technique may also be useful in the analysis of other biological samples

containing a mixture of carbohydrates and polyols. In addition, the measurement of specific radioactivities of various carbohydrates in such samples may be feasible, depending upon their homogeneity following HPLC.

In summary, this technique should be generally applicable where the specific radioactivities of glucose and other carbohydrates and polyols in plasma are to be determined and where measurement of glucose specific radioactivity by conventional methods is made difficult by the presence of other labeled carbohydrates or interfering compounds [3, 18, 19].

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