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ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE CONCENTRATION AND SPECIFIC RADIOACTIVITY OF PHOSPHOENOLPYRUVATE AND URIDINE DIPHOSPHATE GLUCOSE IN TISSUE EXTRACTS

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SUMMARY

A rapid and efficient isocratic high-performance liquid chromatographic method for studying the metabolism of phosphoenolpyruvate and uridine diphosphate glucose (UDPG) has been developed. For each compound this method can measure tissue concentrations in the range 0.1–1000 nmol/g of tissue and determine specific radioactivity. All measurements can be performed in 200 mg of tissue. The recoveries of uridine diphosphate [6-^{3}H]glucose and phosphoenol[1-^{14}C]pyruvate from liver tissue homogenates were 97 and 99%, respectively. Following intra-arterial infusion of [6-^{3}H]glucose and [U-^{14}C]lactate in conscious rat, the concentration and specific radioactivity of phosphoenolpyruvate and UDPG were determined in rat liver. The method may be applied to experimentation in small animals using radiolabelled precursors in order to quantitate in vivo the glycogenic and gluconeogenic fluxes.

INTRODUCTION

The study of gluconeogenesis in vivo has proven very difficult, even though many procedures have been used in an attempt to measure the process [1–8]. Uridine diphosphate glucose (UDPG) and phosphoenolpyruvate (PEP) are in a key position in the glycogen synthetic and gluconeogenic pathways and the determination of their specific radioactivity can significantly improve our understanding of these two pathways. High-performance liquid chromatographic (HPLC) methods have been published for the separation of uridine diphosphate sugars [9–11], while the separation of standard solutions of PEP

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has been accomplished by anion-exchange chromatography [12] and HPLC [13] using borate as complex-forming and eluting buffer. However, these previous methods required long analysis times and did not allow separation of PEP in tissue samples. We have developed a rapid and simple isocratic HPLC method for measuring tissue concentrations and specific radioactivity of both PEP and UDPG in tissue extracts. Using this procedure, the total time of analysis does not exceed 1 h. This method can be adapted to in vivo experiments, using radiolabelled glucose or/and gluconeogenic precursors, in order to collect radiochemically pure compounds for specific radioactivity determination.

EXPERIMENTAL

Chemicals and labelled compounds

PEP and UDPG standards, reagents for tissue treatment, chromatographic procedures and enzymatic assays were purchased from Sigma (St. Louis, MO, U.S.A.); [U-^{14}C]lactate, [6-^{3}H]glucose, phosphoenol[1-^{14}C]pyruvate and uridine diphosphate [6-^{3}H]glucose were purchased from Amersham (Arlington Heights, IL, U.S.A.).

Apparatus and HPLC conditions

The HPLC system consisted of two 510 pumps, a 680 programmable gradient controller, a U6K manual injector and a 484 tunable absorbance detector, all from Waters Chromatography Division, Millipore (Milford, MA, U.S.A.). Samples were injected by syringes of 25 or 100 μl with a blunt 25-gauge needle (Models 802RN and 810RN, Hamilton, Reno, NV, U.S.A.). Solid-phase extraction of the tissue homogenates was performed on 3-ml SPE Supelclean LC-SAX strong anion-exchange columns from Supelco (Bellefonte, PA, U.S.A.). Chromatography was carried out on an HPLC LC-SAX Supelcosil strong anion-exchange column (250 mm$\times$4.6 mm I.D.) from Supelco. Peak areas were integrated on SE120 plotter recorder (BBC, Goetz Metrawatt, Vienna, Austria). Fractions were collected with a Retriever II fraction collector from Isco (Lincoln, NE, U.S.A.).

The isocratic system used an aqueous 0.20 *M* potassium dihydrogenphosphate solution prepared with distilled, deionized water (Milli-Q water purification system, Millipore) adjusted to pH 3.5 with 0.20 *M* ortophosphoric acid, re-filtered and degassed with a 0.45-μm filter. The flow-rate was 1.0 ml/min in all experiments, and the temperature was $20 \pm 1\,^{\circ}$C. Absorbance was measured at 200 nm. The quantitation of the compounds was carried out by external calibration (enzymatic assays [14,15]).

Column equilibration and re-equilibration

Prior to use, the column was flushed for at least 60 min with the mobile phase. After analysis of ten tissue samples, the column was flushed with 20 ml of an aqueous 0.50 *M* potassium dihydrogenphosphate solution and 20 ml of distilled, deionized water, and then re-equilibrated with the mobile phase.

Standard preparation

Standard solutions of PEP and UDPG were prepared by dissolving high-quality pure standards in water to approximate concentration. The same standard solutions of PEP and UDPG were utilized for HPLC calibration and enzymatic assays [14,15]. Stock standard solutions of each compound were stored at −70°C when not in use.

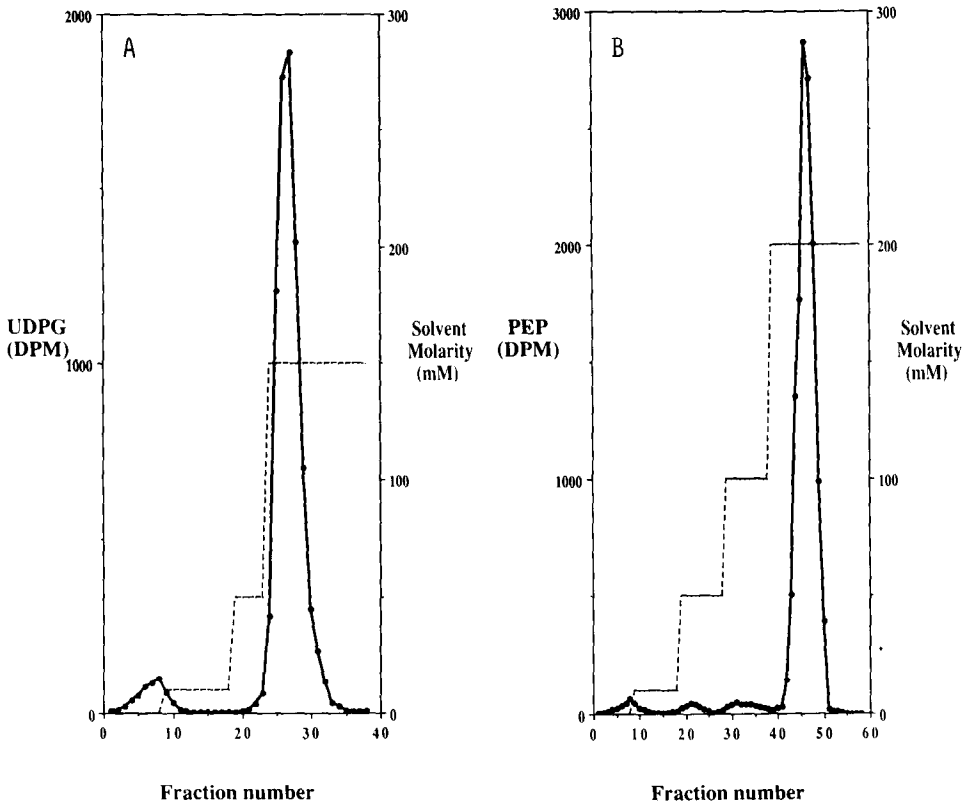

Fig. 1. Solid-phase extraction UDPG (A) and PEP (B). (A) Before sample preparation (see text), [^{3}H]UDPG was added to the tissue, and its elution with dihydrogenphosphate (pH 2.5, variable molarity) determined by counting ^{3}H radioactivity in each 0.5-ml eluted fraction. Fractions 23–32 (150 m*M*) contained 97% of the added tracer. (B) Before sample preparation (see text), [^{14}C]PEP was added to the tissue, and its elution with dihydrogenphosphate (pH 5.0, variable molarity) determined by counting ^{14}C radioactivity in each 0.5-ml eluted fraction. Fractions 42–51 (200 m*M*) contained 99.5% of the added tracer.

Animal preparation and tissue collection

Male Sprague–Dawley rats (Charles River Labs., Wilmington, MA, U.S.A.) were used for the preparation of test samples. Indwelling catheters were inserted into the right internal jugular vein and in the left carotid artery, as previously described [16,17]. One week after catheter placement, the rat received an infusion of [U-^{14}C]lactate (0.4 μCi/min) and [6-^{3}H]glucose (1.0 μCi/min). At the end of the 80-min infusion, the rat was injected with phenobarbital (60 mg/kg body weight), the abdomen was quickly opened, and the liver was freeze-clamped with aluminium tongs precooled in liquid nitrogen. Tissue samples were stored frozen at -70°C for subsequent analysis.

Tissue sample preparation

Tissue samples (200 mg) were homogenized in three volumes ($\sim$600 μl) of ice-cold 0.6 *M* perchloric acid and kept at 0°C for 10 min. The precipitated proteins were removed by centrifugation for 5 min at 13 500 *g* (Microfuge, Beckman Instruments, Palo Alto, CA, U.S.A.) and the supernatant diluted with four volumes of 0.01 *M* potassium dihydrogenphosphate, adjusted to pH 2.5 (UDPG) or to pH 5.0 (PEP) with aqueous 1 *M* dipotassium hydrogenphosphate and finally rediluted with two volumes of aqueous 0.01 *M* potassium

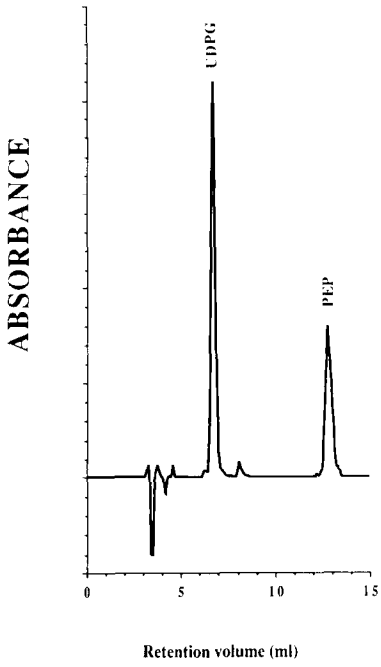

Fig. 2. Isocratic HPLC separation of mixed standard solutions of UDPG (1 nmol, retention volume 6.73 ml) and PEP (1 nmol, retention volume 12.83 ml). HPLC conditions: injection volume, 10 μl; column, LC-SAX Supelcosil strong anion-exchange, 250 mm × 4.6 mm I.D. (Supelco); mobile phase, 0.20 *M* potassium dihydrogenphosphate (pH 3.5); flow-rate, 1.0 ml/min; absorbance, 200 nm; detector sensitivity, 0.05 a.u.f.s.

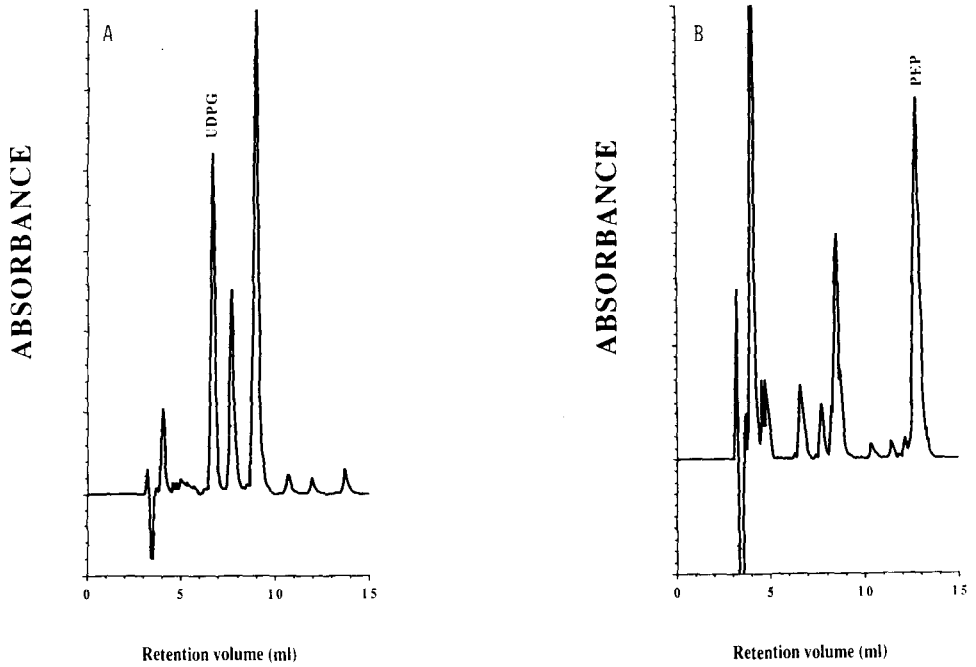

Fig. 3. Isocratic HPLC separation of UDPG (A) and PEP (B). (A) Injected sample was obtained from fraction 27 of UDPG solid-phase extraction. (B) Injected sample was obtained from fraction 46 of PEP solid-phase extraction. HPLC conditions: injection volume, 20 μl; column, LC-SAX Supelcosil strong anion-exchange, 250 mm × 4.6 mm I.D. (Supelco); mobile phase, 0.20 *M* potassium dihydrogenphosphate (pH 3.5); flow-rate, 1.0 ml/min; absorbance, 200 nm; detector sensitivity, 0.05 a.u.f.s. (0.01 for B).

dihydrogenphosphate (again pH 2.5 or 5.0). Prior to the injection into the HPLC system, the compounds of interest from the tissue extracts were pre-separated with solid-phase extraction on a 3-ml strong anion-exchange cartridge (Supelclean LC-SAX, Supelco), using aqueous potassium dihydrogenphosphate solutions of increasing molarity: 10 m*M* (5 ml), 50 m*M* (2.5 ml) and 150 m*M* (10 ml) for UDPG (pH 2.5) (Fig. 1A); 10 m*M* (5 ml), 50 m*M* (5 ml), 100 m*M* (5 ml) and 200 m*M* (10 ml) for PEP (pH 5.0) (Fig. 1B). The eluates were divided in fractions of 0.5 ml; the first ten fractions of the 150 m*M* eluent buffer, containing 97% of the UDPG, and the fractions 3–12 of the 200 m*M* eluent, containing 99.5% of the PEP, were collected for subsequent HPLC analysis. Of the 0.5 ml fractions, 5 μl were injected in duplicate into the HPLC system in order to confirm the elution of the compounds of interest and quantitate their concentrations. Finally, 240 μl of the fractions of interest were injected in duplicate into the HPLC system in order to collect labelled compounds.

Peak characterization

Retention volumes and purity of the substances of interest were confirmed

in different experiments both on solid-phase extraction (Fig. 1A and 1B) and HPLC (see Fig. 4) by (a) consideration of the retention volumes of standard compounds, (b) the addition of standard mixtures to tissue samples, (c) the addition of labelled compounds (phosphoenol[1-^{14}C]pyruvate and uridine diphosphate [6-^{3}H]glucose) to the tissue before the preparation, (d) the determination of the absorption ratio at 220/200 nm and (e) the disappearance of the peaks following specific enzymatic reactions [18]. In particular, the last experiment was carried out by dividing the same solid-phase extraction eluate into two aliquots. One aliquot was chromatographed and the other, following appropriate buffering, was incubated with specific enzymes [14, 15] and then injected into the HPLC system.

Recovery test

Recoveries of PEP and UDPG from liver tissue were quantitated by addition of known amounts of phosphoenol[1-^{14}C]pyruvate and uridine diphosphate [6-^{3}H]glucose to suspensions of the tissue prior to homogenization. The two eluted fractions of UDPG and PEP were collected drop by drop by the pro-

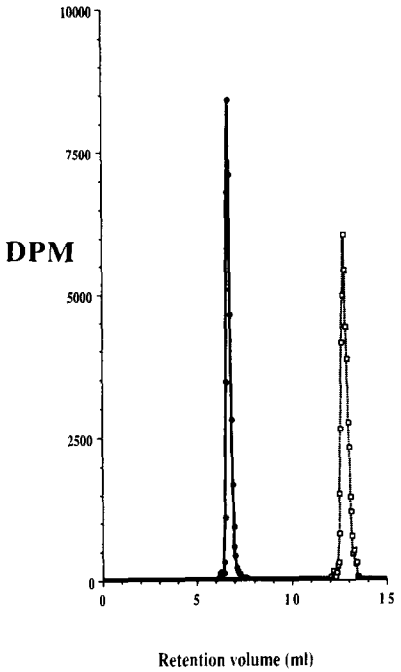

Fig. 4. Isocratic separation of uridine diphosphate [6-^{3}H]glucose (●) and phosphoenol[1-^{14}C]pyruvate (□). Tracers were added to the tissue sample before homogenization. Injection consisted of pooled equal amounts of fraction 27 of UDPG solid-phase extraction and fraction 46 of PEP solid-phase extraction. HPLC eluate was collected drop by drop and then counted. HPLC conditions: injection volume, 100 μl; column, LC-SAX Supelcosil strong anion-exchange, 250 mm×4.6 mm I.D. (Supelco); mobile phase, 0.20 *M* potassium dihydrogenphosphate (pH 3.5); flow-rate, 1.0 ml/min.

grammable fraction collector (taking into account that a volume of 30 μl was enclosed between the UV cell detector and the exit), and their radioactivity was measured by liquid scintillation.

RESULTS

Solid-phase extraction (Fig. 1)

The solid-phase extraction of UDPG and PEP from the tissue homogenate using a strong anion-exchange cartridge and a potassium dihydrogenphosphate solution adjusted to pH 2.5 (UDPG) or 5.0 (PEP) allows to partially separate the two compounds of interest from all non-polar and the most of the polar intracellular substrates with optimal recoveries (UDPG: 97%; PEP: 99%). When the elution patterns were monitored (by means of the addition of phosphoenol[1-^{14}C]pyruvate and uridine diphosphate [6-^{3}H]glucose) in eight solid-phase extractions, no variations were observed.

Chromatographic characteristics

The isocratic HPLC separation of UDPG and PEP using a strong anion-exchange column and a 0.20 *M* potassium dihydrogenphosphate solution adjusted to pH 3.5 (flow-rate 1.0 ml/min) was achieved in less than 15 min, as illustrated on Fig. 2. With the described HPLC conditions, the retention volume for UDPG was 6.73 ml, while that for PEP was 12.83 ml (Fig. 2). The within-day and day-to-day coefficients of variation, after injections of 5 nmol (UDPG) or 1 nmol (PEP), were less than 3%. No interfering peaks were observed when injecting standard mixtures (Fig. 2) or when appropriate preparation of the tissues was carried out (Fig. 3). When specific precolumn enzymatic conversion of UDPG and PEP was carried out, no interfering peaks were eluted within the retention volumes of PEP and UDPG, thus providing evidence for the identity and purity of the chromatographic peaks. The detection limits of UDPG and PEP were 0.05 and 0.1 nmol, respectively, with a coefficient of variation of 5%.

The pH of the mobile phase had opposite effects on the retention volumes of UDPG and PEP: the retention for PEP augmented whereas that for UDPG decreased, as the pH increased from 2.5 to 7.0. Optimal overall resolution for the two compounds was obtained using pH 3.5, as illustrated in Figs. 2–4.

Calibration curves

To determine the response factor of the reference compounds (nmol per peak area and nmol per peak height), the linearity of the HPLC analysis of a known previously determined concentration was verified over the range of physiological interest. Calibration curves for both UDPG and PEP concentrations were linear over the concentration range 0–1000 n*M*. Correlation coefficients between peak height and compound concentration, as determined with enzymatic assays, were 0.99 (UDPG) and 0.99 (PEP) (Fig. 5).

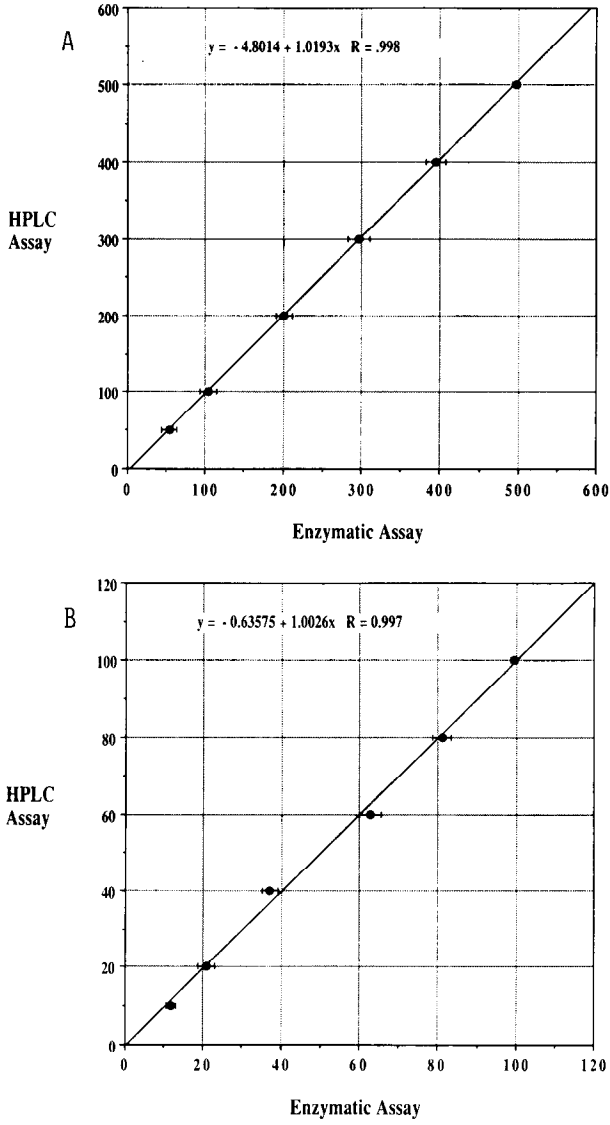

Fig. 5. Comparison of UDPG (A) and PEP (B) concentrations measured by HPLC assay (y-axis) and enzymatic assay (x-axis).

Tissue concentrations

As shown in Fig. 3, UDPG and PEP were separated as sharp peaks in the solid-phase extracts of rat liver. Although some peaks of other biological components appeared, none of these disturbed the peaks of the two substances of interest. The UDPG concentration in the rat liver was 410 ± 12 nmol/g wet weight, while the PEP concentration was 29 ± 2 nmol/g wet weight.

Analysis by HPLC of the specific activity

In order to determine the suitability of our method for in vivo tracer studies we measured the specific activities of [^{3}H]UDPG (280 ± 12 dpm/nmol), [^{14}C]UDPG (196 ± 12 dpm/nmol) and [^{14}C]PEP (232 ± 15 dpm/nmol) in liver extracts following the intra-arterial injection of [6-^{3}H]glucose and [U-^{14}C]lactate in rat.

DISCUSSION

Methods designed for estimation of gluconeogenesis are based on the transfer of ^{14}C atoms from a selected precursor, i.e. lactate or alanine, to glucose [1–4]. Additionally, evidence has been accumulating for a gluconeogenic (indirect) pathway of liver glycogen repletion [5–8]. Since UDPG and PEP are key intermediates for glycogenesis and gluconeogenesis, the determination of their specific radioactivity may improve our understanding of these two pathways.

The absence of a single procedure for the analysis of PEP and UDPG concentration and specific radioactivity in biological samples led us to develop this simple and efficient HPLC method, which allows not only quantitative measurements but also separate recovery of each component without degradation or radioactive contamination.

Our method consists of two chromatographic separations. The first is performed on a solid-phase, strong anion-exchange cartridge, with the use of phosphate buffers of increasing molarity. This separation allows to isolate ca. 97–100% of the compounds of interest from all non-polar and most polar metabolites in the tissue samples. Since this procedure is efficient and reproducible, it can be used to obtain (in less than 30 min) aliquots ready for HPLC analysis from different biological samples.

The second, final step of the separation of PEP and UDPG is an HPLC, isocratic method. Since both UDPG and PEP are present in low concentration in biological samples and PEP is detectable only at low wavelength, it was necessary to measure the absorbance at 200 nm. The two compounds were eluted by our isocratic HPLC method in less than 15 min.

Chromatographic peaks of UDPG and PEP were identified by (a) consideration of the retention volumes of standard compounds, (b) the addition of standard mixtures to tissue samples, (c) the addition of radioisotopic markers, (d) the determination of the peaks following specific enzymatic reactions.

Although UDPG [9–11] and PEP [12,13] separations have been obtained, respectively, from standard mixtures of nucleotides or glycolytic intermediates, the resolution of PEP from tissue samples has not been previously described. Moreover, the available methods for PEP separation [12,13] required long analysis times and complex detection systems.

To demonstrate the potential application of this method to the quantification of these substances in biological samples, deproteinized tissue homogenates from rat were analyzed. The liver concentrations obtained were consistent with previously reported values, obtained with different methodologies.

Finally, the radioactivity present in hepatic UDPG and PEP was determined following the intravenous injection of [6-^{3}H]glucose and [U-^{14}C]lactate in a conscious rat. The possibility of estimating the radioactivity associated with each compound, together with the quantification of its tissue concentration, allows a precise calculation of the specific activity of each compound.

CONCLUSION

We have described a simple and efficient isocratic HPLC method for the measurement of concentration and specific activity of PEP and UDPG in tissue samples. The combination of the present analysis with in vivo tracer infusions should provide a precise and reliable technique for the study of gluconeogenesis and glycogen synthesis in animals.

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