Underestimation of glucose turnover determined using [6-³H]glucose tracer in non-steady state

The role of a tritiated tracer impurity

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Summary. The use of tritiated glucose tracers may result in underestimation of glucose turnover during hyperinsulinaemic clamps giving paradoxical negative endogenous glucose production rates. While mathematical modelling errors in the analysis of tracer data are major determinants of this underestimate in the non-steady state, the relative importance of tracer contamination under these conditions remains in doubt. We have used high performance liquid chromatography to assess the possible contribution to this problem of a labelled tracer impurity found in [6-³H]glucose. In conventional 4 h hyperinsulinaemic clamps performed in six normal subjects, labelled impurity increased as a percentage of the neutral plasma radioactivity fraction from $5.3 \pm 0.9\%$ after a 2 h equilibration period (0 min) to $13.5 \pm 2.2\%$ at 120 min and $15.4 \pm 2.4\%$ at 240 min, as plasma glucose specific activities fell following the infusion of insulin. Negative endogenous glucose production rates were observed both at 90–120 min ($-8.8 \pm 1.6 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and at 210–240 min ($-8.5 \pm$

$1.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) implying a persistent underestimate in isotopically determined glucose appearance rate. Using chromatography data to correct for impurity increased glucose appearance rates by $7.9 \pm 2.1\%$ at 120 min and $11.0 \pm 2.5\%$ at 240 min. Purified tracer was then used for a further six clamps. When the conventional protocol was used with unlabelled glucose infusion an obvious negative error persisted only at 90–120 min. In contrast, labelled infusions gave exclusively positive values for endogenous glucose production. We conclude that a labelled impurity of [6-³H]glucose may be an important source of error in measurement of glucose turnover and endogenous glucose production in the non-steady state. Use of chromatographically pure tritiated glucose tracers is recommended.

Key words: Tritiated glucose, non-steady state glucose turnover, hyperinsulinaemic glucose clamps, HPLC, tracer impurity.

Isotope dilution techniques, using tritiated glucose as a tracer, are commonly employed in clinical research to assess hepatic and peripheral insulin action. However, it is a common observation that during hyperinsulinaemic glucose clamps, glucose appearance rates (Ra) determined with tritiated glucose (using the Steele equations for a modified single compartment model) give a significant underestimate compared with the exogenous glucose infusion rates (GIR) required to maintain euglycaemia [1, 2]. Calculated rates of endogenous glucose production (EGP = Ra - GIR) may then give paradoxical negative values [2]. The need for accurate non-invasive measurements of hepatic glucose production has stimulated interest in identifying the sources of this error and developing means for its elimination.

In most studies short periods of hyperinsulinaemia (< 5 h) are used and measurements are made while GIR and plasma glucose specific activity are changing. Inadequacy of the Steele model is a recognised source of error under these non-steady state conditions [3–5]. Tritiated tracer contaminants which have recently been described

[6] might also cause an error, but their significance in short clamps is disputed [7].

In the present studies we used HPLC to determine whether commercially prepared [6-³H]glucose contained significant tritiated impurities and to examine to what extent, if any, such impurities are responsible for the underestimation in Ra found in normal subjects during 4 h hyperinsulinaemic clamps. We then used HPLC to eliminate tracer impurities and compared estimates of EGP obtained with unlabelled exogenous glucose or labelled exogenous glucose infusions designed to minimize model associated error [8].

Subjects and methods

Written consent was obtained in each case from 10 normal healthy subjects (9 male, 1 female; BMI $23.0 \pm 0.9 \text{ kg/m}^2$; age 29 ± 2 years) with no family history of diabetes mellitus. Two subjects were studied twice (Subjects 1 and 2). Approval for the study was given by the Ethical Committee of the Queen's University of Belfast.

Experimental studies

[6-³H]glucose (TRK 85, Amersham International PLC, Aylesbury, UK) for use in the studies was first aliquoted under sterile conditions into borosilicate glass vials (Duoseal, Amersham International PLC) each containing a single dose at the original concentration. Each was stored at +2 °C, as recommended by the manufacturer, until required for preparation of infusate. Six euglycaemic hyperinsulinaemic glucose clamp studies were performed using this [6-³H]glucose tracer, as supplied by the manufacturer and without additional preparation. All used a conventional unlabelled exogenous glucose infusion. Five of these studies were performed using material from a single batch (Batch 67, Dated 25.4.1988). This was analysed by HPLC at the time of aliquoting and was found to be 89.9% pure (Fig. 1a). One study was performed using material from a subsequent batch (Batch 69, Dated 19.10.1988), obtained after discussion of results with the manufacturers, again with no additional preparation. At the time of aliquoting this was found to be 97.5% pure (Fig. 1b). All tracers were within manufacturer's radiochemical purity specification allowing for expected rate of decomposition. All material was used within six months of manufacture. A further six studies were performed using a pure [6-³H]glucose tracer prepared by HPLC (see below) from material supplied by the same manufacturer (Batch 67 and Batch 72, Dated 8.2.1989).

Euglycaemic hyperinsulinaemic clamp studies

Subjects were admitted at 08.00 hours, after a 12 h overnight fast. A cannula was placed in an antecubital vein and a 6 h, primed (55.8 µCi, 33.2 Ci/mmol) continuous (0.558 µCi · min⁻¹) infusion of

[6-³H]glucose in 0.9% NaCl was commenced. A second cannula was placed in a contralateral wrist vein and the hand was maintained at 55 °C in a thermostatically controlled perspex box (Northern Ireland Technology Centre, Queen's University of Belfast, UK) to allow intermittent sampling of arterialized venous blood. Isotope alone was infused during a 2 h equilibration period, after which a 4 h continuous infusion of insulin (Humulin S, Eli Lilly and Company Limited, Basingstoke, UK) was commenced (1 mU · kg⁻¹ · min⁻¹). During the insulin infusion plasma glucose was determined at 5 min intervals using a glucose oxidase method (Beckman Glucose Analyser II, Beckman RIIC Limited, High Wycombe, UK). Glucose (20%) was infused to maintain plasma glucose at the fasting level. The actual concentration of the infused glucose solution was measured on each occasion. Samples for determination of plasma glucose specific activity were taken at 10 min intervals from -30-0 min, 90-120 min and 210-240 min following the start of the insulin infusion. Additional samples for HPLC of plasma radioactivity were taken at 0, 120 and 240 min.

Analytical techniques

Arterialized venous blood was used for all analyses. Samples for glucose, glucose specific activity and HPLC were collected in lithium heparin fluoride tubes, immediately placed on ice and centrifuged within 30 min. An aliquot was used for duplicate determination of plasma glucose by a glucose oxidase method (see above). The remaining plasma was stored at -20 °C. Samples for insulin were allowed to clot and the resultant serum was stored at -20 °C until radioimmunoassay [9]. Mean between batch coefficient of variation was 5.6% at 8 mU/l and 8.2% at 36 mU/l; samples were diluted where serum insulin > 50 mU/l.

Glucose specific activity was measured after deproteinization of plasma using Ba(OH)₂ and ZnSO₄ by the method of Somogyi [10]. After centrifugation the supernatant was passed sequentially through anion (AG1-X8, BioRad Laboratories, Watford, UK) and cation (AG 50W-X8, BioRad) exchange columns to remove charged molecules. The eluate was collected into glass scintillation vials and lyophilized before being reconstituted to original volume in water. After addition of Biofluor (New England Nuclear Research Products Division, Dupont Limited, Stevenage, UK) samples were counted in a liquid scintillation spectrometer (Tri-Carb 2000 CA, Canberra Packard, Pangbourne, UK). Aliquots of infusate spiked into non-radioactive plasma were processed in parallel with plasma samples to allow calculation of [6-³H]glucose infusion rate, and glucose specific activities were calculated after correction for losses during extraction and using plasma glucose values determined in duplicate as described above.

HPLC analysis of plasma samples

Additional aliquots of plasma and tracer infusate for HPLC were processed in parallel with plasma for glucose specific activity to remove protein and charged molecules. Eluates from ion exchange columns were lyophilized, reconstituted in HPLC mobile phase (0.02 N H₂SO₄) and passed through 0.22 µm filters (Millex G S, Millipore Limited, Harrow, UK) before analysis. The resulting neutral fraction of plasma radioactivity, normally presumed to contain only labelled glucose, was analysed by the HPLC method of Bloch et al. [11]. Aliquots of extracted plasma and tracer infusate (500 µl) were applied to an HPLC system (Waters Chromatography Division, Millipore Limited) with two Aminex HPX 87 H (BioRad) columns in series. The mobile phase was 0.02 N H₂SO₄ (Aristar, BDH Limited, Poole, UK) and the flow rate 0.4 ml/min, at ambient temperature. Eluate was monitored at 190 nm using a variable wavelength spectrophotometer (Model 484, Waters Millipore). Fractions were collected at 20-25 s intervals. These were placed in glass scintillation

Fig. 1a, b. High performance liquid radiochromatographs of [6-³H] glucose isotope preparations used in glucose clamp experiments. Amount of radioactivity in numbered peaks is expressed as a percentage of total radioactivity recovered from the HPLC system (a) Batch 67; 1) 89.9% 2) 7.5% 3) 1.6% 4) 0.6% 5) 0.4% (b) Batch 69; 1) 97.5% 2) 2.0% 3) 0.3%

vials and Biofluor (Dupont) added prior to counting for 20 min each on a liquid scintillation spectrometer.

Calculations

Glucose appearance (Ra) rates were determined during the periods – 30 to 0 min, 90 to 120 min and 210 to 240 min using the non-steady state equations of Steele [12] as modified by De Bodo [13] assuming a pool fraction of 0.5 and an extracellular volume of 190 ml/kg. Endogenous glucose production (EGP) was calculated by subtracting the glucose infusion rate (GIR) from the isotopically determined glucose appearance rate (Ra). Isotopic steady-state was defined as a period when the slope of glucose specific activity vs time was not different from zero (fractional change less than 10% per hour).

The (maximum) amount of radioactivity in glucose was derived from radiochromatograms by dividing the radioactivity in the glucose peak by the total radioactivity (dpm) recovered from the column (glucose and impurities) after correction for background radioactivity, results being expressed as a percentage. These values were then used to obtain corrected values for rate of infusion of labelled glucose and for glucose specific activity at 0, 120 and 240 min. By assuming no significant change in percentage impurity had occurred over 10 min corrected glucose appearance rates (Ra') were calculated for the periods – 10 to 0 min, 110 to 120 min and 230 to 240 min. Plasma clearance rates (ml/min) of [6-³H]glucose and of the non-glucose tritiated impurity were calculated at 0, 120, and 240 min of insulin infusion by dividing their infusion rates (dpm/min) by the respective plasma concentrations (dpm/ml) determined by HPLC for each time point. In one subject (S5) multiple radiochromatograms were obtained for each 30 min period during which plasma glucose specific activity measurements were made to assess reproducibility of HPLC and calculated clearance rates.

Clamp studies using purified [6-³H]glucose tracer

On completion of the initial studies the HPLC system described above was used to purify the [6-³H]glucose tracer. Briefly, D-glucose (Analar, BDH) and H₂SO₄ (Aristar, BDH) were added to the [6-³H]glucose to achieve final concentrations of 2.4 mmol/l and 0.02 N respectively. Aliquots (500 µl) of this solution were applied to the HPLC system and a central portion of the glucose peak was collected using a programmable fraction collector (Microfraction Collector 203, Gilson Medical Electronics Limited, Villiers-le-Bel, France) connected to UV detector signal output (10 mV). [6-³H]glucose prepared in this way eluted as a single peak on repeat HPLC analysis. Appropriate quantities of purified isotope were dissolved in 0.9% NaCl, brought to pH 7.0 by addition of NaHCO₃ (1 mol/l) and sterilized by passing through 0.22 µm filters (Millex G S, Millipore) before use.

Euglycaemic hyperinsulinaemic glucose clamp studies were repeated in 2 subjects (1 male, 1 female) with purified [6-³H]glucose prepared the previous day, according to the protocol already described using conventional primed continuous tracer infusion. Measurements of glucose specific activity and HPLC analyses were performed as before. In a further four studies (four male subjects) purified [6-³H]glucose tracer was also added to the exogenous glucose infusion so as to achieve a glucose specific activity close to that of plasma after 2 h of primed continuous infusion.

This was predicted using a simple formula based upon the relationship observed between basal specific activity and rate of tracer infusion in the earlier clamp studies.

$$\text{Predicted basal specific activity} = \frac{\text{Tracer infusion rate (dpm/min)}}{12 \times \text{body weight (kg)}} \quad \left(\frac{\text{dpm}}{\mu\text{mol}} \right)$$

where 12 was the rounded mean basal Ra (µmol·kg⁻¹·min⁻¹) in the first six clamps after correction for presence of impurity. The primed

continuous infusion, containing a reduced quantity of tracer (0.199 ± 0.011 µCi·min⁻¹) was maintained throughout the hyperinsulinaemic period. A modest monophasic rise in plasma glucose specific activity was anticipated with this labelling strategy which makes no assumptions regarding the time course of endogenous glucose production or final rate of total glucose disposal [8]. In these four studies aliquots of labelled exogenous glucose infusion were spiked into non-radioactive plasma, as for the conventional tracer infusate samples, and processed in parallel with plasma samples for measurement of glucose specific activity, to allow calculation of total tracer infusion rates. Otherwise the study protocol was as for earlier studies.

Statistical analysis

Data in the text and figures are given as mean ± SEM. Statistical analyses were performed using the paired and non-paired two-tailed Student's *t*-tests. *p*-values < 0.05 were regarded as significant.

Results

Clamp studies using unpurified [6-³H]glucose tracer (Table 1)

Before insulin infusion, basal plasma glucose concentrations were 5.1 ± 0.1 mmol/l and basal plasma insulin levels were 4.1 ± 0.8 mU/l (*n* = 6). During the insulin infusion mean plasma insulin concentrations were 58 ± 3 mU/l. Mean plasma glucose concentrations during the hyperinsulinaemic period were 5.0 ± 0.1 mmol/l with mean coefficient

Table 1. Isotopically determined glucose appearance rates (Ra), glucose infusion rates (GIR) and endogenous glucose production rates (EGP) during clamp studies using unpurified [6-³H]glucose tracer (a) uncorrected (b) corrected using HPLC data

a. Uncorrected ^a							
Basal (– 30–0 min)		90–120 min			210–240 min		
Subject	Ra	Ra	GIR	EGP	Ra	GIR	EGP
1	11.9	35.6	47.5	– 11.9	41.5	49.4	– 7.9
2	14.5	35.1	45.0	– 9.9	41.6	43.9	– 2.3
3	12.4	23.3	30.2	– 6.9	30.7	40.5	– 9.8
4	10.4	16.3	20.2	– 3.9	27.6	36.3	– 8.7
5	11.4	32.7	46.9	– 14.2	40.5	52.9	– 12.8
6	13.0	37.2	43.3	– 6.1	52.4	62.1	– 9.7
Mean	12.3	30.0 ^b	38.9	– 8.8	39.1 ^b	47.5	– 8.5
(SEM)	(0.6)	(3.4)	(4.5)	(1.6)	(3.6)	(3.8)	(1.4)
b. Corrected ^a							
Basal (– 10–0 min)		110–120 min			230–240 min		
Subject	Ra'	Ra'	GIR	EGP'	Ra'	GIR	EGP'
1	12.5	41.3	47.2	– 5.9	49.5	47.6	+ 1.9
2	14.5	39.4	44.0	– 4.6	44.6	40.8	+ 3.8
3	11.4	25.9	30.3	– 4.4	32.9	41.7	– 8.8
4	10.2	17.1	20.4	– 3.3	31.0	35.4	– 4.4
5	11.2	40.0	44.1	– 4.1	47.3	50.7	– 3.4
6	12.3	40.0	42.1	– 2.1	55.4	62.1	– 6.7
Mean	12.0	34.0 ^b	38.0	– 4.1	43.5 ^c	46.4	– 2.9
(SEM)	(0.6)	(4.1)	(4.3)	(0.5)	(3.9)	(3.8)	(2.0)

^a All data expressed as µmol·kg⁻¹·min⁻¹; ^b *p* < 0.005 vs GIR; ^c *p* = NS vs GIR

Fig. 2. Plasma concentrations of glucose and insulin, and glucose infusion rates during 4 h glucose clamp experiments. Solid lines, unpurified $[6\text{-}^3\text{H}]$ glucose tracer ($n=6$). Broken lines, purified $[6\text{-}^3\text{H}]$ glucose tracer ($n=6$). Values are given as mean $\pm$ SEM

cient of variation of $4.9 \pm 0.4\%$ (Fig. 2). Time courses of specific activities are shown in Figure 3a. Only subjects 1 and 2 appeared to reach isotopic steady state between 90 and 240 min, however, in the presence of impure tracer this evidence of steady state may be unreliable.

Glucose appearance rates (Ra) determined during the basal and hyperinsulinaemic periods assumed all extracted radioactivity to be in glucose. During the hyperinsulinaemic period, if endogenous glucose production (EGP) is positive or zero, GIR may be approximately equal to, but not greater than Ra. However, isotopically determined Ra was lower than the actual exogenous glucose infusion rate (GIR) required to maintain euglycaemia at both 90–120 min and at 210–240 min. Calculated EGP was thus substantially negative at both 90–120 min and at 210–240 min indicating a persistent underestimate in the isotopically determined Ra.

HPLC of tracer infusates and plasma samples

HPLC of deionized tracer infusates revealed that only $92.9 \pm 1.2\%$ of radioactivity was in the glucose peak (retention time 26.1 ± 0.1 min). The remaining radioactivity (non-glucose) eluted in a second major peak (retention time 30.5 ± 0.2 min) well separated from glucose (Table 2). This peak corresponded to the dominant

Fig. 3a, b. Percentage of basal plasma glucose specific activity during glucose clamp experiments. Basal level was mean of 4 samples from -30 to 0 min. **a** Individual experiments using unpurified $[6\text{-}^3\text{H}]$ glucose tracer and unlabelled exogenous infusion ($n=6$), and **b** Individual experiments using purified $[6\text{-}^3\text{H}]$ glucose tracer. Solid lines, unlabelled exogenous glucose infusion ($n=2$). Broken lines, labelled exogenous glucose infusion ($n=4$)

impurity peak seen on HPLC of the parent $[6\text{-}^3\text{H}]$ glucose preparations. HPLC of the neutral plasma extracts used in the measurement of glucose specific activities revealed that $94.7 \pm 0.9\%$ of total radioactivity was in glucose at the end of the 2 h equilibration period (0 min). The non-glucose radioactivity present in the plasma extract increased during hyperinsulinaemia in all subjects (Table 2). Virtually all the non-glucose radioactivity eluted as a single peak (retention time 30.5 ± 0.3 min) corresponding to the dominant impurity peak present in tracer infusates and the parent $[6\text{-}^3\text{H}]$ glucose preparation (Fig. 4a).

Corrected glucose appearance rates (Ra') and endogenous glucose production rates (EGP') were then calculated using HPLC data (Table 1b). Basal Ra' (-10 – 0 min) was similar to basal Ra, however, following infusion of insulin Ra' was greater than Ra at both 110–120 min (by $2.6 \pm 0.8 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ or $7.9 \pm 2.1\%$)

Table 2. Percentage non-glucose radioactivity found on HPLC analysis of plasma extracts taken during clamps using unpurified $[6\text{-}^3\text{H}]$ glucose tracer. Subjects 1–5 $[6\text{-}^3\text{H}]$ glucose Batch 67; Subject 6 $[6\text{-}^3\text{H}]$ glucose Batch 69

Subject	Non-glucose radioactivity %			
	Tracer infusate	0 min	120 min	240 min
1	5.7	7.2	18.0	21.2
2	7.5	6.5	15.9	14.8
3	9.6	5.9	15.7	13.0
4	8.2	5.7	10.3	16.4
5	9.5	5.5	17.0	21.3
6	2.1	1.1	4.1	5.6
Mean	7.1	5.3	13.5 ^a	15.4 ^{a, b}
(SEM)	(1.2)	(0.9)	(2.2)	(2.4)

^a $p < 0.001$ vs 0 min; ^b $p = \text{NS}$ vs 120 min

Fig. 4a, b. HPLC of radioactivity from representative glucose clamps performed using (a) unpurified (b) purified $[6\text{-}^3\text{H}]\text{glucose}$ tracer. Radiochromatograms were made of tracer infusate solution (1) and of plasma extracts at 0 min (2), 120 min (3), and 240 min (4) following commencement of insulin infusion

and 230–240 min (by $4.3 \pm 1.1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ or $11.0 \pm 2.5\%$). Ra' remained lower than GIR at 110–120 min but was similar to GIR at 230–240 min. EGP' remained negative in all subjects at 110–120 min but at 230–240 min EGP' took on small positive values in two of the six subjects.

Clearance rates of glucose and tritiated non-glucose impurity

Basal clearance rates of the tritiated impurity ($238 \pm 36 \text{ ml/min}$) were higher than the corresponding clearance rates for glucose ($166 \pm 9 \text{ ml/min}$) in all but one subject ($p < 0.05$). Following commencement of insulin infusion the clearance rates of glucose increased (to $497 \pm 67 \text{ ml/min}$ at 120 min and $623 \pm 69 \text{ ml/min}$ at 240 min) while the clearance rates of the tritiated impurity remained unchanged (229 ± 29 at 120 min $p = 0.35$; 246 ± 34 at 240 min $p = 0.35$). In 10 determinations of clearance rates made in subject 5 using HPLC data, the clearance rate of tritiated impurity did not change during the clamp (mean $289 \pm 6 \text{ SEM}$) and overall coefficient of variation for the measurement was 6.5%.

Clamp studies using purified $[6\text{-}^3\text{H}]\text{glucose}$ tracer (Table 3)

Basal plasma glucose concentrations ($5.3 \pm 0.1 \text{ mmol/l}$) and insulin levels ($3.9 \pm 0.7 \text{ mU/l}$) were similar to those in the previous studies. During the insulin infusion mean plasma insulin concentrations were $59 \pm 6 \text{ mU/l}$. Mean plasma glucose concentrations were $5.2 \pm 0.1 \text{ mmol/l}$ with coefficients of variation of $5.1 \pm 0.7\%$ (Fig. 2). Time courses of specific activities are shown in Figure 3b.

In subjects 1 and 2 (clamps using conventional primed continuous infusion of purified $[6\text{-}^3\text{H}]\text{glucose}$) specific activities did not reach isotopic steady state between 90 and 240 min. In studies four to six, which used labelled exogenous glucose infusions, specific activities of the exogenous glucose infusions achieved using the simple formula were slightly greater than observed basal plasma glucose specific activities (mean 114% range 101–122%). Following commencement of the insulin infusion specific activities rose to $132 \pm 4\%$ at 90 min and were $134 \pm 2\%$ of basal levels at 120 min. All four subjects appeared to be in isotopic steady state between 90 and 240 min (mean fractional change $+0.1\%$ per hour; range -0.7 to $+1.6\%$).

In clamps 1 and 2 (conventional protocol) isotopically determined Ra was less than GIR at 90–120 min and calculated EGP was again substantially negative as in the previous studies using unpurified tracer. In contrast, Ra was similar to GIR at 210–240 min and calculated EGP was close to zero in both subjects.

In studies three to six (labelled exogenous glucose infusion) mean basal Ra was $13.3 \pm 0.5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Isotopically determined Ra ($43.8 \pm 3.7 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was greater than GIR ($39.8 \pm 2.8 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) at 90–120 min and calculated EGP was thus greater than 0 ($+4.0 \pm 1.1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) in all subjects. At 210–240 min Ra ($51.5 \pm 3.9 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was again greater than GIR ($48.3 \pm 3.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and calculated EGP was greater than 0 ($+3.2 \pm 0.7 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) in all subjects. No negative values for EGP were seen, and EGP appeared incompletely suppressed at 90–120 min (mean 70% range 56–90%) and at 210–240 min (mean 77% range 67–88%) (Table 3). On HPLC of tracer infusates and plasma samples radioactivity eluted as a single glucose peak and no impurity was detected (Fig. 4b).

Table 3. Isotopically determined glucose appearance rates (Ra), glucose infusion rates (GIR) and endogenous glucose production rates (EGP) during clamp studies using purified $[6\text{-}^3\text{H}]\text{glucose}$ tracer

Subject	Basal ^a (–30–0 min)				90–120 min ^a			210–240 min ^a		
	Ra	GIR	EGP		Ra	GIR	EGP	Ra	GIR	EGP
1 ^b	12.4	34.3	42.1	–7.8	43.3	43.1	+0.2			
2 ^b	15.5	38.1	47.1	–9.0	50.5	50.9	–0.4			
3 ^c	13.1	41.7	37.9	+3.8	56.5	53.6	+2.9			
4 ^c	14.4	52.5	47.3	+5.2	58.4	53.6	+4.8			
5 ^c	12.2	35.1	33.9	+1.2	40.9	39.4	+1.5			
6 ^c	13.6	45.9	39.9	+6.0	50.2	46.6	+3.6			

^a All data expressed as $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; ^b unlabelled exogenous glucose infusion; ^c labelled exogenous glucose infusion

Discussion

In common with previous authors we have found a significant underestimate in glucose appearance rates determined using tritiated glucose under conditions of high glucose flux [2]. Calculated rates of endogenous glucose production remained persistently negative throughout the 4 h hyperinsulinaemic period. The commercially prepared [6-³H]glucose tracer used in the studies was found to contain radioactive impurities when analysed by HPLC. The appearance in plasma extracts of the dominant impurity component was a consistent finding in all studies which used the tracer without additional purification. The amount of the impurity in plasma increased relative to radioactive glucose as plasma glucose specific activities fell during infusion of insulin. Use of the HPLC data to correct for presence of impurity in the measurement of glucose specific activity increased isotopically determined Ra by 8% on average at 120 min and by 11% on average at 240 min.

Conventional methods for measurement of glucose specific activity assume that the extraction procedure employed in processing of samples removes all non-glucose radioactivity. The radioactivity remaining is divided by the quantity of glucose present as measured by a highly specific enzymatic assay. After sample processing, consisting of cationic protein precipitation, anion and cation exchange and removal of tritiated water by lyophilization, the dominant radioactive impurity in these [6-³H]glucose tracers was not removed. The impurity is in effect "mistaken for glucose" and is thus a source of error in the measurement of glucose specific activity, resulting in falsely high values. Thus, a tritiated tracer impurity can cause an underestimation of Ra as previously reported by McMahon et al. in prolonged (7 h) hyperinsulinaemic clamps [6].

The radioactive impurity we have found closely resembles that found by McMahon et al. in [6-³H]glucose tracer prepared by a different manufacturer [6]. The first batch of [6-³H]glucose tracer we tested was more heavily contaminated, however, we have found considerable between batch variation (1–10% impurities). Radioactive glucose (both ³H and ¹⁴C labelled) from several manufacturers has previously been reported to contain radioactive impurities [14]. These bound covalently to proteins thus causing errors in measurement of glucose incorporation and implausibly high rates of non-enzymatic glycation. The greatest errors were found with [6-³H]glucose and differences of up to 26-fold between batches were seen. Unfortunately these earlier observations contain no HPLC data for comparison. Other tritiated tracers may also be contaminated in this way. We have found an identical impurity in [2-³H]glucose tracers (data not shown) and others have reported similar findings on HPLC of [3-³H]glucose tracers [6]. The influence of prolonged storage on the ability to detect these impurities in plasma has not been studied. Any conclusions about the size of impurity-associated error which are based on retrospective analyses performed on stored samples [7] must therefore be regarded as tentative.

HPLC of plasma samples and tracer infusates has provided information on behaviour of the radioactive impurity during these clamp studies. In the basal postabsorptive state, when insulin levels were low, clearance rates of radioactive impurity were in fact slightly greater than clearance rates of radiolabelled glucose resulting in a negligible overestimate of isotopically determined basal Ra. Following commencement of insulin infusion, however, the clearance rates of radioactive impurity did not increase and plasma concentrations of impurity remained constant. In contrast, the concentration of labelled glucose fell as clearance rates of [6-³H]glucose increased to $295 \pm 30\%$ of basal at 120 min and to $370 \pm 27\%$ of basal at 240 min. An underestimate in isotopically determined Ra is thus anticipated. Contrary to the data of McMahon et al. [6], we found no evidence of accumulation of the radioactive impurity in absolute terms, nor any increase in clearance under hyperinsulinaemic conditions.

The size of the underestimate produced in this way will depend on both the quantity of impurity infused and the difference in clearance rates between [6-³H]glucose and impurity. The increment in [6-³H]glucose clearance which follows infusion of insulin exposes the presence of impurity which forms an increasing proportion of radioactivity in plasma extracts and results in a falsely high glucose specific activity. The maximum error due to the presence of radioactive impurity is expected when the difference in clearance rates of radioactive glucose and radioactive impurity is greatest, i.e. during the last 30 min of the 4 h clamp. In contrast, the maximum error due to inadequacy of the model is expected when disturbance of steady state conditions is most pronounced and glucose specific activity changing most rapidly, i.e. during the early part of the hyperinsulinaemic period. The time course of the two errors is therefore different. When glucose specific activity is falling the errors are additive. Obviously a relatively small error in estimation of Ra will cause a much larger error in calculation of EGP.

When the impurity was eliminated from tracer infusates by HPLC, the extraction procedure for measurement of glucose specific activity removed all non-glucose radioactivity. In the two conventional clamps performed using only a primed continuous infusion of HPLC purified [6-³H]glucose tracer, calculated rates of endogenous glucose production were close to zero during the last 30 min of the 4 h hyperinsulinaemic period. The large negative errors observed at 90–120 min may then be attributed exclusively to inadequacy of the single compartment model. When purified [6-³H]glucose tracer was added to the exogenous glucose infusion required to maintain euglycaemia (thereby preventing any fall in plasma glucose specific activity) the calculated rates of endogenous glucose production took on small positive values and no negative errors were seen.

The insulin infusion rates chosen for these studies are usually considered sufficient to suppress EGP almost completely [15]. In the present studies, when purified tracer was used with a labelled glucose infusion, EGP suppressed by only 67–88% after 4 h. As in previous studies, the calculations of EGP are based on indirect measurements, and since the true values are unknown, we cannot

be certain they are correct. A small positive error might be expected with the rising specific activities which occurred in these experiments. The time course of such an error would mirror that of the underestimate due to inadequate modelling in the two conventional clamp studies. Any modelling error should be minimised if measurements are made under near steady state conditions. Indeed, in the four labelled glucose infusion studies specific activities changed very little from 90 to 240 min when compared to the conventional studies. For a true steady state to be achieved, stable euglycaemia should be maintained with a constant GIR, however in these 4 h studies GIR continued to rise throughout (and may do so for 5–8 h in normal subjects) [16]. Further changes in glucose specific activity may occur only very slowly, so a “concealed” non-steady state may exist [8]. However, the relative constancy of the glucose specific activities with the labelled glucose infusion protocol leads us to believe that the small positive EGP values obtained are more likely to be reliable than the zero values obtained using purified tracer in the conventional protocol. In the light of these observations, future studies might reassess suppression of endogenous glucose production using pure [^3H]glucose tracers and improved strategies to eliminate modelling errors.

In conclusion, these studies show that radioactive impurities may contribute significantly to the underestimate in glucose appearance rates determined with [$6\text{-}^3\text{H}$]glucose tracers under non-steady state conditions. When steps are taken to reduce or eliminate model associated error, errors associated with the presence of impurity may assume greater importance. Attention should thus be paid to tracer purity, as verified by HPLC.

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