Journal of Chromatography, 614 (1993) 148–152
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6734

Short Communication

Solid-phase separation of ^{14}C-labelled urea and aminoisobutyric acid for membrane transport studies

Pandora C. Evans*, Li Liu and Jane E. Harding

Research Centre for Developmental Medicine and Biology, Department of Paediatrics, University of Auckland, Private Bag 92019, Auckland (New Zealand)

(First received November 3rd, 1992; revised manuscript received December 28th, 1992)

ABSTRACT

A sorbent extraction method has been developed for separating ^{14}C-labelled urea and aminoisobutyric acid (AIB) in blood. The use of commercial solid-phase extraction cartridges containing aminopropyl-bonded silica provided a convenient and rapid separation of urea and AIB with better than 92% recovery of each and less than 5% cross-contamination. This allows these compounds, together with [^{3}H]methylglucose, to be used as marker compounds for investigating three aspects of membrane transport. The facility to separate any two of the three compounds permits their simultaneous measurement, greatly increasing the amount of data obtainable from each *in vivo* preparation.

INTRODUCTION

In physiological research it is often necessary to investigate the transport of metabolic substrate molecules across biological membranes. The mechanisms of transport include simple diffusion (*e.g.* of urea), facilitated diffusion (*e.g.* of glucose) and active transport (*e.g.* of amino acids). In complex biological systems such as the placenta, these mechanisms have been studied by infusion of non-metabolisable radiolabelled compounds, *e.g.* [^{14}C]urea, [^{14}C]- or [^{3}H]-3-O-methylglucose (MG) and [^{14}C]aminoisobutyric acid (AIB), either alone or in pairs where each of the compounds carried a different radiolabel [1–4]. To date, no method has been described which permits the simultaneous assessment of the above three aspects of membrane transport by measuring all three compounds in blood in a single *in vivo* preparation.

Since complex animal preparations tend to be expensive and time-consuming to maintain we aimed to devise a method which would efficiently separate any two of the three marker compounds (urea, MG and AIB), permitting the use of two compounds with the same label. Preliminary experiments suggested that urea and AIB were sufficiently different in their chemical properties to allow chromatographic separation. The separation described here depends on the affinity of AIB for an amino functionality bonded to the silica packing material of commercial solid-phase extraction cartridges commonly used for sample

* Corresponding author.

0378-4347/93/$06.00

clean-up prior to high-performance liquid chromatography (HPLC). Thus, we have been able to investigate membrane transport using ^{14}C-labelled urea and AIB and [^{3}H]MG in a single preparation.

EXPERIMENTAL

Materials

The labelled compounds [^{14}C]urea (specific activity 2–10 mCi/mmol), [1-^{14}C]-α-AIB (57.6 mCi/mmol), α-[methyl-^{3}H]AIB (13.4 Ci/mmol) and 3-O-[methyl-^{3}H]-D-glucose (79 Ci/mmol) were obtained from New England Nuclear Research Products (Boston, MA, USA). Solid-phase extraction cartridges (1 ml, amino, product No. 211 000), vacuum manifold and accessories were from Alltech Assoc. (Deerfield, IL, USA). Acetonitrile was Chromar grade from Mallinckrodt (Paris, KY, USA) and water was purified by a Milli-Q ultrapure water system (Millipore, Sydney, Australia). All other chemicals were analytical grade.

Deproteinisation of samples

Duplicate 0.1-ml aliquots of blood containing the labelled compounds were mixed with 0.8 ml of 0.04 *M* H_2SO_4 in microcentrifuge tubes, on ice. Sodium tungstate (0.1 ml, 0.3 *M*) was added, the tubes were mixed again, then centrifuged at 15 000 *g* for 4 min at 4°C. Aliquots (0.2 ml) of the supernatant were removed to glass scintillation vials for estimation of [^{3}H]MG and total ^{14}C. A further 0.5 ml was transferred to 5-ml tubes and evaporated to dryness using a vacuum concentration system (Heto Lab Equipment, Copenhagen, Denmark). Tubes were stored dry at −20°C until the separation could be performed.

Separation of AIB and urea

The cartridges were conditioned with 1 ml of acetonitrile followed by 1 ml of acetonitrile which had been equilibrated with Tris buffer (1.0 *M*, pH 7.5) in the ratio of 9:1. The dry, deproteinised samples were redissolved in 0.1 ml of water, diluted to 1 ml with acetonitrile and transferred to the cartridges as completely as possible. The tubes were washed with two 0.5-ml volumes of acetonitrile–water (9:1, v/v) which were also added to the cartridges. The cartridges were then washed with two 0.5-ml volumes of acetonitrile–Tris (9:1, v/v). The loading volume and all the washes, including the volume remaining in the cartridges and the system tubing, were collected in glass scintillation vials for the estimation of [^{14}C]urea. [^{14}C]AIB was eluted into a third set of vials with two 1-ml volumes of 1 *M* HCl. Batches of up to twenty samples were processed thus using a vacuum manifold. The cartridges were not reused, the manifold and all tubing were rinsed thoroughly with deionised water between batches and at least one control sample (described below) was included in each batch.

Scintillation counting

Commercial scintillant (10 ml Ready Safe, Beckman Instruments, Fullerton, CA, USA) was added to each vial. ^{3}H and ^{14}C contents were determined by counting for 10 min in a dual-channel scintillation counter (Model 1219, RackBeta, LKB Wallac, Turku, Finland) using an automatic window setting. External standard quench correction was made using a stored curve obtained by counting commercially prepared quenched standards. Background counts were determined in extracts of unlabelled blood processed with the samples. The ^{3}H and ^{14}C activities in each vial were expressed as dpm/ml of blood following appropriate correction for background counts and dilution.

Control samples

To develop and validate the separation we supplemented blood with approximately 50 000 dpm/ml each of [^{14}C]urea and [^{3}H]AIB, and froze small aliquots. When samples were routinely processed at least one of these was included for every ten samples to determine the efficiency of separation.

Increased sample volume

When the concentration of one or both isotopes was low, larger volumes of blood were processed with similar precision. Protein was pre-

cipitated in triplicate, from 0.25 ml of blood with 2 ml of 0.04 *M* H_2SO_4 and 0.25 ml of 0.3 *M* sodium tungstate in 5-ml tubes with centrifugation at 3000 *g* for 30 min. Supernatants were pooled, with two 0.8-ml volumes taken for total counts and two 2-ml aliquots for evaporation. The subsequent sample treatment and separation were identical to that for the 0.1-ml samples.

RESULTS AND DISCUSSION

The separation of the two compounds was validated using [^{14}C]urea and [^{3}H]AIB. It was assumed that [^{3}H]AIB interacts with the sorbent and solvents in the same way as the ^{14}C-labelled compound. Dried supernatant aliquots from control blood supplemented with [^{14}C]urea and [^{3}H]AIB were redissolved in 0.1 ml of water, diluted to 1 ml with acetonitrile and applied to conditioned cartridges as described in the Experimental section. Sequential fractions corresponding to the loading volume (1 ml), the tube washes (2 × 0.5 ml), the acetonitrile–Tris washes (3 × 0.5 ml) and HCl eluates (3 × 1.0 ml) were collected in separate scintillation vials. A percentage of 95.8 ± 1.4% (mean ± S.D.) of the urea and 4.5 ± 1.1% of the AIB was either not retained or was eluted by washing with acetonitrile–Tris (9:1), while 94.8 ± 4.2% AIB and 2.2 ± 0.8% urea was eluted by 1 *M* HCl (Fig. 1). The third acetonitrile–Tris fraction contained 0.6 ± 0.3% urea and 0.5 ± 0.1% AIB, with 0.2 ± 0.2% urea and 0.9 ± 0.5% AIB in the third HCl fraction, so these were omitted from routine sample separations. However, care was taken to collect all the liquid remaining in the columns and collection tubing associated with each fraction.

The precision and accuracy of the method were calculated from quality control data accumulated over eighteen separate experiments each containing four or five control samples. The mean (± S.D.) percentage of total urea which appeared in the AIB fraction was 3.5 ± 2.5% while 3.2 ± 0.8% total AIB appeared in the urea fraction. For the larger (0.25 ml) samples the spillover was 3.6 ± 2.5 and 4.0 ± 0.9%, respectively.

In thirteen assays the mean (± S.D.) recoveries of label following separation were 92.6 ± 1.3% (urea) and 96.8 ± 3.4% (AIB), inter-assay C.V.s were 2.4 and 5.2% and intra-assay C.V.s were 4.0 ± 1.8 and 3.8 ± 2.4%, respectively. The corresponding values for the 0.25-ml samples (five assays) were: recovery, 94.6 ± 3.2% (urea) and 92.8 ± 1.8% (AIB); inter-assay C.V., 3.6 and 2.4%; and intra-assay C.V., 2.2 ± 1.5 and 1.9 ± 1.2%, respectively.

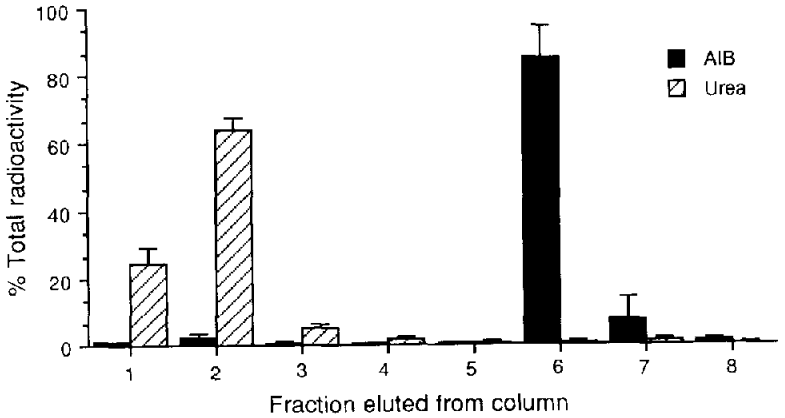

Fig. 1. Separation of [^{14}C]urea and [^{3}H]aminoisobutyric acid using solid-phase extraction cartridges containing amino-bonded silica. Four aliquots of blood supplemented with the radiolabelled compounds were deproteinised with tungstic acid, dried, redissolved in 0.1 ml of water, diluted to 1 ml with acetonitrile and applied to the columns. Fractions were collected in separate scintillation vials and counted to determine the recovery of radioactivity in each. The fractions correspond to: (1) the loading volume; (2) two 0.5-ml washes with acetonitrile–water (9:1); (3–5) acetonitrile–1 *M* Tris, pH 7.4 (9:1) washes, 0.5 ml each; (6–8) 1 *M* HCl eluates, 1 ml each.

The retention of AIB appears to be dependent on both ionic and polar interactions with the bonded phase of the column packing. Of all the bonded silicas screened for the separation, the aminopropyl showed the highest retention of AIB. While it would seem logical to separate urea and AIB by retention of negatively charged AIB on a strong anion exchanger at pH 10–11 we were unable to demonstrate more than 75% retention under these conditions. AIB was better retained by sorbents which had some polar characteristics, *i.e.* diol and aminopropyl. Of these, initial screening of standards diluted in acetonitrile showed 98% AIB was retained by the amino-bonded sorbent equilibrated with acetonitrile–Tris, compared with 90% by the diol under identical conditions.

Maximum retention (and therefore separation) was associated with a low-aqueous component in the loading solvent and we found that more than 10% water in the resuspending solvent led to increased appearance of AIB in the urea fraction. It has been suggested that secondary interactions of polar molecules with the silica sorbent are enhanced in the presence of a Tris buffer [5]. We found that retention of AIB could be further increased by conditioning the cartridges with acetonitrile which had been equilibrated with Tris buffer.

Several methods were investigated for precipitating blood proteins including organic solvents, perchloric acid, barium hydroxide–zinc sulphate and tungstic acid. Of these only the tungstic acid precipitation described in the Experimental section showed consistent recovery of each of the three labelled compounds together with adequate retention of AIB on the cartridges following reconstitution with acetonitrile–water. The mean (± S.D.) recoveries in the supernatant were 97.1 ± 2.8% for urea, 99.2 ± 5.0% for AIB and 101.0 ± 3.6% for MG.

Under the conditions which best separated urea and AIB it was not possible to consistently recover MG in either fraction. Indeed further washing of the cartridge after elution of AIB failed to recover more than 85% MG. We therefore used MG as the only ^{3}H-labelled compound and estimated its concentration in the supernatant prior to separation, together with total ^{14}C which provided a useful check on the recovery of the two ^{14}C-labelled compounds following their separation.

The procedure is convenient and reliable due to the consistent quality of commercial solid-phase extraction cartridges compared with individually packed columns. Taking into account the recoveries of each compound and possible contributions from the other due to spillover, the method would be expected to yield a measured value within 10% of the true result. This is less than the variability of repeated animal experiments using combinations of two tracers with different labels, as well as being significantly faster and cheaper.

The method has proved useful for simultaneously investigating three different mechanisms of transport across the ovine placenta. Owens *et al.* [2] estimated placental transfer by simple and facilitated diffusion using [^{14}C]urea and [^{3}H]MG, respectively, during experimental intrauterine growth retardation. Our ability to separate urea and AIB permitted us to use a similar approach but extended to include the simultaneous observation of the active transport of amino acids using infused [^{14}C]AIB. Such studies have previously been reported only in small animals and in the absence of [^{14}C]urea [1,4]. Thus this method has allowed us to assess the effects of manipulating fetal and maternal nutrition and endocrine status on placental transport by simple diffusion, facilitated diffusion and active transport simultaneously in a single *in vivo* preparation.

ACKNOWLEDGEMENTS

This work was supported by the Auckland Medical Research Foundation and the Health Research Council of New Zealand. Thanks are extended to Dr. P. Kestell, Cancer Research Laboratory, University of Auckland for helpful discussions.

REFERENCES

1 T. Jansson and E. Persson, *Pediatr. Res.*, 28 (1990) 203.
2 J. A. Owens, J. Falconer and J. S. Robinson, *J. Dev. Physiol.*, 9 (1987) 457.
3 J. H. G. Rankin, G. Jodarski and M. R. Shanahan, *J. Dev. Physiol.*, 8 (1986) 247.
4 M. Nitzan, S. Orloff and J. D. Schulman, *Pediatr. Res.*, 13 (1979) 100.
5 K. C. Van Horne (Editor), *Sorbent Extraction Technology*, Analytichem International, Harbor City, CA, 1985.