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Rapid analysis for metabolites of ^{11}C-labelled drugs: fate of [^{11}C]-*S*-4-(*tert.*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one in the dog

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ABSTRACT

Positron emission tomography (PET) requires the use of compounds labelled with short-lived, positron-emitting isotopes (*e.g.*, $t_{1/2}$ of ^{11}C ~120 min). As the concentration of unbound, non-metabolised drug is required as the input function for modeling, this presents particular problems for the study of the kinetics and metabolism of such compounds. We have now developed a rapid extraction procedure, followed by high-performance liquid chromatography using a short analytical column coupled to an on-line γ-detector to determine the metabolism and kinetics of a non-selective β-adrenergic antagonist of high affinity, *S*-4-(*tert.*-butylamino-2-hydroxypropoxy)benzimidazol-2-one. This antagonist is potentially well suited to the non-invasive localisation of β-receptors *in vivo*. The ligand was rapidly taken up into the β-receptor pool or excreted in urine, with less than 5% of the drug converted to metabolites. Plasma protein binding was only 16%. No significant metabolism of the ligand was observed in the anaesthetised dog, and, therefore, no correction for blood metabolite concentration is required for kinetic analysis of the ^{11}C-labelled ligand during PET studies in this species. The analytical method reported here should be widely applicable: quantification of metabolites enables accurate estimation of the input function and is critical to the interpretation of PET data.

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INTRODUCTION

Positron emission tomography (PET) is a powerful method for determining the distribution and kinetics of radiolabelled ligands *in vivo* [1,2]. Positron-emitting isotopes (such as ^{11}C and ^{18}F) are chemically incorporated into a suitable ligand. Following administration, the ligand will distribute between tissue compartments including the plasma and tissue receptor pool. The regional concentrations of radioligand are determined from the PET signal, which arises from radioactive decay of the positron-emitting isotope. The PET signal in plasma or tissue will represent both the parent radioligand and any labelled metabolites formed *in vivo*, either free or bound. However, it is the concentration of unbound ligand in plasma which is required as the input function for modeling receptor ligand kinetics [3,4]. Thus, any metabolites (or impurities from the original synthesis) must be measured and a suitable correction made to the input function.

S-4-(*tert.*-Butylamino-2-hydroxypropoxy)benzimidazol-2-one (*S*-I) (Fig. 1) is a non-selective β-adrenergic antagonist of high affinity [5]. Because of its low lipophilicity, it exhibits low non-specific binding and does not accumulate in cells. The racemic form of the ligand (*RS*-I) has been proposed as a specific antagonist of surface β-receptors [6]. It has been used in competition studies to determine the binding constants of unlabelled β-adrenergic antagonists [5]. It has been labelled with the positron-emitting isotope ^{11}C [7] in order to visualise β-receptor distribution and density in the lung and heart.

In order to determine the input function in PET studies, a knowledge of the clearance and metabolism of ^{11}C-labelled drugs is critical [8,9]. We have developed extraction and analytical techniques, based on high-performance liquid chromatography (HPLC), to investigate the possible metabolism of this ligand in the rat and dog *in vivo*. Initial assessment of the metabolism of the ligand was carried out using both [^{3}H]-*S*-I and [^{14}C]-*RS*-I to enable detailed analysis of any metabolites and to establish the rapid techniques required when using ^{11}C-labelled ligands ($t_{1/2}$ of $^{11}C \sim 20$ min). Subsequently, studies were performed with [^{11}C]-*S*-I using a short analytical HPLC column coupled to an on-line γ-detector.

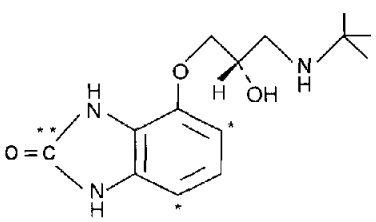

Fig. 1. Structure of *S*-I. The positions of the radiolabels are marked: (*) ^{3}H; (**) ^{11}C or ^{14}C.

EXPERIMENTAL

Radioligands

Isotopically labelled ligand [^{11}C]-4-(*tert.*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one was synthesised in the MRC Cyclotron Unit at Hammersmith Hospital by reacting 1-(3-*tert.*-butylamino-2-*S*-hydroxypropoxy)-2,3-diaminobenzene with [^{11}C]phosgene as previously described [7]. The radioligand was purified by HPLC. The specific activity was ≤ 10 TBq/mmol and the radiopurity greater than 99.98% on all occasions. The ^{3}H-labelled ligand ([^{3}H]-*S*-I) was obtained from Amersham International (Amersham, UK) (specific activity 1.11–2.22 TBq/mmol, radiochemical purity 99.1%). A racemic form of the ligand labelled with ^{14}C ([^{14}C]-*RS*-I, 3.6 MBq/mmol, purity 96%) was a kind gift from Ciba-Geigy (Basle, Switzerland).

Animal studies

Five greyhound dogs were studied. Two females (weight 27.6 and 29.4 kg) and two males (weight 28.5 and 32.6 kg) were infused with [^{11}C]-*S*-I. One female (weight 25.5 kg) was infused with [^{3}H]-*S*-I. Anaesthesia was induced with thiopental sodium (5 mg/kg) and maintained with nitrous oxide–oxygen with 0.75% halothane (tidal volume 20 ml/kg, fifteen breaths per min). For injection of radioligand, a catheter (8F) was placed in the inferior vena cava via the femoral vein. Catheters were placed in the aorta (8F) via a femoral artery and in the pulmonary artery (6F) via an internal jugular vein for blood sampling and pressure monitoring. One animal received 37 MBq [^{3}H]-*S*-I (223 ng/kg) in 10 ml over 20 s and the others 240–750 MBq of [^{11}C]-*S*-I (173–215 ng/kg). Blood samples (5 ml) were withdrawn simultaneously from the aorta and pulmonary artery at 1-min intervals up to 30 min, at 5-min intervals up to 50 min and 10-min intervals to 125 min. The chest was opened 10 min before injection to obtain sequential lung tissue biopsies and the bladder was catherised for collection of urine (^{3}H study only). Seven male Sprague–Dawley rats (220–280 g) were anesthetised with oxygen–nitrous oxide–isofluorane (BOC, London, UK) and a catheter (0.96 mm) was inserted into a tail vein. The rats were allowed to recover consciousness in a restraining cage. The radioligand (^{3}H, $n = 3$, 0.25–2500 μg/kg; ^{11}C, $n = 4$, 1.1–2.8 μg/kg) *S*-I or (^{14}C, $n = 1$) *RS*-I (5 mg/kg) was injected into the tail vein. At intervals (5, 10, 15 and 30 min) following injection animals were reanaesthetised and the maximum possible amount of blood (5–8 ml) was withdrawn by cardiac punture at this time. Urine samples were collected through a needle inserted into the bladder.

Extraction of ^{3}H- and ^{14}C-labelled compounds

All solvents were of HPLC grade and obtained from Rathburn (Walkerburn, UK). Other chemicals were of AnalaR grade (BDH, Poole, UK). All blood samples containing [^{3}H]-*S*-I or [^{14}C]-*RS*-I were cooled in ice immediately on collec-

tion and centrifuged (3 min, 3000 *g*, 4°C) to obtain the plasma, which was then frozen at −80°C for subsequent analysis. Following thawing, 20% trichloroacetic acid was added to each sample in a ratio of 1:2 to precipitate the protein, and the supernatant, after centrifuging, was passed through a solid-phase C_{18} adsorption cartridge (Sep-Pak, Waters Assoc., Chester, UK) and sequentially washed with 5 ml each of water, water–methanol (1:1, v/v) and methanol. Each wash was collected separately and the solvent was removed under vacuum. Lung samples (between 0.5 and 1.1 g) were homogenised in 2 ml of 0.2 *M* hydrochloric acid, centrifuged (10 min, 1000 *g*) and the supernatant removed and treated as above. Urine (10 ml) was extracted directly onto a C_{18} Sep-Pak cartridge as for plasma.

High-performance liquid chromatography

Samples were reconstituted in the mobile phase (1 ml) and HPLC was carried out on a Waters gradient elution system, eluting at 1 ml/min on a μBondapak C_{18} column (30 cm × 0.8 cm, 10 μm particle size, Waters Assoc.) with aqueous acetic acid (5%, v/v) isocratically at ambient temperature for 5 min followed by a 20-min linear gradient to 40% propan-2-ol in aqueous acetic acid. The radioactive HPLC fractions (from blood samples collected at 5, 10, 60 and 120 min) were further purified. HPLC solvent was removed under vacuum and then the samples were reconstituted in 500 μl of aqueous trifluoroacetic acid (0.04%). These samples were then chromatographed on a Nova-Pak ODS column (15 cm × 0.4 cm, 4 μm particle size, Waters Assoc.) eluting at ambient temperature at 1 ml/min with water–trifluoroacetic acid (100:0.04, v/v) isocratically for 5 min followed by a 20-min linear gradient to acetonitrile–water–trifluoroacetic acid (15:85:0.04, v/v). The HPLC eluent was monitored at 254 nm and by scintillation counting in 10-ml Instagel (Packard Instrument, Meriden, CT, USA) per 100 μl. Extraction and HPLC yields were determined by adding [^{3}H]-*S*-I to blood and lung tissue and extracting as above.

Rapid analysis of ^{11}C-labelled compounds

Untreated plasma (2–3 ml) was passed through a Sep-Pak solid-phase C_{18} cartridge, washing with 5 ml water and eluting with 5 ml of methanol. The methanol fraction was reduced to 0.3 ml by rotary evaporation and made up to 1.5 ml with water containing unlabelled *S*-I as a carrier. The sample was then centrifuged (1000 *g*, 2 min) before injection of 1.4 ml onto a Brownlee cartridge containing MCH10 packing (Varian Assoc.). Radioligand was eluted at 2 ml/min with a 5-min linear gradient from 20 to 50% methanol in 5 m*M* aqueous sodium octanesulphonate (pH 6). The eluent was passed through a 1.2-ml PTFE coil inserted into the well of a NaI (Tl) well-type γ-ray detector (NE Technology, Edinburgh, UK) linked to a rate meter.

Over-pressured thin-layer chromatography (OPTLC)

Samples of rat urine and plasma which had been partially purified through a Sep-Pak were also analysed by OPTLC (Chrompress, Newman-Howell Assoc., Winchester, UK) by Dr. David Saynor at Glaxo (Ware, UK) using the method of the Orsay PET group [10]. Samples were dissolved in 100 μl of OPTLC buffer and duplicate 20-μl aliquots were chromatographed on a plastic backed silica TLC plate (Merck, Darmstadt, Germany), developing in acetonitrile–ethylamine–water (100:4:396) buffered to pH 4 with 125 m*M* sodium acetate–acetic acid. Radioactivity on the TLC plate was quantified on a multi-wire proportional counter (Autograph, Oxford Positron Systems, Oxford, UK).

Plasma protein binding

Plasma protein binding was measured by introducing paired aliquots (1 ml) of dog plasma containing 0.125–32 μg/l [^{3}H]-*S*-I into one side of the cells of a multiple equilibrium dialysis system (Dianorm, Berne, Switzerland). Samples were dialysed against phosphate-buffered saline through 0.025-mm Visking tubing for 24 h. Each side of a cell was sampled and the ^{3}H activity measured by liquid scintillation counting.

RESULTS

A simple extraction protocol was developed for *S*-I (using the ^{3}H-labelled ligand) based on C_{18} solid-phase extraction and reversed-phase HPLC on a semi-preparative μBondapak column. The ligand eluted from the Sep-Pak cartridge in methanol–water (1:1, v/v) and eluted on HPLC as a single peak with a retention time of 29 min (μBondapak column) or 25 min (Nova-Pak column). The extraction yields for this ligand throughout the procedure were over 70% for both plasma and lung homogenates and over 80% for urine. Protein binding in plasma was approximately 16% at all concentrations of *S*-I studied. There was no evidence for *ex vivo* metabolism when *S*-I was incubated with plasma or lung homogenate: a single peak of radioactivity post HPLC was always obtained using both the μBondapak and Nova-Pak HPLC system.

When [^{3}H]-*S*-I was infused into an anaesthetised dog, the plasma radioactivity fell rapidly (Fig. 2). At all time points up to 2 h, >95% of the radioactivity recovered in the plasma was found in the methanol–water (1:1, v/v) Sep-Pak wash and chromatographed on HPLC as a single peak with a retention time of 29 min on the μBondapak column (Fig. 3). When this purified substance was chromatographed further on a Nova-Pak column, a single peak of radioactivity was observed, eluting at 25 min. These data show that plasma radioactivity consisted almost entirely of unmetabolised *S*-I. From 60 min, there was evidence for the presence of a more polar species than *S*-I; a radioactive peak appeared in the Sep-Pak water wash and eluted on HPLC (μBondapak) at 19 min. This represented <2.5% of the unmetabolised *S*-I present in plasma at this time (*cf.* Fig. 2)

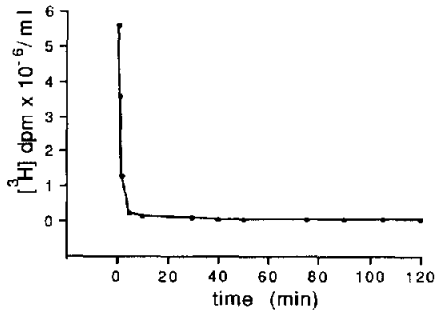

Fig. 2. Radioactivity–time curve for plasma, following infusion of [^{3}H]-*S*-I into a single dog.

and could arise either from a metabolite or from trace impurity in the injected drug. The plasma radioactivity declined bi-exponentially. The initial phase represents distribution of the ligand mainly onto β-receptors (particularly in the lung). The terminal elimination phase was very prolonged. This was due largely to urinary excretion, as >70% of the dose was recovered unchanged in the urine. Although only 6% of the dose was recovered in the urine over 2 h, this was due presumably to the very slow elimination caused by the long off-time from the β-receptors, as the renal clearance of *S*-I in the greyhound (calculated from the ratio of amount of unchanged *S*-I excreted in the urine to the area under the

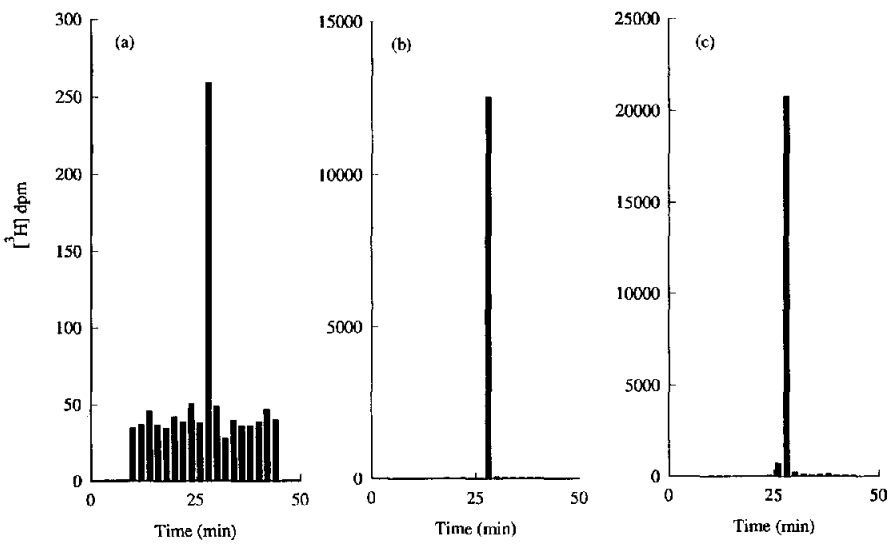

Fig. 3. HPLC radioprofile (μBondapak column) for dog plasma (a) and homogenised lung tissue (b) taken at 60 min following infusion of [^{3}H]-*S*-I. The radioprofile of 60–90 min urine from the same dog is also shown (c). The time for the dead volume to be voided was 11 min. In each case, the radioactivity was contained in only one fraction corresponding to authentic *S*-4-(*tert.*-butylamino-2-hydroxypropoxy)benzimidazol-2-one.

plasma concentration–time curve of radiolabel over the same interval) was similar to creatinine clearance. When uptake to the receptors was blocked, the elimination rate constant of *S*-I increased considerably (data not shown), showing that at the tracer dose used in this study, elimination was distribution limited.

Serial lung biopsies contained only unchanged *S*-I. Similarly, >95% of the urinary radioactivity chromatographed as unchanged *S*-I on HPLC. There was some evidence for small amounts of more polar radiolabelled compounds in the urine, but these accounted for <1% of unmetabolised *S*-I at any time. The overall recovery of radiolabel in each case was >70%, which was similar to recoveries obtained in control studies, making it unlikely that any metabolites had been selectively lost on extraction.

Similar data were obtained from four rats infused with [^{3}H]-*S*-I. Unchanged ligand accounted for >95% of the radioactivity recovered in plasma and lung homogenates at all times. Because of the small plasma volumes (and the consequent low counts) available, it is possible that small amounts of metabolites (<2%) would not have been observed. To determine whether the position of radiolabelling or the use of a racemic mixture could affect the results, a single rat was infused with [^{14}C]-*RS*-I and a sample of blood and urine obtained after 5 min. There was no difference in the recovery of radiolabel during the extraction, and, again, there was no evidence for metabolism at this time.

Samples of plasma (taken at 5 min) and urine (post Sep-Pak) from the rat study were also analysed by normal phase using the technique of OPTLC. A single radioactive spot was present for each sample eluting in the same position as authentic *S*-I (R_F = 0.7). There was no evidence for the presence of metabolites.

The extraction protocol used for [^{3}H]-*S*-I and [^{14}C]-*RS*-I was modified for use with the short-lived ^{11}C-labelled ligand. Plasma was extracted directly with a C_{18} Sep-Pak, and a short reversed-phase Brownlee HPLC cartridge (packed with MCH10) was used for analysis. *S*-I eluted with a retention time of 7 min. The elution system was buffered to pH 6 to overcome any possibility of acid-catalysed degradation. Unlabeled *S*-I was included as a carrier, and also to act as a UV-absorbing marker. An on-line γ-detector was set up to monitor ^{11}C radioactivity. The extraction–HPLC is rapid, taking only 25 min ($\sim$ one half-life) from collection of blood to HPLC quantification of the ligand. The system was evaluated with plasma samples obtained from the dog studied with [^{3}H]-*S*-I; a single peak was observed on HPLC (7 min), with a recovery of >80%.

Samples from four dogs infused with [^{11}C]-*S*-I were analysed with this technique. In each case, only unchanged *S*-I was detected in plasma (Fig. 4). Recoveries in excess of 90% were obtained, indicating that any putative metabolites had not been lost on extraction. In two experiments in the rat, using either ^{11}C-labelled *RS*-I or *S*-I and analysing plasma by the rapid technique, no metabolites were observed at 5 min; however, small amounts (<5%) of a polar material were observed at 15 min post infusion.

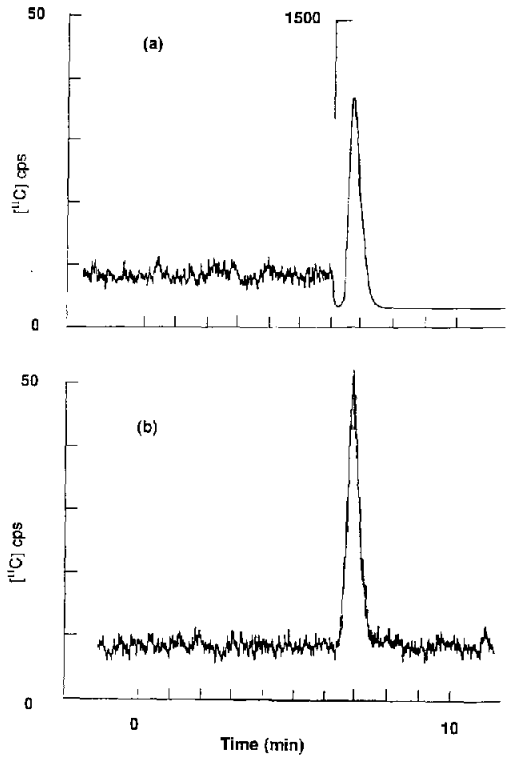

Fig. 4. HPLC radioprofile for dog plasma taken at 10 min (a) and 60 min (b) following infusion of [^{11}C]-*S*-I. A short HPLC column was used and ^{11}C radioactivity was monitored on-line with a NaI (Tl) well-type γ-ray detector linked to a rate meter. The void volume was 3 ml. A single radioactive peak was detected eluting at the same position as the authentic material. The sensitivity of the detector was decreased by 30-fold immediately before the elution position of *S*-I for the 10-min sample.

DISCUSSION

The interpretation of data obtained *in vivo* from the PET scanner is based on mathematical models which allow the determination of receptor density and kinetics [3,11]. This modelling depends critically on the accurate estimation of the input function, which, in the case of the lung, is the free *S*-I present in the mixed venous blood, and for the heart, that present in the arterial blood. Although protein binding will reduce the proportion of free ligand as a fraction of the total radioactivity in the plasma, in the case of *S*-I such binding is minimal. Radioactivity which is present in plasma, either as an impurity co-injected with ligand or as metabolites produced *in vivo*, will, however, lead to inaccuracies in the measured input function. This is particularly important with a ligand such as *S*-I which is thought to be taken up into the β-receptor pool within minutes of administration. Metabolites (or impurities) which are not as avidly sequestered may

consequently be cleared from the circulation more slowly than the parent ligand. The input function for *S*-I would therefore need to be corrected for a progressively decreasing proportion of the parent ligand to other radiolabeled species in plasma.

It is well known that receptor binding, metabolism and clearance of enantiomers can differ [12,13]. Thus, infusion of a racemic mixture may lead to different plasma concentrations of each enantiomer. This is a particularly important consideration for PET studies, because of the dependence on mathematical modelling which requires an accurate input function for the ligand. In this study, we have used the biologically active *S*-I, which has an 80-fold greater affinity for β-receptors than its *R*-enantiomer [6].

No metabolism of *S*-I was observed in the dog during the 2 h of these studies. The high extraction yields excluded the possibility that metabolites had been lost on extraction. No differences were observed when three different isotopically labelled forms of the ligand (^{3}H, ^{11}C and ^{14}C) were used, showing that the position of the radiolabel does not affect the metabolism of the ligand (*e.g.* through an isotope effect on the C–^{3}H bonds). To determine whether our reversed-phase HPLC systems (three used overall) could have failed to resolve metabolites from the parent ligand, an additional study using normal-phase chromatography was carried out on samples of rat plasma and urine using OPTLC: only unchanged *S*-I was observed. In our preliminary extraction procedures, there was a possibility that the acid extractions (using trichloroacetic acid) and HPLC conditions could have resulted in the hydrolysis of a labile metabolite, such as an N-glucuronide, back to the parent compound. Our final rapid extraction procedure (used for the ^{11}C-labelled ligand) was carried out without acid precipitation and using neutral solution. Using this method no metabolites were observed in the dog.

In contrast to our data on *S*-I, Delforge *et al.* [14] using OPTLC to analyse for metabolites, found that infusion of racemic [^{11}C]-*RS*-I into the dog rapidly led to a high metabolite-to-parent drug ratio in blood; at 5 min, less than 12% of the total radioactivity in blood was unchanged *RS*-I. In a single experiment in the rat, also using [^{14}C]-*RS*-I and with analysis by OPTLC, we still did not observe any significant metabolism at 5 min. However, using our rapid HPLC procedure (under neutral extraction conditions), small amounts ($<5\%$) of ^{11}C-labelled *S*-I polar substances were detected in rat plasma after 15 min. The presence of radiolabelled impurities in the ^{11}C preparation would, if the plasma elimination rate constant of the impurity were greater than that of *RS*-I, give results consistent with the formation of metabolites. Quality control of the [^{11}C]-*S*-I used in our study showed that the ligand was essentially pure.

In summary, we have developed a rapid extraction and purification protocol suitable for the analysis of [^{11}C]-*S*-I. This ligand is not metabolised to any significant extent *in vivo* in the dog. Such information is essential in the use of [^{11}C]-*S*-I to investigate the behaviour of the different β-adrenergic receptor subtypes and

the kinetics of unlabelled β-receptor antagonists in this species *in vivo*. The analytical procedures described here should be generally applicable for a number of drugs (and their possible metabolites) labelled with short-lived radioisotopes.

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