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BIOMEDICAL APPLICATIONS

Quantification of the β-adrenoceptor ligand S-1′-[^{18}F]fluorocarazolol in plasma of humans, rats and sheep

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Abstract

Myocardial and pulmonary β-adrenoceptors can be imaged with 2-(S)-(−)-(9H-carbazol-4-yl-oxy)-3-[1-(fluoromethyl)ethyl]amino-2-propanol (S-1′-[^{18}F]fluorocarazolol, I). Quantification of unmodified fluorocarazolol in plasma is necessary for analysis of PET images in terms of receptor densities. We have determined I and its radioactive metabolites in rat, sheep and human plasma, using (1) solid-phase extraction (C_{18}) followed by reversed-phase HPLC and (2) direct injection of untreated plasma samples on an internal-surface reversed-phase (ISRP) column. The two methods were in good agreement. Unmodified I decreased from over 99% initially to less than 5%, 5–10% and 20% at 60 min post-injection in rats, sheep and human volunteers, respectively. Protein binding in sheep and human plasma was determined by ultrafiltration. The fraction of total plasma radioactivity bound to protein and the fraction representing unmodified radioligand were linearly correlated, suggesting that fluorocarazolol was more than 70% protein-bound, whereas its metabolites showed negligible protein binding. Direct injection of plasma on an ISRP column seems a convenient method for quantification of lipophilic radioligands such as fluorocarazolol.

Keywords: Fluorocarazolol

1. Introduction

S-(−)[^{18}F]Fluorocarazolol (I, see Fig. 1), a fluorinated analog of the potent, non-subtype selective β-adrenoceptor antagonist carazolol [1–4], binds to cardiac, pulmonary and cerebral β-adrenoceptors in vivo [5,6] and is currently employed for PET imaging of the heart [7]. The S-isomer of fluorocarazolol

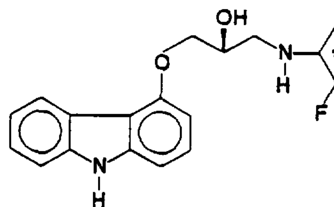

Fig. 1. Position of the fluorine (^{18}F) label in I.

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is used because it shows about 20-fold greater affinity to β-adrenoceptors than the R-isomer [7]. For estimation of myocardial receptor density from PET images using compartment models and iterative adjustment of rate constants, an "input function" is required which consists of the time-dependent concentration of free radioligand in arterial plasma [8,9].

Lipophilic β-adrenoceptor antagonists such as I ($\log P=2.2$, [7]) generally show an extensive first-pass metabolism in the liver [10–12]. Metabolites of I should therefore be measured and their relative concentrations should be subtracted from total plasma radioactivity to obtain the correct input function. Pilot studies in rats have shown that at 60 min post-injection, the target organs contain mainly unmodified I, which suggests that, in this species, metabolites have negligible affinity to β-adrenoceptors [13]. Lipophilic drugs are usually strongly bound to albumin and α-glycoprotein [14,15]. The "free" concentration of I will therefore be smaller than its total concentration because of binding to these plasma proteins.

Here, we describe our efforts to develop chromatographic methods for quantification of I in plasma of experimental animals and humans. Such methods are required to interpret PET images of heart and lungs in terms of receptor densities. A suitable method should have the following characteristics: (i) rapid (because of the short half-life of ^{18}F, 109.8 min); (ii) isocratic (since several samples must be analysed in rapid succession); (iii) quantitative (i.e. all radioactive metabolites should be separated from the radioligand); (iv) reliable (complex extraction procedures are generally undesirable in clinical settings as they increase the chance of analytical errors).

2. Experimental

2.1. Radioligand

S-Desisopropylcarazolol, the precursor to I, was prepared as reported previously [13]. Enantiomeric excess was over 98% (determined by chromatographic separation of the enantiomers on a Chiralcel OD column, mobile phase n-hexane–ethanol–diethylamine (700:100:4 v/v/v), flow-rate 0.8 ml/min). I was synthesized by reacting the precursor with [^{18}F]fluoroacetone [7,13], and purified by HPLC (Zorbax NH_2 column). The specific activity was 37–185 TBq (1000–5000 Ci)/mmol and the radiochemical purity was greater than 99.8%. I was dissolved in ethanol–propylene glycol–0.9% NaCl (1:2:2); before injection 1 vol. of this solution was mixed with 9 vols. 0.9% NaCl and filtered. The solution was sterile and apyrogenic. I passed the test on "acute toxicity" (Dutch Pharmacopeia Ed. IX) at a 10 000-fold higher dose than was ever administered to volunteers.

2.2. Other materials

Unlabelled I was prepared by reacting S-desisopropylcarazolol with commercially available fluoroacetone (Aldrich, Bornem, Belgium). Chemical purity of unlabelled I was over 99% as judged by reversed-phase HPLC (C_{18}). All chemicals were analytical grade and acquired from Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was from Rathburn, Walkerburn, UK. The Radial-Pak C_{18} HPLC column, C_{18} guard column and regular C_{18} Sep-Pak cartridges were from Waters Millipore (Milford, MA, USA). The ChromSpher BioMatrix column and M3 guard columns were purchased from Chrompack International (Bergen op Zoom, Netherlands).

2.3. Animal studies

The studies were performed in compliance with the Law on Animal Experiments of The Netherlands. Rats (males, Wistar strain, body weight 220±20 g) were anaesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.). The carotid artery and a tail vein were cannulated. The radioligand (I, 11 MBq/kg body weight) was injected as a 10-s bolus into the vein; the arterial cannula was used for blood sampling (maximally seven samples). Blood (sample volume 0.2–0.25 ml) was collected in heparinized Eppendorf cups. Plasma and a cell pellet were acquired by short centrifugation (2 min, Hettich mikroliter centrifuge).

Lambs (females, body weight 35 kg) were anaesthetized with 0.5–1.5% halothane in N_2O (2 l/min) and O_2 (4 l/min). The aorta and vena jugularis were cannulated. The radioligand (I, 3.7 MBq/kg body weight) was i.v. injected over a period of 60 s; the arterial cannula was used for blood sampling (28 samples, total volume <75 ml). Lamb plasma was separated from blood cells by short centrifugation in heparinized Vacutainer tubes (5 min, 3000 *g*, MSE Mistral 1000).

2.4. Human studies

Six human volunteers were studied (age 21–55 years, five males, one female). Permission was granted by the Medical Ethics Committee of the University Hospital; all subjects gave informed consent. For injection of the radioligand, a catheter was placed in a brachial vein of the left arm. Another catheter was placed in the radial artery of the right arm for blood sampling. Each volunteer received 74 MBq I. Blood samples (total volume 55 ml) were drawn at 0.5-min intervals from 0 to 5 min and at 10-min intervals from 10 to 60 min post-injection. Human plasma was acquired by centrifugation as described above.

Heart rate, blood pressure and ECG were continuously recorded during the experiments; injection of 74 MBq I did not cause any change in the recorded parameters.

2.5. Extraction of ^{18}F-labelled compounds from plasma

Two different methods were used for sample preparation: (1) in experiments using HPLC, plasma was mixed with 2 volumes of acetonitrile, vigorously shaken (vortex-mixer) and centrifuged (2 min, Hettich mikroliter centrifuge). The supernatant was spiked with unlabelled I (10 μg) and subjected to chromatography; (2) in experiments using internal-surface reversed-phase (ISRP) chromatography, untreated plasma samples were directly applied to a BioMatrix column (see below).

2.6. Solid phase extraction–high-performance liquid chromatography

Reversed-phase HPLC was performed in a system consisting of a Waters 510 pump, Rheodyne injector type 7125, 1-ml sample loop, C_{18} guard column, Waters Radial-Pak C_{18} column (100 × 8 mm I.D., 5 μm) and a Waters 486 tunable absorbance detector.

The procedure was derived from Keukens and Aerts [16]. A C_{18} Sep-Pak cartridge was activated by washing with 5 ml methanol, followed by 5 ml water. Each plasma extract (prepared with acetonitrile as described above) was mixed with 6 vols. 10% NaCl. The resulting mixture was slowly passed through the Sep-Pak and the eluate was collected. The cartridge was now rinsed with 1 ml 0.01 *M* H_2SO_4, followed by 2 ml air, while a second eluate was collected. Finally, the cartridge was eluted with 2 ml acidic acetonitrile (1 ml 0.05 *M* H_2SO_4 in 100 ml acetonitrile), and a third eluate was collected. Polar metabolites appeared in eluates 1 and 2, but authentic I was for >98% retained. Less polar metabolites and I occurred in eluate 3. Radioactivity in all eluates and residual activity on the cartridge (generally <2% of the applied amount) were determined using a calibrated gamma counter (LKB Wallac CompuGamma 1282 CS).

Eluate 3 (500 μl) was then injected onto the HPLC column. The mobile phase was prepared by dissolving 2.46 g anhydrous sodium acetate in 450 ml water, adding 550 ml acetonitrile, adjusting the pH to 6.5 with acetic acid and filtration (Millipore 0.45 μm FH). The flow rate was 1.2 ml/min. Thirty 0.6-ml fractions of the eluate were collected over a period of 15 min using an automatic fraction collector. Radioactivity in the fractions was determined using the gamma counter.

2.7. Internal-surface reversed-phase (ISRP) chromatography

ISRP chromatography was performed using a similar system as described above, but the column was a ChromSpher BioMatrix (150 × 4.6 mm I.D.) with M3 guard column. The mobile phase was 10 m*M* K_2HPO_4–acetonitrile (90:10, v/v, pH 7.5), flow-rate 1.5 ml/min. Untreated plasma samples

(100–150 μl) were directly injected. Twenty-four 0.75-ml fractions of the eluate were collected over a period of 12 min. Radioactivity in the fractions was determined using the gamma counter.

2.8. Plasma protein binding

Plasma protein binding or free fraction of I was determined by the ultrafiltration technique using an MPS-1 reusable micropartition system with molecular mass cut-off of 30 000 Da (YMT membrane, Amicon, Beverly MA, USA). Samples of human and lamb plasma (0.25 ml) were dispensed into MPS-1 units and centrifuged at 2000 × g for 30 min. Radioactivity in the clear ultrafiltrate and activity remaining on the filter were then determined by gamma counting. A sample blank consisting of 300–500 Bq I in saline was run in parallel; non-specific adsorption to YMT membranes was 24±3%.

3. Results

3.1. Extraction efficiencies and chromatographic recoveries

The extraction of ^{18}F during treatment of plasma with acetonitrile was good especially if the pellet was re-extracted (>92% vs. 85±4% for a single extraction). Recovery of applied radioactivity in plasma extracts from cartridge and column during SPE–HPLC was quantitative (98±8%). During ISRP chromatography, column recovery was greater than 93%. Similar values for extraction efficiency and column recovery were observed in all studied species.

3.2. Metabolism in lambs

Lamb plasma was analysed by SPE–HPLC. Representative chromatograms are shown in Fig. 2. Radioactivity eluting at 9–10 min was unmodified I; activity with less than 5 min retention and activity eluting from the Sep-Pak cartridge represented metabolites. Metabolites were already prominent within

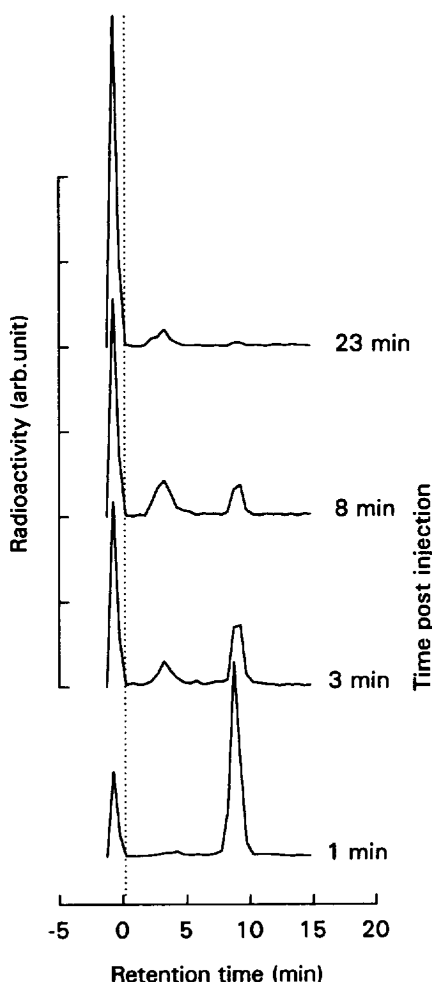


Fig. 2. Radiochromatograms (SPE–HPLC) of lamb plasma extracted at 1, 3, 8 and 23 min post-injection. Samples of a single lamb are shown. Data points at negative retention times represent fractions 1 and 2, eluting from the Sep-Pak cartridge with NaCl and with H_2SO_4; those at positive retention are eluate fractions from the C_{18} HPLC column.

1 min after injection. Time for the dead volume to be voided was about 2 min. The fraction of total plasma radioactivity representing unmodified radioligand decreased from greater than 99.8% initially to less than 10% at 60 min post-injection (see Fig. 5). The amount of radioactivity in column (and Sep-Pak) eluates decreased from 220 000 counts/min at 1 min to 8000 counts/min at 80 min with a corresponding decrease in signal-to-noise of the chromatograms.

3.3. Metabolism in humans

Human plasma was analysed by SPE–HPLC (three volunteers) and by ISRP chromatography (three other volunteers). Representative ISRP chromatograms are shown in Fig. 3. Peak shapes were reasonable but inferior to those in HPLC. Radioactivity eluting at 8–10 min was unmodified I; activity found between 1 and 4 min represented metabolites. Metabolites appeared within 5 min. Time for the dead volume to be voided was 1 min.

Extensive metabolism of I occurred during the time course of a PET scan (i.e. 60 min). Similar pharmacokinetics were observed by SPE– HPLC and by ISRP chromatography; however, due to limited availability of plasma, samples of a single volunteer could be analysed using only one method. The fraction of total plasma radioactivity representing unmodified radioligand decreased from greater than 99.8% initially to 19±8% at 60 min post-injection (see Fig. 5). The amount of radioactivity in column eluates decreased from 50 000 counts/min at 1 min to 1500 counts/min at 60 min (ISRP method).

3.4. Comparison of two analytical methods in rat plasma

Plasma samples from Wistar rats were analysed using both SPE–HPLC and ISRP chromatography. It was thus possible to directly compare the analytical results. An excellent linear correlation was observed (Fig. 4).

Compound I was rapidly metabolized in Wistar rats. The fraction of total plasma radioactivity representing unmodified radioligand decreased from over 99.8% initially to virtually undetectable levels (about 2%) at 60 min post-injection (Fig. 5). Radioactivity in column eluates decreased from 30 000 counts/min at 2 min to 3000 counts/min at 80 min (ISRP method).

3.5. Plasma protein binding

Protein binding of I in lambs and human volunteers was quite significant (about 70% immediately after injection). However, the fraction of plasma radioactivity bound to protein rapidly decreased with time. This decrease was inversely related to the

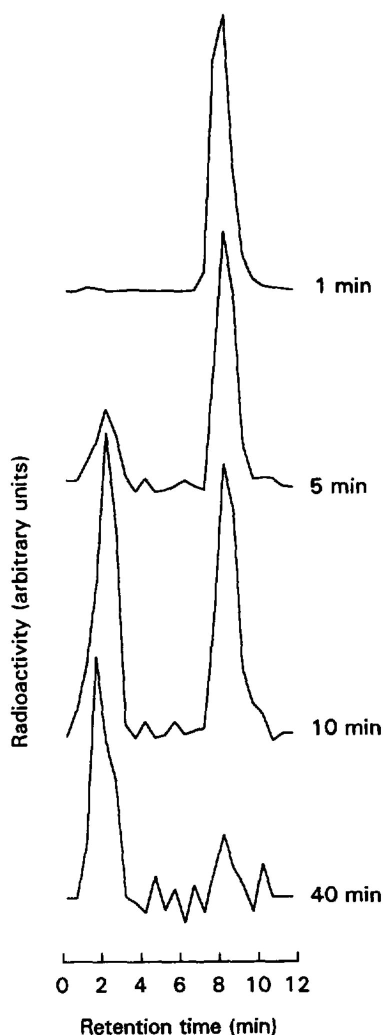


Fig. 3. Radiochromatograms (ISRP, Chromspher BioMatrix) of human plasma extracted at 1, 5, 10 and 40 min post-injection. Samples of a single volunteer are shown.

appearance of radioactive metabolites in plasma (see below).

3.6. Ligand binding to blood cells

Erythrocyte/plasma concentration ratios of radioactivity as a function of time after injection of I are plotted in Fig. 6. Significant association to blood cells occurred in rats; at intervals greater than 30 min, the cellular concentration of radioactivity was

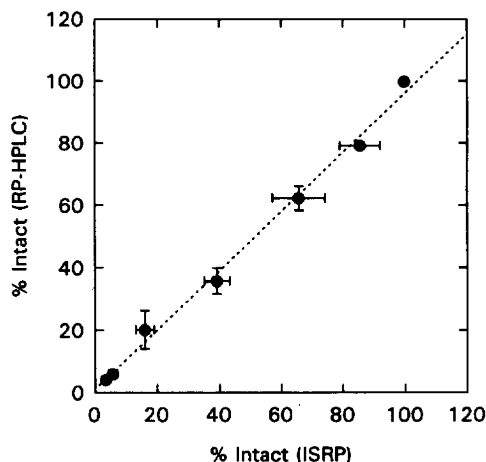
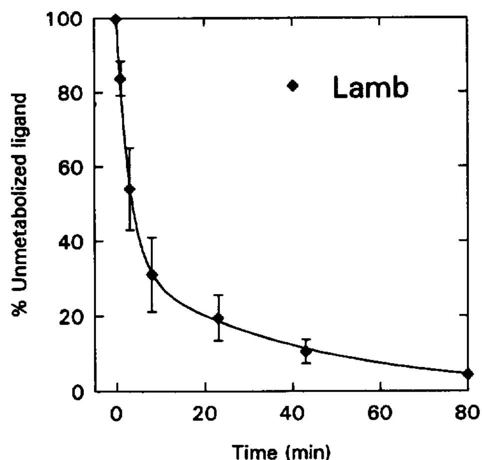


Fig. 4. Correlation between analysis of unmetabolized I in rat plasma by RP-HPLC and by ISRP chromatography.



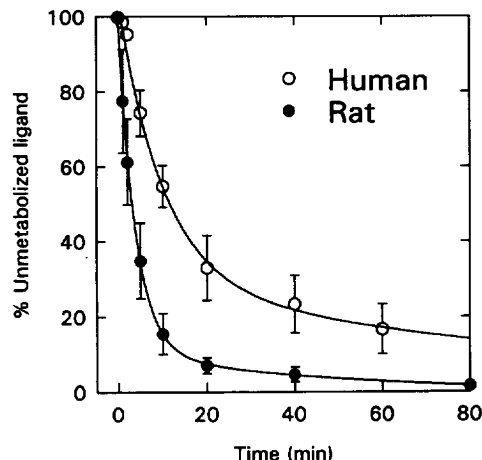


Fig. 5. Plasma pharmacokinetics of I in humans (n = 6; ○), rats (n = 6; ●) and lambs (n = 3; ◆). Data are plotted as mean±S.D.

five times higher than the plasma concentration. Much less radioactivity accumulated in human erythrocytes. Here, erythrocyte/plasma ratios increased from 0.15 to about 0.35.

3.7. Correlation between protein binding and metabolism

Both in lambs and in human volunteers, a linear relationship was observed between the fraction of plasma radioactivity which was bound to protein and the fraction which represented unmetabolized ligand (Fig. 7). A regression line fitted through these data passed through the origin and it showed a y-intercept of about 70% (Fig. 7).

4. Discussion

Literature data on metabolism of I are somewhat controversial. Zheng et al. [7] reported relative stability of the radioligand in mice and pigs, the metabolite fraction in whole blood remaining constant at 29% from 15 to 120 min post-injection. In contrast, we observed rapid metabolism of I in humans, lambs and rats using two different chromatographic techniques (see Fig. 2, Fig. 3 and Fig. 5). These distinctive results may indicate species differences in radioligand metabolism. As details regarding the HPLC method employed by the other workers are not published [7], we could not repeat their experiments. Rapid breakdown of I is in accordance with the lipophilicity of fluorocarazolol (log P=2.2, [7]) which in other β-blockers has been shown to result in extensive first-pass metabolism [10–12]. The metabolic rates which we observed seemed to be inversely related to body weight, i.e. rats > lambs > human volunteers (Fig. 5).

Sample pretreatment with acid or with organic solvents may result in various analytical errors (e.g. formation of radiolabelled artifacts, hydrolysis of labile metabolites, incomplete extraction of radioactivity from plasma [17,18]). Direct analysis of untreated plasma samples with ISRP chromatography is

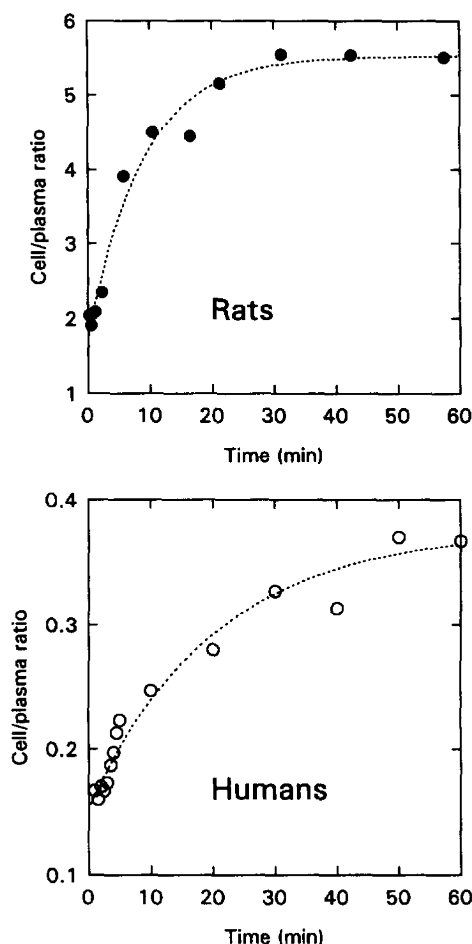


Fig. 6. In vivo association of I to red blood cells of rats and humans. Please note that the scale on the vertical axis for both species is different.

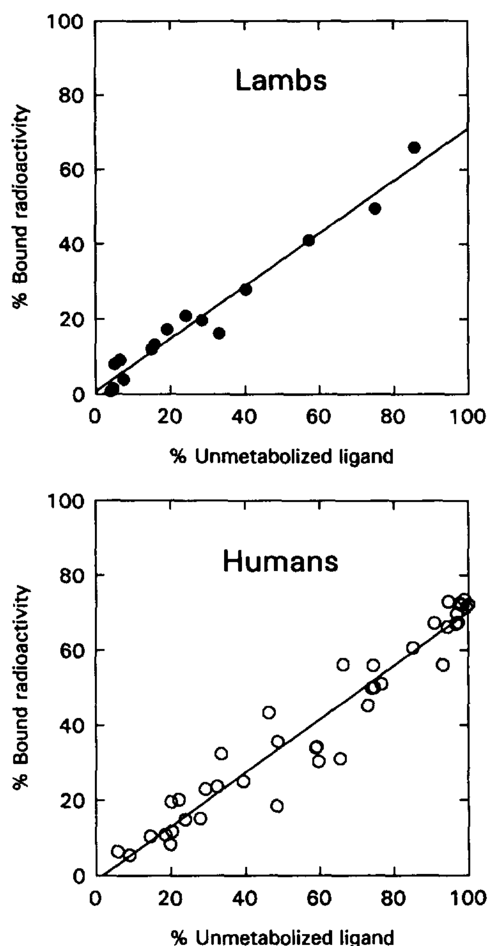


Fig. 7. Correlation between the fraction of total radioactivity bound to protein and the fraction of total radioactivity representing unmetabolized ligand in plasma from lambs and human volunteers.

not prone to such difficulties of interpretation. It is thus quite significant that the SPE–HPLC and ISRP chromatographic methods produced similar results in rats (Fig. 4) and human volunteers. ISRP chromatography seemed a convenient alternative to the more time-consuming SPE–HPLC method.

The amount of plasma which we injected on the BioMatrix column (100–150 μl) was significantly greater than is recommended by the manufacturer (20–50 μl). A large plasma volume was used in order to have acceptable count statistics even in late samples. Plasma was not filtered prior to injection as I (in contrast to its metabolites) shows strong non-specific adsorption to filters. Filtration may therefore lead to erroneous ratios of parent/metabolites in metabolite assays. Skipping the filtration step did not cause problems in ISRP chromatography. Column clogging occurred only once during 72 injections; normal pressure was restored after a short interval of retrograde perfusion with buffer. Between two PET studies (eight injections each), the column was washed with distilled water, methanol and water to keep it in good condition. A 1-ml sample loop was used and plasma was mixed with the mobile phase (final volume 1 ml) before it was injected.

Non-specific adsorption of I also caused difficulties in ultrafiltration. Several types of filter were tried; some brands showed greater than 90% non-specific binding! The YMT membrane proved optimal for this application. The linear relationship which we observed between the fraction of plasma radioactivity which was bound to protein and the fraction which represented unmetabolized ligand (Fig. 7) suggests that I is greater than 70% protein-bound whereas its radioactive metabolites show negligible protein binding. The correlation between ligand fraction and protein binding indicates that the input function of compartment models can be easily corrected for protein-bound ligand. Time-dependent association of I to blood cells in rats and human volunteers (Fig. 6) indicates that plasma data rather than whole-blood radioactivity should be used to estimate input to the tissues under study.

In summary, we have compared two assay methods for the analysis of I in plasma samples. The radiopharmaceutical was rapidly metabolized in three mammalian species (rats, lambs, humans). ISRP chromatography seems a convenient alternative to SPE–HPLC for metabolite assays of I in clinical PET studies of β-adrenoceptors.

Acknowledgments

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