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Quantification of an ^{11}C-labelled β-adrenoceptor ligand, *S*-(−)CGP 12177, in plasma of humans and rats

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Abstract

β-Adrenoceptors in human lungs and heart can be imaged with the radioligand 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-^{11}C-one (CGP 12177, [^{11}C]I). For quantification of receptor density with compartment models by adjustment of rate constants, an 'input function' is required which consists of the integral of the concentration of unmodified ligand in arterial plasma over time. A discrepancy in the literature regarding metabolic stability of [^{11}C]I prompted us to study metabolism in rats by reversed-phase HPLC (RP-HPLC) of trichloroacetic acid extracts of arterial plasma after i.v. injection of [^{11}C]I (>11.1 TBq/mmol, 11 MBq/kg). Some plasma samples were also directly applied to an internal-surface reversed-phase (ISRP) column. In parallel experiments, tritiated [^{11}C]I was employed and methanol extracts of arterial plasma were analyzed by straight-phase TLC. The three methods were in excellent agreement. Unmodified [^{11}C]I decreased from >98.5% (^{3}H) or >99.9% (^{11}C) initially to 57 ± 7% at 80 min post injection due to formation of two polar metabolites. Using the RP-HPLC method, no metabolism was detectable in humans up to 30 min after injection of [^{11}C]I (1851 MBq). Deproteinization of plasma with acetonitrile resulted in the formation of a radioactive species (artifact) which eluted immediately after the void volume in RP-HPLC and which could be mistakenly interpreted as a metabolite. Plasma protein binding was low (ca. 30%) in both humans and rats. Association of the radioligand to blood cells suggested that equilibrium between receptor-bound and free radioligand was reached within 15 min after high-specific-activity injections, but only after more than 30 min after low-specific-activity injections.

1. Introduction

S-(−)[^{11}C]CGP 12177 ([^{11}C]I, see Fig. 1) is a short-lived ($t_{1/2}$ = 20 min) radiopharmaceutical (non-subtype-selective β-adrenoceptor antagonist of high affinity [1,2]) which binds to cardiac and pulmonary surface β-adrenoceptors in vivo [3–5] and is currently employed for PET imaging of human lungs [6] and heart [7,8]. For estimation of 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, unmodified radioligand in arterial plasma [9,10].

Radioactivity in plasma will normally comprise

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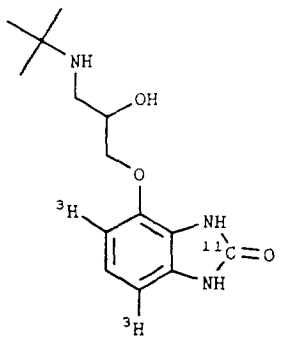

Fig. 1. Positions of the tritium (^{3}H) and ^{11}C labels in I.

both the parent radioligand and labeled metabolites formed in the liver or in extrahepatic tissues. Metabolites should therefore be measured and their relative concentrations should be subtracted from total plasma radioactivity to obtain the correct input function.

Only part of the plasma radioactivity is normally present in the free form. The 'free' concentrations of radioligand and metabolites may be smaller than their total concentrations because of binding to plasma proteins. Especially hydrophobic drugs can be extensively bound to albumin and α-glycoprotein [11,12].

A discrepancy in the literature regarding metabolic stability of [^{11}C]I prompted us to investigate its metabolism. One group reported extremely rapid degradation in the dog and rapid degradation in rats [13,14] whereas other researchers found slow metabolism in rats and hardly any metabolism in dogs and human volunteers [15,16] (see Table 1). A previously published HPLC method [15,16] did not work well in our hands, as radioactive peaks were very broad and structurally related compounds (e.g. the diamine precursor) were not separated from CGP 12177. We employed mainly reversed-phase high-performance liquid chromatography (RP-HPLC) to measure the relative contribution of labeled metabolites to total radioactivity in rat plasma. Some plasma samples were also directly applied to an internal-surface reversed-phase (ISRP) column. In parallel experiments, tritiated I was i.v. administered to rats and methanol extracts of arterial plasma were subjected to straight-phase thin-layer chromatography (TLC) after concentration of the samples at reduced pressure. The long half-life of ^{3}H (11.7 yr) as compared to ^{11}C (20 min) enabled us to use these relatively laborious and time-consuming analytical procedures to validate the RP-HPLC data.

Table 1
Metabolic stability of *S*-I (% unmodified ligand, literature values)

Interval (min)	Analytical technique	
	(OP)TLC [13,14]	HPLC [15,16]
Rat		
5	n.d.[a]	92%
10	35%	n.d.
80	n.d.	60–74%
Dog		
5	10–15%	n.d.
30	< 5%	n.d.
80	≪5%	> 95%
Human		
80	n.d.	> 95%

[a] n.d. = not determined.

Plasma pharmacokinetics of [^{11}C]I was also studied in humans, using HPLC. Protein binding was assessed by ultrafiltration, and ligand distribution between plasma and red blood cells was examined at various intervals post injection.

2. Experimental

2.1. Radioligands

1-(3-*tert.*-Butylamino-2-*S*-hydroxypropoxy)-2,3-diaminobenzene, the diamine precursor to I, was prepared by the method of Aigbirhio et al. [17] and Brady et al. [18]. ^{11}C-Labeled I was synthesized by reacting the precursor with [^{11}C]phosgene according to Boullais et al. [19] and purified by HPLC. Specific activity was ≥ 7.5 TBq/mmol and the radiochemical purity was ≥ 99.95%. Tritiated I (*S*-isomer) was obtained from Amersham International (Buckinghamshire, UK). The specific activity was 1.59–2.18 TBq/mmol and the radiochemical purity was 98.6–98.8%.

2.2. Other materials

Unlabeled, racemic I was purchased from Research Biochemicals (Natick, MA, USA). All other chemicals and solvents were analytical grade and acquired from Merck (Darmstadt, Germany). The Radial-Pak C_{18} HPLC column and C_{18} guard cartridges were from Waters Millipore (Milford, MA, USA). ChromSpher BioMatrix columns were a generous gift of Chrompack International (Middelburg, Netherlands). Thin-layer chromatography plates (silica gel 60, No. 5724) were a product of Merck.

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 per kg body weight, i.p.). The carotid artery and a tail vein were cannulated. Radioligands ([^{3}H]I, 1.59–2.18 TBq/mmol, 1.2 MBq per kg body weight; [^{11}C]I, 7.5–15 TBq/mmol, 11 MBq per kg body weight) were injected as a 10-s bolus into the vein; the arterial cannula was used for blood sampling (maximally 10 samples). Blood (sample volume 0.15–0.2 ml) was collected in heparinized Eppendorf cups. Plasma and a cell pellet were acquired by short centrifugation (2 min, Hettich mikroliter centrifuge).

2.4. Human studies

Five human volunteers were studied (age 21–32 yr, 3 males, 2 females). 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. The volunteers participated in a pilot study for determination of the myocardial β-adrenoceptor density [13]. Each volunteer received two injections of 185 MBq [^{11}C]I at two specific activities (11–22 and 2–5 TBq/mmol) separated by an interval of 40 min. Blood samples (2 ml) were drawn at 0.5-min intervals from 0–5 min and at 10-min intervals from 10–40 min post injection. Plasma was separated from blood cells by short centrifugation in heparinized Vacutainer tubes (5 min, 3000 *g*, MSE Mistral 1000).

2.5. Extraction of ^{3}H- and ^{11}C-labeled compounds

Three different methods were used for sample preparation: (1) in experiments using [^{3}H]I, plasma was mixed with 9 volumes of methanol, vigorously shaken (vortex-mixer) and centrifuged (10 min, 3000 *g*). The supernatant was concentrated under reduced pressure at room temperature (20–22°C), spiked with unlabeled I (10 μg) and subjected to thin-layer chromatography; (2) in experiments using [^{11}C]I, plasma was mixed with 2 volumes of ice-cold trichloroacetic acid (TCA) (7.5%, w/v), vigorously shaken (vortex-mixer) and centrifuged (2 min, Hettich mikroliter centrifuge). The supernatant was filtered (Millipore 0.45-μm HV), spiked with unlabeled I (10 μg) and subjected to HPLC; (3) in some experiments using [^{11}C]I, untreated plasma samples were directly applied to an internal-surface reversed-phase column (see below).

2.6. Thin-layer chromatography

Silica plates were developed over a distance of 15 cm with chloroform–methanol–25% ammonia (8:2:0.1, v/v). The R_f value of authentic I was 0.50; time of development was ca. 75 min. Radioactivity in small sections of the plates was assayed by liquid scintillation counting after elution of the radiolabeled species with isopropanol as described [3].

2.7. Liquid chromatography

Reversed-phase HPLC was performed in a system consisting of a Waters 600 solvent delivery module, Rheodyne injector type 7125, 500-μl sample loop, C_{18} guard column, Waters Radial-Pak C_{18} column (100 × 8 mm I.D., 5 μm) and a Waters 994 or Waters 440 UV detector.

The mobile phase [1% triethylamine-acetate buffer (pH 4)–acetonitrile (85:15, v/v)] was prepared by addition of 10 ml of triethylamine to 990 ml of distilled water and adjustment of the pH to 4.0 with acetic acid. The resulting buffer was filtered (Millipore 0.45-μm HA) and 850 ml were mixed with 150 ml of acetonitrile.

ISRP chromatography was performed in a similar system containing a 100-μl sample loop, C_{18} guard column and ChromSpher BioMatrix column (150 × 4.6 mm I.D.). The mobile phase was 20 m*M* KH_2PO_4 (pH 7)–acetonitrile (92.5:7.5, v/v).

Both the HPLC and ISRP columns were eluted at a flow-rate of 1.0 ml min^{-1}. UV absorption was continuously monitored at 254 nm. Radioactivity in 0.5-ml fractions of the eluate was assayed by gamma counting (LKB Wallac CompuGamma 1282 CS).

2.8. Plasma protein binding

Plasma protein binding or free fraction of [^{11}C]I was determined by the ultrafiltration technique using an MPS-1 reusable micropartition system with molecular mass cut-off of 30 000 Dalton (YMT membrane, Amicon, Beverly, MA, USA). Samples of human plasma (0.25 ml) were dispensed into MPS-1 units and centrifuged at 2000 *g* for 20 min. Radioactivity in the clear ultrafiltrate and activity remaining on the filter were then determined by gamma counting. A sample blank consisting of [^{11}C]I (300–500 Bq) in saline was run in parallel; nonspecific adsorption to YMT membranes was ca. 5%.

3. Results

3.1. Extraction efficiencies and chromatographic recoveries

The extraction of ^{3}H during treatment of plasma with methanol was virtually quantitative (see Table 2). Extraction efficiency of ^{11}C during treatment of plasma with trichloroacetic acid was lower but reproducible (Table 2). Recovery of applied radioactivity in plasma extracts from the column or plate was excellent in all cases (Table 2). If analysed by RP-HPLC, unlabeled I appeared to be stable during 5 h of storage in 5% trichloroacetic acid or 90% methanol at room temperature.

Table 2
Extraction efficiencies/recoveries

Species	Extraction efficiency (%)	
	Methanol	Trichloroacetic acid
Rat	> 95	73 ± 2
Human	92 ± 2	82 ± 2
	Recovery of applied activity from column/plate	
	TLC	HPLC
Rat	> 90	94 ± 8
Human	not determined	94 ± 8

3.2. Comparison of three analytical methods in rat plasma

All methods (methanol extraction + straight-phase TLC, acid extraction + reversed-phase HPLC, direct sample injection on ISRP column) detected two polar, radioactive metabolites in rat plasma, 40 min after injection of I. Peak shapes in ISRP-LC were poor compared to HPLC, but a distribution of radioactivity over three radioactive species was observed in this case as well.

Compound I appeared to be slowly metabolized in Wistar rats. RP-HPLC chromatograms for the ^{11}C-labeled compound are shown in Fig. 2; radioactivity eluting at 9–9.5 min was unmodified I. The two metabolites (retention times 5.5 and 7.25 min, respectively) were barely visible at 10 min, but they became prominent at intervals greater than 20 min. Time for the dead volume to be voided was 3 min. The fraction of total plasma radioactivity representing unmodified radioligand decreased from > 99.9% initially to 57 ± 7% at 80 min post injection. Similar kinetics were observed using TLC (Fig. 3). A sample acquired at 40 min was also analysed by ISRP-LC and the unmodified ligand

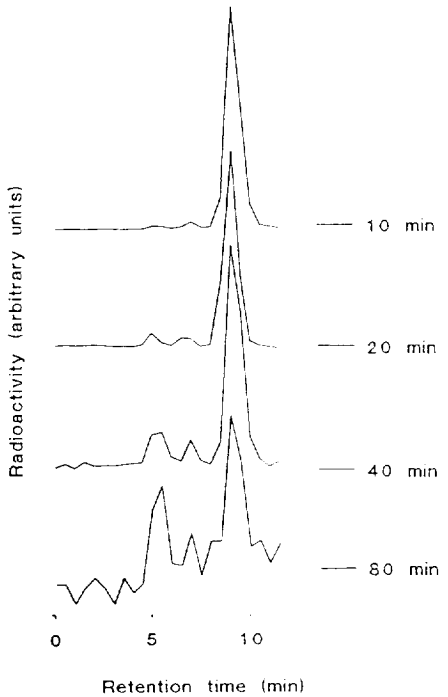

Fig. 2. Radiochromatograms (RP-HPLC) of rat plasma extracted at 10, 20, 40 and 80 min post injection. Samples of a single rat are shown.

fraction (78%) was similar to that determined with the other methods (80 ± 5%, $n = 8$). ISRP was not used more frequently for reasons explained in the Discussion.

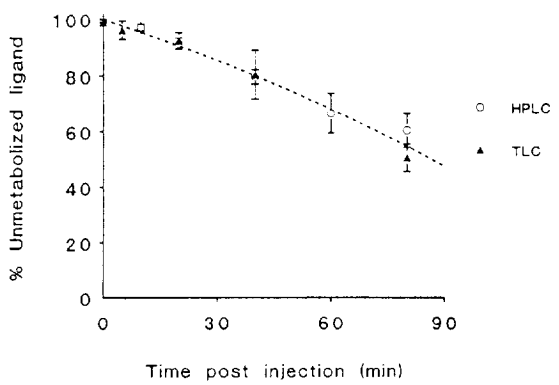

Fig. 3. Plasma pharmacokinetics of *S*-[^{11}C]I in Wistar rats determined with (▲) methanol extraction and TLC, or (○) TCA extraction and RP-HPLC. Data are plotted as a mean ± S.D. ($n = 3$, TLC; $n = 5$, HPLC).

3.3. Metabolism in humans

Human plasma samples were analysed by RP-HPLC only. No metabolism of I was apparent at intervals up to 30 min post injection (Fig. 4). Longer intervals could not be studied for two reasons: (i) circulating levels of radioactivity approached the detection limit after 30 min, resulting in very poor count statistics, and (ii) the volunteers received a second injection of (low specific activity) radioligand at $t = 40$ min. Plasma samples were analysed both after the first (high SA) and after the second (low SA) injection. No metabolites were observed in either case.

3.4. Plasma protein binding

Protein binding of [^{11}C]I was low (30 ± 2% in rats, 31 ± 2% in humans) and it did not change with time after injection.

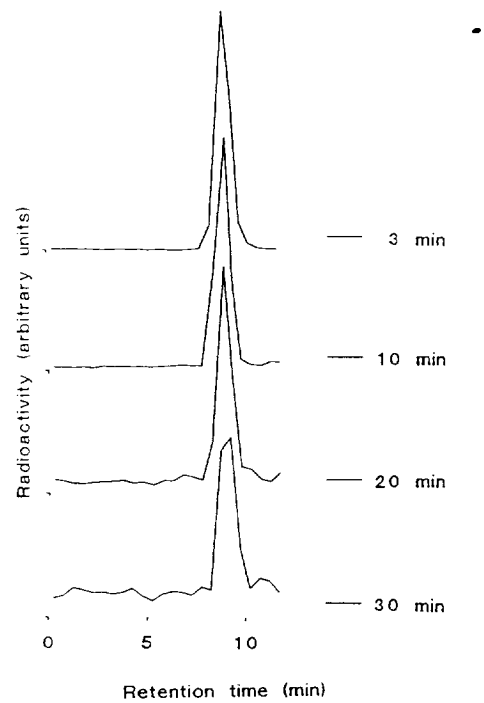

Fig. 4. Radiochromatograms (RP-HPLC) of human plasma extracted at 3, 10, 20 and 30 min post injection (data are an average of 5 volunteers).

3.5. Protein removal

Samples treated with methanol or trichloroacetic acid were suitable for subsequent analysis by TLC or RP-HPLC. However, protein precipitation with acetonitrile resulted in the formation of a radioactive species (artifact) which eluted immediately after the void volume in RP-HPLC while some radioactivity (5–10%) was left at the position of the parent compound. The ratio of parent/artifact was variable and dependent on the ratio of water/acetonitrile in the injected sample (values not shown).

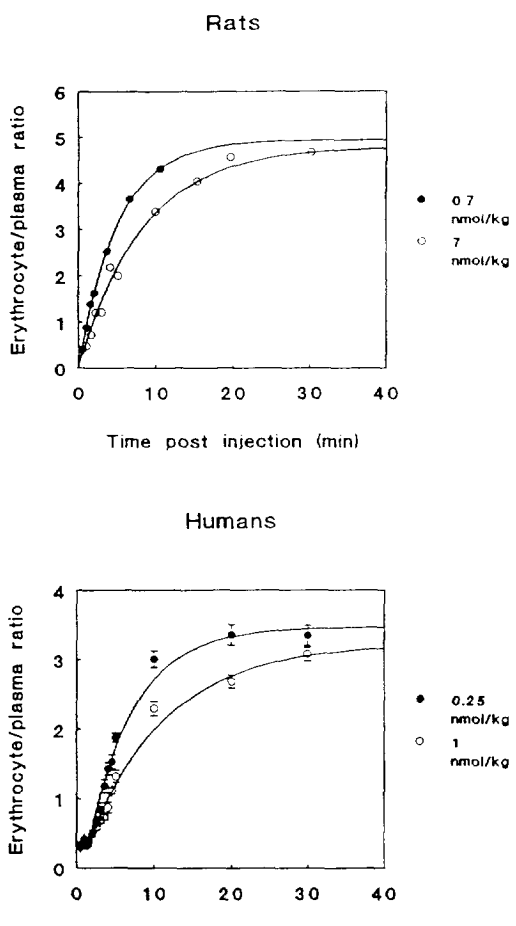

Fig. 5. In-vivo association of S-[^{11}C]I to red blood cells of rats and humans.

3.6. Ligand binding to blood cells

When [^{11}C]I was incubated with whole blood from humans or rats for 2 h at room temperature, no metabolism was apparent if plasma radioactivity was subsequently analysed by RP-HPLC. Extrahepatic metabolism of the drug in blood cells thus seemed to be negligible.

Erythrocyte/plasma concentration ratios of radioactivity as a function of time after injection of [^{11}C]I are plotted in Fig. 5. The initial part of these curves is slightly different in the two species due to the fact that the radioligand was injected as a bolus (within 10 s) in rats whereas it was administered more slowly (over a period of 60–90 s) to human volunteers. Significant association to blood cells occurred in all cases. At intervals $\geq$30 min, the cellular concentration of radioactivity in humans was 3 times higher than the plasma concentration. Erythrocyte/plasma concentration ratios in rats increased from 0 to about 5. The apparent rate of increase was lower when the injected mass was higher, but the eventual steady-state values were similar at the dosages used.

4. Discussion

Quantitative interpretation of PET images in terms of receptor densities is based on mathematical compartment models of radioligand kinetics [9,10]. The solution (i.e. B_{max} in pmol/ml) which is obtained for such models is totally dependent on a correct estimation of the input function; errors in the input will give rise to corresponding (or even magnified) errors in the eventual solution.

The most simple input function would consist of the time-dependent concentration of radioactivity in arterial blood. This may be estimated from PET images when the heart is in the field of view: radioactivity in a region-of-interest corresponding to the lumen of the left ventricle will correspond to activity in the blood pool. Such estimations of tissue input would have the great advantage that patients or volunteers could be

studied without arterial cannulation. However, as shown in Fig. 5, blood pool data cannot be used as input function in PET studies with I due to significant and time-dependent association of the radioligand to blood cells. At intervals >2.5 min, most radioactivity is in cells rather than in plasma. Cellular binding of the radioligand probably occurs largely to β-adrenoceptors. Rat erythrocytes contain a homogeneous population of β_2 sites [20–22]. Significant binding was therefore observed for the non-subtype-selective radioligand *S*-[^{3}H]I, but not for the β_1-selective ligand [^{3}H]CGP 26505 [3]. Human erythrocytes contain a mixed population of β_1 and β_2 binding sites with relative proportions of 33:67 [23].

A more sophisticated input function would consist of the time-dependent concentration of radioactivity in plasma. This approach would be valid if radioligand metabolism were negligible over the period studied. However, if radioactive metabolites are formed (e.g. in the liver) and released into blood, total plasma radioactivity is not a good estimate of ligand input to the tissue under study. The input function should then be corrected for a progressively decreasing proportion of unmodified ligand to other radiolabeled species in plasma.

Literature data on metabolism of I are controversial. Delforge et al. [13] and Mazière et al. [14] reported very rapid metabolism of *RS*-[^{11}C]I in rats and dogs (see Table 1). In contrast, Jones et al. [15] and Luthra et al. [16] claimed that *S*-[^{11}C]I was slowly metabolized in the rat while the ligand was stable in dogs and human volunteers for extended periods of time (see Table 1). Although radioligand enantiomers may show different clearance and metabolism, this is probably not the cause of the conflicting findings, as *RS*-[^{14}C]I and *S*-[^{11}C]I showed similar metabolic stability in the rat [15].

Chromatographic assays of radiopharmaceutical metabolism are prone to various analytical errors (see Ref. [14]): (i) Sample pretreatment may result in the formation of radiolabeled artifacts (e.g. dimers, complexes). The presence of such species will lead to overestimation of the metabolite fraction; (ii) during tissue extraction or protein elimination from the biological sample, labile metabolites (e.g. glucuronides) may be hydrolysed and the parent compound may again be formed. Acid extraction can therefore lead to underestimation of the metabolite fraction; (iii) incomplete extraction of radioactivity from the biological sample may lead to erroneous ratios of parent/metabolites; (iv) incomplete elution of radioactivity from the column/plate (e.g. due to very long retention of a hydrophobic metabolite in RP-HPLC) may lead to underestimation of the number of metabolites; (v) some metabolites may not be separated from the parent compound in the chromatographic system which will also lead to underestimation of the number of metabolites. Finally, (vi) low concentrations of radioactivity in late samples may lead to large counting errors and a poor statistical quality of the analytical results.

In the present study, we have addressed these issues in the following ways:

(i) We compared three forms of sample treatment: extraction with methanol, TCA or acetonitrile. Treatment of a standard solution of *S*-[^{11}C]I with methanol or TCA did not cause formation of artifacts, but addition of acetonitrile produced a polar radiolabeled species with shorter retention time than the parent compound in RP-HPLC. Acid acetonitrile (1 ml 0.05 *M* H_2SO_4 in 100 ml of acetonitrile) gave similar results. Thus, acetonitrile extraction was not used in further experiments.

(ii) TCA extraction and methanol extraction were then employed for protein removal from rat plasma. Since the analytical results were similar (Fig. 3), it is not likely that acid-labile glucuronides were formed. Plasma samples acquired at 40 min were also directly applied to an internal-surface reversed-phase (ISRP) column. This column is of the mixed-mode type; its particles have two surfaces (outer and inner). Large molecules (e.g. proteins) do not enter the particles and they show little retention. Small molecules (e.g. radioligands, metabolites) can enter and they are separated according to their lipophilicity. The resolving power of an ISRP column is less than that of an analytical C_{18}

column and comparable to a C_3 stationary phase (Chrompack, personal communication). After direct sample injection, we observed a similar pattern of parent and metabolites as in the other assays. It is thus improbable that acid-labile glucuronides of I are a significant component of total plasma radioactivity. Since similar (or identical) metabolites were observed upon injection of *S*-[^{3}H]- and *S*-[^{11}C]I, metabolism in rats seems to involve the *tert.*-butyl- or hydroxypropoxy parts of the molecule rather than the benzimidazole-2-one. The IRSP column was only used in a few experiments due to very rapid clogging of the C_{18} guard cartridge. When the column was operated without guard cartridge, *S*-[^{11}C]I was no longer separated from its metabolites and it showed very little retention. Reduction of the amount of organic modifier in the mobile phase or alteration of the pH of the eluent did not result in a useful separation. Probably I and its metabolites are too hydrophilic for routine use of an ISRP column in metabolite analysis. We have successfully applied ISRP columns to the assay of a more lipophilic β-adrenoceptor ligand, *S*-1′-[^{18}F]fluorocarazolol, in plasma from rats and sheep (manuscript in preparation).

(iii) Extraction of radioactivity from plasma was excellent when samples were treated with methanol. Lower but reproducible extraction efficiencies were observed when the samples were treated with TCA (Table 1). Lower extraction efficiency with TCA is probably due to trapping of unmodified I within the pellet, as repeated extraction of the pellet with TCA and subsequent analysis of the supernatant by RP-HPLC indicated the presence of parent compound only ($>90\%$ of radioactivity in human plasma was accounted for in these experiments).

(iv) Since recovery of applied radioactivity from the column or plate was excellent in all cases (Table 1), it is not likely that the presence of a hydrophobic metabolite was overlooked.

(v) Three different assay methods (straight-phase TLC, reversed-phase HPLC, ISRP chromatography) indicated formation of two polar metabolites and similar pharmacokinetics of I in Wistar rats (Fig. 3). It is therefore unlikely that a metabolite was not separated from the parent compound, as this metabolite would have to co-elute with the parent in three chromatographic systems with different mechanisms of retention.

Binding of *S*-[^{11}C]I to plasma proteins was low in rats and human volunteers. Although low protein binding might be expected because of the low lipophilicity of the radioligand [11,12] the plasma free fractions of a drug at tracer and pharmacological dosages can be different. For this reason we measured protein binding at the tracer dosages used in a PET scan. Low and constant values were observed, indicating that the input function can be easily corrected for protein binding.

In summary, we have compared several extraction and assay methods for the analysis of *S*-[^{11}C]I in plasma samples. The radiopharmaceutical appeared to be slowly metabolized in rats and it was not metabolized to any significant extent in human volunteers within the time scale of a PET study. No evidence for rapid metabolism was obtained, but a polar artifact was observed in samples treated with acetonitrile. Well-validated assay methods are essential for estimation of receptor density in intact animals or humans using PET.

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