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Determination of a renin inhibitor in plasma by solid-phase extraction using acetone as protein binding displacer followed by on-line high-performance liquid chromatography*

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Abstract: H 218/54 is a potent inhibitor of human renin activity (pIC_{50} = 8.3 at pH 6) and is therefore a potential agent for blood pressure reduction. This lipophilic compound is highly bound to plasma proteins, e.g. 99.7% in rats and 99.6% in humans. For pharmacokinetic studies, a quantitative assay for ^{3}H-H 218/54 in plasma has been developed.

On top of an AASP phenyl solid-phase cartridge 70 μl of rat plasma or 1 ml of cynomolgus plasma was mixed with 200 μl of water containing 20% acetone. The acetone displaced the substance from plasma proteins without precipitation of the sample and clogging of the extraction column. The mixture was passed through the cartridge, which adsorbed ^{3}H-H 218/54. The cartridge was placed in an AASP autosampler connected to a reversed-phase LC system, with a Vydac C-18 column and CH_3CN–H_2O–TFA (60:40:0.1, v/v/v) as mobile phase. The effluent from the separation column was collected in fractions for radioactivity counting. Recovery, as measured after adding various amounts of tritium-labelled H 218/54 to blank plasma followed by repeated analysis of the samples, was close to 100% with relative standard deviations between 1.4 and 3.0%. At the lowest level tested, 200 dpm per sample, the recovery was 120% with a relative standard deviation of only 10%. The sensitivity of the method will depend on the specific radioactivity of the dose given.

Keywords: *Acetone; protein binding displacer; renin inhibitor; reversed-phase chromatography; advanced automated sample processor; plasma; solid-phase extraction.*

Introduction

The renin–angiotensin system plays an important role in blood pressure regulation [1]. The aspartyl protease renin catalyses the first and rate-limiting step in the cascade formation of the potent vasopressor angiotensin II [2]. Thus, selective inhibition of renin by therapeutic agents should afford an approach for reduction of high blood pressure [3].

H 218/54 (Fig. 1), is a potent inhibitor of human renin, pIC_{50} = 8.3 at pH 6 [4], chemically modified to be stable against proteolytic enzymes, e.g. chymotrypsin and carboxypeptidase.

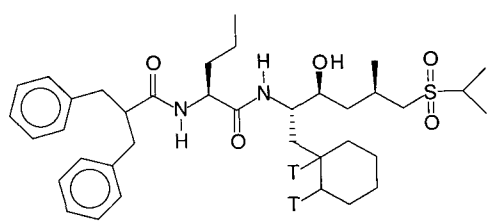

Figure 1
Structure of tritiated H 218/54. The stereochemistry at the four chiral centers is shown.

In this paper, an analytical method is presented for the determination of radiolabelled compound in plasma from rats and cynomolgus monkeys in pharmacokinetic studies. The method involves solid-phase extraction (SPE) and H 218/54 exhibits very high plasma protein binding, e.g. 99.7% in rats and 99.6% in humans. This factor causes low retention of analytes on SPE sorbents [5]. In this assay, acetone was used to displace the compound from plasma proteins avoiding precipitation of the plasma matrix, which could both clog the SPE cartridge and give rise to loss of the renin inhibitor in the work-up procedure.

Experimental

Chemicals

(2*S*,3*S*,5*R*)-1-cyclohexyl-2[(dibenzylacetyl-L-norvalinyl)amino]-3-hydroxy-6-isopropylsulphonyl-5-methylhexane (H 218/54, MW =

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640.9) and ^{3}H-H 218/54, with a specific radioactivity of 2.4 MBq nmol^{-1} and a radioactive purity of >97% as shown by HPLC, were synthesized at the Department of Organic Chemistry, Astra Hässle AB (Mölndal, Sweden). The ^{3}H-NMR spectrum showed that 95% of the tritium label was in the 1 and 2 positions of the cyclohexyl moiety of ^{3}H-H 218/54 (Fig. 1) and the remaining 5% was located in the benzylic position. All solvents were of analytical grade and the water was deionized before use.

Instruments and assay conditions

The Advanced Automated Sample Processor (AASP) and the AASP PrepStation were manufactured by Varian (Walnut Creek, CA, USA). The AASP Phenyl cassettes were purchased from Sorbent AB, V:a Frölunda, Sweden. The AASP run, cycle and valve reset times were 15, 17 and 1.5 min, respectively. The cartridges were purged with 250 μl of water before injection.

The HPLC system consisted of a Spectra Physics pump, SP 8810 (San Jose, CA, USA) with a Waters 484 tunable UV detector (Milford, MA, USA) set at 212 nm and a Spectra Physics integrator, SP 4270. The separation was performed on a Vydac C_{18} column (25 cm × 4.6 mm, 5 μ) fitted with a cyano precolumn (Brownlee Newguard, 7 μ). The mobile phase consisted of acetonitrile–water–trifluoroacetic acid (60:40:0.1, v/v/v). The flow rate was 1.0 ml min^{-1} and the effluent was collected in 1 min fractions with a LKB 2211 SuperRac (Bromma, Sweden). After addition of 4 ml Ready Safe (Beckman) the radioactivity in the fractions was determined in a liquid scintillation counter (Beckman LS 3800, Irvine, CA, USA). Counting efficiency was determined by reference to external standards.

Sample preparation

The stock solution of unlabelled H 218/54, used as a marker, was prepared by dissolving 1.0 mg in 50 ml of water–acetone (20:80, v/v) giving a final concentration of 31 μmol l^{-1}. Acetone was used to displace the analyte from plasma proteins.

Spiked plasma samples were made by addition of ^{3}H-H 218/54 to blank plasma, followed by dilution to the required radioactive concentrations with blank plasma.

The phenyl cartridges, of which each cassette has 10, were prepared at an AASP PrepStation. The cartridges were activated by passing 1 ml of water followed by 1 ml of methanol and finally 1 ml of water through the sorbent under pressure from a nitrogen cylinder. Thereafter, 200 μl of the marker solution of H 218/54 was added to the reservoir on top of the cartridge. Plasma from rats (70 μl) or cynomolgus monkeys (1 ml) was added by pipette and the mixture was allowed to rest for 5 min. H 218/54 was extracted to the solid phase by passing the mixture through the cartridge at a flow rate of 1 ml min^{-1}. The cartridge was washed with 1 ml of acetonitrile–water (20:80, v/v). The prepared cassette was introduced into the AASP and the samples were thereby injected on-line into the HPLC system.

Quantitative evaluation

The substance which eluted from the HPLC column after 10.5 min was collected into scintillation vials in two 1-min fractions. The radioactivity counted in these fractions was added and the radioactivity found in the fractions before and after was subtracted. The plasma concentration of H 218/54 was calculated by dividing the determined radioactivity by the specific radioactivity of the dose given and the volume of plasma used in the analysis.

Recovery and precision

Various known amounts of ^{3}H-H 218/54 were added to blank rat and cynomolgus monkey plasma, followed by repeated analysis of the samples using the method described above.

Pilot study

Following intravenous (iv) administration of ^{3}H-H 218/54 (50 kBq nmol^{-1} and 3.0 μmol kg^{-1}) to a Sprague Dawley rat, repeated blood samples were collected over 12 h. Plasma was prepared and stored frozen at −18°C until analysis by the present method.

Results

Selectivity

The renin inhibitor H 218/54 is mainly cleared by metabolism with pronounced biliary excretion and numerous metabolites can be detected by HPLC (data not shown). Apparently, the specific work-up procedure of plasma samples in combination with HPLC accomplished sufficient separation of

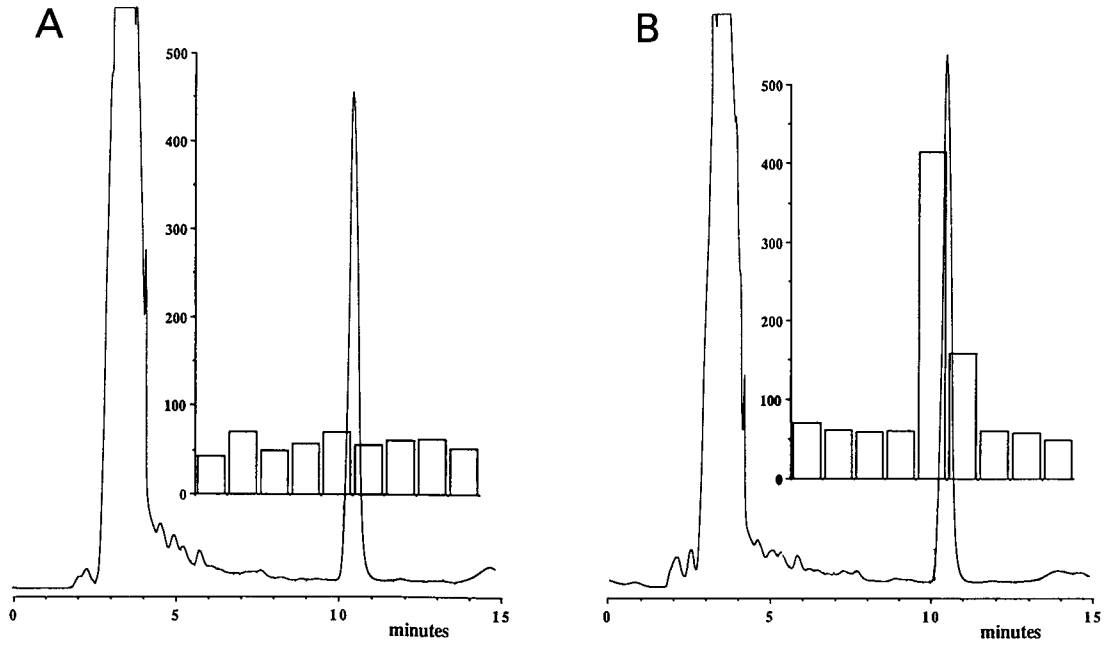

Figure 2
UV-chromatograms at 210 nm and histograms of radioactivity after separation of (A) a blank rat plasma sample and (B) a rat plasma sample collected 6 h after an intravenous dose of ^{3}H-H 218/54. The amount of radioactivity in the histograms is given in dpm per fraction and the amount of ^{3}H-H 218/54 in (B) was determined to 450 dpm. The delay time between the UV signal and the collected fractions was 30 s.

^{3}H-H 218/54 from metabolites. In Fig. 2, histograms of the radioactivity in collected fractions from analysis of blank rat plasma and a rat plasma sample taken 6 h after an iv dose of tritiated compound are given. One metabolite eluting after 6.5 min at low concentrations did not interfere with the determination of H 218/54.

Recovery and precision

In Table 1 the recovery and relative standard deviation (RSD) after repeated analysis of four rat plasma samples spiked with different amounts of radioactivity are given. The recovery for the three highest levels was 100–106%, and the RSD varied between 1.4 and 3.0%. At the lowest level tested, 200 dpm per 70 μl of rat plasma, the recovery was 120% and the RSD was 10%.

Due to the limited access to cynomolgus monkey plasma, the recovery and RSD were determined at one level only, 18,150 dpm ml^{-1}. The recovery and RSD (97 and 1.6%), were comparable to those in rat plasma at the same level of radioactivity, indicating that H 218/54 was equally well extracted from different matrices. Since the remaining assay conditions were identical, it was assumed that the recovery of radioactivity and RSD at all levels would be similar to values for rat plasma.

Table 1
Recovery of ^{3}H-H 218/54 and relative standard deviation after repeated analysis of rat and cynomolgus monkey plasma samples spiked with different amounts of ^{3}H-H 218/54

Amount per sample (dpm)	No. of replicates (*n*)	Recovery (%)	RSD (%)
Rat			
330000	10	105	2.4
19500	9	100	1.4
540	10	106	3.0
200	10	120	10
Cynomolgus monkey			
18150	10	97	1.6

Limit of detection

The sensitivity of the assay is determined by the maximum standard deviation which can be tolerated. With an accepted standard deviation of 10%, the detection limit of the method will be 200 dpm. The limit for concentration determination, however, will depend on the specific radioactivity of the administered dose. Assuming that only radioactively labelled H 218/54 (2.4 MBq nmol^{-1}) will be given to rats

and that a plasma sample of 70 µl will be analysed by this method about 70 pmol l^{-1} can be determined with a RSD of 10%.

Pilot study

The method was applied to plasma samples collected from a rat receiving an iv dose of 3.0 µmol kg^{-1}. Assay sensitivity was sufficient at all time points, and the plasma concentration of H 218/54 could be determined over 12 h at levels shown in Fig. 3. The amount of radioactivity originating from H 218/54 in the 12-h sample was 220 dpm, while the total amount of radioactivity in the sample was 35000 dpm. Although the concentration of radioactive metabolites was more than 150 times higher, the selectivity to determine H 218/54 was adequate.

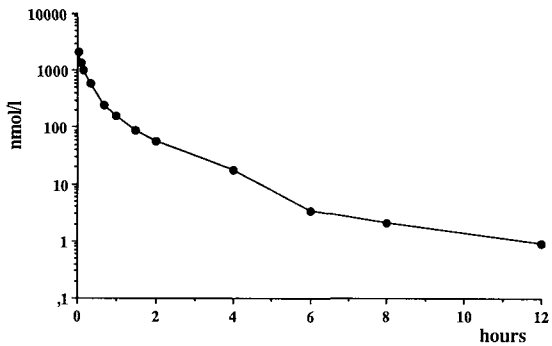

Figure 3
Plasma concentrations of H 218/54 in one rat at various time points after administration of 3.0 µmol kg^{-1} intravenously.

Discussion

A radioimmunoassay (RIA) for the determination of the renin inhibitor ditekiren (U-71,038) in rat serum has been reported [6]. The assay measures the pharmacologically active compounds present in the matrix, including parent compound and active metabolite(s). To avoid incorrect estimates of the disposition of the drug, the selectivity of the RIA method has to be validated with reference to an assay based on chromatography. In the case of ditekiren, however, the RIA technique should give correct concentration data because U-71,038 does not undergo significant systemic metabolism in the rat [7]. In a more recent report, an HPLC method in which samples are prepared by solid-phase extraction using an internal standard and UV detection at 220 nm has been developed to determine ditekiren in monkey serum [8]. After derivatization with dabsyl chloride a specific HPLC assay with column-switching of the renin inhibitor Ro 42-5892/001 is used in toxicokinetic studies in rats and marmosets [9]. Since the analyte and internal standard are optical isomers, chiral separation is done in a column-switching system with β-cyclodextrin in the mobile phase.

In the present method, H 218/54 was isolated from plasma by solid-phase extraction, and the advanced automated sample processor (AASP) PrepStation was used to simplify the work-up procedure. The AASP phenyl cartridge was chosen as the SPE sorbent with desired properties in terms of analyte extraction and elution with the acidic mobile phases used in the analytical C-18 column. Different stationary phases in the extraction and analytical column will improve assay selectivity, because the three ring systems in the H 218/54 molecule should interact variously with the SPE sorbent and the C-18 chains in the separation column.

Initially, SPE was performed without pretreatment or by dilution of plasma with water, which resulted in low and varying recovery of the analyte. H 218/54 is extensively (>99.5%) bound to plasma proteins and during the extraction process the compound was not able to interact with the solid-phase material and followed the plasma matrix through the column. In further attempts, various amounts of methanol and acetonitrile were used as protein binding displacers, which often lead to precipitation of plasma proteins in the reservoir and subsequent clogging of the SPE cartridge. In a paper by Blanchard, the efficacy of different techniques for precipitating plasma proteins was studied [10]. Acetone was among the solvents that could be added to plasma in largest fractions without precipitation. By using acetone as protein binding displacer in the assay of H 218/54, a complete extraction to the SPE cartridge was achieved and precipitation was avoided.

The recovery was close to 100% at three of the four levels tested in rat plasma. At the lowest level, 200 dpm per sample, the recovery was 20% higher than expected. During the analysis the radioactive substance was collected in two fractions, and at this low level the net amount of radioactivity was only 1–3 times the blank level and was therefore difficult to determine. Notably, the relative stan-

dard deviation was still only 10% at 200 dpm per sample.

The recovery and standard deviation of the method were determined by varying the amount of radioactivity in the sample. There was no need to vary the plasma concentration of H 218/54, since the unlabelled compound was added as a marker in constant excess, 89 μmol l^{-1} (rats) and 6.2 μmol l^{-1} (cynomolgus), to the plasma during the sample preparation procedure. The measured UV signal of H 218/54 during LC-analysis can be used as an in-run check for assay reproducibility. The method has been applied successfully during preclinical evaluation in rats and cynomolgus monkeys following administration of tritium-labelled H 218/54 and some preliminary pharmacokinetic data have been presented [11].

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