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Enantioselective high-performance liquid chromatographic analysis of the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin Application to a pharmacokinetic–pharmacodynamic study in rats

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Abstract

A rapid, sensitive and enantioselective HPLC assay for the simultaneous determination of the reference 5-HT_{1A} receptor agonists, *R*-(+)- and *S*-(−)-8-hydroxy-2-(di-*n*-propylamino)tetralin (R-8-OH-DPAT and S-8-OH-DPAT, respectively), in rat blood is presented. A selective extraction procedure was developed using a preliminary sample clean-up followed by isolation of R- or S-8-OH-DPAT on mixed-mode NARC-2 solid-phase columns. Separation of the enantiomers was performed by high-performance liquid chromatography using a Chiracel OD-R column. Detection was obtained using an electrochemical detector set at a voltage of 0.63 V. The mobile phase consisted of a 50 m*M* phosphate buffer (pH 5.5)–acetonitrile (80:20, v/v) mixture. At a flow-rate of 1 ml min^{-1}, the total run time was ~14 min. The limit of detection for R- and S-8-OH-DPAT was 0.5 ng ml^{-1}. In the concentration range between 50 ng ml^{-1} and 1000 ng ml^{-1} intra- and inter-day relative standard deviations were less than 12%. The assay was applied to a pharmacokinetic–pharmacodynamic study in rats in which decrease of body temperature was used as a measure of 5-HT_{1A} receptor-mediated effect. Values for clearance, volume of distribution at steady state and terminal elimination rate constant were 22±2 ml min^{-1}, 1969±473 ml and 156±34 min for R-8-OH-DPAT and 16±1 ml min^{-1}, 3353±347 ml and 334±36 min for S-8-OH-DPAT, respectively. No enantiomeric interconversion was observed in vivo from R-8-OH-DPAT to S-8-OH-DPAT or vice versa.

Keywords: Pharmacokinetics; 8-Hydroxy-2-(di-*n*-propylamino)tetralin; 5-HT_{1A} receptors

1. Introduction

5-HT_{1A} receptors are generally considered as important pharmacological targets for the treatment of various diseases of the central nervous system, such as depression and anxiety [1,2]. Following its synthesis by Arvidsson et al. [3], 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, Fig. 1) has be-

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come the reference agonist for studying 5-HT_{1A} receptor-mediated effects in vitro and in vivo. However, the vast majority of reports on the in vivo actions of 8-OH-DPAT have been limited to dose–response studies, without taking into account its pharmacokinetic profile. Due to the lack of integration between pharmacokinetic and pharmacodynamic data, the mechanisms that determine the time-course of in vivo effects of 8-OH-DPAT are unclear. To date, integrated pharmacokinetic–pharmacodynamic analysis of 8-OH-DPAT has been hampered by the lack of a convenient, rapid and sensitive analytical assay to determine concentrations in small blood samples. With respect to sensitivity it is important to realise that the affinity of 8-OH-DPAT for the 5-HT_{1A} receptor is ~1 ng ml^{-1} [4] and hence the detection limit should be in this range in order to characterise the pharmacokinetic–pharmacodynamic relationship. A particular problem in achieving such limits is the fact that, due to the presence of an acidic phenol and a basic amino moiety, 8-OH-DPAT is charged at almost any pH which impedes selective recovery from blood samples using conventional liquid–liquid or solid-phase extraction. Wood et al. [5] and Yu and Lewander [6] have described simple high-performance liquid chromatography (HPLC)–UV methods using solid-phase and liquid–liquid extraction but reported detection limits of 20–200 ng ml^{-1} for 8-OH-DPAT in plasma. Mason et al. [7,8] and Sonesson et al. [9] did not report detection limits and because their methods involve mass spectrometry and the use of radio-labelled compounds, they are either expensive or less suitable for routine analysis of large numbers of samples. A further drawback of all these methods is the fact that they do not discriminate between the two enantiomers of 8-OH-DPAT (Fig. 1). We considered the development of an enantioselective assay important because *R*-(+)- and *S*-(−)-8-OH-DPAT (R-8-OH-DPAT and S-8-OH-DPAT, respectively) have different pharmacological profiles at the 5-HT_{1A} receptor [4] and it is unknown whether interconversion between the enantiomers occurs in vivo.

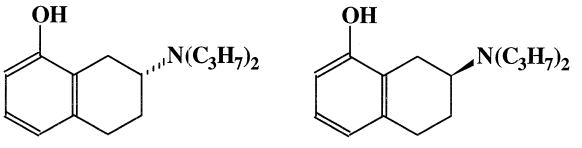

Fig. 1. Structures of *R*-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (right) and *S*-(−)-8-hydroxy-2-(di-*n*-propylamino)tetralin (left).

In this paper, we describe a rapid, sensitive and enantioselective HPLC assay with electrochemical detection for the simultaneous determination of R- and S-8-OH-DPAT in blood samples of 50 μl. A selective extraction procedure was developed using a preliminary sample clean-up followed by isolation of 8-OH-DPAT on mixed-mode (cation-exchange-C_{18}) NARC-2 solid-phase columns [10]. The novel assay was applied to a pharmacokinetic–pharmacodynamic study in rats of R- and S-8-OH-DPAT, in which decrease of body temperature was used as a measure of 5-HT_{1A} receptor-mediated effect [11–14].

2. Experimental

2.1. Chemicals

R-(+)-8-Hydroxy-2-(di-*n*-propylamino)tetralin (R-8-OH-DPAT), *S*-(−)-8-hydroxy-2-(di-*n*-propylamino)tetralin (S-8-OH-DPAT) and *R*-(+)-7-hydroxy-2-(di-*n*-propylamino)tetralin (R-7-OH-DPAT) were purchased from Research Biochemicals International (Natick, MA, USA). Acetonitrile (DNA synthesis grade) was obtained from Biosolve (Valkenswaard, The Netherlands). Methanol (HPLC grade) was purchased from Rathburn (Walkerburn, UK). Dichloromethane Chromasolve was obtained from Riedel-de Haën (Seelze, Germany). All other chemicals used were of analytical grade (Baker, Deventer, The Netherlands).

2.2. Instrumentation

The HPLC system consisted of a Spectroflow 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA), a pulse damper (Antec Leyden, Rijnsburg, The Netherlands), a Waters 717plus autosampler (Waters, Milford, MA, USA) and a digital electrochemical amperometric detector, (DECADE, software version 3.02) from Antec Leyden. The electrochemical detector consisted of a VT-03 electrochemical flowcell using a 25 μm spacer and an in situ Ag/AgCl (ISAAC) reference electrode, oper-

ating in d.c. mode at 0.63 V, at a temperature of 30°C. These conditions were different from those used by Perry and Fuller [15], who used coulometric electrochemical detection to determine 8-OH-DPAT in brain.

Chromatography was performed on a Chiralcel OD-R column (particle size 10 μm, 250×4.6 mm I.D.) (Diacel Chemical Industries, Tokyo, Japan) equipped with a guard column (20 mm×2 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) packed with C_{18} material (particle size 20–40 μm) (Chrompack, Bergen Op Zoom, The Netherlands). Both the analytical and the guard column were maintained at a constant temperature of 30°C.

The mobile phase was a mixture of 50 m*M* phosphate buffer (pH 5.5)–acetonitrile (80:20, v/v) and contained a total concentration of 5 m*M* KCl and 20 mg l^{-1} of EDTA. Mobile phase solvents were filtered through a 0.45-μm nylon filter (Gelman Scientific, Ann Arbor, MI, USA) and mixed. Flow-rate was 1 ml min^{-1} and the mobile phase was degassed continuously using helium.

Data acquisition and processing was performed using the Millennium Chromatography Manager Software version 2.15.01 (Waters, Milford, MA, USA).

2.3. Extraction procedure

To 50 μl of blood hemolyzed in 400 μl water in a glass centrifuge tube, 50 μl of the internal standard solution (1.00 μg ml^{-1} R-7-OH-DPAT) was added. After mixing, 2 ml acetonitrile was added and vortexed for 30 s. To the mixture, 25 μl of a 3 *M* NaOH solution was added and vortexed for another 30 s. After the mixture was centrifuged for 15 min at 4500 *g*, the supernatant was decanted into a clean tube. Subsequently, 3 ml dichloromethane and 750 μl of 0.5 *M* borate buffer (pH 10) were added to the supernatant and vortexed for 15 min. The phase system was centrifuged for 5 min at 4500 *g*. The water phase was disposed by means of suction. The remaining water was removed by freezing and the organic phase was transferred to a clean tube and evaporated under reduced pressure at 37°C. The residue was redissolved in 2 ml of 50 m*M* phosphate buffer (pH 5.5). Bakerbond solid-phase extraction NARC-2 columns (3 ml, 125 mg NARC-2 material) (J.T. Baker, Phillipsburg, NJ, USA) were preconditioned twice with 3 ml methanol, once with 3 ml water and once with 2 ml phosphate buffer (50 m*M*, pH 5.5). Columns were not allowed to run dry. The sample was loaded, and the columns were washed with 2 ml phosphate buffer and 2 ml of a methanol–phosphate buffer (40:60, v/v), after which the columns were allowed to run dry. The sample was then eluted using 3 ml of a 1% NH_4OH in methanol solution. The sample was evaporated under reduced pressure at 37°C and redissolved in 100 μl of a 20% acetonitrile in water solution of which 50 μl was injected into the HPLC system.

2.4. Calibration and validation

Stock solutions of R-8-, S-8- and R-7-OH-DPAT were prepared at concentrations of 100 μg ml^{-1} in water. On each day of analysis a nine-point calibration curve was prepared by spiking 50 μl of blood hemolyzed in 400 μl water with 50 μl of a R-8-OH-DPAT or S-8-OH-DPAT solution and 50 μl of the internal standard R-7-OH-DPAT at 1 μg ml^{-1}. This resulted in R- and S-8-OH-DPAT blood concentration ranges of 0.1–5000 ng ml^{-1}. Samples were processed as described above and peak-area ratios of R-8-OH-DPAT/R-7-OH-DPAT and S-8-OH-DPAT/R-7-OH-DPAT were calculated. Calibration curves were constructed by weighted linear regression [weight factor=$1/(\text{peak-area ratio})^2$]. Quality control samples of fixed concentration (see Table 1) were prepared to determine intra- and inter-variability. Extraction yields were determined at R- and S-8-OH-DPAT blood concentrations of 50 and 1000 ng ml^{-1} with the internal–external standard method.

2.5. Study in rats

Chronically instrumented male Wistar rats, weighing 296±3 g (mean±S.E.M., n=23) were used in the experiments. Eight days before the experiment the abdominal aorta was cannulated by an approach through the right femoral artery for serial blood sampling. The right jugular vein was implanted with a cannula for administration of the infusion solution. Furthermore a telemetric transmitter [Physiotel, implant TA10TA-F40, Data Sciences International (DSI), St. Paul, MN, USA] was implanted in the

Table 1
Validation of the determination of R- and S-8-OH-DPAT: recovery, intra- and inter-assay variability, relative standard deviations of variability and accuracy

Compound	Added (ng ml^{-1})	Recovery (n=4) (mean±S.E.M.) (%)	Intra-assay (n=4)			Inter-assay (n=10)		
			Found (mean±S.E.M.) (ng ml^{-1})	RSD (%)	Accuracy (%)	Found (mean±S.E.M.) (ng ml^{-1})	RSD (%)	Accuracy (%)
R-8-OH-DPAT	49.9	52.8±1.04	49.5±2.86	11.5	99.2	47.7±1.67	10.5	95.4
	999.9	51.1±4.52	1069.3±42.13	7.9	106.9	1010.2±22.62	7.1	101.0
S-8-OH-DPAT	50	49.0±1.70	54.0±2.00	7.4	108.0	54.4±0.91	5.3	108.9
	1000	45.4±0.93	946.6±22.07	4.7	94.7	949.6±11.29	3.8	95.0

abdominal cavity for the measurement of core body temperature. The surgical procedures were performed under anaesthesia of medetomidine HCl (0.1 ml kg^{-1} of a 1 mg ml^{-1} solution, intra muscular) and ketamine base (1 ml kg^{-1} of a 50 mg ml^{-1} solution, subcutaneous). After surgery rats received a single dose of ampicilline trihydrate (0.6 ml kg^{-1} of a 200 mg ml^{-1} solution, A.U.V., Cuijk, The Netherlands).

On the day of experiment the rats (fully recovered, unrestrained, conscious) received an intravenous infusion of either R-8-OH-DPAT (1 mg kg^{-1} in a 5 min infusion or 3 mg kg^{-1} in a 15 min infusion) or S-8-OH-DPAT (5 or 15 mg kg^{-1} in a 15 min infusion). Eighteen 50-μl blood samples were collected over a period of 8 h at predetermined time intervals. Blood samples were directly hemolyzed in a glass centrifuge tube containing 400 μl of water at 0°C and stored at −20°C pending analysis as described above.

Following the administration of either R- or S-8-OH-DPAT core body temperature was recorded and averaged over a 2-s period every 30 s. Body temperature was measured using a telemetric system (Physiotel Telemetry system, DSI), and processed using Dataquest Lab Pro (DSI).

The pharmacokinetics of R- and S-8-OH-DPAT were quantified for each individual rat using the least-squares minimisation algorithm [weight=1/$(y_{\text{predicted}})^2$] of the WinNonlin Pro package V.1.5 (Pharsight, Mountain View, CA, USA). For both R- and S-8-OH-DPAT a standard three-compartment model [16] best described the concentration–time profile, as judged by the Akaike Information Criteria [17].

3. Results and discussion

3.1. Chromatography

The sample pre-treatment with the mixed-mode NARC-2 columns provided a good sample clean-up, as demonstrated by the representative chromatograms in Fig. 2. Retention times for R- and S-8-OH-DPAT and the internal standard, R-7-OH-DPAT, were 11, 9 and 7 min, respectively and the peaks were well separated. The total run time was 14 min. After intravenous administration of R- or S-8-OH-DPAT no additional peaks were observed as compared to spiked blood samples. Furthermore, no enantiomeric interconversion was observed from R- to S-8-OH-DPAT or vice versa. The small peak observed prior to the R-7-OH-DPAT peak was most likely due to an impurity of the internal standard, since it was not observed when blood samples were extracted in the same manner without adding the internal standard.

Table 1 summarises the recovery after extraction, the accuracy and the reproducibility of the analysis. For R- and S-8-OH-DPAT recovery was ~50% in the concentration range 50 ng ml^{-1} to 1000 ng ml^{-1}. When the liquid–liquid extraction was omitted the recovery increased but reproducibility decreased dramatically, probably due to sedimentation of fat and cell fragments on the filter of the solid-phase columns. In the concentration range 50 ng ml^{-1} to 1000 ng ml^{-1} intra- and inter-day relative standard deviations (RSDs) were less than 12%. The weighted linear regression equations (mean±S.E.M., n=20) for R-8-OH-DPAT and S-8-OH-DPAT were y=(1.28±0.109)x+(−36.63±16.384) and y=

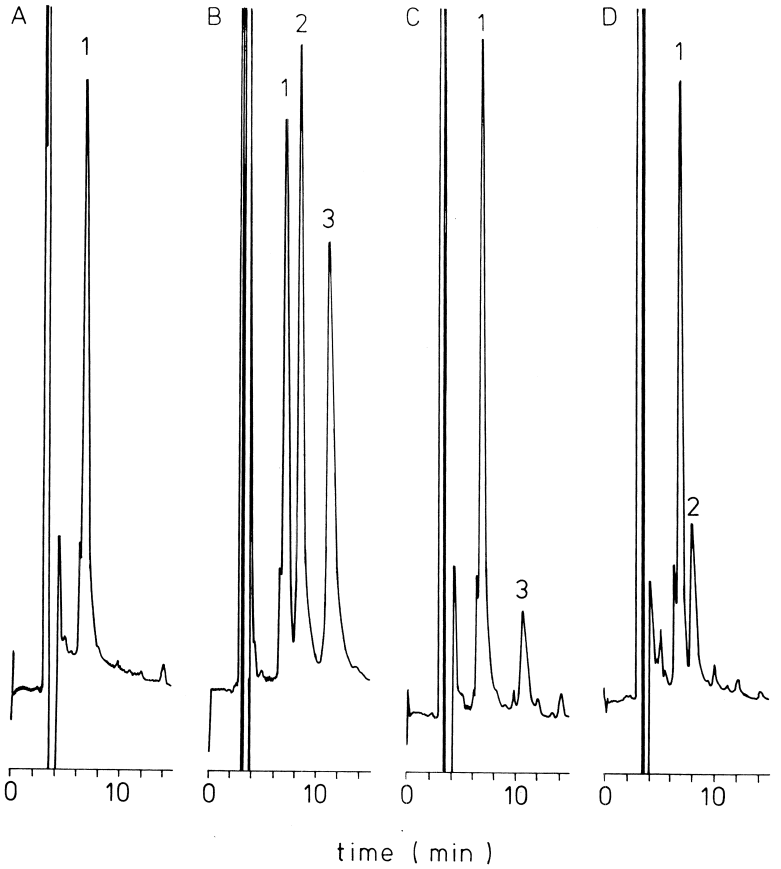

Fig. 2. Chromatograms of an extract of blank blood with internal standard R-7-OH-DPAT (1000 ng ml^{-1}, peak 1) (A), blood spiked with R-8-OH-DPAT (1000 ng ml^{-1}, peak 3) and S-8-OH-DPAT (1000 ng ml^{-1}, peak 2) (B), blood obtained from a rat 30 min after the start of the administration of 1 mg kg^{-1} R-8-OH-DPAT in a 5-min infusion (C), blood obtained from a rat 30 min after the start of the administration of 5 mg kg^{-1} S-8-OH-DPAT in a 15-min infusion (D).

Table 2
Average pharmacokinetic parameter estimates (mean±S.E.M.) obtained with a three-compartment pharmacokinetic model for R- and S-8-OH-DPAT after different infusion rates and doses

Compound	Infusion dose/time (mg kg^{-1}/min)	*n*	Clearance (ml min^{-1})	Volume of distribution at steady state (ml)	Elimination half-life (min)
R-8-OH-DPAT	1/5	6	20±2	2770±917	198±49
	3/15	5	25±3	1329±271	123±45
	Average	11	22±2	1969±473	156±34
S-8-OH-DPAT	5/15	6	15±2	3820±385	381±41
	15/15	6	17±1	2808±326	278±24
	Average	12	16±1	3353±347	334±36

$(1.09\pm0.022)x+(-39.75\pm13.639)$, respectively, y being the peak height ratio and x the blood concentration in ng ml^{-1}. Corresponding coefficients of correlation were between 0.9851–0.9995 for R-8-OH-DPAT and 0.9930–0.9996 for S-8-OH-DPAT, indicating the linearity of the method. Using 50 μl blood, the limit of detection for R- and S-8-OH-DPAT was 0.5 ng ml^{-1} (signal-to-noise ratio=3). Further increment of the voltage did not improve the limit of detection since background noise increases at the same time.

3.2. Study in rats

R- and S-8-OH-DPAT were administered using different doses and infusion rates (Table 2). Fig. 3 shows representative blood concentration time profiles for an intravenous infusion of 1 mg kg^{-1} of

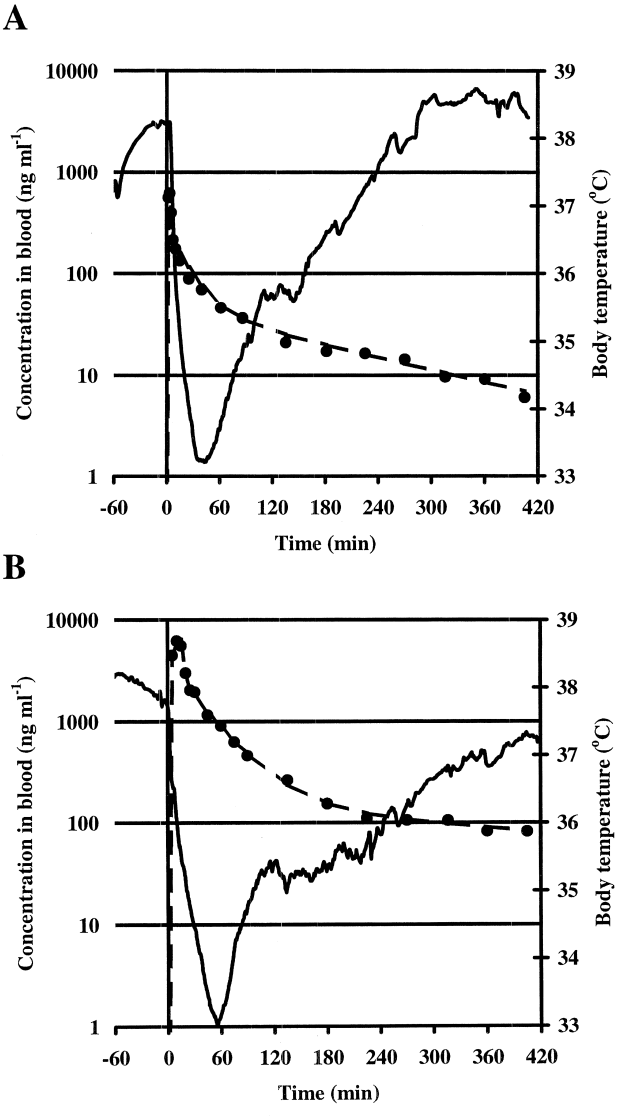

Fig. 3. Typical blood concentration (dashed line, left ordinate) and core body temperature (solid line, right ordinate) time profiles in rats following intravenous infusion of 1 mg kg^{-1} R-8-OH-DPAT in 5 min (A) and 15 mg kg^{-1} S-8-OH-DPAT in 15 min (B). The dashed line represents the best description of the blood concentrations according to a three-compartment pharmacokinetic model.

R-8-OH-DPAT in 5 min and 15 mg kg^{-1} of S-8-OH-DPAT in 15 min. The values for clearance, volume of distribution at steady state and terminal half-life were estimated for each individual rat. For both compounds, no statistically significant differences were found between the different doses and infusion rates, as judged by the unpaired *t*-test. However, a 1.4-fold difference in clearance ($P<0.01$, unpaired *t*-test), and a 1.7-fold difference in volume of distribution at steady state ($P<0.02$, unpaired *t*-test) were found between R- and S-8-OH-DPAT resulting in a two-fold difference in terminal elimination half-life (Table 2). Therefore it can be concluded that 8-OH-DPAT is eliminated from the body in a stereoselective manner.

Fig. 3 also shows the time-course of body temperature measured during administration of R- and S-8-OH-DPAT. After the start of the infusion the body temperature dropped ~3.5–4°C. After reaching a maximal decrease at ~30–80 min, body temperature gradually returned to baseline within 6 h. Combining pharmacokinetic and pharmacodynamic data revealed a complex concentration–effect relationship.

4. Conclusion

We have developed a rapid, sensitive and enantioselective HPLC assay for the determination of the reference 5-HT_{1A} receptor agonist 8-OH-DPAT. The short time of analysis, the sensitivity, the reproducibility and the simplicity of the methodology used make this assay particularly useful for pharmacokinetic–pharmacodynamic studies in which large numbers of samples need to be analysed. No enantiomeric interconversion was observed from R- to S-8-OH-DPAT or vice versa, but the significant difference in clearance indicates a stereoselective mechanism of elimination. In combination with telemetric body temperature measurements, the assay allows for the generation of concentration–effect relationship of 8-OH-DPAT in individual rats which can be used for quantitative analysis of 5-HT_{1A} receptor-mediated responses in vivo.

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