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High-performance liquid chromatography of the adenosine A_1 agonist N^6-cyclopentyladenosine and the A_1 antagonist 8-cyclopentyltheophylline and its application in a pharmacokinetic study in rats

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ABSTRACT

This report describes a rapid and sensitive analysis for the simultaneous detection of the adenosine A_1 receptor ligands N^6-cyclopentyladenosine (CPA) and 8-cyclopentyltheophylline (CPT) in rat blood. The method involved alkaline extraction of the compounds and internal standard N^6-cyclohexyladenosine (CHA) with ethyl acetate, followed by isocratic reversed-phase high-performance liquid chromatography on a 3-μm MicroSphere C_{18} column with UV detection at 269 nm. The mobile phase consisted of a mixture of 10 m*M* acetate buffer (pH 4.0)–methanol–acetonitrile (56:40:4, v/v/v) with a flow-rate of 0.50 ml/min. The total run time was *ca.* 19 min. For CPA and CPT extraction yields were greater than 77 and 66% in the concentration range of 0.010–0.75 μg/ml and 0.025–15 μg/ml, respectively, with intra- and inter-assay variations less than 9%. In 100 μl blood samples the corresponding limits of detection were 3.3 and 6.2 ng/ml (signal-to-noise ratio = 3). CPA was found to be degraded in rat blood *in vitro* with a half-life of 24 min at 37°C. The utility of the analytical method was established by analyzing blood samples from rats which had received an intravenous administration of 200 μg/kg CPA or 12 mg/kg CPT. Due to its rapidity and sensitivity this method is concluded to be particularly useful in pharmacokinetic studies with CPA and CPT.

INTRODUCTION

Adenosine exerts its pharmacological effects via at least two extracellular adenosine receptor subtypes, A_1 and A_2 [1,2]. Recently, several more or less selective agonists and antagonists for these receptor subtypes have been synthesized [3]. These compounds might be effective in the treatment of disorders of the cardiovascular and the central nervous system [4,5].

N^6-Cyclopentyladenosine (CPA) and 8-cyclopentyltheophylline (CPT) (Fig 1.) possess both high affinity and high selectivity for the adenosine A_1 receptor [6]. In studies of the functional effects of adenosine receptor activation, CPA and CPT have been proved to be useful prototypes of agonists and antagonists for the A_1 receptor, respectively. Administration of CPA to the rat has been reported to evoke bradycardia and hypotension [7–9]. CPT has been observed to selectively antagonize the effects of A_1 receptor agonists *in vivo* [10].

So far, no reports on the bioanalysis of synthetic adenosine A_1 receptor ligands have ap-

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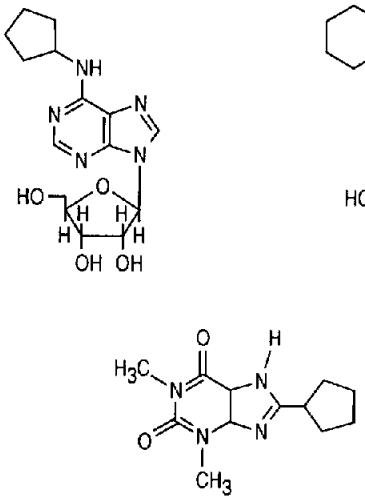

Fig. 1. Structures of N^6-cyclopentyladenosine (CPA) (left), N^6-cyclohexyladenosine (right) and 8-cyclopentyltheophylline (CPT) (bottom).

peared in literature. Only one paper has been published recently describing the bio-assay of an adenosine A_2 agonist [11]. On the bio-analysis of the natural nucleoside adenosine several papers have been published describing reversed-phase high-performance liquid chromatographic (HPLC) methods employing UV [12,13], electrochemical [14] or fluorescence detection [15–19]. However, these methods often have insufficient sensitivity to determine low concentrations of synthetic adenosine derivatives or their applicability to biological fluids has not been demonstrated. Also, the formation of a fluorescent 1,N^6-etheno derivative of CPA is not possible due to the presence of the N^6-cyclopentyl substituent.

In this paper we describe a rapid and sensitive HPLC assay for the simultaneous determination of CPA and CPT in rat whole-blood samples. The procedure relies on liquid–liquid extraction of the compounds in combination with isocratic resolution on a reversed-phase C_{18} column and UV detection. As part of the development of the bio-assay, the stability of CPA and CPT in water, plasma and blood was investigated. In *in vivo* experiments the analytical method was applied to determine the pharmacokinetics of both compounds in rats.

EXPERIMENTAL

Chemicals

N^6-Cyclopentyladenosine (CPA), N^6-cyclohexyladenosine (CHA) and 8-cyclopentyltheophylline (CPT) (for structures, see Fig. 1) were obtained from Research Biochemicals (Natick, MA, USA). Methanol and ethyl acetate were purchased from Baker Chemicals (Deventer, Netherlands) and distilled prior to use. Acetonitrile (HPLC grade) was obtained from Westburg (Leusden, Netherlands). Water was drawn from a Milli-Q system (Millipore SA, Molsheim, France). All other chemicals used were of analytical grade (Baker, Deventer, Netherlands)

Instrumentation

The liquid chromatographic system consisted of an SF-400 pump (Applied Biosystems, Ramsey, NJ, USA), a WISP-712B autosampler (Millipore-Waters, Milford, MA, USA) and a Spectroflow 757 variable-wavelength UV detector (Applied Biosystems) set at 269 nm.

Chromatography was performed on a stainless-steel Microsphere C_{18} 3-μm cartridge column (100 mm × 4.6 mm I.D.) (Chrompack, Bergen Op Zoom, Netherlands) equipped with a guard column (20 mm × 2 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) packed with C_{18} (particle size 20–40 μm) (Chrompack, Bergen Op Zoom, Netherlands). The analytical column was mounted in a laboratory-made water jacket, connected to a F3-CH cooling/heating bath (Haake, Karlsruhe, Germany). A constant column temperature of 27°C was maintained.

The mobile phase was 10 m*M* acetate buffer (pH 4.0)–methanol–acetonitrile (56:40:4, v/v/v). Mobile phase solvents were filtered through a 0.45 μm nylon filter (Alltech Applied Science, Breda, Netherlands), mixed and degassed with helium. Flow-rate was 0.50 ml/min.

Data processing was performed by a Chromatopack C-R3A reporting integrator (Shimadzu, Kyoto, Japan).

Standards

Stock solutions of CPA and CPT were pre-

pared at concentrations of 100 μg/ml and 275 μg/ml in water, respectively. The CPT stock solution contained 75 m*M* ethylenediamine. The CPA and CPT stock solutions were diluted with water to concentrations in the range of 0.010–2.00 μg/ml and of 0.025–50.0 μg/ml, respectively. A stock solution of CHA of 100 μg/ml in methanol–water (1:1, v/v) was diluted with water to a concentration of 1.00 μg/ml for use as internal standard. Stored at 4°C these solutions remained stable for at least two months.

Extraction procedure

To 100 μl of blood hemolyzed in 500 μl of water in a glass centrifuge tube 50 μl of the internal standard solution (1.00 μg/ml CHA) were added. After mixing the sample was alkalinized with 50 μl of 3.0 *M* sodium hydroxide solution and 5 ml of ethyl acetate were added. After 30 s of extraction on a vortex-mixer and 15 min of centrifugation at 2000 *g* the organic layer was transferred to another centrifuge tube using Pasteur disposable pipettes. Ethyl acetate was removed under reduced pressure on a vortex vacuum evaporator (Buchler Instruments, Fort Lee, NJ, USA) at 30°C. The residue was reconstituted in 150 μl of methanol–water (1:2, v/v) of which a volume of 100 μl was injected onto the chromatographic system.

Calibration and validation

On each day of analysis a nine-point calibration curve was prepared by spiking 100 μl of blood hemolyzed in 400 μl of water with 50 μl of a CPA solution and 50 μl of a CPT solution. This resulted in CPA and CPT blood concentration ranges of 0.005–1.00 μg/ml and 0.0125–25.0 μg/ml, respectively. Samples were processed as described above and peak-height ratios of CPA/CHA and CPT/CHA were calculated. Calibration curves were constructed by weighted linear regression [weight factor: 1/(peak-height ratio)] because of the wide range of concentrations.

Extraction yields were determined at CPA blood concentrations of 0.010, 0.150, 0.750 μg/ml and CPT blood concentrations of 0.025, 0.150, 1.50, 15.0 μg/ml with the internal–external standard method. To determine the inter- and intra-assay variability quality control samples were prepared at the same CPA and CPT blood concentrations.

Stability of CPA and CPT in vitro

The stability of CPA and CPT *in vitro* was investigated by incubating the compounds at 37, 24 and 0°C in water, fresh whole blood and plasma, obtained from different rats. Blood was obtained by means of an aorta puncture and directly transferred to heparinized tubes. A volume of 2.95 ml of blood, plasma or water was spiked with 50 μl of a CPA or CPT solution resulting in concentrations of 0.5 μg/ml and 5 μg/ml, respectively.

During the experiment blood, plasma and water were continuously gently shaken. At regular time intervals 100 μl samples were taken and directly transferred to glass centrifuge tubes containing 500 μl of distilled water (0°C). Concentrations of CPA and CPT were assayed as described above.

Study in rats

Chronically instrumented male SPF rats of Wistar descent, weighing 200–250 g, were used throughout the experiments. Two days before the experiment the abdominal aorta was cannulated by an approach through both the left and right femoral artery for the determination of arterial blood pressure and serial blood sampling, respectively. The right jugular vein was implanted with a cannula for administration of the solution. At the day of the experiment the rats (unrestrained, conscious) received an intravenous infusion of 200 μg/kg CPA in 5 min or an infusion of 12 mg/kg CPT in 15 min. Blood samples with a volume of 100 or 200 μl were collected at fixed time points after starting the administration and were directly hemolyzed in glass centrifuge tubes containing 500 μl of distilled water at 0°C. The samples were stored at −35°C pending analysis as described above.

Following the administration of a single dose, heart rate and blood pressure were continuously monitored using a Nihon Kohden Polygraph system (Nihon Kohden, Tokyo, Japan). Hemody-

namic data were converted in a CED1401 interface (Cambridge Electronics Design, Cambridge, England), fed into an 80387 computer (Philips, Eindhoven, Netherlands) and stored on harddisk for off-line analysis. Data acquisition and reduction were performed with Spike2 computer software (Cambridge Electronics).

The pharmacokinetics of CPA and CPT were quantified for the individual rats by iterative computer fitting using Siphar software (Simed, Creteil, France). For CPA a bi-exponentional equation was required to describe the concentration–time course. The concentration–time profile of CPT was fitted best to a two-compartment pharmacokinetic model with nonlinear elimination. Pharmacokinetic parameters were calculated using standard procedures [20].

RESULTS AND DISCUSSION

Chromatography

Fig. 2 shows chromatograms for blank blood with internal standard CHA (A), blank blood which had been spiked with CPA and CPT (B), blood 3.2 min after the start of the CPA infusion (C) and blood 29 min after administration of CPT (D). CPA, CPT and internal standard had retention times of 8.2, 11.4 and 14.3 min, respectively. The peaks in the chromatograms were identified by comparing them with chromatograms of aqueous solutions of the compounds, in which only single peaks were observed. Peaks with retention times 7.5 and 9.9 corresponded to endogenous compounds. Injection of aqueous solutions of inosine and adenosine produced peaks with retention times of 2.6 and 2.9 min, respectively. The total run time was *ca.* 19 min. After injection of *ca.* 200 samples a decrease in retention occurred. This could be solved by flushing the column with 30 ml of methanol and re-equilibration with the mobile phase.

Although liquid–liquid extraction is not a specific sample clean-up procedure, none of the co-extracted substances seemed to interfere significantly with the determination of CPA and CPT in blood. Interference with small peaks from blank blood could be avoided by slight adjustments in the mobile phase composition and the column temperature.

After intravenous administration of CPA to

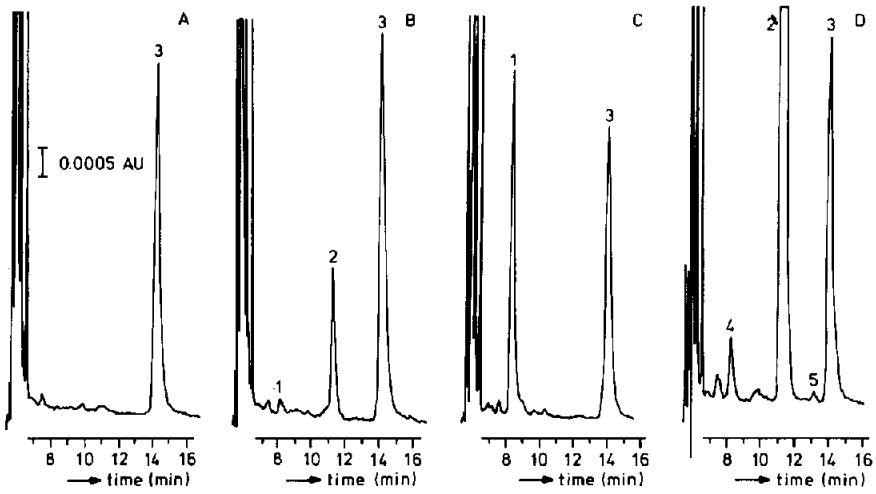

Fig. 2. Chromatogram of an extract of blank blood with internal standard CHA (0.500 μg/ml) (A), blood spiked with CPA (0.0125 μg/ml) and CPT (0.150 μg/ml) (B), blood obtained from a rat 3.2 min after the start of the administration of CPA (CPA concentration was 0.293 μg/ml) (C), and blood from a rat 29 min after having received an infusion of 12 mg/kg CPT during 15 min. CPT concentration was 7.73 μg/ml. The concentrations of the presumed metabolites were 149 ng/ml (8.3 min) and 22 ng/ml (13.3 min), expressed as concentration units of the parent compound (D). Peaks: 1 = CPA; 2 = CPT; 3 = CHA; 4 = CPT metabolite I; 5 = CPT metabolite II.

TABLE I

VALIDATION OF THE DETERMINATION OF CPA AND CPT: RECOVERY, INTRA-ASSAY AND INTER-ASSAY VARIABILITY, ACCURACY AND COEFFICIENTS OF VARIATION

Compound	Added (μg/ml)	Recovery (mean ± S.D., $n=3$) (%)	Intra-assay ($n=6$)			Inter-assay ($n=6$)		
			Found (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (%)	Found (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
CPA	0.0100	82.3 ± 4.5	0.0105 ± 0.0008	7.6	105	0.0101 ± 0.0006	5.9	101
	0.150	80.9 ± 1.4	0.146 ± 0.008	5.5	98	0.147 ± 0.004	2.7	98
	0.750	77.3 ± 1.5	0.758 ± 0.016	2.1	101	0.728 ± 0.033	4.5	97
CPT	0.025	66.7 ± 2.8	0.0254 ± 0.0020	7.9	102	0.0261 ± 0.0023	8.8	104
	0.150	71.2 ± 3.3	0.146 ± 0.005	3.4	97	0.148 ± 0.008	5.4	99
	1.50	84.0 ± 7.4	1.55 ± 0.06	3.9	103	1.51 ± 0.08	5.3	101
	15.0	83.6 ± 1.2	15.2 ± 0.5	3.3	101	14.9 ± 0.5	3.4	99

the rat no extra peaks were observed in the chromatogram, whereas for CPT two additional peaks appeared with retention times of 8.3 and 13.3 min (Fig. 2D). The two peaks are presumably two metabolites of CPT, considering their absence in blank blood and their concentration–time profile after administration of CPT.

Table I demonstrates the recovery after extraction, the accuracy and the reproducibility of the analysis. For CPA and CPT extraction yields were greater than 77 and 66% in the concentration ranges of 0.010–0.75 μg/ml and 0.025–15 μg/ml, respectively. In the same concentration ranges intra- and inter-day coefficients of variation were less than 9%. The weighted linear regression equations (mean ± S.D.; $n = 7$) for CPA and CPT were $y = (3.01 \pm 0.11)x + (0.0034 \pm 0.0049)$ and $y = (2.28 \pm 0.30)x + (0.0107 \pm 0.0112)$, respectively, with y being the peak-height ratio and x the blood concentration in μg/ml. Corresponding coefficients of correlation were at least 0.998 and 0.9995, indicating linearity of the method.

Using 100 μl of blood the limits of detection for CPA and CPT were 3.3 and 6.2 ng/ml, respectively (signal-to-noise ratio = 3). In 200-μl blood samples the corresponding limits of reliable determination were arbitrarily set at 2.5 and 5.0 ng/ml.

Stability of CPA and CPT in vitro

In rat whole blood at 37°C CPA disappeared with a mean half-life ± S.D. of 24 ± 2 min. At 23°C the initial (0–30 min) mean half-life ± S.D. was 62 ± 6 min. No degradation of CPA was observed in blood at 0°C and in plasma and water in the temperature range of 0–37°C. CPT was stable in blood, plasma and water at all three temperatures investigated over a period of 4 h.

Although N^6-substituted analogues of adenosine are claimed to be metabolically stable compounds [21], the half-life of CPA *in vitro* appears to be short with a value of 24 min. The temperature dependency of *in vitro* disappearance suggests that enzymatic degradation occurs. In human blood the half-life of adenosine is 1–2 s at 37°C [22], due to a rapid reduction by either facilitated transport into blood cells, where enzymatic phosphorylation and deamination occur, or degradation by adenosine deaminase in plasma [23]. N^6-substituted adenosine derivatives have been reported to be inhibitors of adenosine uptake by interaction with the nucleoside transporter [24,25]. To our knowledge transport of CPA into blood cells has not been described. Consequently, it remains unclear whether CPA is degraded either by cytosolic enzymes in blood cells or by extracellular enzymes present on the membranes of blood cells.

Study in rats

Fig. 3 shows a representative blood concentration–time profile for an intravenous infusion of CPA. The values of clearance, volume of distribution at steady-state and terminal half-life were 58 ± 2 ml/min/kg, 260 ± 20 ml/kg and 6.2 ± 0.2 min, respectively (mean ± S.E., $n = 5$). In Fig. 3 also the time-course of the heart rate is depicted. After the start of the infusion the heart rate immediately declined to a level of 40% of the pre-infusion level. With decreasing CPA blood concentrations, after termination of the infusion, the heart rate gradually returned to base line level.

Representative blood concentration–time profiles for CPT and its two presumed metabolites are shown in Fig. 4 for a rat which had received

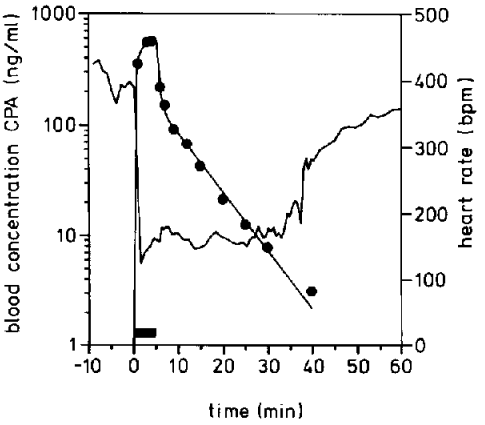

Fig. 3. Blood concentration and heart rate *versus* time profile for a rat which had received 200 μg/kg CPA intravenously during 5 min. The solid line represents the best fit to the blood concentrations (●) according to the two-compartment pharmacokinetic model.

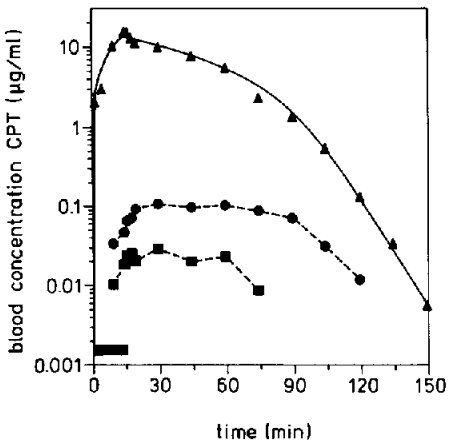

Fig. 4. Representative CPT (▲), metabolites I (●) and II (■) blood concentration *versus* time profiles for a rat which had received an intravenous infusion of 12 mg/kg CPT in 15 min. The solid line represents the pharmacokinetic model fitted to the observed CPT blood concentrations. Metabolite concentrations are presented in concentration units of the parent compound.

12 mg/kg CPT. All individual blood concentration–time curves revealed nonlinear kinetics of CPT with a maximal elimination rate of 5.1 ± 0.5 µg/ml/min/kg, a Michaelis–Menten constant of 1.6 ± 0.2 µg/ml and a volume of the central compartment of 33 ± 4 ml/kg (mean ± S.E., $n = 6$). As an antagonist, CPT showed only slight transient increases in heart rate during the infusion (data not shown). An estimate for the *in vivo* potency of the antagonist CPT can only be obtained after concomitant administration of the agonist CPA. Consequently, the bio-assay will be particularly useful in such a pharmacodynamic interaction study, allowing simultaneous determination of CPT and CPA blood concentrations.

The maximal elimination rate and Michaelis–Menten constant appear to be comparable with those of theophylline [26]. This might indicate similar metabolic pathways for CPT and theophylline. Accordingly, the structure of the two presumed metabolites observed in the chromatogram could correspond to demethylated metabolites of CPT. However, the precise structure of these compounds remains to be identified.

CONCLUSION

This report describes a simultaneous assay of adenosine A_1 receptor ligands CPA and CPT. The short time of analysis, the sensitivity, the accuracy and the reproducibility make this method particularly useful in pharmacokinetic studies of CPA and CPT in the rat. After slight modifications in the mobile-phase composition this bioanalysis may also be applied in disposition studies of other N^6-alkyl or N^6-cycloalkyl substituted adenosine analogues. In our laboratory the analytical method has also been used in pharmacokinetic studies of the N^6-substituted A_1 agonists (*R*)-phenylisopropyladenosine (*R*-PIA) and cyclohexyladenosine (CHA).

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