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Short communication

Determination of the adenosine A_1 agonist N^6-cyclopentyladenosine in rat blood by solid-phase extraction and HPLC

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Abstract

A solid-phase extraction procedure has been developed for the isolation of the adenosine A1 receptor agonist N^6-cyclopentyladenosine from rat blood. The biological samples were spiked with N^6-cyclopentyladenosine and the analogue N^6-cyclohexladenosine (internal standard), diluted with sodium hydroxide, loaded onto disposable cartridges with subsequent desorption with methanol and analysis by HPLC. The performance of columns pre-packed with different C18-bonded silica phases or with a polymeric reversed-phase sorbent (Oasis HLB) was assessed. The highest extraction efficiencies (recovery rates $> 83.3\%$) for the two N6-alkyl substituted adenosines were achieved by the Oasis HLB cartridges. In addition, the polymeric sorbent provided reproducible recoveries (relative standard deviation $< 4.8\%$), whereas large variations (relative standard deviation values, 9–16.3%) in the extraction yields were observed using the conventional silica-based C18 cartridges. The described sample preparation method is rapid, simple, selective and it is suitable for pharmacokinetic studies.

Keywords: Adenosine A_1 receptor agonist; N^6-cyclopentyladenosine; N^6-cyclohexyladenosine; Rat blood; Solid-phase extraction; Polymeric sorbent

1. Introduction

The potential of adenosine receptors as drug targets has been recognized for some time [1,2]. Four receptor subtypes have been identified, A_1, A_{2A}, A_{2B} and A_3, the first two having received the most attention [3]. At present, the only approved adenosine receptor ligand is adenosine itself, used for the treatment of supraventricular tachycardia [2]. Because of adenosine extremely short half-life, adenosine analogues with improved stability to adenosine-metabolizing enzymes have been synthesized [2,4]. The N^6-substituted derivatives of adenosine have been shown to be potent and

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selective agonists for the adenosine A_1 receptor, N^6-cyclopentyladenosine (CPA) being the reference compound [4–6]. Although CPA exhibits higher half-life values with respect to adenosine [4,6,7], its pharmacokinetic profile is not satisfactory [4,7].

Few reports on the bioanalysis of synthetic A_1 adenosine receptor agonists have appeared in the literature. CPA has been determined in rat and human blood by high-performance liquid chromatography (HPLC) after preliminary liquid–liquid extraction [4,6,7]. This pretreatment procedure has inherent disadvantages including possible analyte losses due to emulsion formation [8], time-consuming and laborious sample manipulations, which reduce the assay accuracy and precision and hamper its applicability to pharmacokinetic studies requiring high sample throughput. In addition, the liquid–liquid extraction process is difficult to automate.

The present paper describes a rapid and simple solid-phase extraction (SPE) procedure, using a novel polymeric reversed phase sorbent, for the efficient isolation of CPA from rat whole blood prior to HPLC analysis. The method was used to study the pharmacokinetic of CPA in rat blood and the influence of hydroxypropyl-β-cyclodextrin on CPA degradation.

2. Experimental

2.1. Materials

N^6-CPA and N^6-cyclohexyladenosine (CHA) (see Fig. 1 for structures) were purchased from Sigma-Aldrich (Milan, Italy). Methanol and acetonitrile were HPLC-grade from Merck (Darmstadt, Germany). Water was purified by a Modupure Plus system (Continental Water Systems, San Antonio, TX). All other chemicals were of analytical-reagent grade (Carlo Erba, Milan). Bond-Elut C_{18}-silica cartridges (sorbent mass, 200 mg) were obtained from Varian (Harbor City, CA). Isolute C_{18}- and Isolute MF C_{18}-silica cartridges (sorbent mass, 200 mg) were from International Sorbent Technology (Hengoed, UK). Polymeric Oasis hydrophilic–lipophilic balanced (HLB) cartridges (sorbent mass, 60 mg) were supplied by Waters (Milford, MA).

2.2. High-performance liquid chromatography

The HPLC apparatus comprised a Model LabFlow 3000 pump (LabService Analytica, Bologna, Italy), a Model 7125 injection valve with a 200 μl sample loop (Rheodyne, Cotati, CA) and a Model 975-UV variable wavelength UV–Vis detector (Jasco, Tokyo, Japan) set at 269 nm. Data acquisition and processing were accomplished with a personal computer using Borwin software (JBMS Developpements, Le Fontanil, France). Sample injections were effected with a Model 80665 syringe (Hamilton, Bonaduz, Switzerland). Separations were performed according to the method reported in the literature [6] on a 5-μm Hypersil BDS C_{18} column (150 × 4.6 mm ID; Hypersil, Runcorn, UK) fitted with a guard column (LiChrospher RP-18, 5-μm particles, 4 × 4 mm ID; Merck, Darmstadt) and eluted with aqueous sodium acetate (pH 4.0; 0.01 M)-methanol–acetonitrile (55:40:5 v/v/v). The mobile phase was filtered through 0.2-μm nylon filters (Alltech Italia, Sedriano). The column temperature was maintained at 35°C using a Model 7990 Space Column Heater (Jones Chromatography, Hangoed). Chromatography was performed under isocratic conditions, at a flow-rate of 0.9 ml/min. The identity of the separated peaks was assigned by co-chromatography with the authentic standards. Quantification was on the basis of peak area for the ratio CPA/CHA (internal standard).

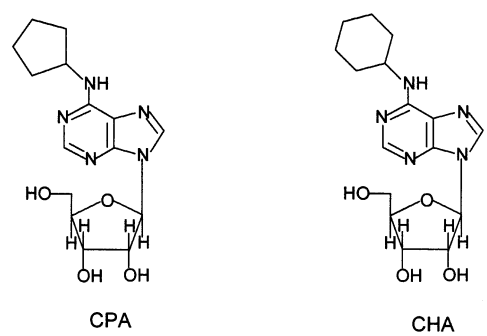

Fig. 1. Chemical structures of CPA and CHA.

2.3. Standard solutions

A stock solution of CPA containing ca. 100 μg/ml of the reference material was prepared in purified water and serially diluted to give working standards in the concentration range of 0.2–30.00 μg/ml. A stock solution of CHA was prepared at a level of 100 μg/ml in methanol–water (1:1 v/v) and diluted with purified water to a concentration of 1 μg/ml. In order to avoid adsorption losses, diluted standard solutions were prepared daily in glass tubes. All standard solutions were kept at 4°C.

2.4. Sample preparation

Heparinized rat whole blood (200 μl) was first hemolyzed in 1.4 ml of purified water (4°C) and 50 μl of the internal standard solution (1.2 μg/ml CHA) were added. After mixing on a vortex mixer, the sample was alkalinized with 2 N sodium hydroxide (250 μl) and applied directly by positive pressure (flow-rate, ca. 1 ml/min) to a pre-conditioned (2 ml of methanol and then 2 ml of water) Oasis HLB cartridge. The extraction column was washed with 4 ml of water and, after light drying by vacuum, eluted with 2 ml of methanol. The latter fraction was reduced to dryness under a nitrogen stream, the residue reconstituted in 400 μl of mobile phase and a portion (100 μl) injected onto the HPLC column.

2.5. Assay validation

The test samples were prepared by spiking rat whole blood (200 μl) hemolyzed in purified water (1.4 ml) with 100 μl aliquots of standard solutions corresponding to CPA blood concentrations of 0.1 and 1.0 μg/ml and CHA blood concentration of 0.3 μg/ml. The samples were processed as described above. The recovery was calculated as the percent ratio of the peak areas of analytes extracted from the test samples to analytes dissolved in the mobile phase.

Calibration standards were prepared by spiking blood extracts with the internal standard (CHA, 0.3 μg/ml) and with known amounts of CPA corresponding to blood concentrations in the range of 0.05–1.2 μg/ml. These solutions were analyzed by HPLC and the response factor relative to the internal standard was determined.

The chromatographic precision was evaluated by repeated analyses ($n = 5$) of the same sample solution obtained from rat whole blood. The method precision was calculated by Oasis HLB extraction and HPLC assay of independent samples ($n = 5$) from the same blood specimen.

2.6. In vitro stability assay

The kinetic of CPA degradation was studied in fresh whole blood obtained from male Sprague–Dawley rats. To 2.95 ml of heparinized whole blood, a 50 μl aliquot of a CPA solution was added resulting in a concentration of 0.6 μg/ml. The obtained sample was incubated at 37°C under gentle shaking. At determined time intervals, 200 μl aliquots were withdrawn from the sample and immediately hemolyzed in glass tubes containing 1.4 ml of purified water (4°C). The resulting solution was then assayed for CPA as outlined above. The degradation half-life ($T_{1/2}$) was obtained by a least-square linear regression analysis of a plot of logarithmic CPA concentration versus time.

3. Results and discussion

3.1. Solid-phase extraction

Previuosly published procedures for the HPLC assay of CPA in blood require a preliminary sample preparation step based on liquid–liquid extraction followed by centrifugation and solvent removal on a vortex vacuum evaporator [4,6,7]. In order to overcome the drawbacks of this pretreatment procedure, in the present work purification methods based on SPE were investigated. Disposable C_{18}-bonded silica cartridges (Isolute C_{18}) were initially selected since they provide the broadest applicability for clean-up of biological fluids [8,9]. Preliminary experiments indicated that CPA and CHA (internal standard) are efficiently extracted from water by the C_{18} sorbent and quantitatively eluted (recovery rates > 82.6%) from the cartridge with methanol.

Table 1
Percent recovery of CPA and CHA from spiked rat blood (0.2 ml) extracted by different cartridges

Compound	Concentration (μg/ml)	% Recovery[a] (relative S.D.)		
		C_{18} cartridges	Tandem C_{18} cartridges	Oasis HLB cartridges
CPA	0.1			83.3 (4.8)
	1.0	28.8 (16.3)	77.7 (15.8)	88.6 (2.5)
CHA	0.3	29.3 (9.1)	81.1 (15.4)	93.1 (4.0)

[a] Each value is the mean of at least four determinations.

Additional experiments were then performed on rat whole blood spiked with CPA (1.0 μg/ml) and CHA (0.3 μg/ml). As shown in Table 1, only 28.8% of CPA and 29.3% of CHA were recovered from the blood samples with the C_{18} sorbent. Losses were traced to incomplete analyte adsorption on the extraction column during sample loading, due to interference from matrix constituents. In order to overcome this problem, the use of two C_{18} cartridges connected in series was examined. This system improved the recoveries of the two adenosine analogues to more than 77.7%, although a high dispersion of the yield values was observed (Table 1). Switching to a C_{18} silica cartridge from a different manufacturer (Bond-Elut C_{18}) did not produce any significant improvement in CPA and CHA recoveries. Non-endcapped monofunctional (lower carbon loading) C_{18}-bonded silica has been reported to enhance the retention of polar compounds through additional interactions associated with the very accessible silanol groups [10]. However, under the elution conditions described in this study, the non-endcapped monofunctional C_{18} sorbent (Isolute MF C_{18}) did not achieve better results (recovery values $< 26.7\%$) than the endcapped trifunctional (higher carbon loading) phases (Isolute C_{18} and Bond-Elut C_{18}). In addition, the former sorbent was unstable at the alkaline pH of the applied sample, producing cloudy extracts due to the stripping of the stationary phase.

At this point of the experimental work, cartridges pre-packed with a novel HLB copolymer became available. Compared with the traditional C_{18}-bonded silica, this sorbent (Oasis HLB) provides greater pH stability and enhanced analyte retention [9]. As reported in Table 1, the use of the Oasis HLB cartridge increased the recoveries of CPA and CHA spiked in rat whole blood to 83.3–88.6% and 93.1%, respectively. Moreover, superior reproducibility was attained by the Oasis HLB sorbent compared to the traditional C_{18} silica packing (Table 1). The proposed SPE procedure produced higher CPA recovery than the liquid–liquid extraction method (CPA mean recovery value, 80.2%) reported in the literature [6] and provided a simpler sample handling scheme. The recovery of CHA, used as internal standard, was not determined in the previously published investigation [6]. Conversely, in this study a 93.1% recovery value for CHA from rat blood was obtained using the Oasis HLB cartridge.

The reproducibility of the procedure presented here was evaluated by replicate assays of a blood sample containing CPA at a level of 0.5 μg/ml. The obtained relative standard deviation values were 2.3 and 2.8%, for the chromatographic and the method precision, respectively.

Calibration curves ($n = 5$) were linear in the range of 0.05–1.2 μg/ml (slope, $177\,421.9 \pm 14\,824.1$) with correlation coefficients greater than 0.998. The intercepts with the y-axis were not significantly different from zero ($P > 0.05$).

Fig. 2 shows the comparative HPLC traces of blank (Fig. 2(A)) and spiked (Fig. 2(B)) rat blood samples extracted according to the method developed in this study. The chromatograms demonstrate that there is no interference from matrix components in the CPA and CHA retention windows. Sample processing by liquid–liquid extraction [6] required slight adjustments in the chromatographic conditions (eluent composition,

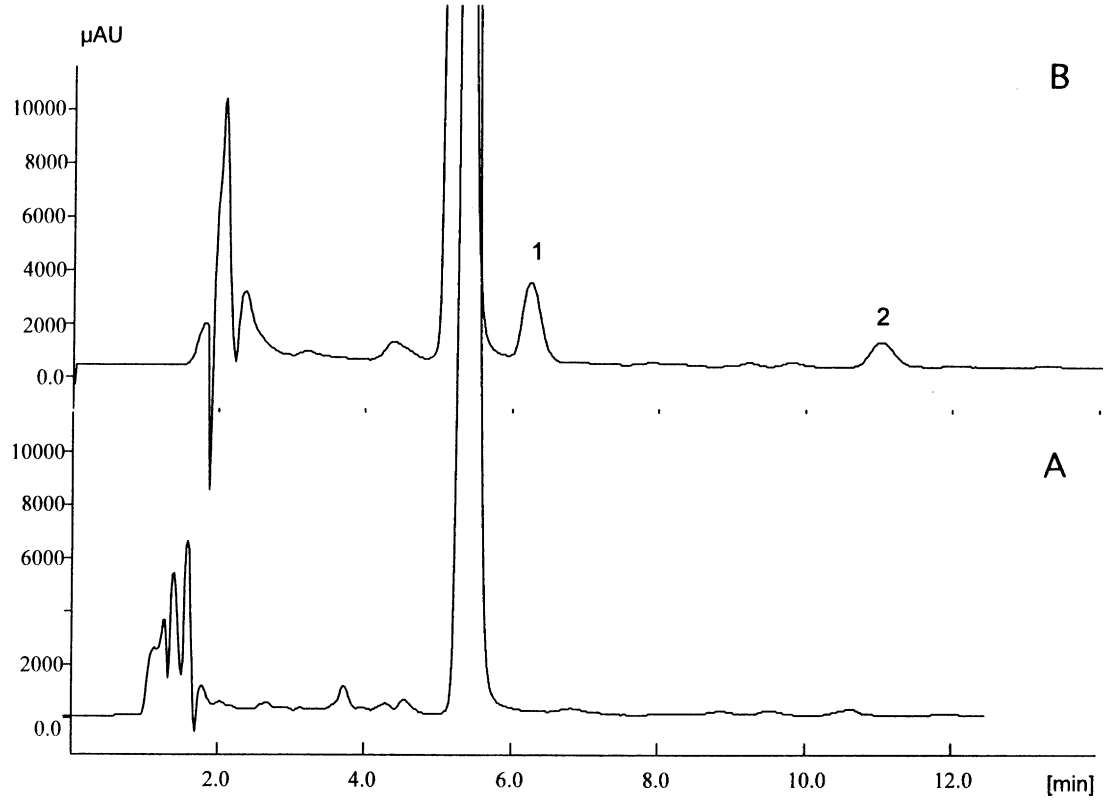

Fig. 2. HPLC chromatograms of (A) blank rat blood extract and (B) the same sample spiked with CPA (1.0 μg/ml) and CHA (0.3 μg/ml). Peaks: 1 = CPA, 2 = CHA. Operating conditions as described in Section 2.

column temperature) to prevent overlapping of small peaks from blank blood with the adenosine analogues. This was not found to be the case with extraction on Oasis HLB cartridges, thus indicating that this technique afforded a more effective purification of the biological matrix.

3.2. Application

The SPE procedure devised in this investigation was applied to the determination of the in vitro pharmacokinetic profile of CPA in rat fresh whole blood. The CPA concentration used corresponded to the level, which elicits maximal activity in pharmacodynamic studies [4]. Peaks corresponding to possible degradation products were not observed in CPA and CHA retention windows. The time course of CPA is described by a first-order kinetic with an half-life of 21.9 ± 1.3 min. This value compared well with those (24 and 25 min) previously reported by Mathôt et al. [6] and Pavan and Ijzerman [4]. This result confirmed the validity of the proposed method.

The influence of hydroxypropyl-β-cyclodextrin (one of the most commonly used cyclodextrin in pharmacokinetic studies [11,12]) on the stability of CPA in rat blood was investigated. The degradation kinetic of CPA as an aqueous solution containing 3% hydroxypropyl-β-cyclodextrin was not different ($T_{1/2} = 20.7 \pm 1.8$ min) from that observed in the absence of cyclodextrin.

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