ELSEVIER

Journal of Chromatography B, 658 (1994) 207–212

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Liquid chromatographic determination of the adenosine receptor agonist CGS 21680 in blood using on-line solid-phase extraction on a phenylboronic acid support and fluorescence detection

H. Irth[a,*], A. Cleton[b], R.A.A. Mathôt[b], M. Danhof[b], A.P. IJzerman[b], U.R. Tjaden[a], J. van der Greef[a]

[a]*Leiden/Amsterdam Center for Drug Research, Division of Analytical Chemistry, University of Leiden, P.O. Box 9502, 2300 RA Leiden, Netherlands*

[b]*Leiden/Amsterdam Center for Drug Research, Division of Pharmacology, University of Leiden, P.O. Box 9503, 2300 RA Leiden, Netherlands*

First received 2 February 1994; revised manuscript received 15 April 1994

Abstract

An analytical method is described for the selective determination of A_1 or A_2 adenosine receptor agonists in blood. By implementing solid-phase extraction using immobilized-phenylboronic acid (PBA) in sample pretreatment, all adenosine derivatives are retained via their intact *cis*-diol group. On-line desorption of the analytes from the PBA support to the C_{18} analytical column is performed by injection of a small plug of perchloric acid. Fluorescence and UV detection are employed for the different adenosine derivatives. The method is applied to the determination of 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-N-ethylcarboxyamidoadenosine (CGS 21680, I) in blood using fluorescence detection. The only off-line sample handling step is the extraction of blood with ethyl acetate and subsequent evaporation of the extraction solvent. The detection limit of the method was 0.25 ng (signal-to-noise ratio 3:1) and the determination limit for I in blood (pretreatment of 100 μl) was 5 ng/ml. The method was validated and used to study the pharmacokinetics of I in rats.

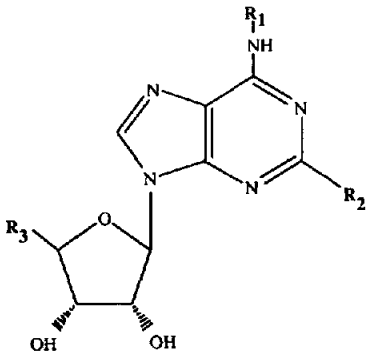
1. Introduction

Adenosine is known to mediate its effects via at least two extracellular adenosine receptor subtypes, A_1 and A_2 [1,2]. In the last decade derivatives of adenosine were synthesized with different potency and selectivity for the two receptors. These compounds may be used for the treatment of different cardiovascular diseases [3,4]. 2-[*p*-(2-Carboxyethyl)phenylethylamino]-5′-N-ethylcarboxyamidoadenosine (CGS 21680, I, see Fig. 1), one of the most potent (K_i = 19 n*M*) and selective A_2 adenosine agonists (74-fold selectivity over A_1 receptors) [5], was shown to reduce the peripheral resistance [6] and to lower blood pressure in spontaneously hypertensive rats [7].

The most effective A_1 or A_2 adenosine re-

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00216-R



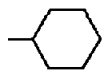
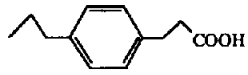
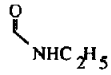
	CGS 21680, I	CHA, II
R_1	H	
R_2	NH  COOH	H
R_3		CH_2OH

Fig. 1. Structure of A_1 or A_2 adenosine receptor agonists I and II.

ceptor agonists are modified at R1, R2 or R3 (see Fig. 1) the only common structural element being the *cis*-diol group of the ribose moiety. The analytical methods described for adenosine derivatives are based on high-performance liquid chromatography (HPLC) in combination with UV absorbance or fluorescence detection [8–11]. Sample pretreatment techniques employed involve liquid–liquid or solid-phase extraction (SPE). In our laboratory, a large number of adenosine derivatives modified at R1, R2 or R3 are under investigation. To avoid the development of new analytical methods for each compound and to increase the selectivity of the extraction method, a sample handling technique was devised which made use of the *cis*-diol group at the ribose moiety. By implementing SPE using immobilized-phenylboronic acid (PBA) in sample pretreatment, all active adenosine derivatives are retained via their intact *cis*-diol group.

Solid-phase extraction using PBA supports is widely applied in the selective isolation of compounds possessing a *cis*-diol group and can easily be used in the coupled-column mode [12–14]. In the present paper the physico-chemical parameters which influence retention on and on-line desorption from the PBA support were studied and a bioanalytical method for the determination of I in blood is described.

2. Experimental

2.1. Chemicals

Compound I was a gift from Ciba Geigy (Dr. R.L. Webb). N6-Cyclohexyladenosine (CHA, II), the internal standard, was obtained from Boehringer Mannheim (Mannheim, Germany). Acetonitrile (Rathburn, Walkerburn, UK) was of HPLC grade. All other organic solvents, perchloric acid, ammonium acetate, citric acid and sodium acetate were of analytical grade purchased from Baker (Deventer, Netherlands). Ethyl acetate was distilled before use. The perchloric acid solution used for on-line desorption was prepared from concentrated acid (80%, v/v) by diluting with deionized water (Milli-Q water purification system, Millipore, Bedford, MA, USA) until pH 0.5 was reached.

2.2. Apparatus

The analytical system (scheme, see Fig. 2) consisted of a Promis (Spark Holland, Emmen, Netherlands) autosampler equipped with a 1.0-ml sample loop, two six-port Rheodyne (Berkeley, CA, USA) injection valves, a 100-μl loop to contain the perchloric acid for the desorption of the adenosine derivatives, a 10×4.0 mm I.D. stainless-steel pre-column and holder (Chrompack, Middelburg, Netherlands) and a Nucleosil C_{18} (Macherey-Nagel, Düren, Germany) column (250×4.0 mm I.D., 5-μm particles). The carrier solution for preconcentration was delivered by a Kratos ABI Spectroflow 400 pump (Ramsey, NJ, USA), the LC eluent by a Gilson 302 HPLC pump (Villiers-le-Bel, France) at a flow-rate of

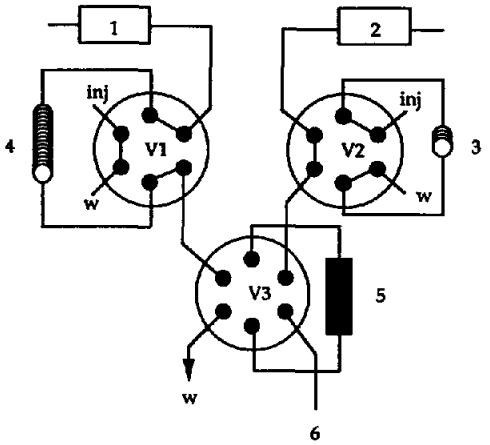


Fig. 2. Scheme of the analytical system. (1) Preconcentration pump; (2) HPLC pump; (3) 100-μl injection loop for perchloric acid; (4) sample injection loop (1.0 ml); (5) PBA-precolumn; (6) to analytical column/detector; (V1,V2,V3) six-port injection valves; (w) waste.

1.0 ml/min. The preconcentration pump was connected to a Rheodyne solvent-selection valve.

The LC eluent was acetonitrile–sodium acetate (50 m*M*, pH 4.0) (67:23, v/v). Fluorescence detection was performed with a Perkin-Elmer LS-4 fluorescence detector (Beaconsfield, UK) operated at an excitation wavelength of 262 nm and an emission wavelength of 345 nm. II was detected with a Kratos Spectroflow 773 UV detector at 269 nm which was switched in series with the fluorescence detector.

2.3. Sample pretreatment

As an internal standard 100 ng of II in 20 μl of methanol was added to 100 μl of blood hemolized in 400 μl of water. After the addition of 200 μl citrate buffer (0.5 *M*, pH 4.0) and mixing for 10 s, this mixture was extracted with 5 ml ethyl acetate for 30 s on a vortex-mixer. After centrifugation for 10 min at 2000 *g*, the organic layer was separated and evaporated in a vortex evaporator (Haake Büchler Instruments, Lenexa, KS, USA) at *ca.* 45°C. The residue was dissolved in 1.25 ml of ammonium acetate (0.25 *M*, pH 8.8) and injected onto the Si100 polyol-phenylboronic acid (PBA) substituted silica pre-column (Serva, Heidelberg, Germany) using a 1.0-ml loop.

The PBA support was slurry-packed into the stainless steel pre-column. The sample was injected into the carrier solution, 0.5 *M* ammonium acetate pH 8.8, and loaded onto the PBA pre-column at a carrier flow-rate of 0.15 ml/min. The column was washed with 2 ml of ammonium acetate. On-line desorption of I to the analytical column was carried out by injecting a plug of 100 μl of 1 *M* perchloric acid into the LC-stream, switching to the pre-column to desorb the pre-concentrated analytes and transferring them to the analytical column [15,16].

2.4. Drug monitoring

To monitor the concentration–time profile of I, arterial blood samples of 100 μl were collected at fixed time intervals after drug administration to a male Wistar rat (225 g). The blood samples were immediately hemolyzed in 400 μl of water and stored at −35°C until analysis.

3. Results and discussion

3.1. Optimization of parameters influencing retention on phenylboronic acid-modified silica

The dependence of the binding of analytes possessing vicinal *cis*-diol groups to boronic acid modified supports on the pH value of the sample solution has been investigated. An optimum pH of 8.8 (ammonium acetate buffer, 0.25 *M*) yielded an average recovery of 97.0% ($n = 5$). After sorption of the analytes to the PBA support, the pre-column could be washed with 3 ml of ammonium acetate buffer without loss in recovery. To decrease non-selective binding to the support backbone, addition of organic modifiers such as methanol to the washing solution was considered. Up to a methanol content of 5% no significant reduction in recovery was observed. At 20% methanol the recovery of I was reduced from 97% to 78% (wash volume 2 ml). For routine analysis of I no organic modifiers were added to the washing solution, since in both

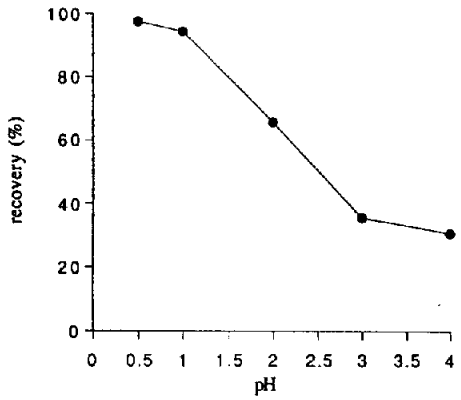


Fig. 3. pH dependence of desorption of I from PBA-silica.

fluorescence and UV absorbance detection no interfering compounds were observed. For the analysis of more polar adenosine derivatives addition of small percentages of methanol may be beneficial for the removal of sample components adsorbed non-selectively to the support backbone.

The efficiency of on-line desorption to the C_{18} analytical column was strongly pH dependent. Fig. 3 shows the recovery of desorption from the PBA-support in dependence of the pH value. At pH values between 2 and 4 recoveries were lower than 65% and rather broad peaks were obtained. Only at strongly acidic pH values (0.5–1) both recovery and peak width were satisfactory. To avoid deterioration of column performance at these low pH values, a plug injection technique [15,16] was used applying 100 μl of 1 *M* perchloric acid to the PBA support.

3.2. Sample pretreatment and chromatography

The solid-phase extraction procedure using a PBA support was automated, the only off-line step being the extraction of blood with ethyl acetate and evaporation of the residue. Compound II was used as internal standard to control the sample pretreatment procedure. Compounds retained by the PBA pre-column were desorbed on-line to the C_{18} analytical column and detected by fluorescence detection (for I) and UV detection (for II), both detectors being connected in series. No deterioration of the efficiency of the analytical column was observed due to the plug injections with perchloric acid. The PBA pre-column could be used for 200 injections before replacement was required.

3.3. Validation

The method was validated by six-fold assay on three consecutive days of blank blood to which different amounts of I and the internal standard had been added. Blood samples of 100 μl were spiked with concentrations ranging from 10 to 1000 ng/ml covering the relevant concentration range.

A typical regression line with the standard deviation of slope and intercept is: $y = (0.0083 \pm 0.0005)x - (0.033 \pm 0.004)$ where y represents the peak-area ratio and x the concentration in ng/ml. The results of the validation are summarized in Table 1. All calculations were performed using weighted regression. The detector response was linear ($r = 0.9990$, $n = 5$) for I up to 1 μg/ml.

Table 1
Validation data for the determination of I in plasma

Concentration (ng/ml)	C.V. (%, $n = 5$)		Accuracy (%)	Recovery (mean ± S.D.)(%)
	day-to-day	within-day		
10	2.0	6.6	106.0	91.7 ± 4.8
100	4.4	5.9	94.3	95.1 ± 3.4
1000	5.0	3.7	106.3	93.2 ± 5.8

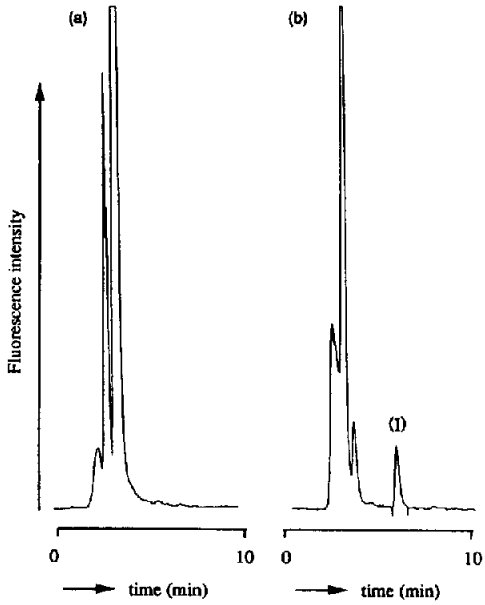


Fig. 4. Chromatogram showing (a) blank blood and (b) blood sample 55 min after infusion of I (concentration, 10 ng/ml).

The detection limit, based on a signal-to-noise ratio of 3, was 250 pg using fluorescence detection and 2.6 ng using UV absorbance detection. The corresponding minimal detectable concentration at a signal-to-noise ratio of 10 in blood (sample size 100 μl) was 5 ng/ml.

3.4. Drug monitoring

A representative chromatogram is shown in Fig. 4 for the determination of I in plasma 55 min after administration using II as internal standard. Fig. 5 presents an example of a concentration *vs.* time curve after a 15-min intravenous infusion of 1000 μg/kg of I. Compound I seems to be a rapidly eliminated drug with a short half-life.

4. Conclusions

By implementing complex formation with immobilized boronic acid during sample handling, adenosine derivatives acting as A_1 or A_2 adenosine receptor agonists could selectively be isolated from a blood extract. Coupled on-line to reversed-phase liquid chromatography, the analytical method provides high recoveries for I allowing its determination in blood at concentrations down to 5 ng/ml. The method can easily be adapted to new adenosine receptor agonists that contain an intact *cis*-diol group.

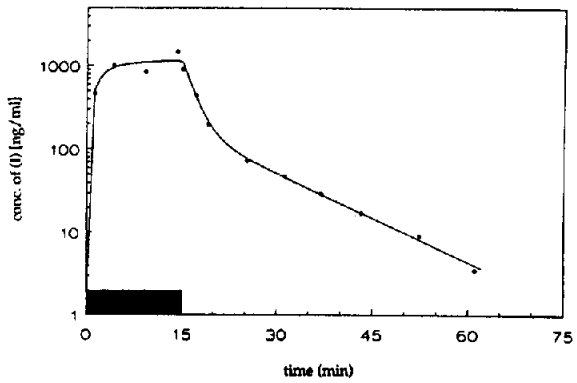


Fig. 5. Concentration–time curve after a 15 min (black box) intravenous infusion of 1000 μg/kg of I.

References

[1] C. Londos, D.M.F. Cooper and J. Wolff, *Proc. Natl. Acad. Sci. USA*, 77 (1980) 2551.
[2] D. van Calker, M. Müller and B. Hamprecht, *J. Neurochem.*, 33 (1979) 999.
[3] M. Williams, *Nucleosides and Nucleotides*, 10 (1991) 1087.
[4] K.A. Jacobson, B.K. Trivedi, P.C. Churchill and M. Williams, *Biochem. Pharmacol.*, 41 (1991) 1399.
[5] A.J. Hutchison, M. Williams and R. de Jesus, *J. Med. Chem.*, 33 (1990) 1919.
[6] J.I. Balwierczak, C.M. Krulan, Z.C. Wang, J. Chen and A.Y. Jeng, *J. Pharmacol. Exp. Ther.*, 251 (1989) 279.
[7] R.L. Webb, B.W. Barclay and S.C. Graybill, *J. Pharmacol. Exp. Ther.*, 259 (1991) 1203.
[8] T.A. Hartwick, A.M. Krstulovic and P.R. Brown, *J. Chromatogr.*, 186 (1979) 659.
[9] A. Colonna, T. Russo, F. Esposito, F. Salvatore and F. Cimino, *Anal. Biochem.*, 130 (1983) 19.
[10] J.P. Chovan, P.A. Zane and G.E. Greenberg, *J. Chromatogr.*, 578 (1992) 77.
[11] R.A.A. Mathôt, S. Appel, E.A. van Schaick, W. Soudijn, A.P. IJzerman and M. Danhof, *J. Chromatogr.*, 620 (1993) 113.

[12] E.H. Pfadenhauer and S.-D. Tong, *J. Chromatogr.*, 162 (1979) 585.
[13] E. Hagemeier, K.-S. Boos, E. Schlimme, K. Lechtenbörger and A. Kettrup, *J. Chromatogr.*, 268 (1983) 291.
[14] E. Schlimme, K.-S. Boos, E. Hagemeier, K. Kemper, U. Meyer, H. Hobler, T. Schnelle and M. Weise, *J. Chromatogr.*, 378 (1986) 349.
[15] C. Lipschitz, H. Irth, G.J. de Jong, U.A.Th. Brinkman and R.W. Frei, *J. Chromatogr.*, 471 (1989) 321.
[16] E. van der Vlis, H. Irth, U.R. Tjaden and J. van der Greef, *Anal. Chim. Acta*, 271 (1993) 69.