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Simultaneous determination of six adenyl purines in human plasma by high-performance liquid chromatography with fluorescence derivatization

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Abstract

A sensitive method was developed for the simultaneous determination of six adenyl purines in human plasma by high-performance liquid chromatography. The adenyl purines (adenine, adenosine, AMP, ADP, ATP and cyclic AMP) were derivatized using 2-chloroacetaldehyde for fluorescence detection, and the reaction and separation conditions were reinvestigated to improve sensitivity for small volume sample analysis. Each derivatized purine was separated on a Capcell Pack SG120A™ column with mobile phase consisting of 0.05 *M* citric acid–0.1 *M* dipotassium hydrogen phosphate (pH 4.0)–methanol (97+3). The detection limits were 100–1000 fmol/ml by fluorescence detection, some 500 times better than previous reports. The proposed method was applied to determine adenyl purines in human plasma. The purine levels were as follows: ATP (9.2–22.2 pmol/ml), ADP (5.5–22.2 pmol/ml), AMP (0.8–3.2 pmol/ml). Other purines, adenine, adenosine, cAMP were lower than 0.1 pmol/ml.

Keywords: Adenyl purines; Adenine; Adenosine; AMP; ADP; ATP; Cyclic AMP

1. Introduction

Adenyl purines are metabolites of ATP from internal and external cellular processes. LC methods with photometric detection [1–4] and fluorimetric methods with 2-chloroacetaldehyde derivatization have been reported [5–9] (Fig. 1). We have reported studies of the relationship between sperm mobility and adenyl purine levels in plasma and sperm extract

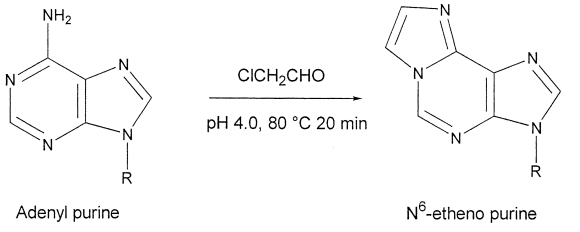

Fig. 1. Derivatization reaction of adenyl purine by 2-chloroacetaldehyde.

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in infertile patients [10,11], and we also studied the effect of caffeine on sperm motilities [9]. Here, we report the determination of five purines (ATP, ADP, AMP, adenosine, cAMP) by liquid chromatography (LC) with fluorescence detection after 2-chloroacetoaldehyde derivatization. The intended application of this method is the large-scale screening of male and female infertile patients. For this application, we had to improve the sensitivity of LC methods for monitoring adenyl purines in small volume samples (plasma, umbilical cord plasma, cerebrospinal fluid) from patients. Furthermore, the analysis time was shortened. We also reinvestigated the derivatizing and LC conditions, e.g. LC column, mobile phase and detection wavelength to improve sensitivity. The proposed method was applied to determine adenyl purines in plasma from a healthy male.

2. Experimental

2.1. Materials

Adenosine-5′-monophosphate (AMP) sodium salt, adenosine-5′-diphosphate (ADP) sodium salt, adenosine-5′-triphosphate (ATP) disodium salt, adenosine (Ado), adenine (Ade) and vidarabine (adenine 9-β-D-arabinofuranoside, internal standard) were purchased from Sigma (St Louis, MO, USA). Citric acid–disodium hydrogenphosphate (pH 3.0–7.5), acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0 and 7.5) were prepared using reagent grade chemicals. LC mobile phase was prepared from LC grade solvent (Wako Pure Chemical Co., Osaka, Japan). The adenyl purine and vidarabine stock/working solution were prepared using distilled water. 2-Chloroacetaldehyde aqueous solution (40 w/v%) was purchased from Tokyo Kasei Co. (Tokyo, Japan), and diluted to 4.0 *M* with distilled water.

2.1.1. Plasma samples

Plasma samples were produced as follows: blood was collected from healthy male. EDTA was added to the blood samples (2.0 mg/ml) and the samples were centrifuged at 3000 *g* for 3 min. These plasma samples were used within 5 min after centrifugation.

2.2. Methods

2.2.1. Derivatization

Plasma (100 μl containing 5 pmol/ml to 5000 pmol/ml adenyl purines and vidarabine), 10 μl of 2-chloroacetaldehyde solution and 100 μl of 1.0 *M* acetate buffer (pH 4.5) were mixed and heated at 80°C for 20 min. Then 10 μl of the reaction solution was injected into the LC apparatus.

2.2.2. Chromatography

LC pump: Shimadzu LC-6A chromatograph (Shimadzu, Osaka, Japan); column: Capcell Pak C18™ SG120 (150×4.6 mm I.D., 5 μm, Shiseido, Tokyo, Japan); Detector: Shimadzu RF-10AXL fluorescence HPLC monitor (excitation wavelength: 280 nm, emission wavelength: 420 nm); system controller: Shimadzu SCL-6B; auto sampler: Shimadzu SIL-6B; integrator: Shimadzu CR-6A; mobile phase: 0.05 *M* citric acid–0.1 *M* disodium hydrogenphosphate (pH 4.5)–methanol (97+3); column temperature: room temperature (ca. 23°C); flow-rate 0.8 ml/min; injection volume: 10 μl.

3. Results and discussion

3.1. Derivatization conditions

The derivatization process was re-investigated. In the literature, acetate buffer (pH 4.0 and 5.0) [5], phosphate buffer (pH 7.0) [6] and citric acid–phosphate buffer (pH 4.0) [8,9] were reported for this derivatization with heating at 80°C between 15 and 40 min. We investigated the effect of reaction buffer and pH with citric acid–disodium hydrogenphosphate and acetate buffers in the pH range from 3.0 to 6.0 and phosphate buffer in the pH range of 6.0 to 7.0 (Fig. 2). It can be seen that 1.0 *M* acetate buffer (pH 4.5) gave the highest detector response, twice that found with citric acid–phosphate buffer. Reaction time was varied from 10 to 60 min at 20 to 80°C (Fig. 2). The highest detector response was obtained by heating for 15 min at 80°C. The concentration of 2-chloroacetaldehyde was varied from 0.1 to 5.0 *M*. The highest detector response was obtained at 3.0 *M*. From these results, samples were

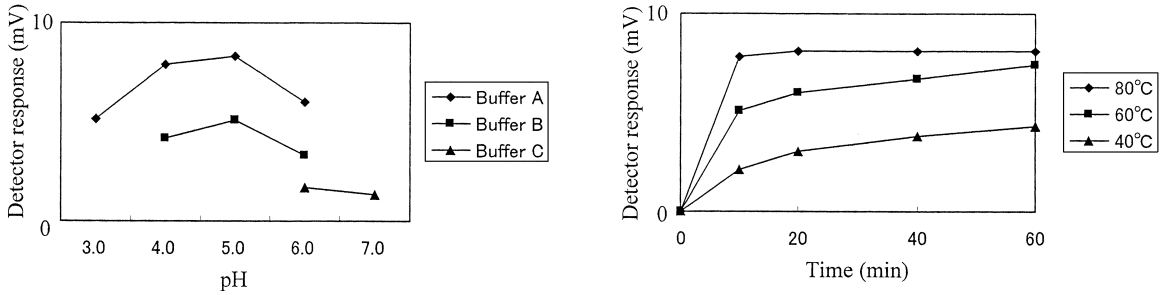

Fig. 2. (1) Effect of derivatization pH. Buffer A: 1.0 *M* acetate buffer, Buffer B: 0.5 *M* citric acid–1.0 *M* disodium hydrogenphosphate, Buffer C: 1.0 *M* phosphate buffer. A 100 pmol/ml ATP solution was used. (2) Effect of time and temperature on the derivatization reaction. A 100 pmol/ml ATP solution was used.

mixed with 1.0 *M* acetate buffer (pH 4.5) and 4.0 *M* 2-chloroacetoaldehyde solutions with heating for 20 min at 80°C. We could shorten derivatization time from 40 to 20 min compared to previous reports [8–11].

3.2. LC condition

Phosphate buffer (pH 7.0) [5] and citric acid–phosphate buffer (pH 3.6–5.0) [7–9] were reported as mobile phase components for separation of adenyl purines. We tested 0.1 *M* acetate buffer (pH 4.0–5.0), 0.05 *M* citrate–0.1 *M* dipotassium hydrogenphosphate buffer (pH 3.0–7.5) and 0.1 *M* phosphate buffer (pH 6.0–7.0) in the mobile phase. 0.05 *M* citrate–0.1 *M* dipotassium hydrogenphosphate buffer (pH 4.0) gave sharper peaks, and methanol gave 10% higher detector response compared to acetonitrile. Therefore, we changed phosphoric acid–ammonium dihydrogenphosphate (pH 4.45) [8–11] to citric acid–dipotassium hydrogenphosphate buffer. We took fluorescent products of adenyl purines and measured the excitation and emission spectra in the proposal mobile phase to decide the optimum detection wavelength. The excitation and emission spectra of ATP are shown in Fig. 3. There is an excitation maximum around 280 nm, and an emission maximum around 420 nm for the six purines, so we changed the detection wavelength to 280 nm excitation and 420 nm emission (previous method, excitation: 305 nm, emission: 420 nm).

3.3. Calibration graphs

A typical chromatogram of adenyl purines in water is shown in Fig. 4. The limits of detection were 0.1 pmol/ml for ATP, ADP and AMP, 0.5 pmol/ml for adenine and adenosine, 1.0 pmol/ml for cAMP ($S/N=5$). The calibration graphs were linear to 5000 pmol/ml of adenyl purines by fluorescence detection (Table 1). The precision varied from 5.3 to

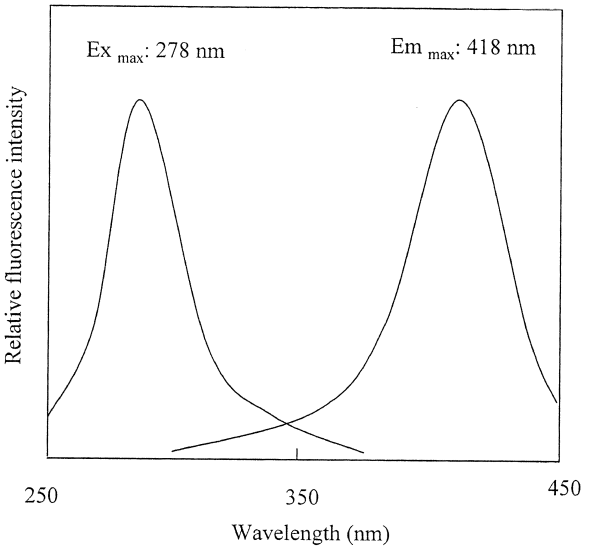

Fig. 3. Excitation and emission spectra of ATP by 2-chloroacetaldehyde. A 100 pmol/ml sample of ATP was used; 0.05 *M* citrate–0.1 *M* dipotassium hydrogenphosphate buffer (pH 4.0) was used as solvent.

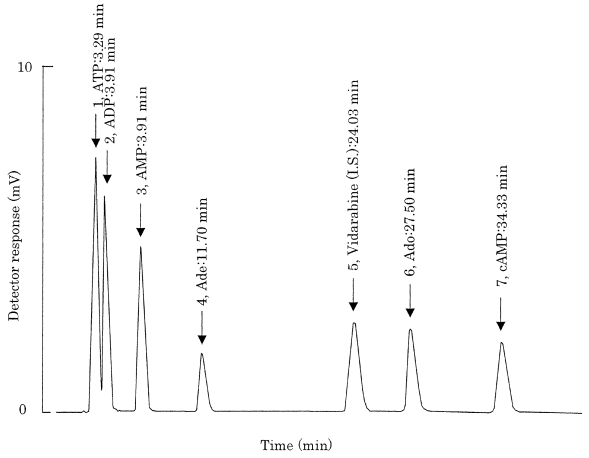

Fig. 4. Chromatogram of adenyl purines (100 pmol/ml) in water.

Table 1
Precision and linearity of adenyl purines (n=6) in water

Purine	Precision (C.V.%), 500 pmol/ml		Linearity (50–5000 pmol/ml)
	Intra-day	Inter-day	
ATP	5.3	5.6	r=0.9992 y=0.185x−2.061
ADP	6.8	7.0	r=0.9989 y=0.164x−0.778
AMP	5.8	5.9	r=0.9991 y=0.077x−2.578
Ade	6.2	6.4	r=0.9997 y=0.053x−0.383
Ado	6.0	6.2	r=0.9997 y=0.070x−0.106
cAMP	6.1	6.1	r=0.9998 y=0.066x−0.194

y, peak area; x, concentration (pmol/ml).

7.0%. The sensitivity of the proposed method was improved over 500 times compared to previous reports [1–9]. Therefore, we could detect 0.1 pmol/ml level adenyl purines using 100 μl plasma, and could also apply the method to monitor low concentration and volume samples, e.g. umbilical cord plasma.

Table 3
Adenyl purines in human plasma

Sample no.	ATP (nmol/ml)	ADP (nmol/ml)	AMP (nmol/ml)
1	9.2±2.1	8.1±2.4	1.1±0.3
2	22.2±4.8	22.2±4.2	3.2±1.3
3	8.2±1.9	7.9±2.0	1.3±0.3
4	8.4±3.2	6.5±1.2	1.0±0.3
5	6.7±1.1	5.5±1.4	0.8±0.2

Adenine, adenosine and cAMP were <1 pmol/ml. Each value was obtained from six runs.

3.4. Monitoring adenyl purines in human plasma

The proposed LC method was applied to the determination of adenyl purines in plasma from a healthy male. The recoveries of spiked purines (200 pmol/ml) to plasma were from 86.2±1.8 to 93.0±3.1% (Table 2). The concentrations of adenyl purines from healthy males were as follows (Table 3): ATP (9.2–22.2 nmol/ml, ADP (5.5–22.2 nmol/

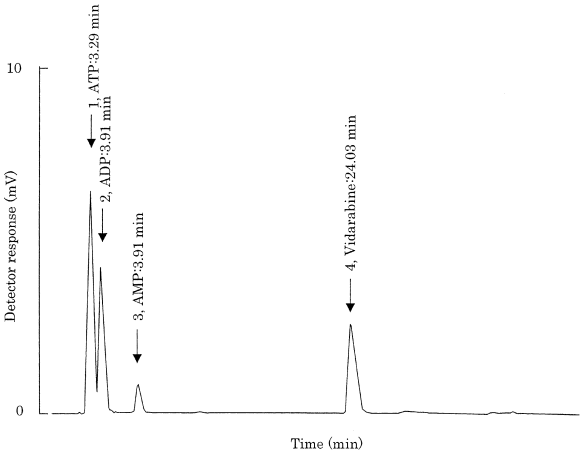

Fig. 5. Chromatogram of adenyl purines in human plasma. Vidarabine was spiked as internal standard (100 pmol/ml).

Table 2
Recovery of spiked adenyl purines (200 pmol/ml, n=6) from plasma

	ATP	ADP	AMP	Ade	Ado	cAMP
Recovery (%)	92.4±2.2	91.3±3.1	93.0±1.9	89.5±2.0	91.3±2.1	86.2±1.8

Ade, adenine; Ado, adenosine.

ml), AMP (0.8–3.2 nmol/ml). Other purines, adenine, adenosine, cAMP were lower than 0.1 pmol/ml. A typical chromatogram is shown in Fig. 5. There are no interference peaks in the chromatogram. As these results show, it was found that the proposed LC method could determine pmol/ml levels of adenyl purines in 100 μl of biological samples. Further applications for determination of adenyl purines in blood and sperm from male-infertile patients are in progress.

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