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Short communication

# Liquid chromatographic determination of 1-adamantanamine and 2-adamantanamine in human plasma after pre-column derivatization with *o*-phthalaldehyde and 1-thio-β-D-glucose

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**Abstract**

We investigated high-performance liquid chromatographic (HPLC) determination of 1-adamantanamine hydrochloride (1-ADA) and 2-adamantanamine hydrochloride (2-ADA) in human plasma after the derivatization with *o*-phthalaldehyde (OPA) and 1-thio-β-D-glucose (TG). Extracted human plasma samples were mixed with OPA and TG at room temperature for 6 min and injected onto HPLC. Retention times of 1-ADA and 2-ADA derivatives were 12.6 and 14.1 min, respectively. The lower limits of detection of 1-ADA and 2-ADA were 0.02 and 0.008 μg/ml, and the lower limits of quantitation of 1-ADA and 2-ADA were 0.025 and 0.01 μg/ml, respectively. The coefficients of variation for intra-day and inter-day assay of 1-ADA and 2-ADA were less than 4.4 and 6.0%, respectively. L-Dopa and dopamine were not found to interfere with the peaks of 1-ADA and 2-ADA derivatives. Human plasma unbound fraction ($f_p$) values of 1-ADA varied between 0.32 and 0.48, while those of 2-ADA varied between 0.38 and 0.68. These results indicate that HPLC assay of 1-ADA and 2-ADA by derivatization with OPA and TG is simple, rapid, sensitive and reproducible for determining 1-ADA and 2-ADA in human plasma.


*Keywords:* Derivatization; LC; Adamantanamine

## 1. Introduction

1-Adamantanamine hydrochloride (1-ADA), amantadine, is clinically used as an antiparkinsonism agent as well as an antiviral drug [1–3]. After prolonged treatment, 1-ADA induces various side effects in the central nervous system (e.g. dizziness, difficulty of thinking and retaining information, dysphoria and occasionally even convulsions) [1,4,5]. Therefore, it is necessary to detect plasma levels of 1-ADA in order to assess the degree of possible toxicity.

For its pharmacokinetic studies, the determination of the levels has been performed by gas–liquid chromatographic procedure employing flame ionization and electron capture detection in biological fluids [6–10]. According to Sioufi and Pommier, the gas–liquid chromatographic procedure with flame ionization detection lacked the sensitivity for reliable determination of plasma levels of 1-ADA [9]. Hesselink et al. have investigated the brain penetration of 1-ADA by microdialysis study using gas chromatographic analysis coupled with mass selective detection [11]. [$^3$H]-1-ADA is frequently utilized for studies on the transport mechanism through the blood–brain barrier and on the reabsorptive mechanism in kidney [12,13]. In the various experiments described earlier, complicated equipment and special facilities for using radioactive compounds are required. Therefore, a more convenient, sensitive and simple method is needed.

1-ADA has no prominent absorption properties in the ultraviolet (UV) spectrum and no fluorescence properties. Therefore, the use of a derivatization reagent which is reactive toward amino group and the more popularized system would seem suitable for determination of 1-ADA concentration in biological fluids. Van Der Horst et al. have shown the determination of 1-ADA in urine using high-performance liquid chromatography (HPLC) by derivatization with 1-fluoro-2,4-dinitrobenzene (DNFB) as an UV-labeled agent [14]. But, the utility of DNFB resulted in producing an amount of precipitation during the derivatization, and interference peaks were detected on chromatograms. Though

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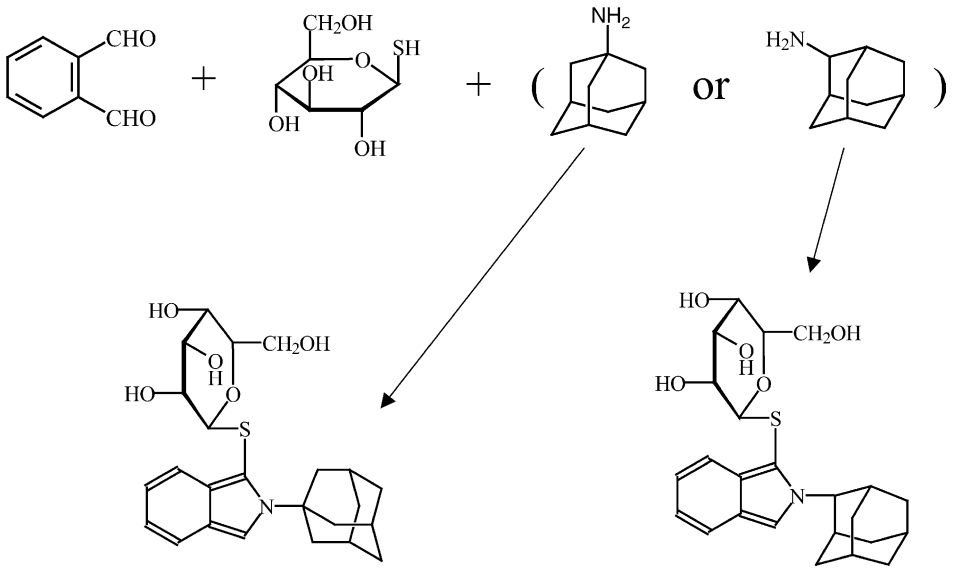


Fig. 1. Formation of fluorescent 1-ADA and 2-ADA derivatives.

Fujino and Goya have reported the method of determination of 1-ADA levels in urine using HPLC by derivatization with 3-(4,6-difluorotriazinyl)amino-7-methoxycoumarin (FAMC) [15], the derivatization by FAMC required severe conditions (140 °C, 15 min). To improve these points, the 3-position of FAMC was substituted for the benzoxazoline-2-thione group. The derivatization using the substitution was satisfactory with respect to simplicity and precision to quantify 1-ADA spiked in the urine [16]. However, the HPLC method using FAMC or its substitution has not been widely utilized, because they are not commercially available. Also, there have been no reports on monitoring plasma 1-ADA levels using HPLC.

It is well known that HPLC analysis by the derivatization with *o*-phthalaldehyde (OPA) and thiol agent for determination of a compound containing the primary amino group in the structure will be useful and sensitive. Amino acids have been sensitively detected and separated with OPA and 1-thio-β-D-glucose (TG) or 2,3,4,6-tetraacetyl-1-thio-β-D-glucopyranoside as a thiol agent in weak alkaline conditions [17,18]. These compounds are easily obtained and the derivatization with them was completed in mild conditions (room temperature, less than 6 min), suggesting that the rapid and simple determination of amino group may be possible.

In the present study, we investigate the quantitative analysis of 1-ADA by HPLC after derivatization with OPA and TG using 2-adamantanamine hydrochloride (2-ADA) as an internal standard (I.S.) in human plasma according to the reaction shown in Fig. 1. In addition, we examine the determination of 2-ADA using 1-ADA as I.S. Moreover, bindings of 1-ADA and 2-ADA to human plasma protein are studied.

## 2. Experimental

### 2.1. Instruments

The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan), and a Rheodyne injection valve (Cotati, CA, USA) with a 20 μl and model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 342 nm and an emission wavelength of 410 nm. The HPLC column (KANTO Chemical, Tokyo, Japan) was 150 mm × 4.6 mm i.d. and with 5 μm particles of $C_{18}$ packing material.

### 2.2. Reagents

1-Adamantanamine hydrochloride, 2-adamantanamine hydrochloride, *o*-phthalaldehyde and 1-thio-β-D-glucose, sodium salt hydrate were obtained from Aldrich (Milwaukee, WI, USA). Methanol for HPLC, L-dopa, dopamine hydrochloride and other general reagents were supplied by Wako Pure Chemical Industries (Osaka, Japan). Ultrafree-MC Centrifugal Filter Units for a protein binding study were purchased from Millipore Corporation (Bedford, MA, USA).

### 2.3. Extraction from plasma

Control plasma was collected from a healthy volunteer. A 50 μl aliquot of plasma sample was rendered alkaline by the addition of NaOH (2 M, 200 μl). 2-ADA and 1-ADA (each 0.25 μg/ml in water, 50 μl) were added as I.S. in showing standard curves of 1-ADA and 2-ADA, respectively. Then, the mixture was vortexed for 1 min and extracted with freshly distilled *n*-hexane (3 ml, two times). Each *n*-hexane phase

was mixed and evaporated, and the derivatization was performed as follows.

### 2.4. Derivatization

Borate buffer (BB, 0.1 M) was adjusted to pH 9.5 by addition of NaOH. A 400 μl aliquot of BB was added to the residue. TG solution (50 mg/ml in water, 50 μl) and OPA solution (40 mg/ml in acetonitrile, 50 μl) were added and vortexed. The mixture was allowed to react for 6 min at room temperature and the derivatized sample (20 μl) was injected on the column.

### 2.5. Chromatographic conditions

Quantification of the peaks was performed with a Chromatopac, model CR-3A integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared by addition of methanol (400 ml) to a 100 ml solution of containing acetic acid (0.2%, v/v) in water at pH 7.0 by NaOH. The derivatives were eluted from the column at 25 °C at a flow rate of 0.4 ml/min.

### 2.6. Calibration curves

Solutions of 1-ADA and 2-ADA (1 mg/ml in water) were added to the human plasma from a healthy volunteer. The range of concentrations of 1-ADA and 2-ADA varied from 0.025 to 2.5 and 0.01 to 2.5 μg/ml, respectively. All samples were extracted and analyzed using procedures described earlier. Calibration curves based on the peak area ratios of 1-ADA or 2-ADA to I.S. were analyzed in duplicate for each sample.

### 2.7. Bindings of 1-ADA and 2-ADA to human plasma protein

Plasma protein binding experiments of 1-ADA and 2-ADA were performed using Ultrafree-MC Centrifugal Filter Units. A 250 μl aliquot of plasma containing 0.05–2.5 μg/ml of 1-ADA or 2-ADA was added to the filters and centrifuged (5000 × *g*, 15 min) at room temperature. The concentration of 1-ADA or 2-ADA in the filtrate samples (50 μl) was analyzed as described earlier.

## 3. Results and discussion

### 3.1. Chromatograms of 1-ADA and 2-ADA derivatives

Fig. 2 illustrates a representative chromatogram of blank plasma (A) and plasma with 0.25 μg/ml of 1-ADA and 2-ADA (B). Drug-free pooled human plasma yielded relatively clean chromatograms with no significant interfering peaks. Retention times of 1-ADA and 2-ADA derivatives were 12.6 and 14.1 min, respectively. The fluorescence

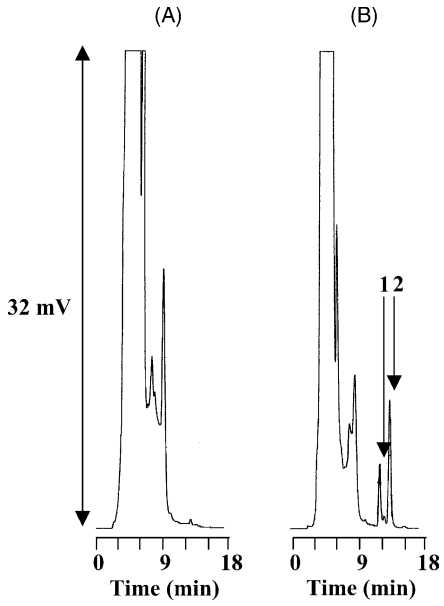


Fig. 2. Chromatograms of blank human plasma and 1-ADA and 2-ADA derivatives. (A) Chromatogram of blank human plasma. (B) Chromatogram of human plasma spiked with 1-ADA and 2-ADA, concentration of each 0.25 μg/ml (peaks: 1 = 1-ADA; 2 = 2-ADA). The attenuation for all chromatograms is 32 mV/full scale.

intensity of 1-ADA derivative was about a half of that of 2-ADA derivative. It was considered that the difference in responses might be involved in the secondary or tertiary structure of derivatives. In this point, further studies are needed.

### 3.2. Standard curves of 1-ADA and 2-ADA

The linearity was displayed for 1-ADA concentrations ranging from 0.025 to 2.5 μg/ml. The regression coefficient was 0.999 ($y = 1.858x - 0.005$). On the other hand, the linearity was displayed for 2-ADA concentrations ranging from 0.01 to 2.5 μg/ml. The regression coefficient was 0.999 ($y = 7.665x - 0.024$). Lower limits of detection for 1-ADA and 2-ADA utilizing this method were established at 0.02 and 0.008 μg/ml (signal-to-noise ratio of 3:1), respectively.

The lower limits of quantification of 1-ADA shown by Belanger and Grech-Belanger was 0.1 μg/ml in human plasma, while plasma samples were extracted three times by 3 ml of diethyl ether in alkaline condition [10]. Zhou et al. have demonstrated that the direct determination of 1-ADA in plasma and urine by solid-phase reagent containing a covalently bound activated ester of 9-fluoreneacetate [21]. Because simultaneous extraction and derivatization of 1-ADA are performed by an automated on-line column reaction, the procedure is very simple and the run time is rapid (13 min). However, the used equipment is not so popularized in a clinical setting. The lower limit of quantification of 1-ADA was 0.2 μg/ml. Although (2-naphthoxy)acetyl chloride was recently used as a simple fluorescent reagent and the run time was rapid (15 min), the detection limit of 1-ADA was about

Table 1
Stability data for 1-ADA and 2-ADA derivatives stored at −18 °C for 5 days

| Concentration (μg/ml) | Measured (μg/ml; mean ± S.D., $n = 5$) | C.V. (%) | Recovery (%) |
|---|---|---|---|
| 1-ADA derivative | | | |
| 0.05 | 0.045 ± 0.003 | 6.7 | 90.0 |
| 0.25 | 0.232 ± 0.015 | 6.5 | 92.8 |
| 2.5 | 2.33 ± 0.14 | 6.0 | 93.2 |
| 2-ADA derivative | | | |
| 0.025 | 0.0241 ± 0.0018 | 7.5 | 96.4 |
| 0.25 | 0.264 ± 0.016 | 6.1 | 105.6 |
| 2.5 | 2.66 ± 0.17 | 6.4 | 106.4 |

0.15 μM (28 ng/ml) and a large amount of sample (300 μl) was required [22]. Since the reagent is not commercially available, it is likely that the method is not widely spread. Also, in a pharmacokinetic study after the oral administration of 1-ADA (100 mg), the plasma levels of 1-ADA varied between 0.20 and 1.05 μg/ml [10]. These values were almost consistent with the range of 0.1–1.3 μg/ml reported by various authors [8,9,19,20]. In addition, in rats at an infusion of 3 mg/kg of [$^3$H]-1-ADA, the range of plasma concentration from 7 to 127 min varied between 0.9 and 0.3 μg/ml [13]. The measurable range of 1-ADA by our method was enough to cover previous values, indicating that our HPLC assay may be very suitable for the pharmakokinetic study of 1-ADA as well as prevent using radioactive compounds. Moreover, the lower limit of quantification of 2-ADA was superior to that of 1-ADA. Detection of 2-ADA in human plasma has not been performed. 2-ADA has been found to be a possible candidate for a drug of a new class of insulin secretagogues in in vitro experiments [23]. It has been shown that its pharmacological effect may be as potent as 1-ADA. We expect that our method will be utilized for pharmacokinetic studies of 2-ADA.

### 3.3. Stability of 1-ADA and 2-ADA derivatives

The storage stability of 1-ADA and 2-ADA derivatives in extracted human plasma was examined by the analysis of human plasma containing known amounts of analytes and the data are listed in Table 1. Tested samples of 1-ADA (0.05, 0.25 and 2.5 μg/ml) were immediately mixed with OPA and TG and allowed to stand at room temperature for 6 min, and then stored at −18 °C for 5 days. The stability of 1-ADA at 0.025, 0.25 and 2.5 μg/ml was 90.0, 92.8 and 93.2%, respectively. On the other hand, when the same procedure was performed, the stability of 2-ADA at 0.025, 0.25 and 2.5 μg/ml was 96.4, 105.6 and 106.4%, respectively. It is considered that 1-ADA derivative is a little inferior to 2-ADA derivative in terms of the stability after the derivatization.

### 3.4. Precision, accuracy and stability of 1-ADA and 2-ADA

Precision and accuracy for intra-day and inter-day assay of 1-ADA and 2-ADA are shown in Tables 2 and 3. In the intra-day assay, relative standard deviation was within 3.2–4.1%, and the recovery was within 98.0–101.2% for 1-ADA. For 2-ADA, relative standard deviation was within 3.2–4.4%, and the recovery was within 98.8–103.6%. In the inter-day assay, the range of coefficient variation was within 4.9–5.8%, and the recovery was within 98.0–104.0% for 1-ADA. For 2-ADA, the range of coefficient variation was within 4.7–6.0%, and the recovery was within 99.2–104.8%.

The two compounds studied showed no significant degradation in human plasma during 1 month of storage at 4 °C. The recovery of 1-ADA at 0.05, 0.25 and 2.5 μg/ml was 98.5, 99.3 and 100.8%, respectively. On the other hand, the recovery of 2-ADA at 0.025, 0.25 and 2.5 μg/ml was 97.5, 103.3 and 101.8%, respectively (data not shown).

Our results of precision, accuracy and stability of 1-ADA and 2-ADA on the measurement by HPLC indicate good reproducibility and negligible degradation of 1-ADA and 2-ADA in plasma during a long term.

Table 2
Intra-day assay reproducibility for determination of 1-ADA and 2-ADA

| Concentration (μg/ml) | Measured (μg/ml; mean ± S.D., $n = 5$) | C.V. (%) | Recovery (%) |
|---|---|---|---|
| 1-ADA | | | |
| 0.05 | 0.049 ± 0.002 | 4.1 | 98.00 |
| 0.25 | 0.246 ± 0.009 | 3.7 | 98.4 |
| 2.5 | 2.53 ± 0.08 | 3.2 | 101.2 |
| 2-ADA | | | |
| 0.025 | 0.0252 ± 0.0011 | 4.4 | 100.8 |
| 0.25 | 0.247 ± 0.008 | 3.2 | 98.8 |
| 2.5 | 2.59 ± 0.09 | 3.5 | 103.6 |

Table 3
Inter-day assay reproducibility for determination of 1-ADA and 2-ADA

| Concentration (μg/ml) | Measured (μg/ml; mean ± S.D., $n = 5$) | C.V. (%) | Recovery (%) |
|---|---|---|---|
| 1-ADA | | | |
| 0.05 | 0.052 ± 0.003 | 5.8 | 104.0 |
| 0.25 | 0.245 ± 0.012 | 4.9 | 98.0 |
| 2.5 | 2.58 ± 0.14 | 5.4 | 103.2 |
| 2-ADA | | | |
| 0.025 | 0.0248 ± 0.0015 | 6.0 | 99.2 |
| 0.25 | 0.253 ± 0.012 | 4.7 | 101.2 |
| 2.5 | 2.62 ± 0.15 | 5.7 | 104.8 |

### 3.5. Interference

Furthermore, we examined the interference of L-dopa and dopamine (each 2.5 μg/ml) on 1-ADA and 2-ADA (each 0.25 μg/ml) with OPA and TG. Because the peaks of the derivatives of L-dopa and dopamine and blank plasma peaks overlapped, these retention times were not determined. When the derivatization of L-dopa and dopamine was carried out in BB, the retention times of their derivatives were 4.8 and 5.5, respectively. Namely, retention times of L-dopa and dopamine derivatives were obviously different from those of 1-ADA and 2-ADA derivatives.

Although L-dopa, used in the treatment of parkinsonism [24,25], possesses an amino group, the values of recovery of 1-ADA and 2-ADA derivatives were 98.0 and 98.4%, respectively. In addition, the interference of dopamine, a main metabolite of L-dopa, was also not expressed. These results indicate that the plasma 1-ADA and 2-ADA levels can be precisely measured when L-dopa and 1-ADA or 2-ADA are co-administered to humans. In general, the plasma concentration–time curve of radioactive compound is not precise because of measuring the intact compound and its metabolites. It has been indicated that 1-ADA was partially metabolized to *N*-hydroxyamantadine, 1-nitrosoadamantane and 1-acetamidoadamantane in animal studies [6,26]. However, the recovery values of 92–95% of the dose of 1-ADA were detected as the intact type in urine [6,10], suggesting that the unchanged type of 1-ADA should be more than 90% in plasma after administration. The metabolites possess no primary amino group, indicating that no or very low derivatization of them with OPA and TG may be completed. Therefore, it is considered that the interference of these metabolites would be negligible.

### 3.6. Bindings of 1-ADA and 2-ADA to human plasma protein

Plasma protein binding study of 1-ADA and 2-ADA was carried out as shown in Fig. 3. The plasma unbound fraction ($f_p$) values of 1-ADA varied between 0.32 and 0.48, while the $f_p$ values of 2-ADA varied between 0.38 and 0.68. Each $f_p$ value of 1-ADA in the examined concentration range was lower than that of 2-ADA.

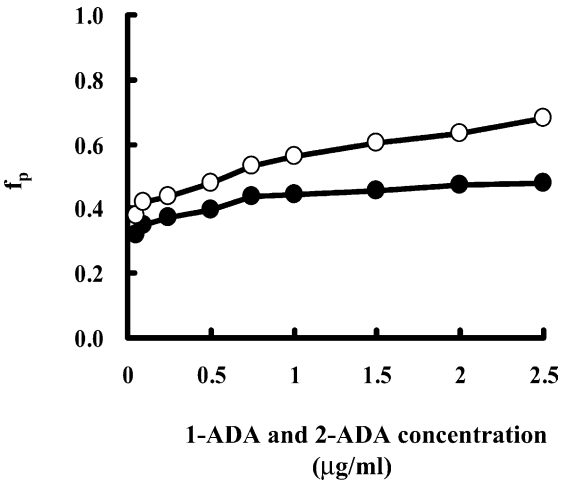


Fig. 3. Binding of 1-ADA (●) and 2-ADA (○) to human plasma protein.

The $f_p$ values of 1-ADA showed the range of 0.32–0.48 in the examined concentration of 1-ADA. Namely, plasma protein binding of 1-ADA of 68–52% was observed. Liu et al. have shown that approximately two-thirds of the 1-ADA was found to be protein bound in the concentration range of 0.1–2 μg/ml [27]. Our results were nearly consistent with previous data, suggesting that our HPLC assay is appropriate for determining the unbound fraction of 1-ADA in the filtrate. In this paper, we expressed the $f_p$ values of 2-ADA using the HPLC technique for the first time. Each $f_p$ value of 2-ADA was higher than that of 1-ADA. Our data indicate that 2-ADA may possess a stronger potency in view of insulin secretion than 1-ADA in in vivo experiments, because the pharmacological effects of administered drug are mainly involved in the unbound concentration in plasma.

## 4. Conclusions

Our HPLC assay of 1-ADA and 2-ADA by derivatization with OPA and TG is simple, rapid, sensitive and reproducible for determining 1-ADA and 2-ADA in human plasma and this method may be applied for the pharmacokinetic studies of 1-ADA and 2-ADA in humans. We strongly expect that our method will be popularized and enable the assessment

or prediction for severe central nervous system side effects based on plasma 1-ADA levels.

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