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Note

# Determination of adenosine in normal human plasma and serum by high-performance liquid chromatography

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Adenosine (ADO), an ubiquitous nucleoside, has many different physiological actions. It is formed by the hydrolysis of adenosine 5′-phosphate (AMP) by 5′-nucleotidase. Subsequently ADO is either reconverted to AMP by adenosine kinase or metabolized to inosine (INO) by adenosine deaminase, an enzyme present in all tissues and red blood cells [1]. The final product of degradation of ADO is uric acid. ADO exerts a vasodilatory effect upon most vascular beds, especially the coronary system [2—4]; however, it exhibits vasoconstrictor properties in the kidney [5, 6]. ADO has been demonstrated to have a neurotransmitter role in the central nervous system [7, 8]; it also inhibits the release of norepinephrine from peripheral nerve terminals [5, 9]. In the adipose tissue, ADO has an insulin-like action [10]. A deficiency of adenosine deaminase leads to high levels of ADO in plasma and tissues which results in an immunodeficiency syndrome [11].

Very few procedures have been developed for the analysis of ADO in normal human plasma. A fluorimetric assay method was developed by Gardiner [12]. In the procedure INO and ADO were enzymatically converted to hypoxanthine and estimated by the rate of appearance of hydrogen peroxide after the addition of xanthine oxidase. A radioimmunoassay has been developed by Schrader et al. [13], which utilizes antibodies directed against an antigen containing ADO. Interfering adenosine deaminase activity was removed from the antisera by treatment with DEAE-cellulose. A few high-performance liquid chromatographic (HPLC) procedures have been developed for ADO assay in plasma and serum. Pfadenhauer and Tong [14] used a boronate affinity column for the isolation of ADO from plasma. The collected eluate was lyophilized to dryness and the reconstituted residue was analyzed by HPLC using a reversed-phase column with phosphate buffer, pH 3.5 as the mobile phase. Circulating

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levels of ADO in six healthy volunteers ranged from 18 to 149 ng/ml with an average of 62 ng/ml. Two other procedures have been developed for the analysis of ADO by HPLC in human serum by Brown et al. [15], and Hartwick and Brown [16]. Both procedures used reversed-phase columns with phosphate buffers at pH 5.8 containing 10% methanol as the mobile phase. Free ADO was not detectable in normal human serum but it was measured in serum samples from a patient suffering from adenosine deaminase deficiency.

An HPLC assay procedure is described here for the analysis of ADO in normal human plasma. 2′-Deoxycoformycin (2′-DCF), a potent inhibitor of adenosine deaminase activity, is added to the samples to inhibit the conversion of ADO to INO. A reversed-phase radial compression column is used for rapid separation of ADO from plasma ultrafiltrate.

## EXPERIMENTAL

### *Materials*

INO, ADO and adenosine deaminase were obtained from Sigma (St. Louis, MO, U.S.A.); [$^3$H]ADO (49 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.SA.), and heparin (1000 units/ml) was procured from Upjohn (Kalamazoo, MI, U.S.A.). The 2′-DCF was a gift from Developmental Therapeutics Program, Chemotherapy, National Cancer Institute (Bethesda, MD, U.S.A.). Stock solutions were prepared by dissolving 0.1 mg 2′-DCF in 10 ml normal saline (final concentration, 10 ng/$\mu$l).

### *Instrumentation*

A Waters Assoc. (Milford, MA, U.S.A.) 202 high-performance liquid chromatograph with a U6K injector and a radial compression column unit was used with a 11.5 cm × 0.8 cm, 10-$\mu$m $C_{18}$ $\mu$Bondapak column. The guard column contained 10-$\mu$m $C_{18}$ $\mu$Bondapak reversed-phase packing. A variable-wavelength detector (Instrumentation Specialties, Lincoln, NB, U.S.A.) set at 254 nm was used for detection.

### *Operating procedures and conditions*

The mobile phase consisted of 0.05 *M* $NaH_2PO_4$ (in distilled water) adjusted to pH 7.4 and degassed for 30 min. Standards were prepared by dissolving ADO and INO (final concentration 20.0 $\mu$g/ml) in the mobile phase. The column was at ambient temperature (approximately 22°C) and the flow-rate was set at 8.0 ml/min (14 MPa). Sample volumes of 180 $\mu$l were injected per analysis.

### *Sample collection*

Blood samples were drawn via venipuncture in the morning from normal adult male and female volunteers, into 7-ml vacutainer tubes containing 10 $\mu$l of heparin (10 units) and 140 $\mu$l of 2′-DCF (1400 ng). The samples were immediately cooled in ice and the tubes were centrifuged at 2500 rpm (1400 *g*) for 15 min; then 3.5 ml of plasma were transferred to an ultrafiltration cone (CF-50, Amicon, Lexington, MA, U.S.A.) and centrifuged at 1400 *g* for 2 h. An aliquot (0.8 ml) of the ultrafiltrate was dried under a stream of air and reconstituted with 200 $\mu$l of the mobile phase.

The recovery of ADO was determined by adding 0.05 $\mu$Ci of [$^3$H] ADO to blank plasma prior to ultrafiltration. After 2 h of centrifugation, [$^3$H] ADO was measured in the ultrafiltrate and in the residue remaining in the cone (Beckman 255 Scintillation Spectrometer, counting efficiency for $^3$H $\approx$ 40%). The recovery of ADO was found to be essentially quantitative.

## RESULTS AND DISCUSSION

Fig. 1a shows a chromatogram of ADO and INO isolated from normal human plasma containing 2'-DCF. The retention times for INO, 2'-DCF and ADO were 104, 207 and 400 sec, respectively. In chromatograms of plasma samples which did not contain 2'-DCF, the ADO peak was not observable (Fig. 1b) and the INO peak was more pronounced. Positive identification was achieved by peak superimposition, i.e. by adding INO and ADO standards (200 ng) to ultrafiltrate samples and observing increased peak height at the corresponding retention times. Identification was also confirmed by the absence of the ADO peak and the enhancement of the INO peak in the samples that did not contain 2'-DCF or in samples to which adenosine deaminase (5—20 units) had been added.

The limit of detection (2:1 signal-to-noise) of this procedure is 10 ng of ADO. Repetitive injections of standards gave good reproducibility of retention times (S.D. = ± 2%) and peak heights (S.D. = ± 2.3%). Standard curves were

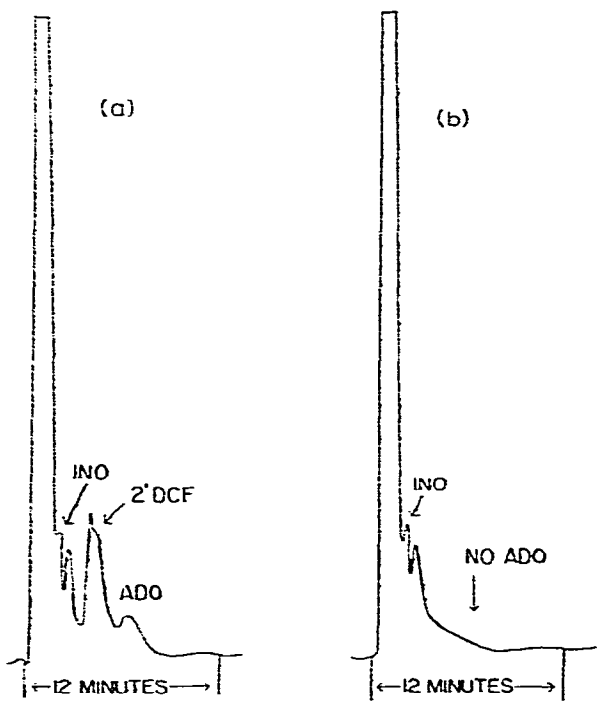


Fig. 1. (a) Chromatogram of INO and ADO from normal human plasma. The peak at 207 sec is that of 2'-DCF. Blood was collected in the presence of heparin and 2'-DCF. (b) Chromatogram of an extract of normal human plasma obtained from blood collected in the presence of heparin and absence of 2'-DCF. Note that only INO is detectable.

linear in the range of 20—1000 ng ADO and the day-to-day reproducibility varied less than 3.2% (S.D.). The working portion of the standard curve is in the range of 30—300 ng ADO.

After approximately 110—135 injections of plasma samples, the column resolution starts to decrease and this deterioration is noted even with the use of a guard column. The retention time of ADO increases considerably and the ADO peak becomes too broad to be quantitated. If 5% methanol is added to the mobile phase, the ADO peak becomes sharp with a retention time of 400 sec but the INO peak can not be resolved from the solvent front. At this stage the column can be used for only 10—20 injections before it is deteriorated beyond use.

Variations were not found in the levels of endogenous ADO in blood samples collected and allowed to stand for 15 or 30 min prior to workup. If release and uptake of ADO occurs by red blood cells, it does not appear to affect the final determination in a 30-min time span. The effect of short-term local ischemia, obtained by use of a tourniquet, upon ADO levels was studied. Blood samples were collected immediately and 2 min after the tourniquet had been applied; no significant difference was noted between the levels of ADO in the two samples.

In the absence of added 2'-DCF, ADO was not detected in any plasma sample. The use of 200 ng of the inhibitor per ml blood collected provided maximal inhibition of adenosine deaminase; concentration of 2'-DCF as high as 2500 ng/ml blood gave the same values of ADO.

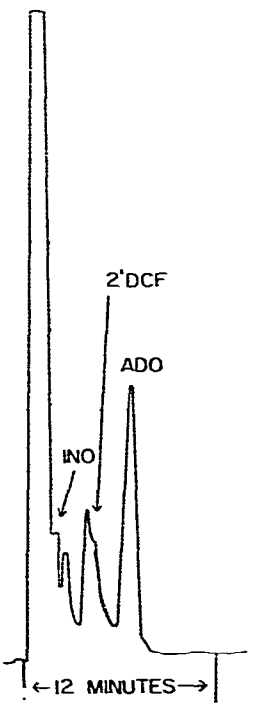


Fig. 2. Chromatogram of an extract of normal human serum obtained from blood collected in the presence of 2'-DCF and the absence of heparin. The sample clotted in the collection vial producing a very large quantity of ADO.

Clotting could not be prevented in the blood collection vials or ultrafiltration cones without the use of heparin. Fig. 2 presents a chromatogram of a sample which clotted in the collection vial; it shows very large amounts of ADO to be present in the serum, probably due to the breakdown of ATP which occurs in the normal clotting process.

The ADO levels in plasma and serum of normal adult male and female volunteers are presented in Tables I and II, respectively. Although the number of people tested is too small for a definitive conclusion, there appears to be no significant difference between the two sexes. The ADO levels reported in this study are slightly higher than those published by previous investigators [14]; this is probably due to the use of the adenosine deaminase inhibitor in these experiments. It is observed that a large amount of ADO is present in serum probably as a result of the normal clotting process. This could lead to falsely increased levels of ADO and limit the clinical usefulness of the method. Heparin prevents clotting from taking place and it is felt that plasma determination would be of greater clinical utility since it is more likely to represent true circulating levels of ADO.

Previous publications have reported the measurement of ADO only in patients with adenosine deaminase deficiency [15, 16] and ADO was not

TABLE I

ADENOSINE LEVELS IN PLASMA OF NORMAL ADULT VOLUNTEERS

| Subject | Age | Sex | Plasma (ng ADO per ml) |
|---|---|---|---|
| 1 | 30 | M | 81 |
| 2 | 24 | M | 121 |
| 3 | 30 | M | 150 |
| 4 | 32 | M | 281 |
| 5 | 33 | M | 142 |
| 6 | 32 | M | 134 |
| 7 | 32 | F | 132 |
| 8 | 28 | F | 136 |
| 9 | 23 | F | 173 |
| 10 | 25 | F | 73 |
| 11 | 34 | F | 62 |
| Mean ± S.D. | | | 135 ± 38 |

TABLE II

ADENOSINE LEVELS IN SERUM OF NORMAL ADULT VOLUNTEERS

| Subject | Age | Sex | Serum (ng ADO per ml) |
|---|---|---|---|
| 1 | 30 | M | 1278 |
| 3 | 30 | M | 1611 |
| 10 | 24 | F | 830 |
| 12 | 24 | F | 2330 |
| Mean ± S.D. | | | 1512 ± 458 |

detectable in normal human plasma. It has been noted in this study that the elapse of time between sample collection and centrifugation was sufficient to allow breakdown of ADO to INO beyond the limit of detection of this HPLC procedure. Only by the use of the adenosine deaminase inhibitor (2'-DCF), at the time the blood was drawn, were levels of ADO quantitated in normal human plasma.

## CONCLUSIONS

This HPLC method for the determination of ADO levels in the plasma of normal human subjects is simple, rapid and reproducible. The use of 2'-DCF to prevent degradation of ADO is necessary. This procedure can be applied to study ADO in different physiological and pathological conditions in man.

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## REFERENCES

1 J.R.S. Arch and E.A. Newsholme, Essays Biochem., 14 (1978) 82.
2 F.J. Haddy and J.B. Scott, Physiol. Rev., 48 (1968) 688.
3 M. Katori and R.M. Berne, Circ. Res., 19 (1966) 420.
4 R. Rubio, V.T. Wiedmeier and R.M. Berne, J. Mol. Cell. Cardiol., 6 (1974) 561.
5 P. Hedqvist and B.B. Fredholm, Naunyn-Schmiedeberg's Arch. Pharmacol., 293 (1976) 217.
6 H. Tagawa and A.J. Vander, Circ. Res., 26 (1970) 327.
7 M. Huang and J.W. Daly, Life Sci., 14 (1974) 489.
8 S.P. Hunt and H. Kunzle, Brain Res., 112 (1976) 127.
9 A.R. Wakade and T.D. Wakade, J. Physiol., 282 (1978) 35.
10 U. Schwabe, P.S. Schönhöfer and R. Ebert, Eur. J. Biochem., 46 (1974) 537.
11 E.R. Giblett, J.E. Anderson, F. Cohen, B. Pollara and H.J. Meuwissen, Lancet, ii (1972) 1067.
12 D.G. Gardiner, Anal. Biochem., 95 (1979) 377.
13 J. Schrader, S. Nees and E. Gerlach, Pfluegers Arch., 378 (1978) 1967.
14 E.H. Pfadenhauer and S.-D. Tong, J. Chromatogr., 162 (1979) 585.
15 P.R. Brown, A.M. Krstulović and R.A. Hartwick, Monogr. Hum. Genet., 10 (1978) 135.
16 R.A. Hartwick and P.R. Brown, J. Chromatogr., 143 (1977) 383.