Journal of Chromatography, 529 (1990) 437–441
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5324

Note

Determination of adenosine and cyclic adenosine monophosphate in urine using solid-phase extraction and high-performance liquid chromatography with fluorimetric detection

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(First received January 8th, 1990; revised manuscript March 9th, 1990)

Adenosine (ADO) has an established role as an endogenous vasodilator and neuromodulator [1]. Katholi et al. [2] demonstrated in animal experiments that the intrarenal levels of ADO may play a physiological role in the regulation of renal afferent nerve activity. As a part of an ongoing project, investigating the possible role of intrarenal ADO in humans, we developed a rapid and sensitive method for the determination of ADO and cyclic adenosine monophosphate (cAMP) in urine.

EXPERIMENTAL

Materials

ADO, cAMP and adenosine deaminase were Boehringer-Mannheim, (Mannheim, F.R.G.). Chloroacetaldehyde (50–55% in water) was from Fluka (Buchs, Switzerland). Sep-Pak Light C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). Tetrahydrofuran (HPLC grade) was from E. Merck (Darmstadt, F.R.G.). All other chemicals were analytical-grade reagents. The water used was deionized.

0378-4347/90/$03.50

Apparatus

The chromatographic equipment consisted of a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector with a 20-μl loop, an Ultrasphere ODS 150 mm × 4.6 mm I.D. column (5-μm particles; Beckman, Berkeley, CA, U.S.A.) and a Schoeffel FS970 LC fluorimeter (Kratos, Ramsey, NJ, U.S.A.) at an excitation wavelength of 275 mm and with an emission filter with a cut-off wavelength of 418 nm.

Chromatographic conditions

The eluent was 50 m*M* acetate buffer pH 4.5 (acetic acid + sodium hydroxide) containing 1% (v/v) tetrahydrofuran. The flow-rate was 1.5 ml/min. The temperature was ambient.

Sample preparation

A 1.0-ml volume of urine was mixed with 1.0 ml of 0.5 *M* ammonium sulphate adjusted to pH 9.3 with ammonia. The mixture was passed through a Sep-Pak Light C_{18} cartridge (pretreated with 1 ml of methanol and 1 ml of water). The cartridge was washed with 1.5 ml of 5 m*M* ammonium sulphate adjusted to pH 9.3 with ammonia. ADO and cAMP were eluted with 1000 μl of a 10% methanol solution in 10 m*M* phosphoric acid. The last 800 μl of this eluate were sampled in a 10-ml polystyrene tube. The flow-rate was approximately 1.5 ml/min in all steps. A 75-μl volume of 0.5 *M* acetate buffer pH 4.8 and 75 μl of chloroacetaldehyde were added. The tube was capped and immersed in a boiling water-bath for 20 min. After cooling, 20 μl of the solution were injected on to the column.

RESULTS AND DISCUSSION

Calibration curves were obtained for ADO and cAMP in water, since these compounds naturally occur in all urines. The peak areas were determined by triangulation (peak height × peak width at half height) at 0.5 μA full scale on the recorder. The calibration curves were linear in the range 0.5–2.5 μ*M* (five calibration points). The correlation coefficients were 0.9992 and 0.9994 for ADO and cAMP, respectively. Fig. 1 shows a chromatogram of a standard mixture containing 1.0 μ*M* ADO and 1.0 μ*M* cAMP. The coefficient of variation for ADO in urine was 2.1% at a concentration of 1.20 μ*M* ($n=7$). The coefficient of variation for cAMP in urine was 3.5% at a concentration of 1.18 μ*M* ($n=7$). Minimum detectable concentrations (three times the background noise) were ca. 50 n*M* for both ADO and cAMP.

Concentrations between 0.30 and 3.42 μ*M* for ADO and between 0.28 and 2.75 μ*M* for cAMP were measured in urines from healthy volunteers without drug treatment. During treatment with diuretics, concentrations as low as 0.09

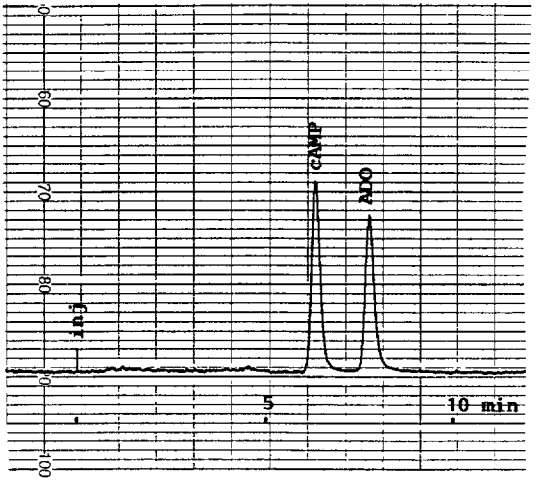

Fig. 1. Chromatogram of a standard mixture of 1.00 μM ADO and 1.00 μM cAMP in water.

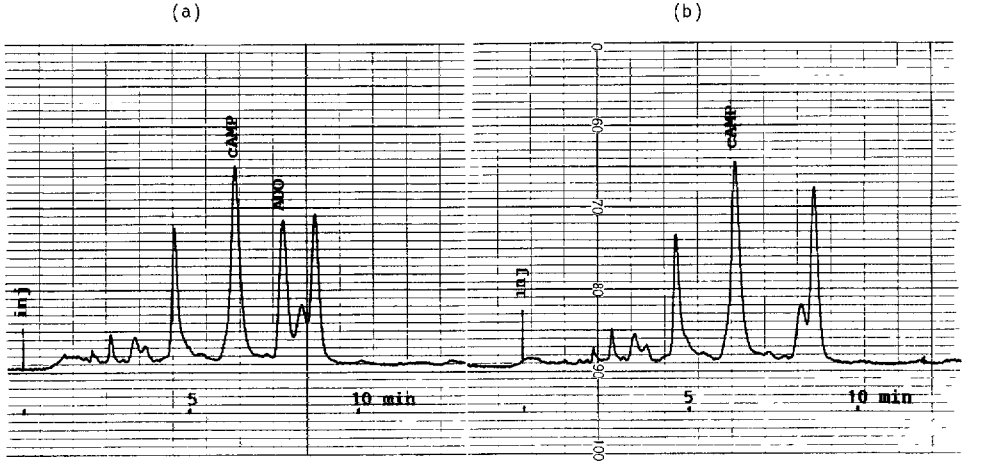

Fig. 2. Chromatograms of a urine sample from a healthy volunteer (a) before and (b) after treatment of the sample with adenosine deaminase. The concentrations of ADO and cAMP in the sample were calculated to be 1.00 and 1.16 μM, respectively.

μM for ADO and 0.08 μM for cAMP were detected. To test the purity of the ADO peak in urine, the sample was treated with 2 U/ml adenosine deaminase at room temperature for 20 min. Fig. 2 shows chromatograms of a urine sample found to contain 1.00 μM ADO and 1.16 μM cAMP and the same sample after treatment with deaminase. At room temperature the concentration of ADO did not change in 3 h indicating a low level of adenosine metabolism in urine. Further, ADO levels were stable for at least 24 h at 8 °C.

Recovery of the solid-phase extraction, checked at a concentration of 1.0 μM, was ca. 97% for both ADO and cAMP. Polystyrene tubes, instead of glass, were used for the derivatizing step because reproducibility was poor with glass. The recovery was also higher with polystyrene tubes.

Derivatization of adenine compounds with chloroacetaldehyde to form fluorescent etheno derivatives was reported by Kochetkov et al. [3]. The fluorescence characteristics of these derivatives of ADO and cAMP were examined by Secrist et al. [4]. Several methods have been published combining this reaction with high-performance liquid chromatographic separation and fluorescence detection [5–7]. Our derivatizing procedure is similar to that used by Yoshioka and Tamura [5] but uses a higher concentration of chloroacetaldehyde (ca. 0.63 M) to assure that the reaction is complete after 20 min. As direct derivatization of urine gave high background interference, sample purification was necessary. Kuttesch et al. [6] used an anion-exchange column for this purpose. This method seems tedious and results in a considerable dilution of the sample. Davies et al. [8] and Hirotoshi et al. [9] developed methods for the determination of ADO, including affinity solid-phase extraction and UV detection, with minimum detectable concentrations of ca. 100 nM in both cases. cAMP cannot be measured with these methods.

We have previously used solid-phase extraction successfully in the isolation of several highly polar compounds from urine, e.g. morphine and metabolites [10], isoniazid [11] and benzoylecgonine [12]. Therefore, we attempted to use the same principle in this case. Sep-Pak Light cartridges have, compared to the standard Sep-Pak, smaller diameters (0.5 cm instead of 1.0 cm) and much smaller inlet and outlet void volumes. This leads to smaller volumes for sample, washing and eluting solution (25% of standard Sep-Pak) and higher efficiency. The packing material is the same. Adsorption on the Sep-Pak, and washing, was carried out at pH 9.3 to assure retention of both cAMP and ADO while removing water-soluble acidic compounds. The best compromise for selective elution of both compounds was 10% methanol in 10 mM phosphoric acid (pH ca. 2.0).

In conclusion, this paper presents an easy, rapid and sensitive method for the determination of ADO and cAMP in urine.

ACKNOWLEDGEMENTS

We wish to thank Ms. Gunilla Wallenberg and Britt Wicksell for skilfull technical assistance. This work was supported by Swedish Medical Research Council (MFR 9068, 8873), the funds of Karolinska Institutet, Claes Groschinsky, Tore Nilsson (TNF) and Swedish Heart Lung Foundation.

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