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Simultaneous determination of adenosine, *S*-adenosylhomocysteine and *S*-adenosylmethionine in biological samples using solid-phase extraction and high-performance liquid chromatography

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Abstract

A sensitive and rapid method for measuring simultaneously adenosine, *S*-adenosylhomocysteine and *S*-adenosylmethionine in renal tissue, and for the analysis of adenosine and *S*-adenosylhomocysteine concentrations in the urine is presented. Separation and quantification of the nucleosides are performed following solid-phase extraction by reversed-phase ion-pair high-performance liquid chromatography with a binary gradient system. N^6-Methyladenosine is used as the internal standard. This method is characterized by an absolute recovery of over 90% of the nucleosides plus the following limits of quantification: 0.25–1.0 nmol/g wet weight for renal tissue and 0.25–0.5 μM for urine. The relative recovery (corrected for internal standard) of the three nucleosides ranges between 98.1±2.6% and 102.5±4.0% for renal tissue and urine, respectively (mean±S.D., $n=3$). Since the adenosine content in kidney tissue increases instantly after the onset of ischemia, a stop freezing technique is mandatory to observe the tissue levels of the nucleosides under normoxic conditions. The resulting tissue contents of adenosine, *S*-adenosylhomocysteine and *S*-adenosylmethionine in normoxic rat kidney are 5.64±2.2, 0.67±0.18 and 46.2±1.9 nmol/g wet weight, respectively (mean±S.D., $n=6$). Urine concentrations of adenosine and *S*-adenosylhomocysteine of man and rat are in the low μM range and are negatively correlated with urine flow-rate.

Keywords: Adenosine; *S*-Adenosylhomocysteine; *S*-Adenosylmethionine

1. Introduction

The purine nucleoside adenosine (ADO) has been recognized as a regulator of a wide variety of organ functions. Its role as an endogenous vasodilator of smooth muscles is well established. In contrast, in the kidney, ADO causes vasoconstriction and reduces glomerular filtration rate. This effect is enhanced proportionally to elevated plasma renin activity ([1,2] and see [3] for a review). ADO inhibits renal renin secretion [4] and, in addition, was shown to decrease norepinephrine release from intrarenal nerve endings [5].

ADO is generated by two pathways depicted in Fig. 1 [6]. Following the catabolic pathway of ATP, the enzyme 5′-nucleotidase hydrolyzes AMP to ADO. In the reverse direction ADO can be phosphorylated via ADO kinase to AMP [7]. The second pathway of ADO generation is the hydrolysis of *S*-adenosylhomocysteine (SAH), which is the de-

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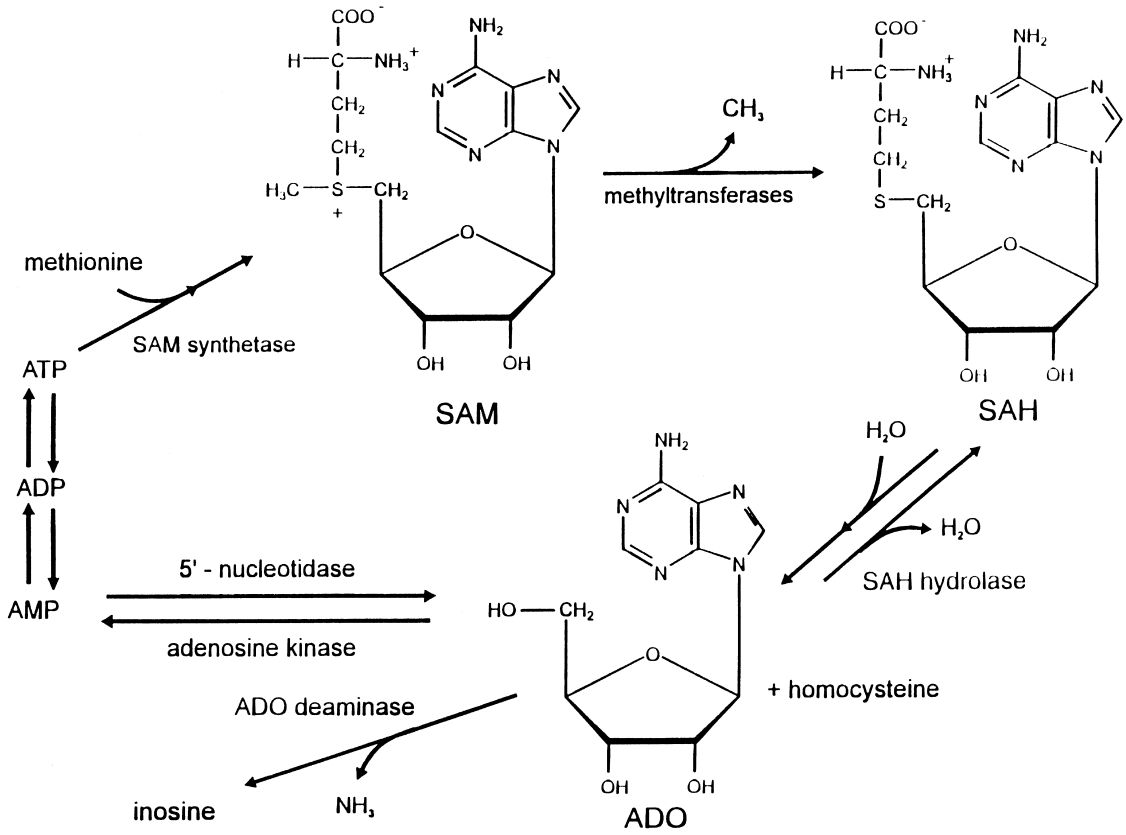

Fig. 1. Metabolic pathway of adenosine (ADO), *S*-adenosylhomocysteine (SAH) and *S*-adenosylmethionine (SAM). Enzymes: SAM synthetase (EC 2.5.1.6), methyltransferases (EC 2.1.1.*), SAH hydrolase (EC 3.3.1.1), ADO deaminase (EC 3.5.4.4), ADO kinase (EC 2.7.1.20), 5′-nucleotidase (EC 3.1.3.5). See text for further explanation.

methylated product of *S*-adenosylmethionine (SAM). The enzyme SAH hydrolase, responsible for this hydrolytic step, in the reverse direction catalyzes SAH synthesis. In vivo, due to ADO deaminase activity, the hydrolytic direction predominates since the intracellular ADO concentration is held at a low level. In vitro, however, the thermodynamic equilibrium of SAH hydrolase favours SAH synthesis [8]. The cytosolic ADO concentration depends upon the phosphorylation potential, i.e. the ATP/ADP ratio. It also modulates intracellular SAH levels. SAH is a potent inhibitor of SAM-dependent methyltransferases and therefore the activity of these enzymes is influenced by the ADO concentration. SAM, on the other hand, serves as a methyl donor in several transmethylation reactions. These include the metabolism of nucleic acids, proteins, phospholipids and biogenic amines. Thus, the ratio of intracellular SAM/SAH, termed the methylation potential, may be taken as a quantitative parameter of intracellular methylation reactions [9]. Under conditions of energy deficit, such as hypoxia or ischemia, an increased rate of ADO formation is observed in the kidney [10]. Therefore, adenosine has been suggested to be a mediator in the pathogenesis of acute renal failure [11]. In addition renal ischemia enhances SAH tissue levels by inactivation of the SAH hydrolase [12]. On the other hand, elevated intracellular levels of ADO and SAH, may lead to a significant increase in renal excretion of adenine nucleosides. Therefore, their urine concentration or excretion rate may represent a diagnostic or prognostic parameter in the course of acute renal disease.

Several methods have been described to determine ADO, SAH and SAM in biological samples. However, previously reported data on adenine nu-

cleosides showed great variations [13,14]. In addition, the analytical procedures did not meet requirements with respect to chromatographic quality or laboratory efficiency [15]. Therefore, an analytical method originally reported by Uziel et al. [17] and later by Davies et al. [18] was modified. A sensitive and rapid procedure is described using high-performance liquid chromatography (HPLC) after solid-phase extraction for the simultaneous quantification of ADO, SAH and SAM in renal tissue as well as ADO and SAH in urine samples.

2. Experimental

2.1. Sample collection and storage

Male Sprague-Dawley rats (weight 260.7±7.5 g) were anesthetized with thiopental (80 mg kg^{-1} i.p.). The left kidney was exposed by flank incision and carefully freed of connective tissue. An equilibration period of 30 min was used to stabilize systemic hemodynamics and the activity of central and peripheral nerve system. Subsequently, the kidney was removed and shock-frozen within 1–2 s using a Wollenberger-clamp precooled in liquid nitrogen.

Sulfosalicylic acid was added to urine samples of humans and rats (final concentration approximately 0.8 g/100 ml) to prevent potential enzymatic degradation of the nucleosides. All samples were stored at –80°C until analysis.

2.2. Sample preparation

2.2.1. Renal tissue

As previously described [14], the frozen kidney was powdered under liquid nitrogen in a mortar, transferred into a preweighed vial containing 3 ml precooled 0.6 *M* perchloric acid and shaken vigorously. The exact amount of tissue was assessed gravimetrically and a non-physiological analogue of adenosine, N^6-methyladenosine (M-ADO, 50 nmol in 50 μl of 0.02 *M* Tris buffer) was added as internal standard. After centrifugation at 11 000 *g*, the supernatant was recovered and adjusted to a pH between 5.5 and 6.5 by adding potassium carbonate (2 *M*). SAM is characterized by chiral instability at the sulfur atom at pH values above 7.0. In accordance with previous reports [15], SAM appeared as a double peak, due to isomer separation at neutral pH and above. Racemization of SAM results in two diastereoisomers [16], which are different in their physical properties and thus two peaks may occur. This phenomenon did not arise if the pH was adjusted as described above. Therefore the relatively acidic pH was chosen although the recovery of the nucleosides was maximal between pH 5 and 8. The precipitated potassium perchlorate was discarded after centrifugation at 11 000 *g* and 2 ml of the supernatant was applied onto the solid-phase extraction column.

2.2.2. Urine

In 2 ml of urine M-ADO (20 nmol in 100 μl of 0.02 *M* Tris buffer) was added as internal standard. Thereafter, the sample was adjusted to a pH between 5.5 and 6.5.

2.2.3. Solid-phase extraction

BondElut columns (ICT, Bad Homburg, Germany) containing 100 mg phenylboronic acid bonded silica, were rinsed with 5 ml HCl (0.1 *M*). Solvation of the sorbent was performed with 5 ml formic acid (0.1 *M*) to facilitate its covalent interaction with the nucleosides. The column was rinsed again with 5 ml Tris buffer solution (0.02 *M* in 30% methanol, pH 7.4). Thereafter, the renal tissue extracts or urine samples were applied onto the columns. Then the columns were washed with 1 ml sodium citrate (10 m*M*, pH 8.8). Once bound to the phenylboronic acid, SAM remained stable during the rinsing step with sodium citrate, in contrast to its chiral instability in alkaline solutions. Elution of the compounds was performed by acidification of the boronate complex with 500 μl HCl (0.1 *M*). A 50 μl volume of the eluate was injected into the HPLC system.

2.3. HPLC analysis

2.3.1. Instrumentation

The HPLC system consisted of a high-pressure pump (flow-rate 1.0 ml min^{-1}) equipped with a low-pressure gradient system (S 1000, Sykam Gilching, Germany); a UV absorbance detector (model 204, Linear Instruments, Fremont, CA, USA) set to 254

nm; an automatic sample injector (Marathon, Spark Holland, Emmen, The Netherlands). Remote control of the HPLC system, data acquisition and calculation of peak areas (peak AUC) were performed via a microcomputer-based data system (Axxiom 737, Calabasas, CA, USA). Two types of reversed-phase HPLC columns were used: (1) Grom-Sil 120 ODS-3 CP (5 μm, 125×4 mm I.D.) to measure renal tissue content of ADO, SAH and SAM; and (2) Nucleosil 100 C18 (3 μm, 125×4 mm I.D.) to assess concentration of ADO and SAH in urine. Both columns were purchased from Grom (Kayh-Herrenberg, Germany). The column temperatures were maintained constant at 30°C.

2.3.2. Mobile phase

A binary low-pressure gradient elution was used. For analysis of renal tissue, eluent A consisted of ammonium dihydrogenphosphate (10 m*M*) and heptanesulfonic acid sodium salt (0.6 m*M*) as the ion-pair forming agent in 3% methanol. Solvent B contained, in addition, 10 % (v/v) acetonitrile. After sample injection, the mobile phase was kept at 100% solvent A for 11 min. To increase solvent B to 40% at 24 min, a linear gradient was started at 12 min. Subsequently, solvent B was raised to 100% for 2 min. Before the next sample injection, solvent A had to be kept at 100% for 6 min to reequilibrate the system. Thus, the chromatograms were completed within 35 min. Concentrations of ADO and SAH in urine were determined using identical solvents A and B as described above but neither solution contained heptanesulfonic acid.

2.3.3. Peak identification and calculations

Peaks representing ADO, SAH, SAM and M-ADO were identified according to their retention times (Table 1). In some experiments ($n=4$), identification of ADO and SAH was verified by the disappearance of their peaks after addition of SAH hydrolase and ADO deaminase, respectively. Urine concentrations and tissue contents of the nucleosides were calculated by comparing the areas under the peak (AUC) with that of the internal standard (M-ADO). Tissue content of the nucleosides was expressed as nmol/g wet weight and their concentrations in urine as μ*M*.

2.4. Chemicals

ADO and adenosine deaminase were purchased from Boehringer (Mannheim, Germany), methanol and acetonitrile from Merck (Darmstadt, Germany). SAH, SAM and N^6-methyladenosine from Sigma-Aldrich Chemie (Deisenhofen, Germany) and heptanesulfonic acid sodium salt from Fluka Chemika-Biochemika (Buchs, Switzerland). All chemicals used were of HPLC-grade or the highest purity grade available.

3. Results and discussion

3.1. HPLC analysis: Precision and variability

Injection of aqueous solutions containing the three nucleosides and the internal standard M-ADO showed linearity of the peak areas in concentrations between 5×10^{-7} and 10^{-4} *M* (correlation coefficient $r=0.9998$). The present detection limit in standard solution was 10^{-7} *M* (data not shown). Since a sample volume of 50 μl was injected into the HPLC system, 5 pmol of each nucleoside could be detected without concentration of the sample. The methodological variability was examined by repeated measurements of the same standard mixture on 20 consecutive days. Table 2 presents the results.

Table 1
Retention times (min) of analytes and internal standard (data represent mean values±S.D. of six measurements)

	SAM	SAH	ADO	M-ADO
Urine	–	9.5±0.2	11.7±0.3	20.6±0.1
Kidney	5.5±0.1	7.8±0.2	10.2±0.2	19.1±0.1

Table 2
Methodological variability, with repeated determination of a standard mixture (10 μ*M*) on 20 consecutive days (data represent mean values±S.D. of seven measurements)

	SAM	SAH	ADO
Coefficient of variation (%)	−0.4±1	0.6±3	−0.7±2

3.2. Sample preparation: Precision and recovery

To examine sensitivity, recovery and reproducibility of the complete analytical procedure, a standard solution containing ADO, SAH, SAM and M-ADO at five concentrations (0.5–100 μ*M*) was subjected both to solid-phase extraction and HPLC separation. Fig. 2 depicts the AUC values of the UV responses in comparison to the concentrations given. Linearity was observed over the range of concentrations studied. The correlation coefficient for all four nucleosides was $r=0.99999$ (Fig. 2).

Absolute recovery of ADO undergoing the solid-phase extraction was assessed by adding tritium (^{3}H)-labelled ADO to a standard sample. Comparison of the ^{3}H-radioactivity in the original sample to that in the eluate revealed a recovery of 92±3% ($n=3$). Therefore, based on the similar AUC and the linearity of the peak areas of the four nucleosides, a similar absolute recovery can be assumed for SAH, SAM and M-ADO.

Quantitative analysis by HPLC, though a sensitive analytical assay, can be impeded, if only small amounts of biological material are available, (i.e. urine of laboratory animals or samples from biopsies). This is especially so when the nucleoside concentrations are low, as found for the SAH content in normoxic rat kidney (see below). Therefore a sample preparation prior to HPLC chromatography is mandatory in order to purify and concentrate the analytes in those cases. Affinity chromatography, using immobilized phenylboronic acid as presented here, proved to be a suitable sample preparation method for the extraction of ADO, SAH and SAM from biological material. The nucleosides are characterized by *cis*-diol groups, which bind to the hydroxyl groups of the phenylboronic acid [18]. Elution is performed by acidification which renders the immobilized phenylboronic acid neutral and thus releases the nucleosides. Under the present conditions, the optimum volume to elute the analytes from the phenylboronic acid was 500 μl. This resulted up to a 4-fold concentration of the nucleosides when 2 ml of urine or tissue extract were available. Comparative series employing different compositions of the elution media were performed because other media, such as formic acid with or without methanol, have been described to be suitable solutions for elution [19]. HCl (0.1 *M*) was clearly shown to possess the best elution properties in terms of recovery, stability and HPLC separation of the adenine nucleosides (data not shown).

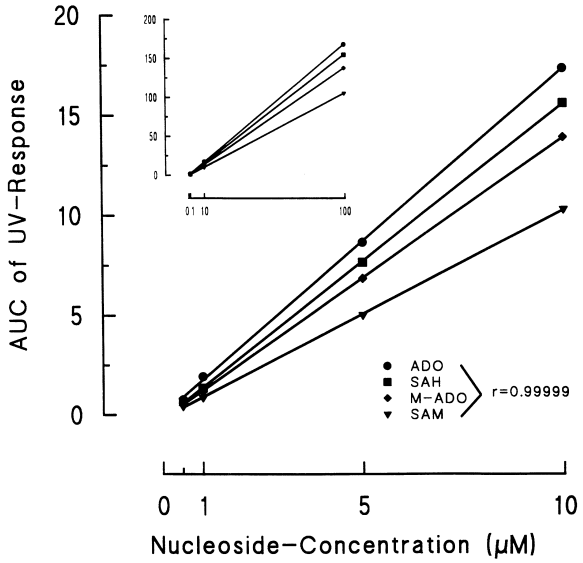

Fig. 2. Area under the curve (AUC) of the UV absorbance detector response for ADO, SAH, SAM and the internal standard M-ADO at various concentrations after sample preparation and HPLC analysis. *r* is the correlation coefficient. See Fig. 1 for abbreviations.

3.3. Validation of the assay

Fig. 3 shows representative chromatograms of samples from rat kidney and human urine. To validate the analytical method, the relative recovery of given levels of ADO, SAH and SAM was determined. Various amounts of the nucleosides were added to pooled samples of rat renal tissue homogenate and of urine at concentrations corresponding to the physiological and pathophysiological range observed in preliminary experiments. Thereafter, sample preparation and HPLC analysis were performed as described above using M-ADO as internal standard.

3.3.1. Validation in renal tissue

0.25–10 nmol of SAH and 1–50 nmol of ADO and SAM were added per 1 g wet weight to kidney

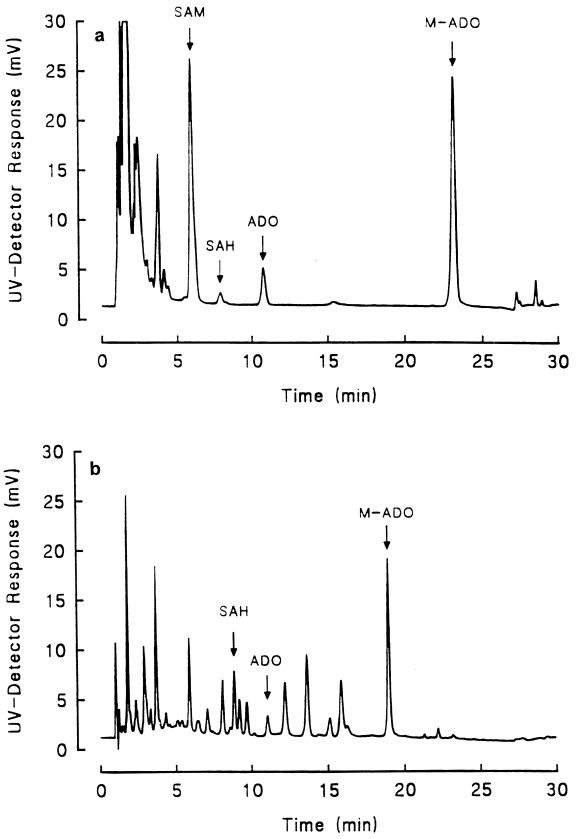

Fig. 3. Representative chromatograms of a perchloric extract of rat kidney tissue (a) and of human urine (b). After solid-phase sample preparation, 50 μl of the eluates were injected into the HPLC system. See Fig. 1 for abbreviations.

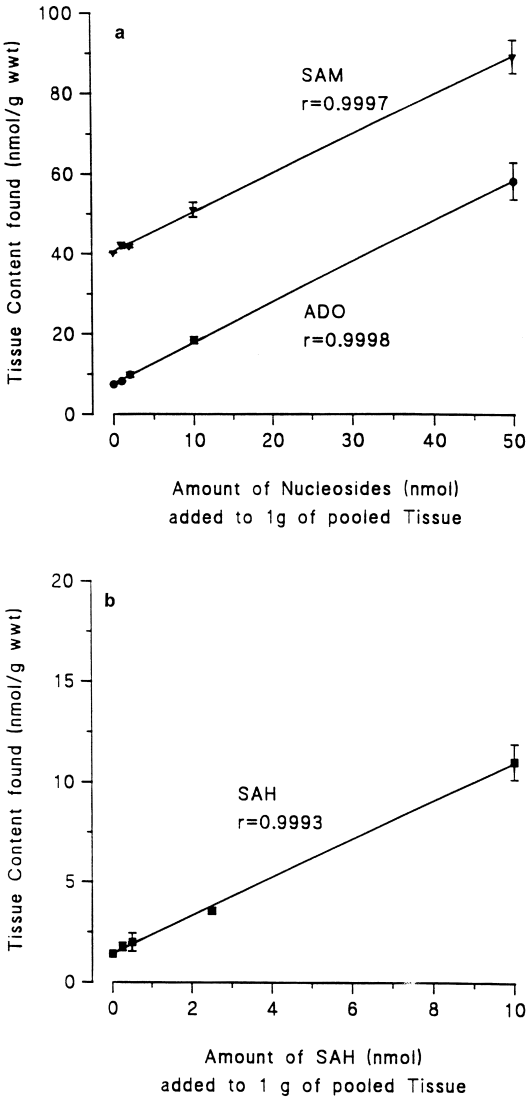

Fig. 4. Analytical recovery of ADO and SAM (a) and of SAH (b) added in various amounts to pooled homogenates of rat renal tissue (per 1 g wet weight). r is the linear correlation coefficient. Symbols represent mean values±S.D. obtained in three different experiments. See Fig. 1 for abbreviations.

homogenates. The comparison of values expected with those measured revealed linear recovery with correlation coefficients (r) of 0.9998, 0.9993, and 0.9997, for ADO, SAH and SAM, respectively, as shown in Fig. 4a and Fig. 4b. After correction for internal standard the following relative recoveries were observed (mean±S.D., $n=3$, range in brackets): ADO, 102.5±4.0% (98.1–105.5%); SAH, 99.8±9.7% (91.0–106.8%); SAM, 100.5±2.2% (99.2–102.1%). The limit of quantification for tissue content of ADO, SAH and SAM was 0.5, 0.25 and 1.0 nmol/g wet weight, respectively.

3.3.2. Validation in urine

As described above ADO and SAH were determined in urine. To 1 ml of pooled urine of humans or rats, 0.25–25 nmol ADO and SAH were added. Fig. 5 depicts the data obtained in these experiments performed in three series. Comparison of both ADO and SAH concentrations expected with those measured revealed linear recovery with a correlation coefficient of $r=0.9999$. The relative recoveries (after correction for internal standard) of

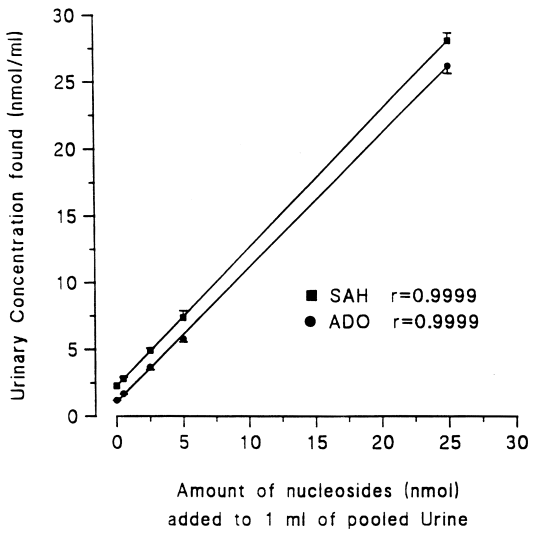

Fig. 5. Analytical recovery of ADO and SAH added in various amounts to pooled urine samples. Symbols represent mean values±S.D. of three different experiments. See Fig. 1 for abbreviations.

ADO and SAH were (mean±S.D., $n=3$, range in brackets) 98.1±2.63% (93.3–100.1%) and 102.2±3.4% (101.4–103.1%) respectively. Limit of quantification was 0.25 nmol ml^{-1} (ADO) and 0.5 nmol ml^{-1} (SAH).

3.3.3. Biological variability

Two triplicate measurements ($n=6$) of nucleoside content in pooled homogenates of renal tissue showed the following ranges of variation: ADO between −7.7 and +10%; SAH between −10.7 and +19.6%; SAM between −1.8 and +1.2%. In pooled urine, two triplicate measurements of ADO and SAH concentrations showed a variability between −4.7 and +3.4%, and between −3.5 and +4.2%, respectively.

3.4. Tissue content of ADO, SAH and SAM in rat kidney

In the present study, tissue content of ADO, SAH and SAM in rat kidney ($n=6$) was found to be 5.64±2.2, 0.67±0.18 and 46.2±1.19 nmol/g wet weight, respectively. Similar ADO values were reported earlier using enzymatic quantification following thin-layer chromatography by Osswald et al. [14]. However, in the same study, a marked and time-dependent increase in ADO content (up to 38.1±6.3 nmol/g wet weight) was demonstrated during renal artery occlusion. This manœuvre is also accompanied by a rapid decrease in ATP [20]. These observations most likely explain up to 15-fold variations of ADO, SAH and SAM in renal tissue reported in mice [13]. As in that study, the organs were analyzed after an uncontrolled duration of ischemia. This might also apply to human renal tissue obtained by biopsy from nephrectomized donor kidneys, in which an approximately 10-fold higher ADO content was reported compared with the values observed in the normoxic rat kidney [21]. The renal adenine metabolism is influenced instantly and significantly by ischemia. Therefore, a standardized procedure for instant interruption of the metabolism is mandatory if ADO, SAH and SAM levels in renal tissue have to be assessed under normoxic conditions. The freeze-clamp-technique used in the present experiments, ensures termination of metabolic reactions within 1–2 s.

3.5. ADO and SAH excretion into the urine

In conscious rats, kept in metabolic cages ($n=6$), urine ADO concentration was found to be 6.01±1.10 μ*M* which is in agreement with values previously reported in rats and dogs [22–24]. Urine SAH concentration of conscious rats was found to be 3.06±0.86 μ*M*. Previously, no data on urine SAH concentrations in rats have been reported.

Data on ADO concentrations in human urine differ from ≤1≥5 μ*M* [19,25,26]. Even higher variations were observed in the present measurements. However, urine ADO concentrations clearly depended on the urine flow-rate. In hydrated individuals urine ADO was as low as 0.35 μ*M* while volunteers subjected to fluid restriction showed values of up to 7.24 μ*M*. Therefore, the excretion rate of ADO into the urine appears to be a more valid parameter of its renal release rather than the urine concentrations. Yet, ADO excretion rate might also be influenced by urine flow-rate. In healthy volunteers, ADO excretion rate was 2.77±1.55 nmol min^{-1} during fluid restriction. A significant increase to 5.04±2.48 nmol min^{-1} was present after hydration with 0.75 l per 1.73 m^2 tap water ($n=4$, $p<0.05$). Similar observations were made for SAH in human urine. Its concentrations varied between 1.16 μ*M* and 16.9

μ*M*. SAH excretion rates during fluid restriction and volume load were 6.16±1.18 and 11.4±5.63 nmol min^{-1}, respectively.

In conclusion, we have presented a simple and sensitive method using solid-phase sample preparation and HPLC which enables the simultaneous analysis of adenine nucleosides in tissue homogenates and in urine. As the tissue content of adenine nucleosides in the kidney rapidly increases under ischemic conditions, a standardized procedure for collection of organ samples which instantly interrupts metabolism is essential. The underlying mechanisms for the negative correlation between urine flow-rate and the concentrations of ADO and SAH in the urine have to be further investigated.

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