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# A novel HPLC procedure for detection and quantification of aminoacetone, a precursor of methylglyoxal, in biological samples

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**Abstract**

Increase in methylglyoxal is thought to be involved in different pathological conditions. Deamination of aminoacetone by semicarbazide-sensitive amine oxidase (SSAO) leads to production of methylglyoxal. We have synthesized aminoacetone and developed a novel HPLC procedure for its quantitative determination. The urinary excretion of aminoacetone is approximately 20–30 μg/mouse/day, and the concentration is about 0.5 μg/g in mouse liver and small intestine. SSAO inhibitor increases aminoacetone levels in both tissues and urines. Results confirm that aminoacetone is an endogenous substrate for SSAO. However, data also indicate that deamination is not the only catabolic pathway for aminoacetone.


*Keywords:* Aminoacetone; SSAO; Methylglyoxal; HPLC

## 1. Introduction

Semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) is a group of copper containing enzymes present in many mammalian tissues including humans, with relatively high activities present in vascular smooth muscle, cartilage and adipose tissues [1–3]. The enzyme has been independently identified as an endothelial surface adhesion molecule regulating lymphocytes trafficking [4]. Adipose SSAO has also been shown to regulate glucose transport [5]. Methylamine and aminoacetone are thought to be physiological substrates for SSAO [6–8]. SSAO catalyzes the oxidative deamination of these primary amines and lead to production of formaldehyde, and methylglyoxal, respectively, as well as hydrogen peroxide and ammonia [1]. Since these products are quite toxic, the potential pathological implications of deamination of methylamine has been addressed [1,9,10]. It is known that methylglyoxal is increased and related to protein glycation in different pathological conditions, such as diabetes and aging [11,12]. Oxidative deamination of aminoacetone to methylglyoxal has been shown using extracts of human umbilical artery [8] and plasma of goat and bovine [13,14]. Interestingly, administration of aminoacetone was shown not only to increase the urinary excretion of methylglyoxal but also malondialdehyde, an oxidative stress marker, in mice [15]. Methylglyoxal is primarily synthesized by the phosphorylated triose pathway [12]. It is unclear how important is deamination of aminoacetone contributing to methylglyoxal production. Aminoacetone is synthesized from threonine and glycine [16,17]. Although aminoacetone exhibits high affinity for SSAO [15], its presence in tissue has not been reported. Treatment with a SSAO inhibitor reduces the urinary excretion of methylglyoxal in rodents [18]. Investigation on aminoacetone is hampered, since the

*Abbreviations:* SSAO, semicarbazide-sensitive amine oxidase; AA, aminoacetone; MA, methylamine; MDL-72974A, (*E*)-2-(4-fluorophenetyl)-fluoroallylamine; FMOC-Cl, fluorenylmethyl chloroformate

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compound is not available commercially. In the present study, aminoacetone has been synthesized and an HPLC procedure for its measurement in urine and tissues established. This is the first report showing the presence of aminoacetone in tissues.

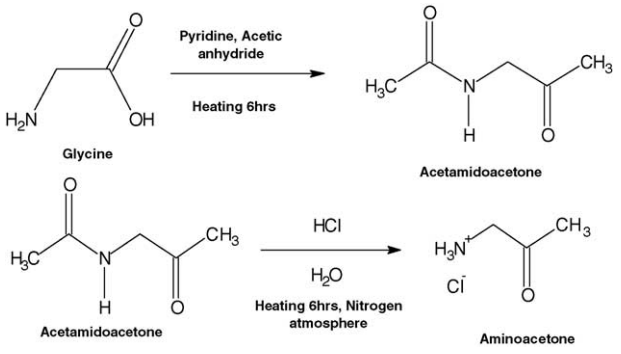
## 2. Experimental

### 2.1. Chemicals

Glycine, pyridine, acetic anhydride, acetone, tetramethylammonium chloride, boric acid, sodium hydroxide, phosphorus pentoxide, citric acid, phosphoric acid, hydrochloric acid, acetic acid, sodium acetate, ethanol, diethyl ether and 9-fluorenylmethyl chloroformate (FMOC-Cl) were purchased from Sigma–Aldrich. HPLC-grade acetonitrile, methanol, hexane and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Unless stated otherwise, all reagents were of analytical grade. Potassium borate buffer was prepared from boric acid (0.8 M) adjusted to the desired pH 10 with 5 M potassium hydroxide. (*E*)-2-(4-Fluorophenetyl)-fluoroallylamine MDL-72974A was kindly provided by Marion-Merrell Dow (Cincinnati, Ohio, USA).

### 2.2. Synthesis and purification of aminoacetone hydrochloride

Aminoacetone hydrochloride was synthesized and purified using a modified procedure according to Hepworth [19] (Scheme 1). Acetamidoacetone was prepared by mixing glycine (37.5 g, 0.5 mol), pyridine (242.5 g, 3.0 mol), and acetic anhydride (595 g, 5.835 mol) heating to boiling, refluxing and stirring for 6 h. The reflux condenser is replaced by one set for downward distillation and the excess pyridine, acetic anhydride, and acetic acid were removed by distillation under reduced pressure. The residues yielded a pale yellow oil (bp 120–125 °C, 1 mmHg). The acetamidoacetone (26 g) was further mixed in concentrated hydrochloric acid (87.5 ml) and water (87.5 ml), and then boiled under reflux in a nitrogen atmosphere for 6 h. The resulting solution containing aminoacetone product is concentrated obtaining a dark red oily residue which is then dried under reduced pressure over phosphorus pentoxide to crystal aminoacetone hydrochloride. Aminoacetone HCl salt was re-crystallized with absolute ethanol and diethyl ether. Yields for acetamidoacetone and aminoacetone after re-crystallization were 45% and 4.1%, respectively. Under a stream of dry nitrogen the solid residues were rapidly filtered through a sintered glass funnel, then washed with ether again and immediately transferred to sample vials, dried again, and sealed in dry nitrogen. The melting point of aminoacetone HCl crystal is sharp at 72–73 °C. Nuclear magnetic resonance (NMR) and HPLC were used to substantiate the identity of aminoacetone. NMR was performed in $D_2O$ solvent using a Varian Germini 400 MHz NMR spectrometer. The data indicate the presence of protons of methyl and methylene groups in acceptable proportion: $\delta$H 2.18 (s, 3H), 3.99 (s, 2H). HPLC analysis was conducted as described bellow. Authentic aminoacetone from Dr. Etelvino Bechara was used for quantitative comparison. The synthesized aminoacetone is therefore highly a pure compound. The chemical is kept in a cool and dark place in bottles filled with nitrogen gas. For the preparation of a working standard, aminoacetone was dissolved in absolute alcohol (1 mg/ml) and stored in a freezer (−20 °C), under these conditions it was stable for at least a month.




Scheme 1. Chemical synthesis of aminoacetone from glycine via acetamidoacetone.

### 2.3. Animal experiments

Male CD1 Swiss white mice weighing 30 g and Wistar rats (260–300 g) were used in the experiments. The animals were housed in hanging wire cages with free access to food and water on 12 h light/dark cycle (lights on at 6 a.m.) at temperature 19–20 °C. The experimental protocol has been designed according with the guidelines of the Canadian Council on Animal Care and approved by University of Saskatchewan Animal Care Committee.

Mice were treated with saline (100 μl, i.p.), SSAO inhibitor (MDL-72974A) (5 mg/kg, i.p.), and subsequently two hours later with aminoacetone (10 mg/kg, i.p.). After the last injection, the mice were placed in metabolic cages for urine collection over a period of 24 h. The collecting vessels were positioned in Styrofoam boxes containing dry ice to freeze the urines immediately after excretion.

Regarding analyses of aminoacetone in tissues animals were killed 2 h after injection of saline or MDL-72974A (5 mg/kg, i.p.) and aminoacetone (10 mg/kg, i.p.). The liver and small intestine were dissected, washed in saline and stored at −70 °C. For the ex vivo catabolism experiments the freshly dissected liver and small intestine were sliced using a McIlwain tissue chopper (Michle Laboratory Engineer, Surry, UK).

Rats were used for the assessment of aminoacetone concentrations in tissues and ex vivo catabolism studies. The collected tissues ($n = 3$) were further divided into three independent parts for independent analyses.

### 2.4. Preparation of samples

Animal tissues were homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in 0.1 M phosphate buffer (pH 7.3; 1:5, w/v). Aminoacetone was partially purified by ion exchange chromatography. Aliquots of tissue homogenates or urine samples were applied to small CG-50 Amberlite columns packed in glass Pasteur pipette (2 cm). After washing the column with 10 ml HPLC-grade water the column was eluted with 2 ml of hydrochloric acid (1 M), aminoacetone was collected in the second ml of eluent and neutralized by adding 100 μl sodium hydroxide (10 M). These samples were used for derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) and HPLC-analysis.

### 2.5. FMOC derivatization of aminoacetone

For derivatization 500 μl potassium-borate buffer (0.8 M; pH 10) was added to 1.0 ml of samples and vortexed for 60 s. One millilitre of FMOC-Cl reagent solution (10 mM in acetonitrile) was then added to the buffered samples and vigorously vortexed for 1 min. The reaction was terminated by extraction of excess reagent (FMOC-Cl), its hydrolysis product FMOC-OH, and acetonitrile with 5.0 ml hexane. The upper hexane layer was discarded and this procedure was repeated twice. The potassium-borate buffer was neutralized by adding 0.1 ml 20% (v/v) acetic acid, aliquots of these samples (injection volume was 250 μl) were injected into a HPLC.

### 2.6. HPLC

The HPLC system composes a Shimadzu solvent delivery module (LC-10 AD*vp*), a Shimadzu auto injector (SIL-10AD*vp*), and a Shimadzu DGU-14A degasser. For spectrophotometric detection a Hewlett Packard fluorometer (HP1046A) and a BIO-RAD UV monitor model 1305 (Shimadzu SPD-10A*vp* UV-VIS detector) were used. A reversed-phase HPLC column (4.6 mm × 250 mm; Beckman Ultrasphere IP; 5 μm, C18) was used.

A modified tertiary gradient system was based on previous method for analysis of amino acids [22]. Solvent A was 20 mM citric acid containing 5 mM tetramethylammonium chloride (TMA), adjusted to pH 2.85 with 20 mM sodium acetate containing 5 mM TMA. Solvent B was 80% (v/v) of 20 mM sodium acetate solution containing 5 mM TMA adjusted to pH 4.5 with concentrated phosphoric acid and 20% (v/v) methanol (100%). Solvent C was acetonitrile (100%). The elution gradient of mobile phase is graphically shown in Fig. 1. The flow rate was maintained at 1.4 ml/min throughout the analysis. The separation was performed at a column temperature of 25 °C. Absorbance was measured at 265 nm; fluorescence was monitored at 254 and 630 nm (excitation and emission wavelengths, respectively).

### 2.7. Statistics

The results were assessed using one way analysis of variance (ANOVA) followed by multiple comparisons (Newman-

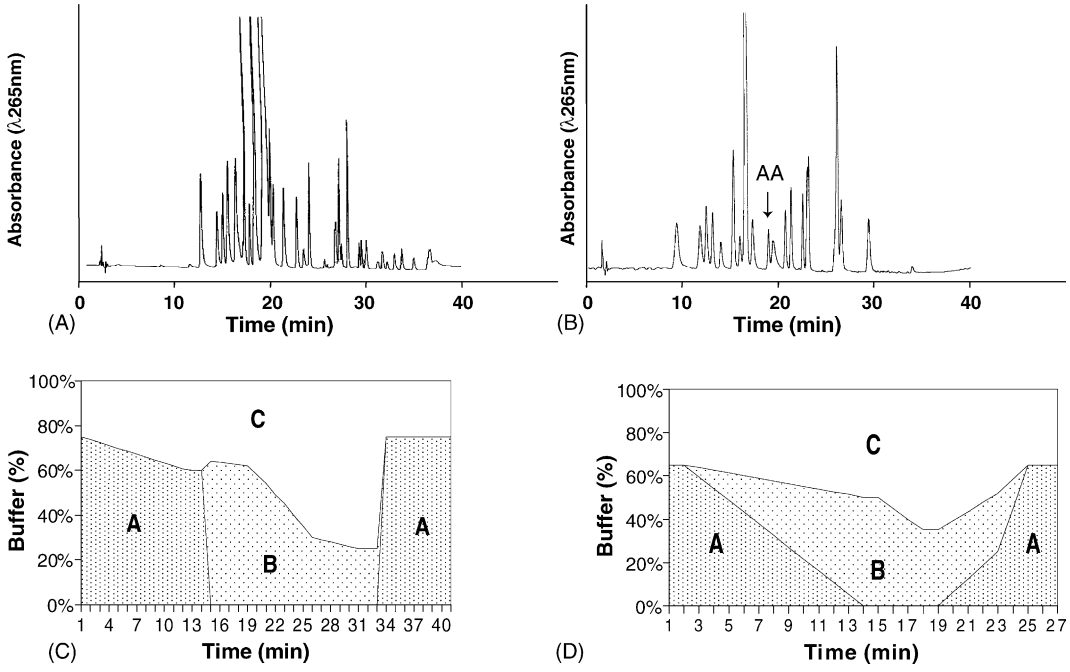



Fig. 1. HPLC separation of FMOC derivative of aminoacetone and amino acids. (A) Gradient system according to Bank [20]; (B) gradient system developed specifically for aminoacetone; (C) gradient system according to Bank; (D) gradient system developed in the present investigation. Eluent A: 20 mM citric acid; 5 mM tetramethylammonium chloride (TMA); pH 2.85 adjusted with 20 mM sodium acetate contains 5 mM TMA. Eluent B: 80% 20 mM sodium acetate; 5 mM TMA. 20% methanol pH 4.5 adjusted with phosphoric acid. Eluent C: 100% acetonitrile.

Keuls). The null hypothesis used for all analyses was that the factor has no influence on the measured variable and significance was accepted at >95% confidence level.

## 3. Results and discussion

### 3.1. HPLC chromatography of aminoacetone-FMOC adduct

The separation of aminoacetone and amino acid FMOC derivatives are shown in Fig. 1A and B. Although according to the original tertiary gradient system described by Bank et al. [20] (Fig. 1C) the resolution of standard amino acids is satisfactory, it is not applicable for aminoacetone due to interference with valine-FMOC. The solvent system has therefore been modified [see Fig. 1D] after numerous redesigns and tests of the gradient system. As can be seen in Fig. 1B, under the modified chromatographic condition aminoacetone-FMOC is resolved from valine-FMOC, although the change of the solvent system cause merger of different amino acid FMOC adducts.

### 3.2. Pre-purification of aminoacetone by CG-50 chromatography

The chromatography conditions for aminoacetone detection were not suitable for analysis of aminoacetone in urine or tissue extracts. It is necessary to partially purify the tissue extracts before derivatization with FMOC. To obtain this, a weak anion ion exchange column (CG-50 Amberlite) was employed, which effectively remove all neutral, acidic amino acids and possible also some unidentified amines or small peptides. In the final acid eluates from the CG-50 column, basic amino acids such as lysine and arginine were eluted along with aminoacetone and methylamine with hydrochloric acid (1 M). These basic amino acids and amines do not interfere with the assessment of aminoacetone. Fig. 2A shows a typical separation of aminoacetone in mouse small intestine extracts.

### 3.3. Validation of the analysis

A series of concentrations of aminoacetone were prepared (from 0.01 to 5 μg/ml). A linearity of the calibration curves of aminoacetone was obtained. For analysis of aminoacetone in the biological materials, we were unfortunately, not able to find a suitable basic amine as an internal standard. Estimation of the amount of aminoacetone is therefore based on external aminoacetone standard curves. Regarding the calibration curves for aminoacetone the correlation coefficients were higher than 0.99 in all cases. The recovery of aminoacetone from urine or tissue homogenate samples were analyzed from aminoacetone spikes (e.g. Fig. 2B). The results were 72 ± 4% and 91 ± 2% following CG-50 and FMOC-Cl derivatization procedure respectively. Aminoacetone-FMOC

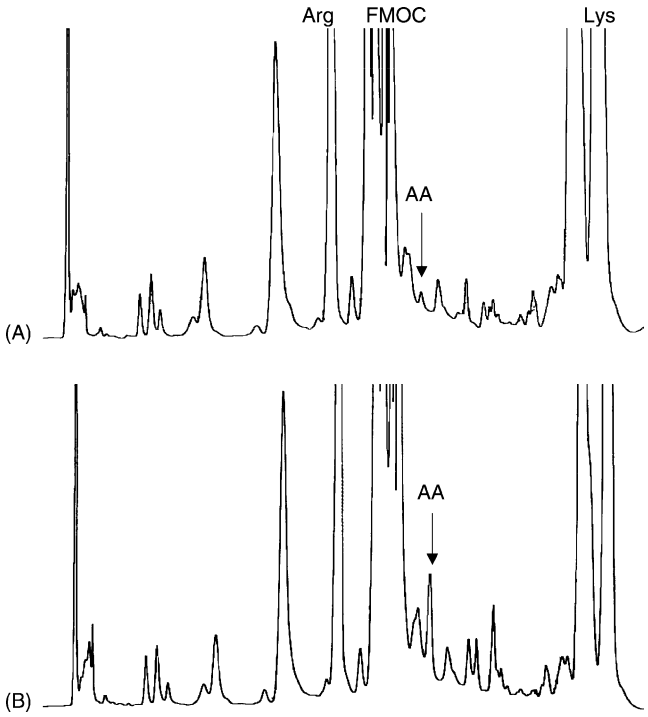



Fig. 2. HPLC chromatogram of aminoacetone-FMOC obtained from mouse small intestine. (A) Aminoacetone in the small intestine tissue extracts were partially purified by an ion-exchange column (CG-50 Amberlite) before derivatization with FMOC-Cl. (B) Authentic aminoacetone was included in the tissue homogenates.

derivative is extremely stable. Therefore the intra- and inter-day variations are negligible. Aminoacetone is quite stable storage at −70 °C and during the process of analyses. The limit of detection for aminoacetone was estimated at 0.01 μg/ml.

### 3.4. Aminoacetone levels in urines and tissues

The average concentrations of aminoacetone in urine ranged between 20 and 30 μg/mouse/24 h (equivalent to 15–25 μg/mg creatinine/24 h). These values are comparable to the levels of methylamine. After a single intraperitoneal administration of MDL-72974A, the concentration of aminoacetone in urine significantly increased by 2.5-fold (see Fig. 3A) within 24 h. The urinary aminoacetone levels returned to the baseline on the second day.

Fig. 3B shows the effect of administration of aminoacetone and SSAO inhibitor on urinary excretion of aminoacetone. Urinary aminoacetone increased following administration of aminoacetone, in particular, when SSAO activity was blocked. Methylamine levels, which were also detected in the same HPLC system, were increased by SSAO inhibitor but not by aminoacetone. It is interesting to note that overall less than 10% of the administered aminoacetone was recovered in the urine. SSAO inhibitor only partially increased the recovery. It appears that other pathways might be involved in degrading aminoacetone in vivo.

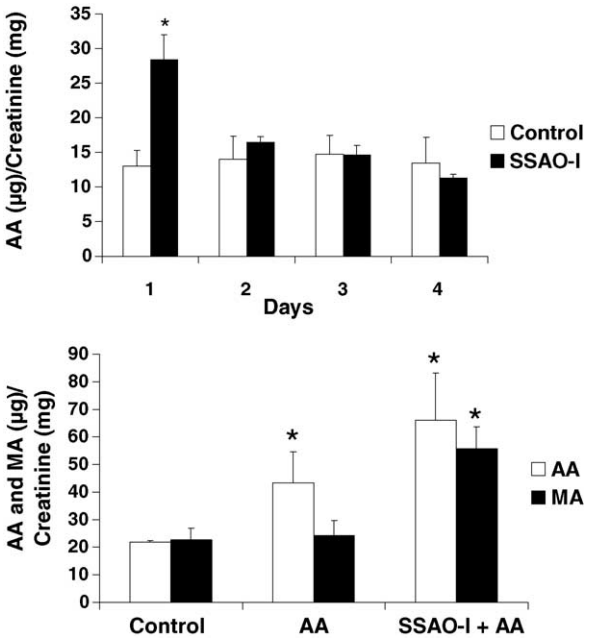



Fig. 3. Effect of MDL-72974A and aminoacetone on urinary excretion of aminoacetone and methylamine in mice. (A) MDL-72974A (10 mg/kg, i.p.) (solid bars) or saline (open bars) administered by intraperitoneal injection, 24 h urines were collected daily after a single injection. (B) Aminoacetone (AA) (10 mg/kg, i.p.) in the presence or absence of SSAO inhibitor on urinary excretion of aminoacetone and methylamine (MA). Data are mean ± S.E. ($n$ = 3–5), $^{*}p < 0.05$ indicates statistical significance compare with control.

Aminoacetone was also assessed in tissue homogenates. As can be seen in Fig. 4, the concentrations of aminoacetone in liver and intestine approximately 0.45 and 0.4 μg/g of tissue, respectively. When SSAO was inhibited, aminoacetone concentration was increased but only significant with respect to intestine tissue.

### 3.5. SSAO-mediated catabolism of aminoacetone

Data above seem to suggest that the catabolism of aminoacetone is not only via SSAO-mediated deamination. The present investigation shows in vitro and ex vivo catabolism of aminoacetone in the liver (with high general

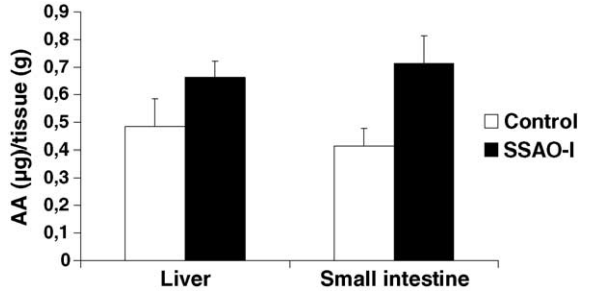



Fig. 4. Effect of MDL-72974A on aminoacetone (AA) levels in liver and small intestine tissues. Tissues were dissected 2 h after injection of saline or MDL-72974A (5 mg/kg, i.p.).

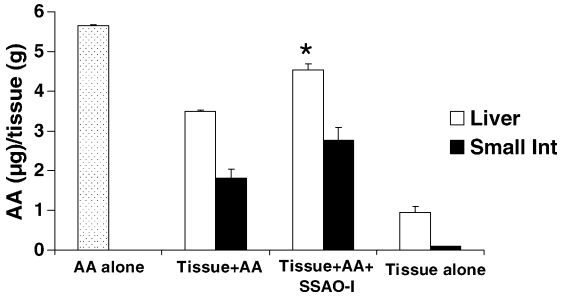



Fig. 5. Degradation of aminoacetone in vitro. One millilitre aminoacetone (AA) (150 μM) was incubated in saline in the presence of 1 g rat liver or intestine tissue slices at 37 °C for 4 h. The effect of pretreatment of SSAO inhibitor, tissue slices were pre-incubated with 10 μM MDL-72974A for 30 min. Untreated tissue was incubated twice with saline only. The residual aminoacetone was analyzed as described in text. Data are mean ± S.E. ($n$ = 5), $^{*}p < 0.05$ indicates statistical significance compare with control.

catabolic activities) and the small intestine (possessing high SSAO activity). Fig. 5 shows the change of aminoacetone concentrations after incubation with saline alone, with rat liver and intestine tissue slices, with tissue slices pretreated with SSAO inhibitor and tissue alone without aminoacetone. Aminoacetone is chemically quite stable in saline. The original concentration was maintained after 4 h incubation. Following incubation with mouse liver and intestinal tissues approximately 39% and 68% aminoacetone was lost, respectively. Percentage of degradation of aminoacetone was roughly estimated from the amounts of aminoacetone remaining subtracted the amounts of aminoacetone in tissues only and divided by the added amounts of aminoacetone. MDL-72974A was capable of reducing the rate of disappearance of aminoacetone, i.e. 21% and 51% in the liver and intestine tissues, respectively. The disappearance of aminoacetone cannot be completely blocked by inhibition of SSAO activity. This suggests that in addition to SSAO-mediated deamination aminoacetone might be metabolized via other pathway.

In the above experiment SSAO inhibitor MDL-72974A at 10 μM moderately blocks aminoacetone degradation. It is perhaps possible that the inhibitor was not efficiently transported to the SSAO compartment in these tissue slices. In the subsequent experiment MDL-72974A was injected to the mice and the liver and small intestine were then dissected 30 min after the drug treatment. Fig. 6 shows the reduction of aminoacetone levels after incubation for 3 h. The results are in principle similar as the in vitro experiment shown in Fig. 5. Without treatment of SSAO inhibitor there was approximately 52% and 80% disappearance of aminoacetone following incubation with mouse liver and intestine tissue slices, respectively. When SSAO activity was inhibited, the loss of aminoacetone was reduced to 27% and 61%.

Aminoacetone can be converted to highly toxic products, such as methylglyoxal, hydrogen peroxide and ammonium, by SSAO [1]. Evidence suggests that SSAO-mediated

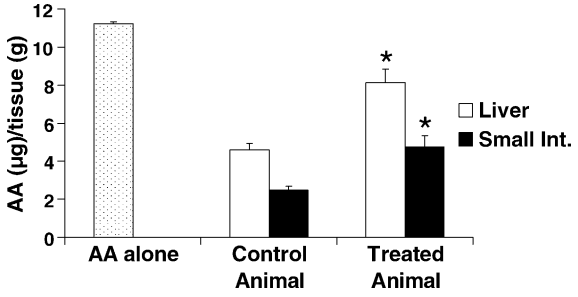



Fig. 6. Effect of MDL-72974A injection on aminoacetone degradation in mouse liver and small intestine tissue slices in vitro. Liver and intestine tissues were collected from animals 30 min after injection of MDL-72974A (5 g/kg) or saline as control. Aminoacetone (100 μM) was incubated with tissue slices at 37 °C for 3 h. The residual aminoacetone was analyzed as described in text. Data represent mean ± S.E. ($n = 5$). $^{*}p < 0.05$ indicates statistical significance compare with control.

deamination may be related to vascular disorders [1]. Most information up-to-date about this hypothesis is based on deamination of methylamine, which leads to formation of formaldehyde [6,7]. Both formaldehyde and methylglyoxal are extremely reactive and capable of cross-linking with proteins [21,22]. Interestingly, methylglyoxal has been found increased in diabetic patients and methylglyoxal-protein adducts has been identified in these patients [23,24]. However, methylglyoxal can be synthesized from different pathways, i.e. via enzymatic or non-enzymatic fragmentation and elimination of phosphate of glycerolaldehyde-3-phosphate and dihydroxyacetone-phosphate from glycolysis [12]. The contribution of deamination of aminoacetone towards production of methylglyoxal remains to be established. Although we have previously employed an HPLC procedure analyzing the 2,4-dinitrophenylhydrazine (DNPH) derivatization of aminoacetone for the analysis of its enzymatic deamination in vitro [15], this method was found not suitable for the assessment of aminoacetone in tissues or urine. Using the newly developed method described above the presence of aminoacetone in tissues has now been first demonstrated.

The concentrations of aminoacetone in tissues are rather low. Although SSAO inhibitor can cause a significant increase in aminoacetone, the magnitude of increase is limited. Completely inhibition of SSAO can merely partially block the degradation of aminoacetone; therefore, deamination is not the only route of degradation. Other pathways are involved in aminoacetone catabolism. It has been shown that aminoacetone is also subject to non-enzymatic oxidation to methylglyoxal in the presence of irons [16]. The fast turnover of aminoacetone may explain that no large quantity of aminoacetone was detected in tissues. Despite the fact that SSAO only partially involved in the conversion of aminoacetone to methylglyoxal, it cannot rule out the importance of this pathway, since SSAO is localized on the outer surface of vascular endothelial and smooth muscle cells. Methylglyoxal produced in situ, i.e. via SSAO-mediated deamination the extracellular surface, would not be readily detoxified as in the intracellular compartment. Such methylglyoxal may readily interact with the adjacent protein structures, namely, the outer membrane of the vasculatures and thus cause chronic protein misfolding of the blood vessels contributing to vascular disorders.

## 4. Conclusion

We have developed an HPLC procedure for the assessment of aminoacetone in urine and tissues. Results substantiate that aminoacetone is an endogenous substrate for SSAO; in addition, aminoacetone is also subject to active metabolism via other pathways. Its low concentrations in tissues and moderate increase by SSAO inhibitor do not suggest that deamination of aminoacetone is a major route for synthesis of methylglyoxal. However, in the vascular compartment, where SSAO is abundant, the potential involvement of SSAO-mediated production of methylglyoxal in vascular disorders cannot be ruled out.

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