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Kinetics of the Degradation of N^G-Nitro-*L*-Arginine and Its Methyl Ester in Human Umbilical Vein Blood and Amniotic Fluid[1])

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Summary: The kinetics of the degradation of the inhibitors of the nitric oxide synthesis, N^G-nitro-*L*-arginine methyl ester and N^G-nitro-*L*-arginine, were examined in human amniotic fluid and umbilical vein blood. The reaction rate constants were calculated or estimated using the time-controlled concentration course of both substances. These concentrations were measured by high-performance liquid chromatography with two different separation systems: ion-exchange chromatography and ion-pair chromatography. Using this method, either N^G-nitro-*L*-arginine methyl ester and/or N^G-nitro-*L*-arginine were added to 18 samples of amniotic fluid, 33 samples of plasma and 21 samples of uncentrifuged umbilical vein blood samples; subsequently these samples were used for measurement. The degradation of the two individual study substances can be described by a uni-unimolecular two-step consecutive reaction. Thereby, N^G-nitro-*L*-arginine methyl ester decomposes to N^G-nitro-*L*-arginine. Although N^G-nitro-*L*-arginine decomposed further, the decomposition product could not be identified. The average of the reaction rate constants for N^G-nitro-*L*-arginine methyl ester/N^G-nitro-*L*-arginine was determined, yielding the following values: 0.032 h^{-1}/ 0.00047 h^{-1} in amniotic fluid, 0.029 h^{-1}/0.00384 h^{-1} and 0.00074 h^{-1} in plasma and 0.80 h^{-1}/0.00060 h^{-1} in uncentrifuged umbilical vein blood. During the first hours after sampling, these reaction rate constants could be used to approximate the concentrations of N^G-nitro-*L*-arginine methyl ester and N^G-nitro-*L*-arginine at the time of sampling.

Introduction

Nitric oxide, also known as endothelium-derived relaxing factor, plays an important role as a regulator of cell functions and of communication in the body (1–3). It is produced in the body from the terminal guanidino nitrogen of *L*-arginine (3–5) and has a half life of only a few seconds. Previous studies have shown that there is an impotant relationship between nitric oxide and the regulation of blood pressure during pregnancy. In pathological cases, changes have been demonstrated in the concentrations of nitrite and nitrate (6, 7). In patients with preeclampsia, for example, the concentration of nitrite in the blood is reduced. This reduction could be due to the body's decreased production of nitric oxide or an inhibition of the arginine metabolism. All N^G-substituted *L*-arginines are inhibitors of nitric oxide synthesis (4). These substitutes can be one or two methyl groups or a nitro group. These inhibitors also exhibit changes in concentration in various body fluids. In previous studies (8, 9) two very differing half lives are given for the degradation of N^G-nitro-*L*-arginine methyl ester in blood plasma: 7.5 minutes and 222 minutes. This reaction represents a hydrolysis of N^G-nitro-*L*-arginine methyl ester to N^G-nitro-*L*-arginine.

With the intention to determine the concentrations of these inhibitors at a later stage in patients with pathological pregnancies, for example complicated by preeclampsia or hypertension, we examined the concentrations of both inhibitors, N^G-nitro-*L*-arginine methyl ester and N^G-nitro-*L*-arginine, in the body fluids, particular to and during pregnancy. We determined kinetics, such as the reaction rate constants of these inhibitors. Consequently the concentrations were measured time-dependently with two different ion-chromatographic high-performance liquid chromatography (HPLC) systems.

Materials and Methods

Sample collection

Amniotic fluid and venous umbilical cord blood were obtained from the labour ward during labour or drawn from the umbilical cord immediately following delivery. Heparinized tubes (Monovette AH with canules 21G 1½"TW from Sarstedt, Nümbrecht, Germany) were used to collect the blood samples. The samples were stored in a refrigerator at −20 °C and were analysed as soon as possible.

Sample handling

To obtain plasma, the whole blood samples were centrifuged in a Labofuge A centrifuge (Heraeus Christ GmbH, Osterode am Harz,

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Germany) for 10 minutes at 3000 min^{-1}; this corresponds to a g-force of about 1050.

The test tubes used for adding inhibitors to the sample were prepared as follows: 50 mg albumin (as a matrix, Sigma Chemie GmbH, Deisenhofen or Fluka, Neu-Ulm, Germany), 10 mg sodium chloride (as a solubility promotor, Merck, Darmstadt, Germany), about 1 mg N^G-nitro-*L*-arginine, and approximately 2 mg N^G-nitro-*L*-arginine methyl ester (both from Fluka, Neu-Ulm, Germany) were dissolved in 10 ml distilled water, well homogenized and portioned in accordance with the desired initial concentration. Thereafter, the portions were freeze-dried, sealed and stored at −20 °C. Some test tubes were only filled with either N^G-nitro-*L*-arginine methyl ester or N^G-nitro-*L*-arginine instead of both substances. The volume of amniotic fluid or blood that was added was 10 ml and 2.5 ml, respectively. After adding blood or amniotic fluid, the solution was thoroughly mixed and could immediately be prepared for the first measurement. The samples were stored at room temperature during the day and at night they were stored in a refrigerator at a temperature of about 8 °C.

HPLC analysis and sample preparation

The apparatus consisted of two HPLC sets. The preparation of the samples was carried out directly prior to measurement. The two HPLC systems together with the data analysis were described in detail in an earlier paper (10). One of the HPLC systems based upon the principle of ion-exchange chromatography with a strong cation exchanger and was first developed by *Raberger* and his colleages (8, 9, 11, 12). The second HPLC system based upon the principle of ion-pair chromatography with a reversed phase column and was developed by *Tabrizi-Fard & Fung* (13). The concentrations of the two arginine derivatives in the samples were determined by the use of both separation systems in parallel studies, where this was possible.

Mathematics and statistics

The series of measurements were evaluated with the programme TableCurve Windows version 1.0 (curve fitting, Jandel Scientific, San Rafael, California, USA) and with Excel version 5.0a (Microsoft Corporation, Incline Village, Nevada, USA).

The average value used in the statistical evaluation was the arithmetical mean. The standard deviation was based upon the parent population. Both of these calculations were included in Excel as statistical functions.

Results

Figure 1 gives examples of three chromatograms, each featuring ion-exchange and ion-pair chromatography of the plasma sample 193 recorded at different times. The evaluation of such series of measurements produces the time-controlled concentration course of N^G-nitro-*L*-arginine [ARG(NO)] and its methyl ester [L-NAME] which is shown in figure 2 (in this case for amniotic fluid C). The position of these plotted points is similar to the theoretical concentration reaction of the reactants in a uni-unimolecular two-step consecutive reaction:

$$A \xrightarrow{k_A} B \xrightarrow{k_B} C \,,$$

which is shown in figure 3. These curves can be calculated with equations [1] and [2] which is the result of integrating the kinetic equations [3] for substance A, and [4] for substance B (14, 15).

$$c_A = c_{A,0} \cdot e^{-k_A \cdot t} \qquad \text{Eq. [1]}$$

$$c_B = c_{B,0} \cdot e^{-k_B \cdot t} + c_{A,0} \cdot \frac{k_A}{k_B - k_A} \cdot (e^{-k_A \cdot t} - e^{-k_B \cdot t}) \qquad \text{Eq. [2]}$$

$$\frac{d}{dt}(c_A) = -k_A \cdot c_A \qquad \text{Eq. [3]}$$

$$\frac{d}{dt}(c_B) = -k_B \cdot c_B + k_A \cdot c_A, \qquad \text{Eq. [4]}$$

with

k_A, k_B — reaction rate constants and
c_A, $c_{A,0}$, c_B, $c_{B,0}$ — concentration of A or B at time t and t = 0

The coefficients could be approximated by fitting the curve using a special programme which incorporates the equations [1] or [2] and is applied to the measuring points. These coefficients represent the reaction rate constants and the initial concentration at the time t = 0. Contrary to equation [1] no available programme succeeded in calculating realistic coefficients for equation [2], thus the reaction rate constant k_B could be estimated only by comparison of the position of the plotted points and the shape of the curve.

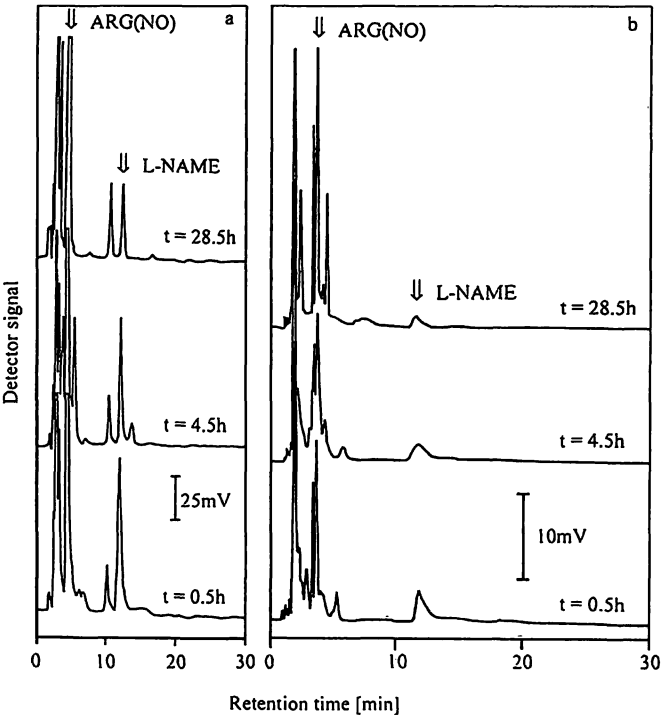

Fig. 1 Chromatograms (detection UV 268 nm) of umbilical vein blood plasma 193 for different storage periods t:

a) ion-exchange chromatography – Nucleosil 100-5 SA columns (250 mm × 4 mm + 30 mm × 4 mm) with 50 mmol/l sodium dihydrogen phosphate with methanol, volume fraction 0.125, pH 2.3 at 35 °C, 1 ml/min and

b) ion-pair chromatography – Eurospher 100-5 C8 column (250 mm × 4 mm) with 18.5 mmol/l heptane sulphonic acid with methanol, volume fraction 0.10, pH 2.7, 1.5 ml/min.

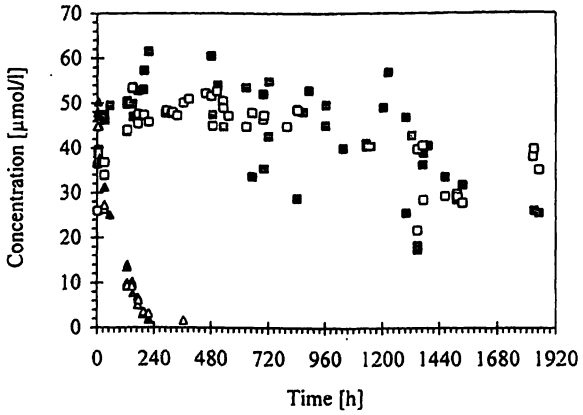

Fig. 2 Time dependence of the concentration of N^G-nitro-*L*-arginine methyl ester (triangles) and N^G-nitro-*L*-arginine (squares) in amniotic fluid C; solid – ion-exchange chromatography and hollow symbols – ion-pair chromatography.

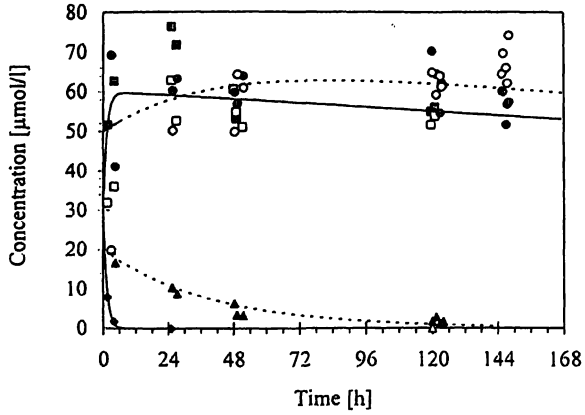

Fig. 4 Plotted points from umbilical vein blood 189: plasma – N^G-nitro-*L*-arginine methyl ester (triangles), N^G-nitro-*L*-arginine (circles) and uncentrifuged – N^G-nitro-*L*-arginine methyl ester (rhombi), N^G-nitro-*L*-arginine (squares) and the concentration curves (constants see tabs. 1 and 2); solid symbols – ion-exchange chromatography and hollow symbols – ion-pair chromatography.

Figure 4 shows the measuring points and the curves with the calculated or estimated reaction rate constants (see tabs. 1 and 2) as well as the initial concentrations from the umbilical vein blood sample 189 and its respective plasma.

Although N^G-nitro-*L*-arginine methyl ester can be measured in plasma with an initial concentration of 20 µmol/l even after 120 hours or 5 days, no N^G-nitro-*L*-arginine methyl ester was detected in uncentrifuged blood after 6 hours in spite of an initial concentration of about 30 µmol/l. However, N^G-nitro-*L*-arginine could still be found in amniotic fluid (fig. 2) with the same initial concentration even after 240 hours or 10 days. On the other hand, N^G-nitro-*L*-arginine having approximately the same initial concentration, could still be detected weeks later.

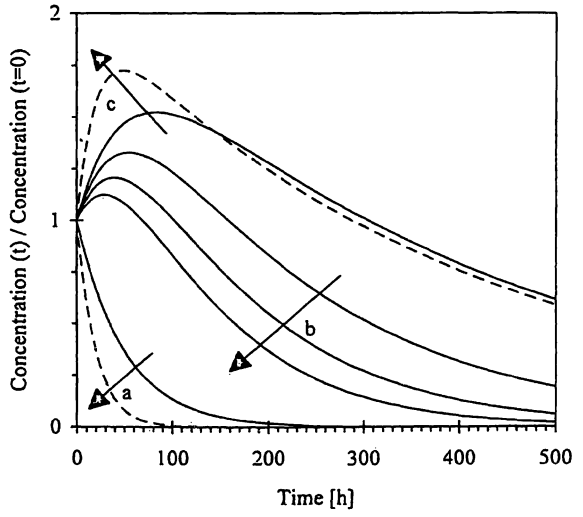

Fig. 3 Calculated concentration curves, related to the initial concentration, with variation of the reaction rate constants for a consecutive reaction with
(a) equation [1]: k_A = 0.02, and 0.05 h^{-1},
(b) equation [2]: k_A = 0.02 h^{-1}, and
k_B = 0.0100, 0.0075, 0.0050 h^{-1}, and
(c) equation [2]: k_A = 0.02, 0.05 h^{-1}, and k_B = 0.0025 h^{-1}

Hence the stoichiometric equation of the two-step consecutive reaction can be written as follows:

$$L\text{-NAME} \xrightarrow{k_{L\text{-NAME}}} \text{ARG(NO)} \xrightarrow{k_{ARG(NO)}} ???$$

By means of amino acid analysis with *o*-phthaldialdehyde pre-column derivatization (10) it was not possible to determine to which amino acid N^G-nitro-*L*-arginine decomposes. Considering the concentrations of both arginine derivatives that were used, a clear increase in the concentration of one of the amino acids should have been traceable. Thus, the question arises whether the degradation product of nitro arginine is an amino acid at all.

Tables 1 to 3 demonstrate the calculated or estimated reaction rate constants for all examined samples. In some samples only N^G-nitro-*L*-arginine was added. Thus, equation [1] (in this case being valid for N^G-nitro-*L*-arginine) could be used not only to estimate but also to calculate the constant.

Discussion

HPLC measurements

In both separation systems used, approximately the concentrations were measured for both substances. Since the peak of N^G-nitro-*L*-arginine methyl ester stands very isolated in the chromatogram, inaccuracies may have arisen during sample preparation. In spite of using the same pipette, there were small differences in the volumes used for deproteinizing. In the case of N^G-nitro-*L*-arginine errors occurred due to the peak not being sufficiently separated from the peaks of other eluting substances immediately before or after it (see fig. 1). As illustrated in figure 2, the points plotted for N^G-nitro-*L*-arginine produce a scattered cloud in contrast to those of N^G-nitro-*L*-arginine methyl ester. The separation of

N^G-nitro-*L*-arginine from the other "disturbing" substances also depends on their concentrations. Furthermore, the unwanted but unavoidable inconsistancies of some chromatographic conditions are also present in these measurements, such als fluctuations in the measuring temperature or in the composition of the eluents, thereby also affecting the separation performance.

Kinetics

A further reason for the appearance of this scattered cloud is attributable to the fact that the samples were purposely not stored at a constant temperature (at night and over the weekend in the refrigerator at 8 °C but at the time of measuring at room temperature between 20 and 30 °C). This, in fact, constitutes an error in the determination of kinetic data. However, this was deliberately done here in order to obtain values which are not "controlled" by temperature and hence values which are valid for a particular temperature range only, since a sample cannot be assumed to have been stored at a constant temperature in normal daily clinical routine.

Tab. 1 Results for the reaction rate constants in umbilical vein blood plasma.

Plasma no.	$k_{L\text{-NAME}}$ [h^{-1}]	$k_{ARG(NO)}$ [h^{-1}]	
		Group 1	Group 2
3	0.033	n. a.[1])	
5	0.048	n. a.[1])	
7	0.032	0.00750	
11	0.027		0.00100
13	0.040	0.00250	
22	0.021	0.00200	
23	0.021	0.00250	
25	0.035	0.00750	
29	0.029	0.00750	
30	0.026	0.00750	
34	0.017	0.00250	
35	0.048		0.00150
36	0.039	n. a.[1])	
42	0.018	0.00200	
46	0.036	0.00250	
47	0.036	0.00250	
48	0.066	0.00200	
49	n. a.	0.00340	
50	0.020		0.00075
53	0.034		0.00100
55	0.012		0.00125
56	0.016		0.00075
57	0.015		0.00100
59	0.014		0.00075
61	0.014		0.00075
181	0.025		0.00075
189	0.025		0.00075
193	0.033		0.00025
214	0.017		0.00025
216	n. a.[1])		0.00020
221	n. a.[1])		0.00020
250	0.030	0.00400	
251	0.046		0.00040
Average	0.029	0.00384	0.00074
Standard deviation	0.012	0.00217	0.00036
Number	30	14	16

[1]) n. a. = not added

Tab. 2 Results for the reaction rate constants in uncentrifuged umbilical vein blood.

Blood no.	$k_{L\text{-NAME}}$ [h^{-1}]	$k_{ARG(NO)}$ [h^{-1}]
108	n. a.[1])	0.00010
109	n. a.	0.00010
117	n. a.	0.00075
118	n. a.	0.00050
122	n. a.	0.00050
123	n. a.	0.00025
130-a[2])	0.80	0.00050
130-b[2])	n. a.[1])	0.00100
140	0.80	0.00075
144	0.80	0.00075
145	0.80	0.00075
146	0.80	0.00075
174	0.80	0.00075
176	0.80	0.00075
178	0.80	0.00075
181	0.80	0.00075
182	0.80	0.00075
183	0.80	0.00075
189	0.80	0.00075
204	0.72	0.00050
212	0.93	0.00050
216	n. a.[1])	0.00020
Average	0.80	0.00060
Standard deviation	0.04	0.00024
Number	14	22

[1]) n. a. = not added
[2]) different measurement series of one sample

Tab. 3 Results for the reaction rate constants in amniotic fluid.

Amniotic fluid no.	$k_{L\text{-NAME}}$ [h^{-1}]	$k_{ARG(NO)}$ [h^{-1}]
A	0.023	0.000700
B	0.010	0.000500
C	0.011	0.000500
D	0.034	0.000500
E	0.035	0.000300
F	0.035	0.000650
G-a[2])	n. a.[1])	0.000625
G-b[2])	n. a.[1])	0.000625
G-c[2])	n. a.[1])	0.000625
H	n. a.[1])	0.000625
I	n. a.[1])	0.000625
J	n. a.[1])	0.000625
K	0.060	0.000250
L	0.034	0.000250
M	0.034	0.000250
N	n. a.[1])	0.000500
O-a[2])	0.041	0.000250
O-b[2])	n. a.[1])	0.000250
P	0.036	n. a.[1])
Q	0.042	0.000250
R	0.019	0.000500
Average	0.032	0.000470
Standard deviation	0.013	0.000166
Number	13	20

[1]) n. a. = not added
[2]) different measurement series of sample

Nevertheless, the similarity between the position of the points plotted and the theoretic curves of concentrations is quite obvious when comparing figure 1 with figure 2. In figure 2 there is not only a theoretical pair of curves to be seen but simultaneously also changes in the shape of the curves due to the variation of a reaction rate constant. If sufficient measurements are made, the reaction rate constants for N^G-nitro-*L*-arginine methyl ester can be calculated in equation [1] without any problems. No programme succeeded in calculating the constants for N^G-nitro-*L*-arginine in equation [2], since these are probably present in the equation too often. Hence, these constants could only be obtained by a visual comparison of the position of the measured values and a single curve which includes the values calculated for N^G-nitro-*L*-arginine methyl ester and then estimated from these given values. Thus, a good agreement could be achieved and the reaction rate constants could be determined (see fig. 4). The measurement of N^G-nitro-*L*-arginine on its own without N^G-nitro-*L*-arginine methyl ester confirmed this agreement. In this case the kinetic equation is valid, in which the constants can be calculated, as shown in equation [1]. Several constants for N^G-nitro-*L*-arginine methyl ester had to be estimated by comparison as well, particularly those for the uncentrifuged blood, as not enough data could be obtained.

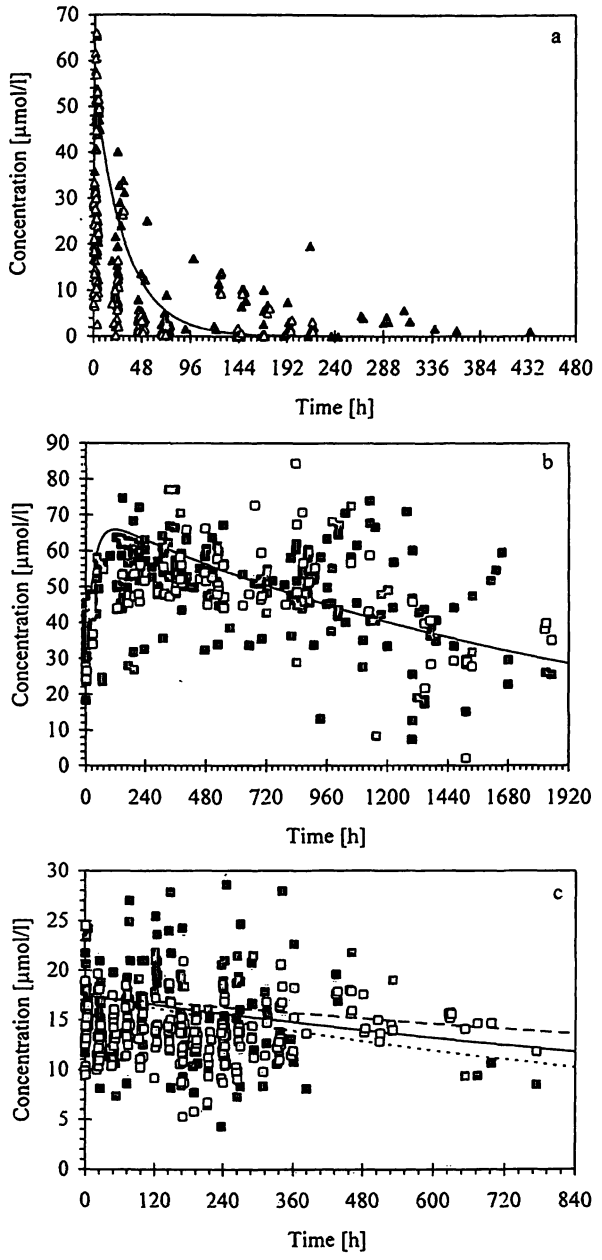

Fig. 5 Plotted points of all amniotic fluid samples: a) N^G-nitro-*L*-arginine methyl ester, b) N^G-nitro-*L*-arginine where N^G-nitro-*L*-arginine methylester was added, and c) N^G-nitro-*L*-arginine only – sample no. G-a to J, N, and O-b; solid ion-exchange chromatography and hollow symbols – ion-pair chromatography; and the concentration curves calculated with the mean value (solid line), mean value minus standard deviation (dotted line), and mean value plus standard deviation (dashed line) of the reaction rate constants (for the numerical values see tab. 3).

The values in the tables, whether calculated or estimated, do not vary much from each other except in the case of the plasma samples (tab. 1). Nevertheless, differences can be recognized which certainly arise due to the samples having been handled in different ways. However, each sample (whether amniotic fluid, plasma or uncentrifuged blood) is an individual substance in inself. Table 1 demonstrates that the constants of N^G-nitro-*L*-arginine in plasma can be divided into two groups. A measurement with umbilical artery blood, the constant of which would be equal to those of group 1, gives a possible explanation. Perhaps only the blood from group 2 is venous blood and that of group 1 is in fact arterial blood (contrary to the declaration)? This is only one possible explanation. It may also be possible that the samples of one group contain substances that act in plasma either as an accelerator or certain catalyst (group 1) or as a retardant or species of inhibitor (group 2).

In figure 5 all points for N^G-nitro-*L*-arginine methyl ester and N^G-nitro-*L*-arginine as well as those for N^G-nitro-*L*-arginine alone have been taken from all the amniotic fluid samples listed. The continuous curves have been calculated using the average values of the reaction rate constants for amniotic fluid and the assumed "average" initial concentrations. The fact that the plotted points are scattered around the curve is due to the varying concentrations among the individual samples. However, the shapes of these curves show very accurately the concentration to be time dependent in individual cases. Part C of figure 5 shows two extra curves calculated with the mean value plus or minus standard deviation, respectively (dashed line or dotted line) in addition to the curve calculated using the average of the constants. During the first hours after addition of the arginine derivative, there are only small differences visible between the three curves. Thus, the mean can be used to calculate the concentrations, in spite of the large standard deviations of up to approximately 60% of the average.

$$t_{1/2,i} = \frac{\ln(2)}{k_i} \qquad \text{Eq. [5]}$$

In order to compare our results with those in the literature (8, 9), the half lives were calculated using the mean of the reaction rate constants $\bar{k}_i$ according to equation [5]. In uncentrifuged blood the half lives for N^G-nitro-*L*-arginine methyl ester were 51.75 min and 180 h (group 1) or 39 d (group 2), for N^G-nitro-*L*-arginine alone in plasma 24 h and 13 d and in amniotic fluid 22 h and 61.5 d. The half lives given for human body fluids are longer than those in blood samples from rabbits in vivo 7.5 min (8) and in plasma from canine blood in vitro 222 min (9).

Conclusion

The mean values determined for the reaction rate constants in the degradation of N^G-nitro-*L*-arginine methyl ester and its N^G-nitro-*L*-arginine are confirmed to be suitable for calculating the concentrations of one or both substances at the time of sampling in the first approximation using equations [6] and [7], derived from [1] and [2]. Equation [7] is only required for calculating N^G-nitro-*L*-arginine if both substances are present in a sample.

$$c_{i,0} = c_i \cdot e^{k_i \cdot t} \qquad \text{with} \quad i = A \text{ or } B \qquad \text{Eq. [6]}$$

$$c_{B,0} = c_{B,t} \cdot e^{k_B \cdot t} - c_{A,0} \cdot \frac{k_A}{k_B - k_A} \cdot \{e^{(k_B - k_A) \cdot t} - 1\} \qquad \text{Eq. [7]}$$

Hereby the time interval between sampling and analysis must be as short as possible, not exceeding a few hours. The best example would be the uncentrifuged blood if the concentration of the N^G-nitro-*L*-arginine methyl ester (if present) is to be determined. Some half life values do point to this. Also having some determinations of the concentration made at short intervals, would render the calculations even more reliable. In this case the individual differences are not clearly distinguishable.

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