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Postmortem Stability of the Rat Atrial Natriuretic Peptide in Blood and Atrial Tissue

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The postmortem stability of the rat Atrial Natriuretic Peptide (ANP) has been studied as a necessary and previous step to be applied in the forensic field as a postmortem marker. This peptide —whose extreme sensitivity to slight changes in blood volume is well known— could have great importance in thanatochemistry to establish a correct diagnosis when macroscopical observations and classical parameters are not conclusive or cannot be employed. The results show high stability in atrial tissue, where values are similar from 0 hours (108.99 pm/mg) to 8 hours (109.41 pm/mg) and decrease uniformly until 15 pm/mg) at 32 hours, time of our last determination. Blood ANP showed similar stability from 0 h (105.43 pg/ml) to 8 h (106.62 pg/ml).

Key words: Atrial natriuretic peptide, Rat, Thanatochemistry.

Biochemical parameters are increasingly used in the postmortem study of many cadavers, mainly when there has been a special difficulty in diagnosing the exact cause of death. Forensic science demands the use of postmortem markers with high specificity and sensibility, so that the most sensitive technics are being employed to obtain as much information as possible. The study of glucose, adrenalin, serotonin, histamine and many other parameters are now common, often offering the key for a correct diagnosis.

Rat atrial natriuretic peptide (ANP) (Ser99-Tyr126) belongs to a group of closely related endogenous peptides whose physiological activities were first detected and described by DEBOLD *et al.* (2). The increasing of the plasmatic levels of ANP has been demonstrated in different chronic and acute pathologies processes that have in common the distension of the auricular walls (6-8).

Plasma half-life of ANP is also known to last only a few minutes (10) while kallicrein is known to be one of the substances with a high inactivating ability (1).

Since there is no reference about the postmortem stability of this peptide, the

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aim of this study was to determine its postmortem behaviour in order to improve the planning of future experimental designs.

Furthermore, this peptide could be of forensic interest as a biochemical parameter in those cases where the cause of death is not well known but is suspected to be related to changes in blood volume, as in vital drownings.

Materials and Methods

Adult Wistar rats (n = 48) weighing 200-250 g have been employed. They were grouped in six different series of eight animals each, according to the period of postmortem evolution (0, 4, 8, 16, 24 and 32 h). These rats were anesthetized with sodium penthobarbital (40 mg/kg, i.p.) and, after a stabilization period of 20 min, were sacrificed with an overdosis of the same barbiturate (150 mg/kg, i.p.). Once dead, rats were left for evolution in supine position at ambient conditions (20° C; humidity 65 + 5 %). When the time corresponding to their series elapsed, rats were autopsied and blood and atria collected.

Blood samples (2 ml) were taken from abdominal aorta into plastic syringe containing Aprotinin (500 KIU), Soybean Trypsin Inhibitor (50 BAEE), and EDTA (1 mg/ml) (9). Blood was immediatly centrifuged at 4° C (30 m, 5,000 rpm) and supernatant was stored at −30° C until assay. Blood extraction procedure: one milliliter of plasma was applied on a previously activated Sep-Pack C_{18} cartridge (Millipore, R); the retained peptide was eluted with 6 ml of absolute ethanol: acetic acid (86:14) and evaporated under nitrogen stream (9).

Both atria from every rat were homogenized in 2 ml of 0.1 N HCl with Triton X-100 for 60 s and centrifuged for 20 min at 12,000 rpm (4). The supernatant was frozen at −30° C, thawed and centrifuged again at 12,000 rpm for 20 min. The pellet was discarded and supernatant fractionated in 100 µl aliquots and stored at −30° C until assay.

Radioimmunoassay: the standard diluent (pH: 7.4) was 0.02 M monosodic phosphate, 0.15 M sodium chloride, 0.01 % albumin, 0.1 % gelatin powder, 0.01 % Triton X-100 and 0.01 % azide. Fifty µl of standard rat ANP or sample were incubated for 72 h at 4° C with 250 µl of the standard diluent, 50 µl of antiserum and 50 µl of the labelled peptide. The separation of the antibody-bound and free peptide was accomplished by adding 300 µl of dextran-coated charcoal (1.6 g of Norit and 0.16 g of dextran T-40 in 50 ml of the standard diluent); after 5 s of agitation the tubes were centrifuged at 3,000 rpm at 4° C for 10 min, and the supernatant promptly decanted and its radioactivity counted in an LKB Multigamma counter programmed with a LKB Laboratory computer.

Statistical analysis was carried out with ANOVA-test (mean compared with a *t*-Student test) and with a Linear Regression test calculating the Correlation Coefficient.

Results

Atrial ANP shows a period of stability from 0 to 8 h and from 8 to 32 h it decreases uniformly. Comparing to levels at 0 h, there are statistically significative dif-

Table I. *Atrial and blood levels of atrial natriuretic peptide in rat (media ± SD, n = 8).*

Series	Time (h)	Atrial (pm/mg)	Blood (pg/ml)
I	0	108.99 ± 4.81	105.43 ± 5.79
II	4	114.41 ± 11.49	111.63 ± 7.01
III	8	109.41 ± 8.20	106.62 ± 3.89
IV	16	80.12 ± 4.22	(n.d.)
V	24	63.61 ± 4.82	(n.d.)
VI	32	15.00 ± 1.52	(n.d.)

(n.d.) = not determined.

ferences ($p < 0.001$) at 16 h, 24 h, and 32 h.

These values decrease according to a linear regression equation mathematically expressed as $y = 110.87 - 6.132x$ ($p < 0.001$) and with a correlation coefficient of $r = -0.98$.

Blood ANP were stable from 0 to 8 h. Blood from series IV, V, and VI (16, 24, and 32 h) could not be obtained due to postmortem coagulation of blood.

Discussion

The postmortem stability of the ANP levels, both at rat atria and blood, show a stability period from 0 to 8 h. There is a light increase at 4 h (although statistically not significant) that may be due to intra or interassay coefficients of variation (5.1 and 7.7 respectively).

Nevertheless it must be taken into account the fact that few minutes after death the autolytic and putrefactive processes, start, originating pH changes that could cleavage precursors of the rat 99Ser-126Tyr ANP, giving rise to a long series of sequences of this peptide and many others closely related that could originate radioimmunological cross-reactivity.

It is not possible to compare these results with others since there are no publications related to this topic. Some autors have employed human atria —obtained from autopsies within 10 h postmortem— to isolate and purify this peptide with good results (3, 5), although neither the ways of degradation nor the levels have been estimated.

The ANP levels in plasma are kept constant and, when increased, the excess is quickly degraded to the original basal levels (10). Therefore there is a fast degradation of the excess and a former equilibrium. When the animal dies it has a level (both in plasma and atria), the exact mechanism of degradation from this moment on being unknown.

Unfortunately, the postmortem stability of peptides similar to ANP has never been studied in forensic science, so that these values cannot be compared to others.

It could be concluded that the rat Ser99-Tyr126 ANP levels are stable in rat atria and blood until 8 h postmortem. This postmortem stability and detectability in atrial tissue —at least— until 32 h, determine the ability of this biochemical parameter as a marker for Forensic application in cases of death where either changes in the volume of circulating blood take place or other pathological processes known to increase the blood ANP levels while diminishing the atrial ones.

In addition, the knowledge of these results may be useful in many experimental designs where a period of time before sampling is necessary.

The exact mechanism of the postmortal degradation should be studied to know the real causes of initial stability until 8 h and rapid degradation from this time on.

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Resumen

Se estudia la estabilidad postmortal del péptido natriurético auricular de rata como paso previo y necesario a su aplicación como marcador postmortal en medicina forense. Los resultados demuestran una gran estabilidad en el tejido auricular, donde hay valores similares desde las 0 h (108,99 pm/mg) hasta las 8 h (109,41 pm/mg), decreciendo uniformemente hasta 15 pm/mg a las 32 h, momento de la última determinación realizada. Los niveles sanguíneos muestran una estabilidad similar desde las 0 h (105, 43 pg/ml) hasta las 8 h (106,62 pg/ml). Este péptido podría tener un gran interés en tanatoquimia para establecer el diagnóstico de la muerte en aquellos casos en que la observación macroscópica o los parámetros

clásicos no son concluyentes o no pueden ser empleados.

Palabras clave: Péptido natriurético auricular, Rata, Tanatoquimia.

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