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Rapid paper chromatographic separation of [^{14}C] angiotensin II from some metabolites: application to organ distribution

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A rapid, inexpensive method for the separation of 5-1-isoleucyl[^{14}C] angiotensin II (A-II) from its various metabolites has been devised. A-II was extracted from tissues with absolute methanol (recovery 96%) and paper chromatographed in a butanol–acetic acid–water (18:2:5) medium for two ascents at 60° C. The resulting R_F for A-II of 0·45 was then compared with the R_F values of three A-II metabolites produced by enzymatic degradation of the ^{14}C-A-II and [^{14}C]isoleucine. Trypsin degradation produced the [^{14}C]hexapeptide metabolite, chymotryptic degradation produced the [^{14}C]tetrapeptide metabolite and carboxypeptidase A degradation produced the [^{14}C]heptapeptide. Increases in temperature produced a continuous increase in R_F values for all the substances examined but the resolution decreased above 60° C. Similarly, increases in the temperature caused the appearance of secondary peaks with some but not all peptides. The tryptic digest (hexapeptide) and the chymotryptic digest (tetrapeptide) are apparently acid- and heat-stable under the experimental conditions. All of the peptides examined failed to produce secondary peaks when heated at neutral pH. The method was used to study the tissue distribution of ^{14}C-A-II after intravenous injection.

Most current research into the metabolism and distribution of angiotensin II (A-II) involves the use of radioimmunoassays to detect the presence of endogenous peptides (Fuxe et al 1976; Oparil 1974; Reed & Day 1978; Schoeneberger et al 1977). One major problem of the technique is that the lack of antibody specificity makes differentiation of the parent octapeptide from some of its larger hexapeptide metabolites difficult. Furthermore, since the interpretation of the experiment depends on displacing ^{125}I-A-II from antibody binding sites, the destruction of the labelled peptide by endogenous peptidases may produce false positive results (Reed et al 1977). One solution to the problem employs exogenous radioactive A-II followed by chromatography and/or electrophoretic separations. However, these methods are either time consuming, expensive, or both, rendering daily application difficult. Consequently, a method has been devised that is both rapid and inexpensive but adequately separates A-II from some important metabolites.

METHODS

Distribution procedure

Male Texas Inbred rats (200 g) were injected via the femoral vein with 4·4 μg kg^{-1} ^{14}C-A-II (240 mCi mM^{-1}) dissolved in 0·1 M phosphate buffer pH 7·4. The animals were decapitated 5 min after injection. Heart, liver, kidney, brain and lung were removed and each homogenized in 5 ml of ice cold methanol using a Tissuemizer homogenizer. The homogenate was centrifuged at 2000 *g* for 20 min and the methanolic supernatant evaporated to dryness. 1 ml of ice cold methanol was added to the sample which was then chromatographed.

Chromatographic procedure

500 μl of the above extract was spotted on Whatman 3 MM paper and placed in a sealed cast aluminum autoclave containing the n-butanol–acetic acid–water (18:2:5) medium. The container was kept at 60° C until the first ascent was complete (approx. 4 h) and after air drying the paper was subjected to the same procedure. It was then cut into either 1 cm strips or subdivided into fractions (Table 1), and each piece placed in a counting vial containing 10 ml of a scintillation mixture [2,5-diphenyloxazol, 5 g litre^{-1}; 1,4-di-(5-phenyloxazolyl)-benzene, 0·9 g litre] and 5 ml of Triton X-100. Radioactivity was counted using a Packard liquid scintillation counter. Recoveries ranged between 78–82%.

Preparation of standards

Since A-II is metabolized by many endogenous peptidases, any study of exogenously administered ^{14}C-A-II must be capable of separating the parent

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Table 1. The chromatographic separation of [^{14}C]-peptides. The chromatographic paper was cut into four fractions; each fraction representing a given number of cm from the origin. The labelled peptides standards found on each fraction are shown.

Fraction No.	Paper (cm)	Peptides
1	origin-2	—
2	3–5	des-Phe^8 A-II
3	7–11	A-II
4	13–17	des-1-4 A-II, des-Asp^1-Arg^2 A-II, isoleucine

octapeptide from its smaller labelled metabolic fragments. To assess the effectiveness of the separation radioactive standards representing selected A-II metabolites were synthesized using a modification of the method of Oparil & Baile (1973). This involves the enzymatic digestion of ^{14}C-A-II (0·88 μg) under the following incubation conditions.

(1) Incubation with carboxypeptidase A (CPA, 500 μg Sigma) in 0·1 M ammonium acetate, pH 8·0 at 37° C. This reaction hydrolyses the peptide bond adjacent to the free carboxyl group producing the des-Phe^8 A-II heptapeptide metabolite.

(2) Incubation with trypsin (TR, 500μg Sigma) in 0·2 m NaH_2PO_4, pH 7·0 at 37° C to cleave the agrinine valine bond producing the des-Asp^1-Arg^2 A-II hexapeptide metabolite.

(3) Incubation with chymotrypsin (CT, 500 μg Sigma) in 0·1 m NH_4HCO_3, pH 8·0 at 37°C. This reaction hydrolyses the peptide bond at the tyrosine residue producing a labelled tetrapeptide.

All of these procedures were carried out for 3 h in a total incubation volume of 0·3 ml. The reaction mixture was dried, redissolved in 100 μl of methanol and an aliquot chromatographed as above. [^{14}C]Isoleucine was also used as a standard since it represents the amino acid end point of ^{14}C-A-II metabolism carrying the radioactive label. No other metabolites were considered.

Extraction procedures

In some experiments either ^{14}C-A-II or its ^{14}C-metabolic fragments were added to incubation medium containing a sample of rat tissue (liver) previously boiled for 10 min to inactivate endogenous enzymes. Various extraction solvents were used (absolute ethanol, 70% ethanol, methanol, 10% *p*-chloroacetic acid, butanol and acetone) and the recoveries of the parent octapeptide and its metabolites were assessed.

RESULTS AND DISCUSSION

Extraction experiments

Recoveries with different extraction solvents were compared by adding 10 ml of each solvent to a mixture of boiled tissue and radioactive peptide. Methanol produced the most complete extraction (94%, range 91–98) for each peptide or metabolite studied. Each solvent extracted each peptide to about the same extent. The amounts extracted varied from 70–20%.

Paper chromatography: effect of solvent polarity

Each of the peptides and isoleucine were chromatographed at various temperatures in various solvents. The n-butanol–acetic acid–water system at 60° C produced the best separations in the 18 : 2 : 5 mixture proportions. The R_F values were: A-II, 0·45; CPA, 0·15; T, 0·75; CT, 0·70; ISO, 0·70.

Effect of temperature

Increases of the temperature from 30° to 70° C produces an increase in R_F values for both the ^{14}C-A-II and the CPA digest (Fig. 1). No change was seen for the TR or CT digestion products.

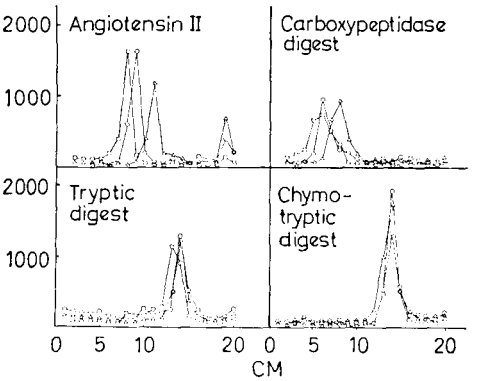

FIG. 1. A-II, the tryptic chymotryptic and carboxypeptidase A digests were subjected to ascending chromatography at different temperatures (30°: □, 60°: ○, 70 °C: △).

In experiments at 60° C, A-II produced a secondary peak on the chromatogram. This was negligible at 30° C and increased to approximately 40% of the primary peak at 70° C. Because peptide hydrolysis could be occurring as a result of the high temperatures over 4 h, each peptide was heated in distilled water at 30°, 60° and 100° C for 4 h and chromatographed at 60° C. These peptides proved heat stable. But when the water was replaced with the n-butanol–acetic acid–water (18 : 2 : 5) chromatographic solvent, increases in the temperature produced a progressive

diminution of the A-II peak while simultaneously increasing the size of the secondary peak (Fig. 2). Incubation at 100° C produced a secondary peak that was 70% of the height of the primary peak and had a R_F value of 0·9. Apparently, hydrolysis of A-II occurs on the chromatographic paper producing breakdown products with R_F values that overlap the curves for the tryptic and chymotryptic digestion products. Consequently, the chromatographic procedure was conducted at 60° C to minimize this degradation and to maximize the differences between R_F values for the various peptides.

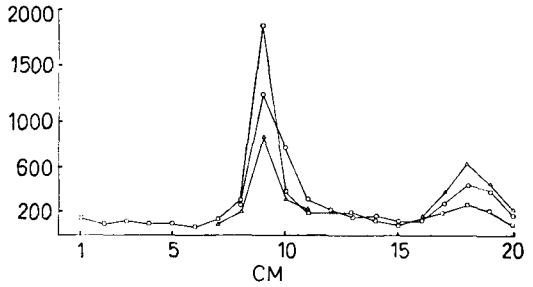

Fig. 2. ^{14}C-A-II was subjected to various temperatures (30°: □, 60°: ○, 100 °C: △) for 4 h and chromatographed as in methods.

Multiple ascents

Single ascents produced inadequate separation but allowing the paper to dry and subjecting it to a second ascent produced good resolution which was not improved by a third ascent (Fig. 3). This procedure adequately separates A-II from the des-Phe^8 A-II product and from the tryptic and chymotryptic products. It does not resolve the latter peptides nor does it separate isoleucine from these smaller metabolic fragments.

Tissue distribution of exogenous ^{14}C-A-II

^{14}C-A-II (4·4 μg kg^{-1}) was injected into the femoral vein of pentobarbitone anaesthetized rats which were killed 5 min later. Various tissues were removed and assayed as above (Table 2). The relative order of total radioactive peptide found in the tissues was: heart > lung = liver > kidney > brain. The heart (2396 pg g^{-1}) and the lungs (1007 pg g^{-1}) contained the most radioactivity which is not surprising considering that these are the tissues which receive the largest concentration of the injected drug after an intravenous injection. Neither is it surprising that the brain has the lowest concentration of radioactivity (226 pg g^{-1}) since the molecule would not be expected to cross the blood brain barrier. However,

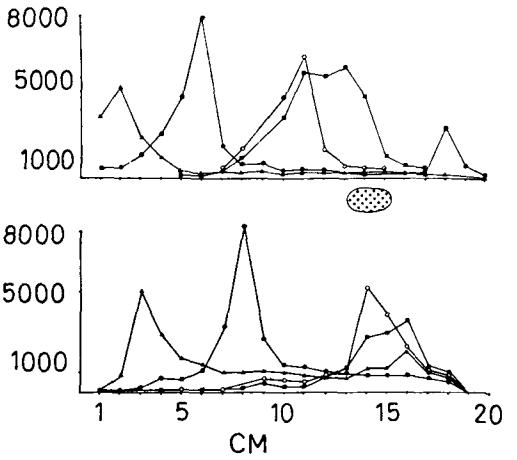

FIG. 3. ^{14}C-A-II and the tryptic, chymotryptic and carboxypeptidase A standards and [^{14}C]isoleucine were subjected to ascending paper chromatography for one (upper graph) or two ascents (lower graph). Carboxypeptidase A △—△, A-II ●—●, tryptic ■—■, chymotryptic ○—○ digests.

total tissue radioactivity is often misleading, therefore, the relative metabolites of the various tissues must be considered. Five minutes after the injection, approximately 85% of A-II appears as metabolic fragments in all of the tissues examined. The des-Phe^8

Table 2. Organ distribution of exogenous ^{14}C-A-II. ^{14}C-A-II (4·4 μg kg^{-1}) was injected into the femoral vein of pentobarbitone-anaesthetized rats and the animals killed 5 min post injection. Various organs were assayed for ^{14}C-A-II and its metabolites. n = 13.

Tissue	Fraction No.	pg g^{-1} Tissue wet wt	Tissue %
Brain	1	0	0
	2	8 ± 8	4
	3	36 ± 11	16
	4	182 ± 33	81
	Total	226 ± 46	
Lung	1	7 ± 5	1
	2	52 ± 12	5
	3	98 ± 21	10
	4	850 ± 123	84
	Total	1007 ± 16	
Heart	1	4 ± 3	0
	2	30 ± 7	1
	3	136 ± 25	6
	4	2226 ± 24	93
	Total	2396 ± 28	
Liver	1	12 ± 6	1
	2	51 ± 20	5
	3	150 ± 17	15
	4	776 ± 104	78
	Total	989 ± 152	
Kidney	1	2 ± 2	0
	2	25 ± 7	5
	3	116 ± 23	23
	4	373 ± 71	72
	Total	516 ± 107	

A-II metabolite produced by carboxypeptidase activity represents only 5% of the total metabolites with the remaining 95% being made up of smaller fragments. Earlier studies (McDonald et al 1974) indicated that cathespin C is the predominant liver lysosomal enzyme responsible for the degradation of A-11. Cathepsin C has tryptic like activity; i.e. it is a dipeptidyl-aminopeptidase producing a des-Asp^1-Arg^2 A-II metabolite. This metabolite would be indistinguishable from the tryptic digest in our system. The predominance of this and smaller metabolites in all the tissues studied may suggest that this enzyme is active in other tissues as well as the liver.

The relative distribution of intact A-II follows the distribution of total radioactivity; the heart and the liver contain the most A-II and the brain contains the least. However, 5 min post-injection the kidneys contain high concentrations of intact A-II while containing comparatively little total peptide. Therefore, if we consider the relative percentage of the total radioactivity made up of intact A-II, the distribution is altered. The kidneys contain the greatest percentage of A-II (23%) and the heart (6%) the least. Thus, while the heart does contain large amounts of radioactivity, it has comparatively little ability to extract or store intact A-II. At this time the kidneys contain relatively little radioactivity but seem to sequester A-II from the blood. Previous studies have also suggested a role for a kidney as a site of accumulation of injected A-II (Khairallah et al 1962). Conversely, in vivo injections of ^{14}C-A-II into the renal artery show little binding activity (Oparil & Baile 1973).

The brain, which contains the least radioactivity, has a relatively large amount of intact A-II (16%). Previous studies have demonstrated brain radioactivity (Volicer & Loew 1971) after peripheral injection, but the nature of the radioactivity is controversial since the presence of intact A-II was not demonstrated. More recent studies examining the cerebrospinal fluid of animals previously injected with A-II found only metabolic fragments present (Ganten et al 1975). The present study does find intact A-II which may be explainable in the following manner (1). Since whole brains were studied, the ^{14}C-A-II may be in the cerebral vascular system rather than in the brain tissue itself (2). The ^{14}C-A-II may be concentrated in brain areas outside the blood-brain barrier; i.e., the area postrema, a proposed site of action of the peptide (Joy & Lowe 1970; Ferrario et al 1972) (3). The injections produced a sudden (40 mm Hg) rise in mean blood pressure. Previous studies have demonstrated that sudden increases in blood pressure increase the permeability of the blood-brain barrier (Johansson et al 1970), perhaps allowing access of the peptide into the brain tissues.

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