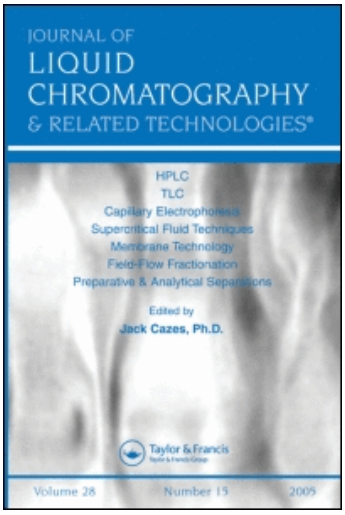

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Plasma Angiotensins by Reversed Phase HPLC and Radioimmunoassay

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To cite this Article Doris, Peter A.(1985) 'Analysis of Plasma Angiotensins by Reversed Phase HPLC and Radioimmunoassay', Journal of Liquid Chromatography & Related Technologies, 8: 11, 2017 — 2034
To link to this Article: DOI: 10.1080/01483918508074113
URL: http://dx.doi.org/10.1080/01483918508074113

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(11), 2017-2034 (1985)

ANALYSIS OF PLASMA ANGIOTENSINS BY REVERSED PHASE HPLC AND RADIOIMMUNOASSAY

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ABSTRACT

The analysis of biologically active angiotensin peptides in blood plasma by high performance liquid chromatography in a weakly non-polar reversed phase (C_2) chromatographic system combined with quantification of chromatographically isolated peptides by radioimmunoassay has been developed. This system is able to resolve each of seven closely-related peptides of the angiotensin group. The chromatographic system was applied to plasma samples which have been prepared for chromatographic analysis by C_{18} cartridge extraction. Samples were reconstituted in HPLC solvent prior to injection into the HPLC system. Separated angiotensin were collected by fraction collector and the volatile components of the solvent system were blown off under an air stream. The content of several of the various angiotensin peptides in the fractions was then determined by radioimmunoassay using an appropriate antiserum. Antiserum to angiotensin II (octapeptide) was used to quantify the biologically active components angiotensin II, angiotensin III (heptapeptide) and C-terminal hexapeptide. Recovery of angiotensin II in the C_{18} cartridge extraction has been assessed at 85.0 $\pm$ 0.9% (mean $\pm$ SEM) using I^{125}-labelled angiotensin II, and 82.2 $\pm$ 4.45% using synthetic unlabelled angiotensin II. Recovery of standard

 0148-3919/85/0811-2017$3.50/0

preparations of angiotensin II in the HPLC system have been estimated at 57.5 ± 6.08%. The application of this technique to evaluating some components of the angiotensin system in normal plasma is presented.

INTRODUCTION

The vasoactive peptide hormone, angiotensin II (AII) is one of a family of peptides produced extracellularly, chiefly in the circulation. Its formation results from the action of a cleavage enzyme, renin, on angiotensinogen, which yields the decapeptide angiotensin I (AI). Normal production of AII is by conversion of AI by the action of a C-terminal dipeptidase, angiotensin-converting enzyme (1). It is also possible that AII may be formed directly from angiotensinogen by the action of another enzyme which has been called tonin (2). Recent evidence suggests that some tissues may be capable of producing AII by an intracellular process (3,4,5,6).

Circulating levels of AII are typically in the femtomole/milliliter range and quantitation of AII in biological samples has depended principally on bioassay or radioimmunoassay (RIA) techniques. Both techniques, however, present serious impediments to accurate quantification of AII. In the first instance, bioassay techniques can be subject to interference from other vasoactive substances. Secondly, estimates of AII by RIA have been hampered by both non-specific and angiotensin-specific

cross-reactive interference (7,8). Removal of non-specific interference has been accomplished in the past by multistep absorbtion extraction processes (7), though recently a technique using C_{18} reversed phase minicolumns has been described (9). Removal of cross-reactive interference from other, related angiotensin peptides, however, has not been so readily accomplished. In addition to potential cross-reactive interference from AI, antisera to AII may cross-react with des. Asp^1. angiotensin I, angiotensin II (AIII), angiotensin C-terminal hexapeptide and C-terminal pentapeptide. While some of these angiotensins may have biological actions parallel to AII or may give rise to other angiotensins with shared activity, it is likely that most possess either no or only partial biological activity and thus contribute to an overestimation of true AII (8). What is more, AIII has been shown, in some species, to play a disproportionately greater biological role in some processes, for example, regulation of adrenocortical mineralocorticoid secretion (10), than AII so that a means of assessing AIII levels in biological samples independent of AII would be desirable.

The study below reports the development of a technique which permits the isolation of numerous angiotensin peptides from plasma or other neutral biological fluids in such a way as to permit isolated peptides to be subsequently quantified by radioimmunoassay.

MATERIALS

Peptides

Standard peptides were synthetic and in excess of 95% pure. Angiotensin I, angiotensin III, angiotensin C-terminal hexapeptide and C-terminal pentapeptide were obtained from Cambridge Research Biochemicals (Atlantic Beach, NY). Des. asp.[1] angiotensin I, angiotensin II and angiotensin C-terminal tetrapeptide were obtained from Bachem (Torrance, CA). Amino acid sequences of these peptides are shown in Table 1. Radioiodinated angiotensin II (^{125}I-AII) was obtained from New England Nuclear (Boston, MA).

Chromatographic Materials

Biological samples were extracted prior to HPLC on 1 ml C-18 reversed phase mini-columns (Bond Elut, Analytichem Inc., Harbor City, CA). HPLC was performed over a C-2 weak non-polar silica-

Table 1

Angiotensin Amino Acid Composition

Peptide	N-terminal	C-terminal
C-term. tetrapep.		H-Ile-His-Pro-Phe-OH
C-term. pentapep.		H-Tyr-Ile-His-Pro-Phe-OH
C-term. hexapep.		H-Val-Tyr-Ile-His-Pro-Phe-OH
AIII	H-Arg-	Val-Tyr-Ile-His-Pro-Phe-OH
AII	H-Asp-Arg-	Val-Tyr-Ile-His-Pro-Phe-OH
des. Asp' AI	H-Arg-	Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH
AI	H-Asp-Arg-	Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH

term = terminal
pep = peptide

bonded column, (silica particles spherical, 5 μm diameter), dimensions 4.6 mm x 25 cm (Analytichem Inc., Harbor City, CA).

Solvents, Chemicals

Sample extraction used ACS grade methanol (Fisher, Plano, TX), reagent grade formic acid (Aldrich, Milwaukee, WI), Tris-(hydroxymethyl) aminomethene, reagent grade (Sigma Chemical Co., St. Louis, MO), and double glass distilled water. HPLC solvents comprised ACS grade methanol, reagent grade formic acid, double glass distilled water and ammonium formate (Sigma Chemical Co.). Prior to analysis blood samples (20 mls) were collected into 0.5 mls 0.1M disodium EDTA (Sigma Chemical Co.) and 0.5 mls 0.0025 M 1,10 ortho-phenanthroline (Aldrich).

High Performance Liquid Chromatograph

A Waters system comprising twin solvent pumps (Model 6000A), a Model U6K Injector, Waters Gradient Controller and Waters Model 440 Ultraviolet absorbance detector were employed in gradient elution of peptides. Fractions were collected from the column into an automatic fraction collector (LKB) at timed intervals of 12 sec. beginning with the injection of sample.

METHODS

Extraction of Angiotensins from Blood Plasma

Blood samples were collected from a forelimb vein of conscious adult mongrel dogs. Twenty milliliters of blood were

rapidly added to 0.5 mls 0.1M disodium EDTA and 0.5 mls 0.0025 M ortho-phenanthroline in a heparinized tube, mixed, placed on ice, and transported to a refrigerated centrifuge where plasma was separated from blood cells by centrifugation for 15 mins at 4°C and 3000g. Separated plasma was extracted over C18 mini-columns which had been prepared by wetting with 1 ml methanol followed by 3 mls deionized water. For direct RIA, 1 ml of plasma was then drawn over the column. Plasma samples for HPLC were between 4-5 mls in volume and were extracted by repeatedly drawing aliquots of 1 ml of plasma over the column. After addition of plasma, the columns were washed by passing through 1 ml of Tris base (10 mM, pH 7.4). Elution of retained peptides from the column was accomplished with 1 ml of a mixture of methanol and 10% aqueous formic acid (80%/20% vol/vol). Recovery of several angiotensin peptides as well as radioiodinated AII was assessed by addition of peptides to plasma with subsequent extraction and quantification either by RIA (unlabelled peptides) or direct gamma-scintillation counting (^{125}I-AII).

High Performance Liquid Chromatographic Separation of Angiotensin Peptides

The seven angiotensin peptides studied were separated with a linear gradient elution followed by an isocratic step. Initial solvent (Solvent A) was a 70%/30% by volume mixture of 20 mM aqueous ammonium formate/methanol with 1 ml 97% formic acid added to each liter of solvent. Solvent flow rate was 1 ml/min. Upon

injection of sample a linear gradient elution changing from 100% solvent A to 100% solvent B was performed over a 7-minute period. Solvent B comprised a 60%/40% by volume mixture of 20 mM aqueous ammonium formate/methanol with 1 ml 97% formic acid added to each liter of solvent. Upon termination of the gradient Solvent B was run isocratically for a further 7 minutes during which elution of peptides was completed.

Peptide Radioimmunoassay

A radioimmunoassay for AII was developed by using ^{125}I-AII and an antiserum raised in rabbits. The antiserum was generated by conjugating angiotensin II to bovine serum albumin by the method of Goodfriend et al (11). Dialyzed conjugate was then homogenized with an equal volume of Freund's complete adjuvant and 0.5 mls of the homogenate were injected subcutaneously over the back of New Zealand white rabbits. Subsequent to immunization animals were boosted with the same mixture monthly for three months. Significant binding of radioiodinated AII was present in most animals after the second boost. Ten days after the third boost a pool of blood was drawn from one animal which had previously showed significant antibody titers. This blood was clotted and serum removed. This serum has proved to be most suitable for angiotensin radioimmunoassay and is used at a final dilution of 1:100,000. The assay is performed with Tris-HCl buffer (pH 7.4, 50 mM containing 0.2% normal rabbit serum, 0.1%

sodium azide) as vehicle. Each assay tube has a final volume of 500 µl. Standard AII is synthetic (Bachem) and assay detection range is 1.6 pg to 200 pg. Assay of plasma extracts is by reconstitution of each 1 ml plasma extract with 250 µl of Tris-HCl buffer. Two x 100 µl aliquots of the reconstituted sample are assayed. The assay is incubated in the presence of ca. 4000 cpm ^{125}I-AII for 24 hrs. Separation of bound from free is by the second antibody method (goat anti-rabbit gamma globulin, Research Products International) with 50 µl of 25% aqueous polyethylene glycol (M. Wt. range 5,000-6,000 D) added to enhance precipitation.

RESULTS

Recovery of Angiotensins During Extraction

Two means of estimating angiotensin recovery during C_{18} cartridge extraction of plasma were used. Recovery of radioiodinated AII added to 1 ml of inhibited plasma was 85.0 ± 0.9% (mean ± SEM, n=12). Recovery of unlabelled angiotensin II was assessed by addition of 100 pg AII to 1 ml replicates of a pool of inhibited plasma and comparison by RIA of AII immunoreactivity (AII-IR) in 6 samples to which 100 pg of AII had been added and 6 samples (1 ml) from the sample pool to which AII was not added. Recovery of unlabelled AII was 82.2 ± 4.5%. Recovery of unlabelled AIII and C-terminal hexapeptide were estimated in a similar manner and found to be 87.0 ± 3.5% and

86.1 ± 4.6%, respectively. Thus, C_{18} extraction appears to yield good recovery for angiotensin peptides in a limited molecular weight range. Although no estimates have been made of recovery of other angiotensin peptides, the level of selectivity in C_{18} cartridge extraction is broad enough to expect similar recovery rates for the other angiotensins listed in Table 1. Full validation of recovery for other angiotensins awaits the application of appropriate RIA antisera and/or radiolabel availability.

Separation of Angiotensin Standards with UV Monitoring

A typical UV chromatogram resulting from the injection of approximately 10 µg of each of 7 angiotensin peptides into the gradient elution system is shown in Fig. 1. All peaks were well separated and showed good peak symmetry with only a small degree of peak tailing. A small, spurious peak, which was found consistently, emerged prior to the elution of the first angiotensin peptide. Subsequent studies revealed that this peak was associated with the standard preparation of pentapeptide. Since this preparation was analyzed to be 97% pure and showed a consistent and larger peak later in the elution profile it was presumed that the initial peak constituted an impurity with relatively high UV absorbance at the monitoring wavelength (254 nm). No further attempt to determine the chemical nature of this impurity has been made.

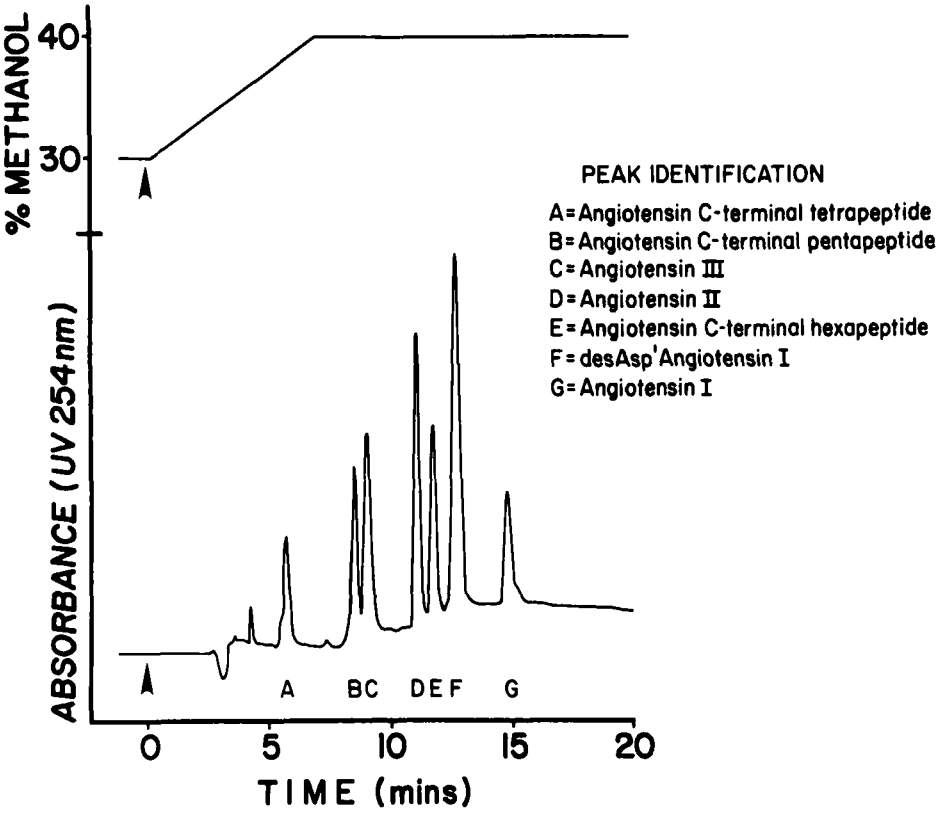

FIGURE 1. Ultraviolet absorbtion elution profile of seven angiotensin peptides. See text for details of stationary and mobile phases.

Separation of Biological Quantities of Angiotensins with Radioimmunoassay Detection

In order to ascertain the applicability of HPLC separation in the concentration range at which angiotensins are present in blood the HPLC system was tested by application of low doses of standard angiotensins. Detection of peptides at this level necessitated use of RIA. Doses of peptide studied were 200 fM

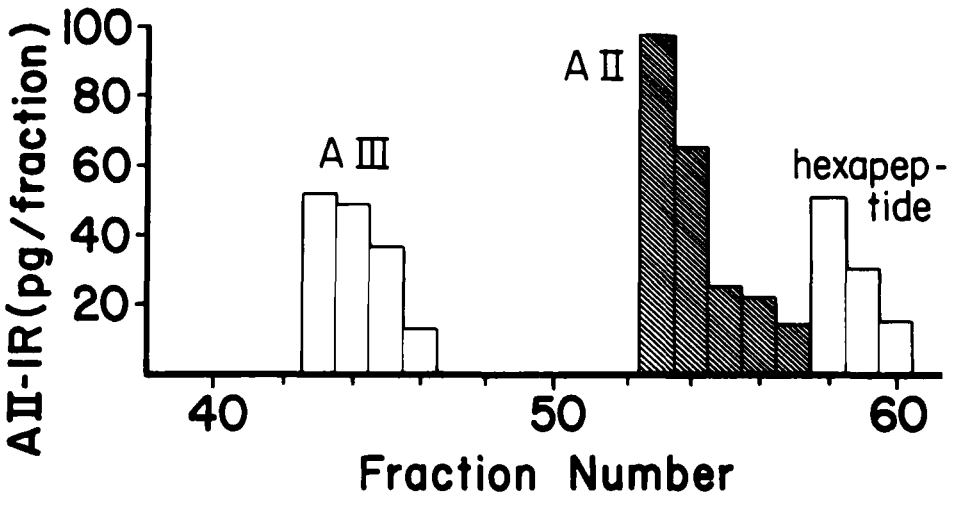

FIGURE 2. Elution of three angiotensin peptides detected by RIA using anti-AII antiserum.

injected into the HPLC system, dissolved in 20 µl of solvent. Peptides selected for study were those showing sufficient cross-reactivity with AII antiserum to permit RIA detection at this concentration. Therefore, only AII, AIII and C-terminal hexapeptide were studied.

Resolution of angiotensins at low concentration is shown in Figure 2. The time course of elution is very similar to that obtained at higher concentration with UV absorbance detection. However, it would appear that tailing of the peaks was somewhat exaggerated at low dose levels. To what extent this can be accounted for by the additional tubing required to carry solvent

from the outlet of the column to the fraction collector is uncertain.

Recovery tests of AII standard peptides were also performed to evaluate what proportion of added AII was recovered in the AII peak. Recovery has been estimated at 67.5 ± 6.08% (n=3). Some experiments have been performed which suggest that the major source of loss occurs at injection; addition of 100 pg of AII in the same volume (10 µl) and collection at the point of connection to the column gave an average recovery of 73.9% in two replicate experiments. Undoubtedly, the use of small volumes as well as the inaccuracy inherent in RIA may account for some of the apparent loss.

Angiotensin in Extracts of Canine Blood Plasma

Table 2 shows the relative proportions of AII, AIII and C-terminal hexapeptide in venous plasma extracts subjected to HPLC separation of angiotensins. The values obtained are close to data obtained from human venous blood using paper chromatography by Semple et al (12).

Table 2

Sample	n	%AII	%AIII	C-terminal hexapeptide
Dog venous plasma (C_{18} extract)	4	78.2 ± 5.0	13.7 ± 3.8	7.2 ± 2.7

(Values are % of total AII-IR in sample appearing under the peak corresponding to each of the three peptides).

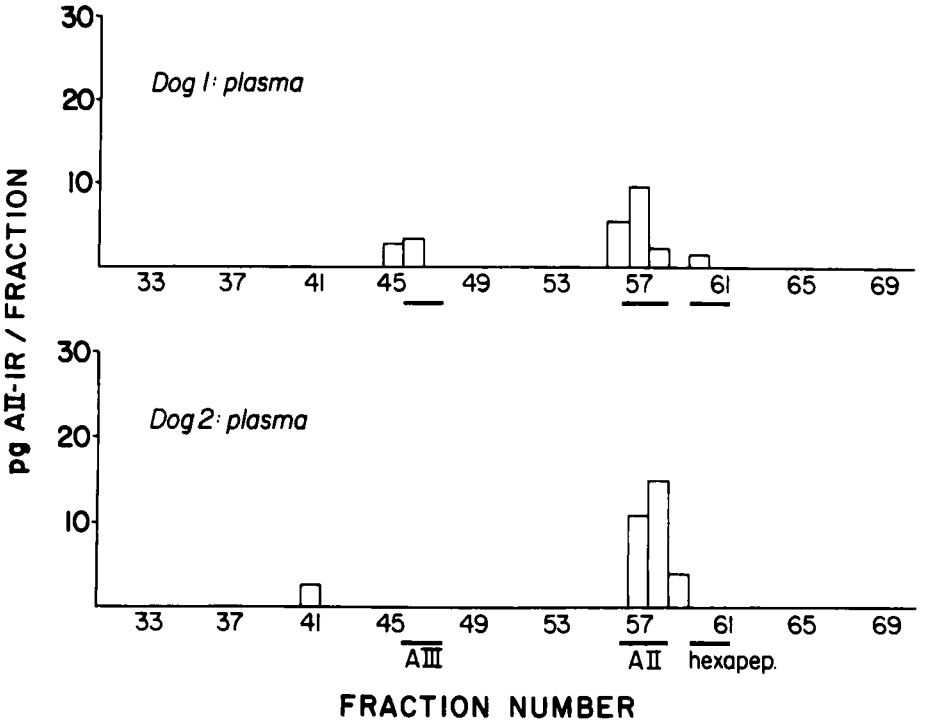

FIGURE 3. Angiotensin detected by HPLC and RIA in extracts of 4 mls of canine venous plasma.

Figure 3 shows the chromatographic profile of two of these samples. One animal showed peaks corresponding to the elution time of all three standard peptides. In the other animal the only substantial AII immunoreactivity corresponded to AII. No peaks corresponding to AIII or C-terminal hexapeptide were observed; this was not the case with the other animals studied. One spurious peak appeared in this animal's sample prior to the elution of AIII. This peak does not appear to correspond to the

elution times of other angiotensin peptides studied and, in any case, would have to be present in high concentration to have sufficient cross-reactivity with the AII antiserum to permit detection. It is concluded that this peak probably does not represent an authentic angiotensin and may be an RIA artifact, perhaps due to inadequate drying of the HPLC fraction.

DISCUSSION

High performance liquid chromatography offers a rapid, efficient and reproducible method for analyzing biologically occurring and active angiotensin peptides. The system that has been developed uses a preliminary extraction method with C_{18}-silica cartridges which permit concentration of samples prior to analysis. For the peptides tested, this extraction procedure gives good recovery and reproducibility. Furthermore, it can be applied to up to 10 samples at once and uses relatively inexpensive and easily obtained materials.

Resolution of peptides in the HPLC separation is good with adequate peak definition and acceptable peak shape as determined by UV absorbance monitoring of microgram quantities of peptide. Monitoring of picogram quantities of peptides by RIA was limited in the present study to those angiotensin peptides which cross-reacted sufficiently with AII antiserum to be detectable at these quantities in RIA. These were AII, AIII and the C-terminal hexapeptide. Once again resolution at these low dose levels was

also adequate, though peaks appeared to show a greater degree of tailing.

Other laboratories have reported a variety of methods to separate biologically occurring angiotensins. These have included paper chromatography (12), thin-layer chromatography on silica gels (13), and HPLC (14,15,16). In addition, some of these methods have demonstrated their applicability to analysis of angiotensins in biological samples (12,14). However, no reports exist of systems employing HPLC techniques which resolve the broad range of closely related and biologically significant angiotensins studied here and which have been evaluated as to suitability for determination of angiotensins at levels normally present in blood samples. Preliminary studies in this laboratory revealed that ion pairing techniques on C_{18} HPLC columns were unable to produce the degree of resolution which has been achieved using the much weaker non-polar C_2 stationary phase. It would appear that selection of a weaker non-polar stationary phase has contributed substantially to the separation of peptides sharing close structural homology.

The data that has been collected in our analysis of peptides fractionated from canine venous blood extracts compares closely with equivalent data obtained by Semple et al (12). One difference lies in the ability of the present separation to resolve C-terminal hexapeptide from C-terminal pentapeptide, which was not achieved in the study of Semple et al. However,

both the data of Semple et al and the present data indicate that venous levels of both of these peptides are quite low. The C-terminal amino acid structure is essential for interaction with angiotensin receptors (17) and the potential biological significance of such metabolic products of AII has been suggested by other studies (18,19). An illustration of this point lies in the finding that at least one metabolite of AII, namely AIII, has biological actions in common with AII (vasopressor and adrenal mineralocorticotrophic), at least in some species (10). The capability of the present system, therefore, not only provides the potential for evaluating the biological role of angiotensins under different conditions, but also provides a ready means of assessing, in combination with appropriate RIA's, the metabolism of angiotensins in normal and disease conditions.

ACKNOWLEDGMENTS

The author thanks the Department of Biochemistry, TTUHSC, for generous equipment loans. The secretarial assistance of Carol McLain is gratefully appreciated.

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