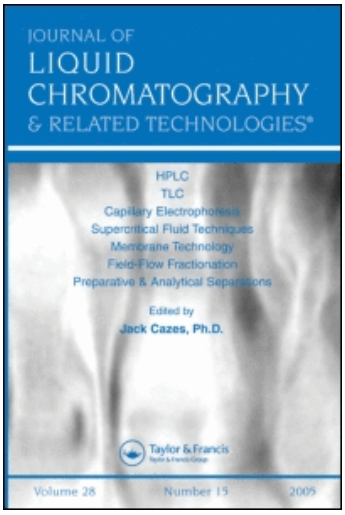

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THIN-LAYER RADIOCHROMATOGRAPHIC DETERMINATION OF CAPTOPRIL (SQ 14,225) AND ITS DISULFIDE DIMER METABOLITE IN BLOOD

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ABSTRACT

A reliable thin-layer radiochromatographic (TLRC) assay has been developed for quantitation of radiolabeled captopril (CP), a new sulfhydryl-containing orally active antihypertensive agent, and its disulfide dimer metabolite (CPD) in blood. CP, which is chemically unstable in blood, was immediately converted to a stable derivative by addition of N-ethylmaleimide (NEM) to freshly collected samples. Aliquots of whole blood samples were analyzed for total radioactivity, and NEM-treated aliquots were extracted with methanol. Reconstituted residues of the extracts were applied to silica gel GF plates, developed with chloroform/ethyl acetate/glacial acetic acid (4:5:3), and analyzed for radioactivity associated with CP and CPD by zonal analysis.

INTRODUCTION

Captopril (CP), D-3-mercapto-2-methylpropanoyl-L-proline (Fig. 1), is a potent and specific inhibitor of the enzyme which catalyzes the conversion of angiotensin I to angiotensin II (1), and has been shown to be an orally effective antihypertensive agent in extensive clinical trials (2-4). CP contains a free sulfhydryl group and is not chemically stable in biological samples. Numerous approaches to stabilization of CP in biological samples were investigated, including the use of antioxidants,

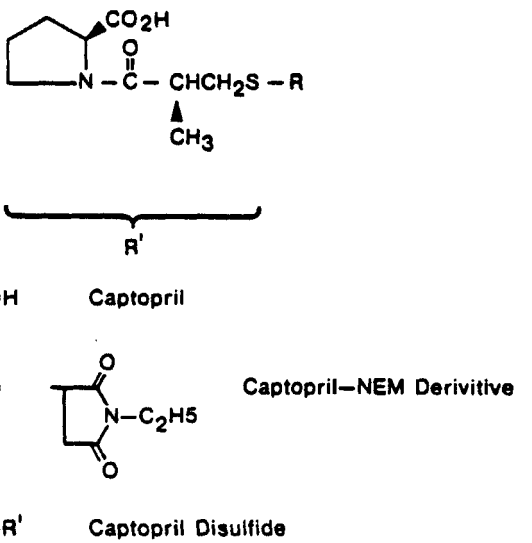

FIGURE 1. Structures of captopril (CP), the N-ethylmaleimide derivative of captopril (CP-NEM) and captopril disulfide (CPD).

chelating agents and derivatizing agents. Formation of a derivative of CP with N-ethylmaleimide (NEM) (Fig. 1) was found to be the most suitable approach to the stabilization of CP in blood. CPD, the disulfide dimer metabolite of CP (Fig. 1) was found to be stable in biological fluids, and CPD in blood was unaffected by the addition of NEM. Many difficulties were encountered during the development of a chemical assay for CP in blood, and alternative assays utilizing radiolabeled CP were investigated. A thin-layer radiochromatographic assay (TLRC), for both CP and its disulfide dimer metabolite (CPD) (5,6) in blood, has been developed and has been utilized in several clinical studies of CP, in studies of CP in vitro (7) and in drug disposition studies in several animal species (8-10). ^{35}S-CP was used initially in clinical studies (5,6) but more recently ^{14}C-CP has been utilized (11,12). Subsequent to the development of the TLRC assay, a chemical assay was developed for CP (as the methylated NEM derivative)

in blood. The assay utilizes GC/MS with the MS in the selected ion monitor mode (13). In this report, the TLRC assay is described and compared with the GC/MS assay.

EXPERIMENTAL

Materials

^{14}C-CP, universally labeled with ^{14}C in the proline moiety, was employed. Clinical 100-mg doses of ^{14}C-CP (tablets, capsules, or dry powder for solutions) were prepared, each of which contained approximately 50 μCi. Radiochemical and chemical purity were approximately 98% with about 2% of ^{14}C-CPD present as an impurity.

Blood Sample Handling

Samples of blood (5 ml) were collected into heparinized Vacutainers® from a suitable forearm vein. The 5-ml blood samples were subdivided and treated as follows: (a) a 3-ml portion of each blood sample was transferred immediately to a test tube containing approximately 25 mg of NEM and mixed thoroughly for subsequent analysis of unchanged CP, CPD, and other metabolites (collectively), and (b) a 2-ml aliquot of each blood sample was set aside for quantitation of total radioactivity. All samples were refrigerated until analyzed.

Extraction

Aliquots (ca. 3 ml) of NEM-treated blood were extracted three times with 9 ml of methanol. In control experiments, it was determined that this procedure quantitatively extracts CP and CPD from blood. Aliquots (1.0 ml) of the combined methanol extracts were analyzed for total radioactivity. The remainder was filtered through glass wool and concentrated to dryness in vacuo at about 35°C. The residue was reconstituted in 0.60 ml of methanol for TLRC.

Thin-Layer Radiochromatography (TLRC)

Aliquots of the reconstituted methanol extracts of blood samples (100 µl to 200 µl) were chromatographed on 0.25-mm silica gel GF plates (Analtech, Inc., Newark, DE) in chloroform/ethyl acetate/glacial acetic acid (4:5:3 by volume). CP, CPD, and CP-NEM were used as reference compounds. Chromatograms were visualized by exposure to iodine vapor.

Each chromatogram was divided into four zones, as indicated by dotted lines in the schematic diagram shown in Fig. 2. All the silica gel in each zone was scraped from the plate, mixed with 1 ml of water and counted in 15 ml of the scintillation cocktail. The radioactivity in zone C represented the CP-NEM derivative and any material at the R_f of unchanged CP; zone B represented CPD; and zone A represented other metabolites (collectively). Negligible amounts of radioactivity were present in zone D.

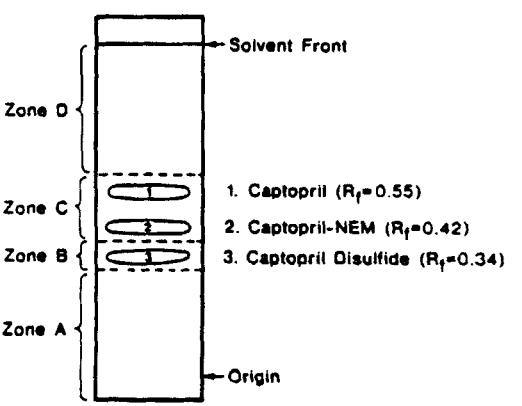

FIGURE 2. Schematic diagram of a thin-layer chromatogram illustrating the areas included in each zone. Reference compounds shown are 1) captopril; 2) captopril-NEM derivative; and 3) captopril disulfide.

Liquid Scintillation Counting

The scintillation cocktail of Anderson and McClure (14) was used to count all blood samples. This cocktail contained 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)benzene], 3 g of 2,5-diphenyloxazole, and 250 ml of Triton X-114 (Ruger Chemical Co., Irvington, NJ), q.s. to 1 liter with xylene. PGM, a mixture that consisted of a saturated solution of sodium pyruvate in methanol, glacial acetic acid, and methanol in the ratio of 4:3:1 (by volume) was used to neutralize the contents of the vials before they were counted. Individual samples were prepared as described below. All samples were counted in duplicate.

Samples of whole blood (0.2 ml) were digested in 1 ml of Soluene-350® (Packard Instrument Co., Downers Grove, IL) and bleached with 1 ml of a 20% solution of benzoyl peroxide in toluene. Samples were then neutralized with 0.1 ml of PGM and mixed with 15 ml of the scintillation cocktail. Samples of methanol extracts of NEM-treated blood (1.0 ml) were counted directly in 15 ml of the scintillation cocktail.

All samples were counted in a Model 2425, 3375, or 3380 Packard Tri-Carb® liquid scintillation spectrometer (Packard Instrument Co.). Counting efficiency was determined with automatic external standardization and the use of previously prepared quench curves. Some of the samples prepared from the chromatograms were also counted in an Intertechnique Model SL-4200 scintillation spectrometer (IN/US Service Corp., Fairfield, NJ). This spectrometer used external standardization, stored quench curve coefficients, and a computer program to calculate the percentage of radioactivity found in zones A, B, C, and D of the thin-layer chromatogram (Fig. 2).

Preparation of Spiked Control Blood Samples

Control blood samples containing NEM were "spiked" with ^{14}C-CP to give concentrations of 2.8, 1.0, 0.6, and 0.2 µg/ml in blood or with ^{14}C-CPD to give a concentration of 0.5 µg/ml.

Calculation and Analysis of Data

The percentages of CP (zone C), CPD (zone B), and other metabolites (zones A and D) in each sample were calculated based on a value of 100% for the total radioactivity in zones A, B, C, and D combined (Fig. 2).

RESULTS AND DISCUSSION

When control samples "spiked" with ^{14}C-CP were analyzed for CP in the same manner as the clinical blood samples, the following results were obtained: 95.4 ± 1.1% (mean ± S.E.M., n=4), 94.6 ± 1.2% (n=6), 94.4 ± 0.3% (n=3), and 92.8 ± 0.5% (n=5) of the radioactivity was found in zone C (CP and CP-NEM), and 1.9 ± 0.4%, 3.0 ± 1.0%, 1.6 ± 0.3%, and 2.8 ± 0.8%, of the radioactivity was found in zone B (CPD) for samples spiked with ^{14}C-CP at concentrations of 2.8, 1.0, 0.6, and 0.2 µg/ml, respectively. It should be noted that ^{14}C-CP material used in these control experiments contained approximately 2% CPD. In samples spiked with ^{14}C-CPD 93.5 ± 2.1% (n=5) of the radioactivity was found in zone B and 1.1 ± 0.3% in zone C (CP and CP-NEM).

The TLRC assay described is suitable for analysis of CP and CPD in clinical blood samples after administration of radiolabeled (^{3}H, ^{35}S, and ^{14}C) CP. The presence of CPD in blood samples is believed to be primarily the result of its formation *in vivo* and not simply the appearance in blood of an impurity in the radiolabeled drug. This statement is based on numerous *in vivo* and *in vitro* studies which demonstrate the interconversion of CP and CPD (15,16).

The analysis of spiked samples indicated the satisfactory stabilization and recovery of CP as its NEM derivative, and of CPD. In a preliminary comparison of the TLRC and GC/MS assays for CP in actual clinical samples obtained after an oral 100-mg dose of ^{14}C-CP, the TLRC assay gave greater precision than the GC/MS assay. For duplicate samples, the coefficients of varia-

tion (C.V.) were 8% and 7% at ca. 700 ng/ml (1 hr samples) for the GC/MS and TLRC assays, respectively. At ca. 25 to 50 ng/ml (4 hr samples), the C.V. were 33% and 10%, respectively. The limit of detection of the TLRC assay was ca. 10 ng/ml for CP, a value corresponding to approximately 10 cpm/ml of blood. The detection limit of the GC/MS assay was approximately the same.

The TLRC gave somewhat higher values than the GC/MS assay, especially at time points several hours after the maximum concentration of CP in blood was attained. Since this may have been due to minor metabolite(s) interfering in the TLRC assay, a minor modification of the TLRC assay might improve specificity. The most recent experiments in our laboratories indicate that CP is quantitatively converted to its NEM derivative in blood under the conditions used in our studies. Thus, in our current studies, only the zone corresponding to the CP-NEM standard is considered to represent unchanged CP in the original sample. The zone at the R_f of CP is being analyzed separately. The TLRC assay is not only easier to carry out, but is competitive with the existing GC/MS assay in specificity and sensitivity, and shows superior precision. The complexity of the GC/MS assay, and the need for expensive instrumentation and sophisticated technology required for its implementation, indicate that the TLRC assay is a suitable alternative in animal and human studies in which radiolabeled CP can be administered.

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