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DETERMINATION OF THE ANGIOTENSIN CONVERTING ENZYME INHIBITOR BENAZEPRILAT IN PLASMA AND URINE BY AN ENZYMIC METHOD

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SUMMARY

An enzyme inhibition assay for the angiotensin-converting enzyme (ACE) inhibitor benazeprilat is described. Plasma and urine samples were diluted and endogenous ACE was inactivated by heating. After incubation of the plasma samples with hippuryl-histidyl-leucine as substrate and blank plasma as the source of ACE, released hippuric acid was measured by high-performance liquid chromatography. Urine samples were incubated with [^{3}H]hippuryl-glycyl-glycine and with rabbit lung extract as the source of ACE. Released [^{3}H]hippuric acid was quantified by liquid scintillation counting. Drug standards for the standard curve were prepared in the biological matrix. A cross-check with a gas chromatographic–mass spectrometric method showed good agreement, demonstrating that this enzymic method is suitable for assessing drug bioavailability and pharmacokinetics.

INTRODUCTION

Benazepril·HCl (CGS 14824 A), 3-([1-ethoxycarbonyl-3-phenyl-(1*S*)-propyl]-amino)-2,3,4,5-tetrahydro-2-oxo-1-(3*S*)-benzazepine-1-acetic acid hydrochloride (I), is a new prodrug that is being developed for treatment of hypertension. It is hydrolysed in vivo to the diacid benazeprilat (CGS 14831, II), a strong inhibitor of angiotensin-converting enzyme (ACE). This report describes enzymic methods for measuring benazeprilat in plasma and urine. In a well characterized incubation system with defined concentrations of ACE and substrate, the rate of enzyme reaction depends only on the amount of inhibitor present. The rate of reaction or the concentration of the released enzyme product can thus be plotted against the inhibitor concentration for standard samples spiked with inhibitor, and from this standard curve the inhibitor concentrations of unknown samples can be read.

Our procedure for plasma is a modification of the method of Hajdú et al. [1].

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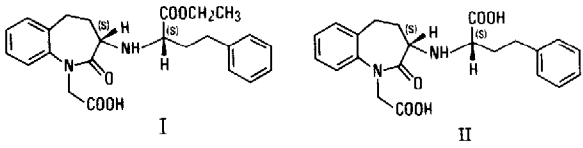


Fig. 1. Chemical structures of the prodrug benazepril (I, CGS 14824 A, mol.wt. 424.50) and of its active metabolite benazeprilat (II, CGS 14831, mol.wt. 396.44).

The time-consuming extraction of inhibitor was replaced by heat-inactivation of endogenous ACE. Plasma samples were first diluted with buffer and heated to inactivate ACE. Then they were incubated with human blank plasma as the source of exogenous ACE and with hippuryl-histidyl-leucine (hip-his-leu) as substrate. Hippuric acid, liberated by the action of ACE, was quantified by high-performance liquid chromatography (HPLC) with a LiChrosorb-NH_2 column and UV detection.

The procedure for urine is a modification of the method of Swanson et al. [2]. Diluted urine was incubated with dissolved rabbit lung extract as source of ACE and [^{3}H]hippuryl-glycyl-glycine ([^{3}H]hip-gly-gly) as substrate. Released [^{3}H]hippuric acid was extracted with a water-immiscible scintillation cocktail, whose composition was chosen to make physical separation of the organic phase from the aqueous phase unnecessary.

MATERIALS AND METHODS

Reagents

Hippuryl-L-histidyl-L-leucine was from Sigma (St. Louis, MO, U.S.A.), [phenyl-4(n)-^{3}H]hippuryl-glycyl-glycine from Amersham International, and N-hippuryl-glycyl-glycine from Bachem (Bubendorf, Switzerland). 2-(4-*tert.*-Butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD), benazepril·HCl and benazeprilat were from Ciba-Geigy. Rabbit lung acetone powder was from Sigma. Acetonitrile (HPLC grade) and D,L-mandelic acid were from Fluka (Buchs, Switzerland). LiChrosorb-NH_2 (10 μm) was from Merck, (Darmstadt, F.R.G.).

The incubation buffer (buffer A) consisted of 0.25 M sodium hydrogenphosphate and 0.75 M sodium chloride, adjusted to pH 8.3 with 1.0 M phosphoric acid. The elution buffer (buffer B) was 10 mM potassium dihydrogenphosphate, adjusted to pH 3.0 with 1.0 M phosphoric acid. Methanolic phosphoric acid was a 2:1 (v/v) mixture of methanol and 8% aqueous phosphoric acid. Rabbit lung ACE solution was prepared from lung acetone powder as described in ref. 2. The substrate solution for the plasma assay was hip-his-leu (4.1 mM) in water, and for the urine assay [^{3}H]hip-gly-gly was added to 7.5 mM hip-gly-gly in water to give 37 kBq/ml. The scintillation cocktail consisted of 6.4 g of butyl-PBD in 800 ml toluene and 200 ml ethyl acetate.

Preparation of standards

A stock solution was prepared by dissolving benazeprilat in 0.7% ammonia solution to a concentration of 10 nmol/ml. Twelve standard samples, containing

10–600 pmol of benazeprilat per ml of native plasma or urine, were prepared by diluting stock solution with buffer A and adding 5% blank plasma or blank urine. If possible, the blank urine used was from the same subject as the urine samples for analysis.

Preparation of spiked samples

Spiked samples were prepared by adding 20 µl of aqueous stock solution of benazeprilat to 980 µl of plasma or urine and further diluting with plasma or urine to yield samples containing 10–500 pmol/ml of benazeprilat. Spiked samples were divided into portions and kept frozen until used as control in each assay.

Sample preparation

Urine and plasma samples were diluted 1:20 with buffer A. Plasma samples were then placed in a water-bath at 75 °C for 5 min to inactivate endogenous ACE. Samples with a high concentration of benazeprilat were further diluted in steps of 1:10, plasma samples with buffer A and urine samples with buffer A containing 5% of blank urine.

Procedure for plasma

All incubations were carried out in 55×11 mm polypropylene test-tubes at 37 °C in a water-bath under shaking. Diluted and heat-treated plasma or standard samples (100 µl) were preincubated with blank plasma as the source of exogenous ACE (100 µl) for 12 min before the addition of 50 µl of hip-his-leu substrate solution. After an additional incubation period of 80 min, the enzymic reaction was stopped with 200 µl of methanolic phosphoric acid. Acetonitrile (1000 µl), containing mandelic acid (30 mg/l) as internal standard, was added and the test-tubes were centrifuged at 5000 *g* for 5 min. Clear supernatant was transferred into HPLC vials for analysis.

High-performance liquid chromatography

The chromatographic system consisted of an automatic sample injector WISP 712 (Waters Assoc.), an SP 8700 pump and an SP 4270 integrator (Spectra-Physics) and a Spectroflow monitor (Kratos). The column was a 250 mm×4.6 mm I.D. steel column packed with LiChrosorb-NH_2 (10 µm) (Merck). The mobile phase was a 1:4 (v/v) mixture of acetonitrile and buffer B. The flow-rate was 2.3 ml/min, and the sample size was 15 µl. The effluent was monitored at 228 nm. The analysis time was ca. 9 min. The peak height of the hippuric acid signal, corrected to a fixed concentration of mandelic acid, was taken as a measure of ACE activity in the incubation system.

Procedure for urine

The assay had to be modified for analysis of urine owing to the presence of endogenous hippuric acid. The following changes were made: blank plasma was replaced by rabbit lung ACE solution (100 µl) as the source of exogenous ACE; [^{3}H]hip-gly-gly solution (50 µl) was used as substrate; the enzymic reaction was stopped with 2 *M* hydrochloric acid (200 µl), saturated with sodium sulphate;

after addition of 2.2 ml of scintillation cocktail, the mixture was equilibrated on a vortex mixer for 2 min.

This procedure could also be used to analyse plasma samples, with blank plasma substituted for rabbit lung ACE solution as the source of ACE. Combining blank plasma as the source of ACE with urine samples proved unsatisfactory owing to frequent build-up of persistent emulsions in the equilibration step on the vortex mixer.

Liquid scintillation counting

To measure released [^{3}H]hippuric acid, the samples were counted in a liquid scintillation spectrometer (Packard 460 CD) without separation of the organic phase from the aqueous phase. Disintegrations per minute (dpm) values were corrected for the blank value and taken as a measure of ACE activity in the incubation system. Samples free of inhibitor gave ca. 500 Bq, blank samples without enzyme activity gave ca. 22 Bq.

Computations

Raw results were collected by the HPLC integrator and the liquid scintillation counter, and batch-wise written onto a hard disk of a personal computer (IBM-XT) by means of a communication program. All subsequent operations, e.g. construction of a standard curve, reading of sample values off the curve and printing of results, were controlled by means of a menu in a calculation template (Lotus 1-2-3). A linear transformation (logit/log) of the standard curve was unsatisfactory, therefore a cubic spline function with a smoothing factor [3] was used to approximate the sigmoid form of the standard curve. This subroutine, written in BASIC, displayed the fitted curve on screen to allow selection of an appropriate smoothing factor.

RESULTS AND DISCUSSION

Fig. 1 shows the structures of the prodrug benazepril (I) and its active metabolite benazeprilat (II). Fig. 2 shows liquid column chromatograms of incubated plasma free of inhibitor (A) and containing 600 pmol/ml of benazeprilat (B). The elution sequence was unchanged tripeptide (a), hippuric acid (b) and mandelic acid (c).

Accuracy and day-to-day precision of the methods were determined by measuring portions of six spiked plasma or urine samples with each assay on eight different days (Table I). Plasma concentrations were calculated from single measurements, urine samples were assayed in duplicate and the mean of the dpm values was used for calculation. Mean recoveries were in the range 92.9–105% ($n=8$) and the coefficients of variation (C.V.) were below 11% for concentrations larger than 20 pmol/ml. Standard curves could be used over a range of 20–600 pmol of benazeprilat per ml of native plasma or urine. The limit of detection was estimated from spiked samples and from blank samples to be 10 pmol/ml for urine and 20 pmol/ml for plasma.

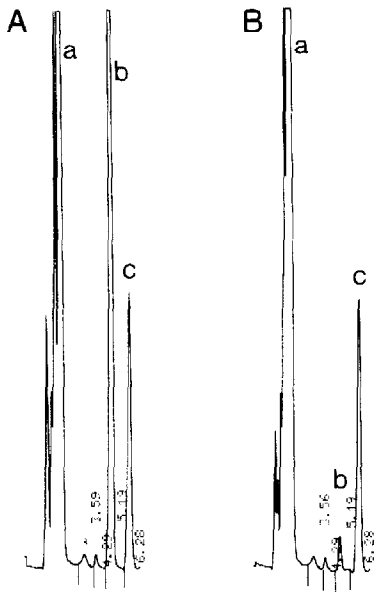


Fig. 2. HPLC of incubated (A) blank plasma and (B) plasma spiked with benazeprilat (600 pmol/ml). Peaks: a = hip-hist-leu; b = hippuric acid (RT = 5.19 min); c = mandelic acid (RT = 6.28 min).

TABLE I

ACCURACY AND DAY-TO-DAY PRECISION

Benazeprilat was determined in six spiked plasma samples (single measurement) and six spiked urine samples (mean of duplicate measurements) on eight different days. C.V. = 100 × standard deviation/mean.

Plasma			Urine		
Added (pmol/ml)	Recovery (%)		Added (pmol/ml)	Recovery (%)	
	Mean	C.V. (n=8)		Mean	C.V. (n=8)
20	104	13.5	14	116	16.0
30	99.7	5.4	35	92.9	0.6
50	103	6.0	70	100	3.6
75	103	6.1	105	105	3.1
138	97.1	6.0	210	102	5.4
250	99.6	4.8	350	100	7.4

Variation of ACE activity

The amount of ACE in the blank plasma of eighteen healthy male volunteers measured in one assay was found to cover a range of 59–178% of the mean value. The activity of a pool of blank plasma measured on 11 different days was found to cover a range of 79–125% of the mean value (C.V. 16.4%, n=11).

Stability of benazeprilat

Kaiser et al. [4] found benazepril·HCl and benazeprilat to be stable in plasma for at least 1 year if stored below −18 °C. To determine the stability of benazepril against hydrolysis under conditions of the assay, plasma was spiked with benazepril·HCl and assayed as described. Samples containing 5000, 1000 and 200 pmol/ml of benazepril yielded a mean inhibition ($n=3$) equivalent to 13, 3 and 0.1 pmol/ml of benazeprilat, respectively. This interference is the sum of inhibition by benazepril itself and by benazeprilat, produced by hydrolysis during incubation.

Inactivation of endogenous ACE

Enzyme inhibition assays allow quantitation of inhibitor only if concentrations of enzyme and substrate in the incubation system are well defined. To achieve this with biological samples, the inhibitor has to be isolated or the endogenous enzyme has to be inactivated. To check inactivation of ACE by heat-treatment, human blank plasma (100 µl) was diluted with buffer A (100 µl) and placed in a water-bath for 5 min at 37–78 °C. After cooling and addition of 50 µl of hip-his-leu substrate, the assay procedure for plasma was followed. The amount of hippuric acid released during the incubation for 80 min at 37 °C was taken as a measure of residual ACE activity in the plasma samples and plotted against temperature of the water-bath (Fig. 3). ACE activity was destroyed completely by temperatures in excess of 65 °C.

Counting of [^{3}H] hippuric acid in the presence of [^{3}H] hip-gly-gly

Water-immiscible scintillation cocktails were tested for their suitability to assess [^{3}H] hippuric acid in the presence of [^{3}H] hip-gly-gly without separation of the scintillator phase from the aqueous phase. Urine samples were incubated with labelled tripeptide and with lung ACE as described under Materials and Methods, and were counted in a liquid scintillation spectrometer with different cocktails. Fig. 4 shows extraction of hippuric acid (A) and tripeptide (B) with toluene-based cocktails containing various amounts of ethyl acetate. Samples for curve A were incubated for 4 h to achieve near complete hydrolysis of labelled tripeptide

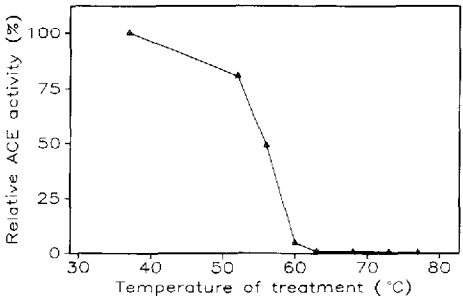


Fig. 3. Inactivation of ACE by heat-treatment. Human plasma was diluted with an equal amount of buffer A and placed for 5 min in a water-bath at different temperatures prior to incubation with hip-his-leu. Released hippuric acid was quantified by HPLC and taken as a measure of residual ACE activity in the plasma.

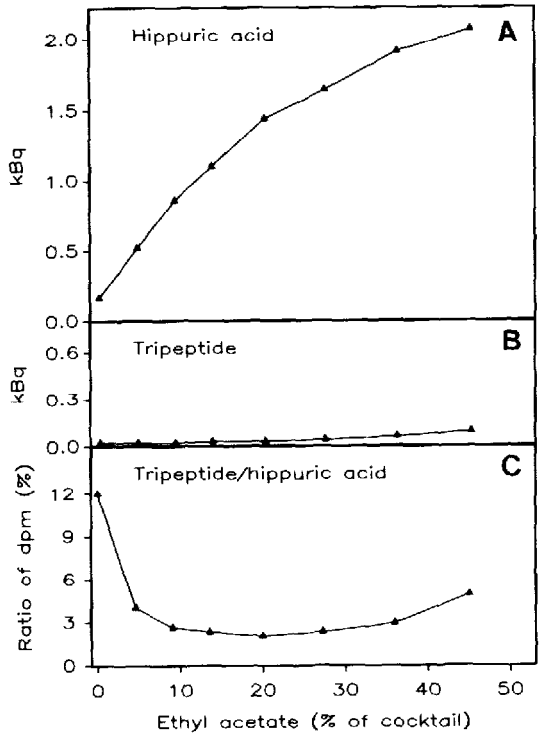
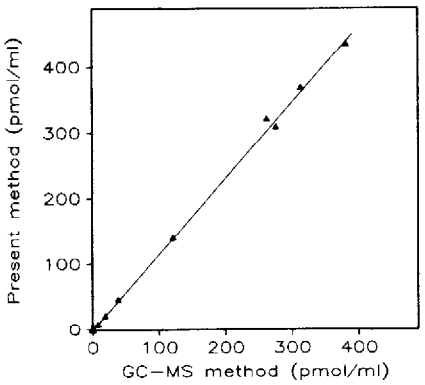


Fig. 4. Extraction of [^{3}H]hippuric acid (A) and [^{3}H]hip-gly-gly (B) from aqueous incubation solution with a scintillation cocktail containing various amounts of ethyl acetate. In (C), counts from tripeptide are expressed relative to those from hippuric acid. Urine was incubated with labelled tripeptide and lung ACE, for 4 h in the case of A to get nearly complete conversion of tripeptide into hippuric acid, and after heat-inactivation of added ACE in case of B to avoid any conversion. Samples were counted without physical separation of the organic phase from the aqueous phase.

to [^{3}H]hippuric acid. Samples for curve B were heat-treated prior to incubation to inactivate added lung ACE, thus preventing enzymic release of [^{3}H]hippuric acid from [^{3}H]hip-gly-gly. Curve C shows counts from tripeptide expressed relative to those from hippuric acid. A scintillation cocktail containing 20% of ethyl acetate allows optimal counting of [^{3}H]hippuric acid in the presence of [^{3}H]hip-gly-gly without separation of the organic phase from the aqueous phase. Interference by an equal amount of the tripeptide is less than 3%.

Cross-check with a gas chromatographic–mass spectrometric (GC–MS) method

Concentrations of benazeprilat in biological samples obtained with the present assays were compared with those measured in the same samples by GC–MS [4]. Plasma and urine of volunteers who had taken 20 mg of benazepril·HCl were collected at different times after dosing and analysed. Plasma results are single measurements, urine samples were analysed in duplicate. Figs. 5 and 6 show the results obtained for plasma and urine samples, respectively. Linear least-squares regression analysis yielded a correlation coefficient of 0.998 for the plasma samples ($n=9$) and of 0.984 for the urine samples ($n=23$). In both urine and plasma,



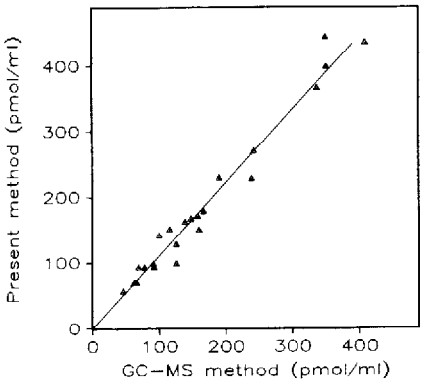


Fig. 5. Correlation of benazeprilat concentrations measured by the enzyme inhibition–HPLC method and by GC–MS [4] in plasma of volunteers administered benazepril·HCl. $y = 1.16x - 1.90$; $r = 0.998$; $n = 9$.

Fig. 6. Correlation of benazeprilat concentrations measured by the enzyme inhibition–liquid scintillation counting method and by GC–MS [4] in urine of volunteers administered benazepril·HCl. $y = 1.11x - 0.86$; $r = 0.984$; $n = 23$.

concentrations of benazeprilat obtained by the enzymic assay were 11–16% higher than those measured by GC–MS. This could be due to a second active metabolite other than benazeprilat being present or, more likely, due to additional benazeprilat being freed from an inactive conjugate during incubation.

CONCLUSIONS

The present assays for the ACE inhibitor benazeprilat in plasma and urine are modifications of previously described methods. They offer easier sample preparation for either HPLC analysis or liquid scintillation counting. Enzymic assays require separation of active drug from endogenous enzyme before incubation with exogenous enzyme. This separation step was replaced by heat-inactivation of endogenous ACE. If appropriately labelled substrate is used, the [^{3}H]hippuric acid released can be quantified by liquid scintillation counting. Usually this is done after separation of the scintillator phase from the aqueous phase containing excess labelled substrate. Here the scintillation cocktail was modified to allow counting of hippuric acid in a two-phase mixture without physical separation of the two phases.

The assay of benazeprilat by enzyme inhibition correlated strongly with the independent assay of the same biological samples by a GC–MS technique. Enzymic assays cannot discriminate between different active metabolites. This lack of specificity can be an advantage when assessing bioavailability of the active principle or pharmacodynamics. A major advantage is that enzymic assays can easily be adapted to different inhibitors.

ACKNOWLEDGEMENTS

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