Sequential Electrochemical Reduction, Solvent Partition, and Automated Thiol Colorimetry for Urinary Captopril and its Disulfides

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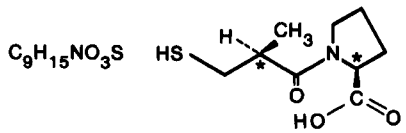
Abstract □ Analysis of urinary captopril was necessary for dosage form bioavailability and dose titration studies. The necessity for long-term storage of samples prior to analysis and the presence of an oxidation-prone thiol of captopril required development of an acid-chelate stabilization method for urinary captopril. An electrochemical reduction released disulfide-conjugated captopril for thiol colorimetry. Of several rugged reduction cells evaluated, one with a porous glass disk separating the anode and the mercury pool cathode was preferred. Methylene chloride partitions from acidified salt-saturated urines, before and after reduction, allowed the measurement of free and disulfide-conjugated captopril. The drug partitioned into the solvent, whereas the aqueous phase retained acid protonated, amino group-bearing thiols like cysteine. Subsequent solvent evaporation volatilized other potential colorimetric interferences. An automated thiol colorimetry of 25 samples/hr was developed for analysis of the aqueous reconstitutes. Results were confirmed by a subsequently developed HPLC method with electrochemical detection.

Keyphrases □ Captopril—urinary, disulfides, automated thiol colorimetry, solvent partition, sequential electrochemical reduction □ Colorimetry—automated thiol, urinary captopril and disulfides, solvent partition, sequential electrochemical reduction □ Electrochemical, reduction—sequential, solvent partition, automated thiol colorimetry, urinary captopril, disulfides

Captopril, 1-(3-mercapto-2-D-methyl-1-oxopropyl)-L-proline (*S,S*)



MW 217.29

is the first clinically potent oral hypotensive agent designed for highly selective inhibition of the angiotensin-converting enzyme (1). Captopril has been found effective for treatment of hypertension and congestive heart failure (2–4).

BACKGROUND

Captopril[1] is readily oxidized in human urine. Acidification minimizes excessive oxidation for only 1–2 days. However, an anticipated large sample load required storage for several weeks before analysis. This provided the impetus for a urinary captopril stability study to determine sample stability under various storage conditions prior to analysis. Satisfactory stabilization was attained by acidification, trace metal removal, and quick refrigeration. A very simple, direct automated colorimetry of pre- and postdosage urines, developed first, appeared adequate. Attempts were made to minimize the predosage urine blank by automating additional thiol colorimetries. These included the alkaline nitroprusside (5) and *S*-nitroso-Bratton–Marshall (6) colorimetries. An automated version of the reaction of Ellman and Lysko (7) gave the lowest blanks.

This simple, direct analysis was quickly found to lack sensitivity by comparison with thin-layer radiochromatographic analysis (8, 9) performed on divided samples from subjects dosed with radioactive captopril. Consequently, the direct colorimetry was preceded by a solvent partition clean-up. To assay for total captopril, which includes captopril, its disulfide, and captopril mixed disulfides (*e.g.*, captopril–cysteine), the urine sample was electrochemically reduced prior to solvent extraction.

The present method, intended as an interim method until development of an HPLC–electrochemical detection (ECD) procedure (10), offers an alternative to laboratories not equipped for HPLC-ECD.

EXPERIMENTAL

Reagents and Apparatus—The reagents included captopril, captopril disulfide, captopril–cysteine disulfide, 8% diethylenetriamine pentaacetic acid (I) in 0.8 *N* NaOH (refrigerate), 10.5% citric acid monohydrate in 6.3% oxalic acid dihydrate, acid-chelating mixture of 0.4% I and 1.05% citric acid monohydrate in 0.63% oxalic acid dihydrate (constituted from the preceding concentrates), freshly water washed methylene chloride, sodium sulfate (anhydrous, fine, granular, or ball milled for rapid dissolution), 1.86% disodium ethylenediaminetetraacetic acid dihydrate (II) plus 0.02% polyoxyethylene sorbitan monooleate[2] in 20% triethanolamine (III)[3], 0.08% 5,5′ dithio-bis(2-nitrobenzoic acid) (Ellman's reagent) in 50% methanol–50% 0.01 *M* sodium acetate–acetic acid buffer[3] (pH 4.7), 0.1 *N* HCl[3], 10% mercuric chloride in 1 *N* HCl, aqueous saturated sodium chloride, and 0.5 *M* Na_2CO_3. In constituting Ellman's reagent, refrigerated buffer was used to minimize heat generated on dilution of the methanol, since heat hydrolyzes the reagent to produce the same yellow color characteristic of its colorimetric reaction with thiols. Since it is also sensitive to light, the reagent was dispensed from an amber container. To further minimize yellow background the triethanolamine used was water-white rather than the yellow of amines stored for long periods of time. Water referred to in this report is distilled water.

The apparatus included centrifuge tubes (50-ml round-bottom, screw capped, with flat polytetrafluoroethylene[4] or polyethylene cone liners), nitrogen evaporation assembly with a 40° bath, ultrasonic bath, plastic caps[5], and an autoanalysis system[6] as illustrated in Fig. 1. The system included an automatic sampler[7] equipped with a 50-2/1 cam. The cam facilitates 50 sample transfers per hour, with samplings twice as long as intermediate washes. The asterisks in Fig. 1 identify solvent resistant tubing[8], whereas the other tubing[9] was not required to be solvent resistant. The peristaltic pump transferred Ellman's reagent, the II–III mixture, and other solutions as indicated in Fig. 1 and in the *Reagents* section.

Colorimetric Standards—Exactly 25.0 mg of captopril was accurately weighed into a 100-ml volumetric flask, dissolved, and diluted to volume with the acid-chelating mixture. Ten-milliliter portions of this primary standard were transferred to 25-, 50-, and 100-ml volumetric flasks. Each flask was diluted to volume with the acid-chelating mixture. These secondary working standard solutions contained 100, 50, and 25 μg of captopril/ml. The primary standard solution was refrigerated up to 1 week. Fresh secondary working standards were prepared daily.

Sample Collection—Five milliliters of I concentrate was added to each subject's plastic, urine collection container. Subjects voided into these containers and the contents were mixed by swirling. The total volume was immediately measured and recorded. Without delay, each

[1] Capoten, Lopirin, SQ 14,225, E. R. Squibb, Princeton, N.J.

[2] Tween 80, I.C.I. Americas, Inc., Wilmington, Del.

[3] Autoanalysis reagents were gravity filtered through a funnel with coarse sintered disk prior to use.

[4] Teflon, E. I. duPont de Nemours & Co., Wilmington, Del.

[5] From polyethylene containers, Nalgene No. 6250, Nalge Co., Rochester, N.Y.

[6] Technicon Autoanalyzer, Technicon Industrial Systems, Tarrytown, N.Y.

[7] Technicon Sampler II, Technicon Industrial Systems.

[8] Solvaflex, Norton Plastics and Synthetics Divison, Akron, Ohio.

[9] Tygon, Norton Plastics and Synthetics Division.

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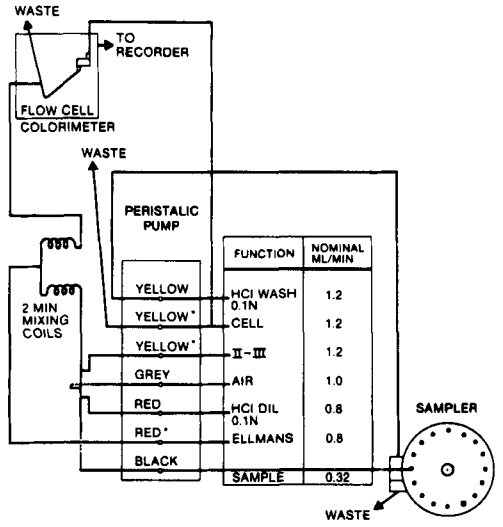

Figure 1—*Autoanalysis of Ellman colorimetry. See Reagents and Apparatus section.*

sample was then poured into central, plastic storage vessels (one for each subject), containing 100 ml of cold citric–oxalic concentrate. The solutions were mixed by swirling and the sample rapidly chilled to about refrigerator temperature (3–5°). Each subject's urine voided during a desired collection period was treated as described and collected in that individual's central storage vessel. The volume of urine plus stabilizers in the central storage vessel was measured and recorded. About 50 ml of the settled, measured urine was transferred to a suitably labeled tube. All such tubes were refrigerated during storage or transportation pending assay. The collections for each subject included a predosage control urine.

Electrochemical Reduction Cell—The cell in Fig. 2 was a scale-up from that of previous study (11), and was used for most of the studies. The 5.05 × 1.85-cm i.d. tube contained a magnetically stirred mercury pool in electrical contact with a platinum wire extending to the exterior of the tube. An inverted U side arm (3.5-mm i.d.) was attached ~2 cm from the bottom of the tube. The latter contained a saturated sodium chloride 2% agar bridge constituted and maintained as described previously (12). The agar salt bridge isolates the negative reductive mercury pool cathode from the positive oxidative platinum anode. The anode is positioned at the tip of the bridge in a small container of saturated sodium chloride. The cells were connected in series with miniature alligator clip jumper leads through a 0–5 milliammeter and a power supply[10] capable of supplying 2.5 mA/hr. The integrity of the agar bridge was maintained optimally by storage with saturated sodium chloride solution at both

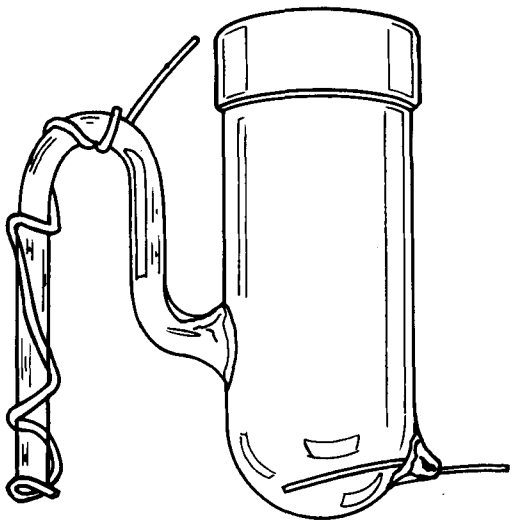

Figure 2—*Saetre-Rabenstein (see Ref. 11) agar bridged electrochemical reduction cell.*

[10] Power Supply No. 6212A, Hewlett-Packard Co., Palto Alto, Calif.

ends. During electrolysis the mercury pool–urine interface was magnetically stirred[11] minimizing vortexing or dispersed mercury droplets. Two cells were stirred over each stirrer. As many as 12 cells were operated in series. To minimize chlorine generation at the anode the saturated sodium chloride electrolyte was made alkaline, as recommended previously (13). Five to six drops of 0.5 *M* Na_2CO_3 were mixed in 9 ml of the anode electrolyte, and the reduction assembly was operated in a hood to sweep chlorine away. The alligator clips were placed well away from the anodic electrolyte to minimize corrosion by chlorine. To minimize chlorine retention in the gel, the bridge tip was removed from the electrolyte soon after a run. Near quantitative disulfide reduction was obtained without widely recommended (13–18) nitrogen blanketing of the cathode compartment. Almost complete sulfhydryl recovery was ensured by l-citric–oxalic thiol stabilization, slight electrolytic hydrogen generation, and a minimum headspace beneath a pinhole vented plastic cap.

Electrochemical Reduction of Sample—Prior to analysis, the saturated sodium chloride and three subsequent water washes were aspirated from the mercury in each cell. The wet mercury and stirring bar were then poured into a 100-ml beaker containing a generous piece of filter paper on the bottom. The mercury was cleaned and dried by swirling it several times over the paper. The mercury and stirring bar were returned to the cell. The cell was then clamp mounted over a magnetic stirrer with its salt bridge exit tip immersed in a small container of alkaline saturated sodium chloride. Washed, dried, and mounted cells were filled with urine samples, leaving minimal headspace beneath the pinhole vented plastic caps[5]. Moderate magnetic stirring was initiated. The cells were securely connected in series to the milliammeter and power supply with the miniature alligator clip jumper leads as follows. The black negative terminal of the power supply was attached with a clip lead to the platinum–mercury cathode of the first cell, then its positive salt bridge platinum anode was clip attached to the platinum–mercury cathode of the next cell in the series. This series clip lead connection between adjoining cells was continued until the last platinum salt bridge anode was connected to the red positive terminal of the power source through the milliammeter. The power supply current was adjusted to 2.5 mA. The voltage across each cell was measured to locate those with abnormally high voltage drops due to a high resistance, deteriorated agar bridge. When a cell had a voltage drop greater than 5 V it was replaced. The electrolysis proceeded for 1 hr with adjustment of the 2.5-mA current, if required. The reduced urine samples were solvent partitioned as subsequently described.

Cells were prepared for the next electrolysis by first shutting off the power supply and stirrers. The urine and three successive, briefly stirred water washes were aspirated. Saturated sodium chloride was added to each cell. The electrolysis as just described for urine samples was performed for 30 min. After 30 min, the electrolysis leads at the power supply terminals were reversed for 10 sec. The power source was then turned off. Leads were disconnected. Cells were demounted for storage in large covered beakers with salt bridges moistened with saturated sodium chloride at both ends.

Solvent Partition—This was performed on reduced urine (section above) to determine free plus disulfide conjugated captopril and on nonreduced urines to determine free captopril. To prepare for the solvent partition 1.0 ± 0.1-g portions of the fine, granular, anhydrous sodium sulfate were weighed into screw-capped 50-ml round bottom tubes. In addition, methylene chloride was freshly water washed (250 ml of methylene chloride washed with 3 × 150 ml of water).

Reduced urine samples were handled as follows: the electrolysis was allowed to proceed while exactly 5.0 ml of the urine was removed by pipet then gradually and vigorously swirled into the sodium sulfate in the 50-ml screw-capped tube to effect salt dissolution[12]. Nonreduced urine samples were likewise transferred to other sodium sulfate containing tubes. Ten milliliters of the washed methylene chloride was gradually added to each tube over the surface of the aqueous phase. Each tube was firmly capped. A pair of tubes was gently rocked to effect emulsion-free extraction[13]. The tubes were centrifuged for ~2 min at ~2500 rpm. With tissue-wiped

[11] Micro V magnetic stirrers, Cole-Parmer Instrument Co., Chicago, Ill.

[12] This was done with a vigorous, circular motion to prevent refractory caking. This vigorous swirling was successively repeated down a line of tubes to effect a gradual salt dissolution. To aid the dissolution the lower portion of the tubes was agitated in the active portion of an ultrasonic bath immediately after the initial swirling.

[13] The extraction was effected by enclosing a pair of tubes within both hands. The tubes were gently rocked 3× within 60° from the horizontal then placed vertically in a rack for at least 30 sec to allow maximum phase separation. The rocking extraction-standing sequence was then repeated two more times. This gentle rocking technique was developed when ordinary shaking oxidized captopril and yielded very stable emulsions.

Table I—Percent Recovery of Captopril [a] in Pooled, Refrigerated, and Frozen Human Urine

Storage Time, days	Stabilized by: Citric–Oxalic-I pH 2 Refrig	Citric–Oxalic-I pH 2 Frozen	Perchloric Acid pH 1 Refrig	Perchloric Acid pH 1 Frozen
Initial	101.0	—	94.9	—
6	97.1	96.8	—	—
7	—	—	84.6	85.3
9	92.6	91.9	—	—
21	92.5	92.8	—	—
27	91.8	86.1	73.2	57.6
29	91.6	—	—	—
42	87.5	82.4	70.5	52.0
63	84.3	—	—	—

[a] 25 and 50 μg/ml.

10-ml pipets and using a pipet filler all of the possible lower phase was carefully transferred to another 50-ml screw-capped tube. Two additional extractions were performed. The combined extracts were evaporated under nitrogen at 40°. Residues were reconstituted in 5.0 ml of the acid chelating mixture, sonicated, and vortexed briefly to dissolve any particles. The tubes were then centrifuged for 5 min at ~2500 rpm. Automated colorimetry with associated blanking was then performed as described.

Automated Colorimetry—Figure 1 shows the apparatus for automated colorimetry. The centrifuge clarified samples were transferred to sample cups[6] by careful decantation[14]. The filled cups were then placed in sampler turntable slots with each sample cup bracketed by full washout water cups. In the same manner single cups containing the acid chelating mixture (reagent blank) and duplicate cups containing 25-, 50-, and 100-μg/ml standards were placed in the turntable prior to and after the sample sequence. The automated analysis was allowed to proceed while preparing the mercuric treated samples as follows. Two milliliters of the above clarified urine sample and reagent blanks was transferred into suitable test tubes containing 0.2 ml of mercuric chloride solution. These were capped, mixed, then centrifuged to optical clarity. Each supernate was carefully transferred[14] (to avoid disturbing sediment) into a sample cup. These were placed on the sampler, bracketed by water cups, following the second set of standards. Five sample cups containing the primary standard dilution followed the mercuric blanked samples. The latter solutions were used to remove trace, potential sulfhydryl blocking mercuric ion from the autoanalysis system (absorbance data were discarded).

Calculations—Percent dose voided = $[100\,(X - B)\,SV]/(1000\,D) = [(X - B)\,SV]/(10\,D)$, where X is absorbance of sample minus the reagent blank, B is 1.1 (absorbance of mercuric treated sample minus the mercuric treated reagent blank), where 1.1 is the mercuric solution sample dilution factor, S is average of three standards calculated as (micrograms per milliliter)/(absorbance of standard), V is urine + stabilizers volume, ml, D is captopril dose, in milligrams.

RESULTS AND DISCUSSION

Urinary Captopril and Its Disulfides—This method was used to analyze captopril in 8-hr postdosage urine of normal subjects before and after electrochemical reduction. Results with 10 subjects expressed as percent of dose (100 mg) were:

$$\text{Free Captopril} - 30.3 \pm 7.74\ SD$$

$$\text{Captopril + Disulfides} - 54.9 \pm 14.6\ SD$$

Thin-layer radiochromatography (8) indicated that human urinary captopril was primarily free (unchanged) and, in almost equal proportion, disulfide conjugated with cysteine. Relatively small amounts of captopril disulfide were observed. Consequently, the present results suggest that ~25% of the dose in the 8-hr urine is principally captopril–cysteine.

Urinary Captopril Stabilization—Long-term urinary captopril stabilization studies were performed by the simple, direct automated analysis on pooled human urine in screw-capped tubes containing 25 and 50 μg of captopril/ml at room, refrigerator, and freezer temperatures. Direct automated analysis of the unfortified pooled urine served as a blank. Data obtained are presented in Table I.

Note that long-term freezing seemed to enhance degradation relative to refrigeration storage particularly with perchloric acid acidified urine. Acid–chelate stabilized urine freezing did not seem to increase stabilization, and in the long-term slightly enhanced degradation.

The stabilization attained by the acid–chelate mixture was attributed to efficient (19) trace metal binding by I at the pH of human urine (pH 5–6), acidification, and refrigeration.

Selectivity, Recovery, Precision, Sensitivity—Selectivity was shown by analysis of predosage 8-hr urines collected from 34 individuals. All yielded essentially zero colorimetric response. Interference from occasionally extracted urinary pigments is obviated by subtracting the colorimetric response of the mercuric blank from that of the sample (see Calculations). Mercuric ion added to a portion of the sample (reconstituted from the methylene chloride extract–nitrogen evaporation residue) prevents captopril colorization of Ellman's reagent by formation of thiol mercaptides. This allows measurement and correction for nonsulfhydryl color.

Pooled predosage and postdosage urines from six individuals were fortified with captopril at 25 and 50 μg/ml, respectively. In two experiments the recoveries with predosage urine were 96.4 and 98.8%. The analysis of the unfortified postdosage pool was subtracted from that fortified to 50 μg/ml to yield a recovery for added captopril of 96.6%. The precision and sensitivity were evaluated by pooling blank urine from 12 subjects. Six replicate assays of this pool determined the apparent micrograms of captopril per milliliter. The pooled urine was fortified with captopril at ~46 μg/ml. Two chemists each performed eight replicate assays of thiol content as described.

Chemist 1 achieved a mean of 45.1 ± *SD* 0.26 with an *RSD* of 1.2. Chemist 2 obtained 45.4 ± *SD* 0.72 with an *RSD* of 2.0. The apparent micrograms per milliliter of captopril in the blank pooled urine was 0.24. The average blank value (subtracted for the recovery data) was calculated from absorbances of 0.002–0.003. Zero and negative absorbance values in this range were frequently obtained and were considered essentially zero blanks. The sensitivity of this method is conservatively estimated at 0.5 μg/ml, which is approximately twice the average blank found in this precision study.

Solvent Partition—To reduce the large predosage blank obtained in direct colorimetry of the urine, the multiple methylene chloride extraction and evaporation was performed, as described in *Experimental* section. Solvent partition of highly polar captopril required salting out from the acidic urines. Appreciable captopril oxidation was observed when using sodium chloride as a salting out agent instead of sodium sulfate. This oxidation was attributed to chlorine from metal catalyzed, solvent mediated air oxidation of chloride (20). Attempts were made to substitute a column solvent partition in which the acidified urine–sodium sulfate was held by a column of polar stationary phase (*e.g.*, diatomaceous earth, silica gel, or cellulose) and a readily evaporated mobile phase (*e.g.*, ethyl acetate, methylene chloride, or 10% isopropanol in methylene chloride) percolates through the column to efficiently perform continuous, multiple extractions. If successful, this column partition would have considerably decreased operator time. However, incomplete recoveries (60–80%), possibly attributable to irreversible adsorption and/or oxidation, were obtained. Attempts at a reversed-phase partitioning into particulate polystyrene divinylbenzene[15] or octadecylsilane bonded to silica particles[16] followed by solvent elution also resulted in recoveries of 85–90%, short of the goal of 95% or better.

Automated Colorimetry—In the design of the colorimetry from a preexisting manual colorimetry (7), the sequence of adding Ellman's reagent before the alkali was reversed to maintain solubility of the reagent within the autoanalysis system. In addition, methanol and acidic aqueous buffer were added to Ellman's reagent to enhance both solubility and stability.

Electrochemical Reduction—The electrical characteristics of the cell included the resistance across the agar bridge of 1600–1700 ohms, a voltage drop across the cell of 3–4 V, and a cathode voltage, *versus* silver–silver chloride, of −0.9 to −1.0 V. These characteristics allowed efficient disulfide reduction by minimizing parasitic reductions of hydrogen ion, oxalic acid (14), and I–metal complexes.

Part of a pool of five normal human urine, stabilized with I–citric–oxalic acids, was fortified with captopril disulfide at 50 μg/ml. Table II presents results of a time and current study of the electrochemical re-

[14] When commercially available pasteur pipets were used to transfer the reconstituted acid captopril solutions to autoanalyzer cups, the first transfer yielded appreciably lower results than the second transfer with the same pipet. This was not encountered when previously washed and dried volumetric pipets or decantation transfers were made.

[15] XAD-2 Resin, Rohm and Haas Co., Philadelphia, Pa.

[16] SEP-PAK Columns, Waters Associates, Inc., Milford, Mass.

Table II—Effect of Time and Current on Reduction of Captopril Disulfide [a] in Normal Human Urine Pool

Min	Percent Recovery at Current, mA		
	2	2.5	5
20	53.4	—	68.7
40	84.4	—	92.0
60	97.3	99.3, 98.1, 100.1	93.0

[a] 50 μg/ml.

duction of this fortified urine followed by the extraction analysis. The unfortified human urine pool (control) reduced electrochemically at 2.5 mA for 1 hr yielded a colorimetric response, through the extraction analysis, of 0.003 absorbance units. This is equivalent to a blank (see preceding discussion on sensitivity of the solvent partition).

Table II indicates a possible fall off of reduction at 5 mA. Subsequently, a current of 2.5 mA for 1 hr was used with the encouraging results indicated. Whereas a 1-hr reduction was necessary here, Saetre and Rabenstein (11, 13) reported a complete 10-min reduction with cystine and the penicillamine and the glutathione disulfides. Possibly the amino groups of the latter three disulfides facilitate disulfide reduction by shifting electrons from the disulfide linkage in a manner described previously (21). The 1-hr electrochemical reduction was also valid for captopril–cysteine disulfide. However, a time–current study comparable to that in Table II was not done with the mixed disulfide.

In view of the agar bridge fragility in routine use, several substitutes were considered including a sturdy polyacrylamide gel (22), sintered glass disks (17), tightly rolled paper wads in each end of a glass U tube containing the saturated sodium chloride[17], and 4-mm porous glass[18] disks[19]. The porous (pore diameter 4 μm) glass[18] acts as a semipermeable membrane providing ultra-low liquid leakage rates and minimum IR drop through the tip (23). A rather simple porous glass[18] disk bridge[19] (Fig. 3) had the same resistance as the agar bridge and gave captopril analyses very comparable to that with the agar bridge. It is considered a preferable substitute for the agar bridge. Incorporating the porous glass[18] disk at its end, a tapered glass tube enters a hole in the previously needle-punctured plastic cap (from a 5-ml plastic container)[5] at the top of the cell illustrated. The 4 × 2.9 mm (diameter × width) porous glass disk in heat shrink polytetrafluoroethylene tubing[19] was previously slipped over the glass taper. The heat shrink tubing, well away from the porous glass[18] disk, was then heat shrunk with a heat gun. The assembly was completed with a platinum electrode clipped to the wide top of the glass tube, which contained saturated aqueous sodium chloride. The electrochemical reduction was chosen over other disulfide reduction procedures since it is performed at an acid pH which stabilizes the sulfhydryl. It adds no reducing agents (dithiothreitol, mercaptoethanol, or sulfite) which colorize Ellman's reagent. It is not toxic as is the highly effective disulfide reductant tributylphosphine (24). Finally, several reports (13–16) indicated that borohydride and zinc-acid reductions yielded incomplete reduction or reoxidations in biological samples, whereas electrochemical reductions were quantitative.

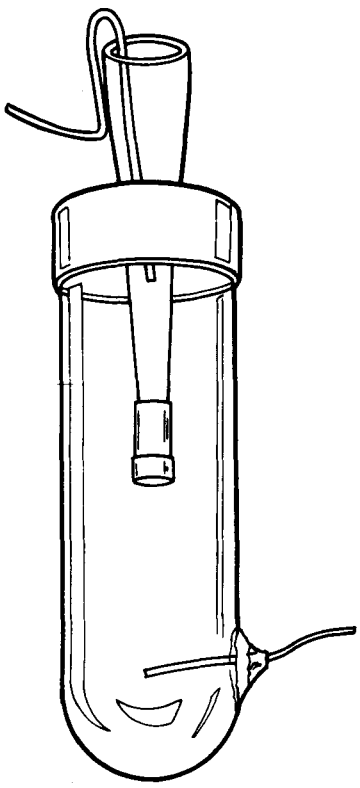

Figure 3—*Porous glass*[18] *bridged electrochemical reduction cell.*

[17] Dr. M. Szyper, Squibb Institute for Medical Research, New Brunswick, N.J., personal communication.

[18] Vycor, Corning Glass Company, Corning, N.Y.

[19] The disk and heat shrink tubing were obtained as Catalog No. G0100 from Princeton Applied Research, Princeton, N.J.

Table III—Analysis of Captopril and Its Disulfides in 0–4 Hr Predosage Urine by HPLC–ECD and the Present Method [a]

Subject No.	μg/ml			
	Captopril		Captopril Plus Disulfides	
	HPLC-ECD	PM	HPLC-ECD	PM
2D	13.7	14.7	22.0	23.4
3B	30.6	32.5	47.0	49.3
4A	32.0	33.1	50.5	51.3
5C	25.1	33.5	39.4	32.9
6C	29.7	28.7	52.6	50.7
7B	45.6	43.3	74.2	73.5
8B	34.4	33.2	58.1	59.6

[a] Present method = PM.

The reported electrochemical reduction utilizes a meter, a relatively sophisticated power supply, and an integrated reduction cell. It should be pointed out that simpler versions using a battery, a variable resistor, beakers, and detached agar U-bridges have been implemented (18).

Confirmatory Analysis *via* HPLC–Electrochemical Detection (ECD)—Analysis performed by the presently reported method and the subsequently developed HPLC method (10) are compared in Table III. Agreement is very satisfactory. The assay conformity appears more remarkable when it is learned that the HPLC–ECD assays were performed on samples stored for 90–120 days after their colorimetric analyses.

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Intestinal Absorption of Amino Acid Derivatives: Importance of the Free α-Amino Group

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Abstract ■ The intestinal absorption of L-lysine-*p*-nitroanilide, L-alanine-*p*-nitroanilide, and glycine-*p*-nitroanilide was studied in the presence of competitive inhibitors in a perfused rat intestine. It was observed that L-lysine-*p*-nitroanilide absorption was inhibited by L-lysine methyl ester and L-arginine-β-naphthylamide but not by N_α-acetyl-L-lysine methyl esters. L-Alanine-*p*-nitroanilide absorption was inhibited by L-alanine methyl ester but not by β-alanine methyl ester. It was further observed that N_α-benzoyl-L-arginine-*p*-nitroanilide and N_α-succinyl-L-phenylalanine-*p*-nitroanilide were poorly absorbed. It was concluded that the peptidase in the brush border region that serves as the hydrolysis site requires a free α-amino group (an aminopeptidase), and that passive absorption of these compounds occurs only to a small extent.

Keyphrases □ Absorption, intestinal—amino acid derivatives, importance of the free α-amino group □ Amino acid derivatives—intestinal absorption importance of the free α-amino group □ α-Amino groups—importance of the free α-amino group in the intestinal absorption of amino acid derivatives

Recent investigations have demonstrated that brush border hydrolysis of soluble derivatives of insoluble drugs may significantly increase the absorption rate of the drug (1). Since the approach is based on enzymatic hydrolysis, the specificity of the hydrolytic enzymes in the brush border region must be investigated. While many enzymes have been reported to be present in this region (1), the aminopeptidases represent a likely site for reconversion of compounds previously studied (2–4). Furthermore, a free amino group on the substrate is required for aminopeptidase activity. The objective of this study was to determine the importance, for absorption, of a free α-amino group in amino acid derivatives of drugs.

EXPERIMENTAL

Materials—α-*N*-Benzoyl-DL-arginine-*p*-nitroanilide, *n*-succinyl-L-phenylalanine-*p*-nitroanilide, L-lysine-*p*-nitroanilide, L-lysine methyl ester, N_α-acetyl-L-lysine methyl ester, L-arginine-β-naphthylamide, L-alanine-*p*-nitroanilide, L-alanine methyl ester, β-alanine-*p*-nitroanilide, L-lysine methyl ester, β-alanine methyl ester, glycine-*p*-nitroanilide[1], and glycine methyl ester were used as received.

[1] U.S. Biochemical Corp., Cleveland, Ohio.

Perfusion Experiments—Drug absorption was measured in a perfused rat intestine segment as previously reported (1, 5). The inlet (C_o) and exit (C_m) concentrations were measured spectrophotometrically for the *p*-nitroanilide derivatives. The free *p*-nitroanilide was released by overnight (12 hr) hydrolysis of the syringe and perfusate samples after addition of sodium hydroxide (pH 11–12). A three-point analysis of the spectrum (350, 375, and 400 nm) was used in order to subtract the usually small protein background at these wavelengths.

THEORETICAL

The method of data analysis is the same as previously reported (1, 6). The method is appropriate as long as the boundary condition is linear:

$$J_w = -D\left.\frac{dc}{dr}\right|_{r=R} = P_w C_w \qquad \text{(Eq. 1)}$$

where J_w is the flux at the wall, P_w the wall permeability, and C_w the wall concentration. The dimensionless wall permeability (P_w*) is:

$$P_w^* = \frac{P_w R}{D} \qquad \text{(Eq. 2)}$$

where R is the radius of the intestine and D the solute diffusivity. The wall permeability ($^\circ P_w$*) is calculated using the measured ratio of exit to inlet concentrations (C_m/C_o) and the Graetz number (Gz) where $Gz = \pi DL/2Q$ and where L is the length of the intestinal segment and Q the fluid flow rate. The calculations follow the method previously reported (6). Diffusion coefficients are given in Table I. Analysis is based on the uncorrected $^\circ P_w$* values since the correction is small (6).

For the test compounds used in the present report (*e.g.*, L-lysine-*p*-nitroanilide) it is assumed that hydrolysis at the wall provides the driving force for transport to the wall, with the released *p*-nitroanilide being taken up by the wall (1). The wall permeability is, consequently, a heterogeneous reaction rate constant. The general case has been discussed (1) and gives:

$$P_w = \alpha D \operatorname{Tanh}(\alpha\delta_E) \qquad \text{(Eq. 3)}$$

or

$$P_w^* = \alpha R \operatorname{Tanh}(\alpha\delta_e) \qquad \text{(Eq. 4)}$$

where

$$\alpha^2 = k/D \qquad \text{(Eq. 5)}$$

where k is the first-order reaction rate constant, and δ_E is the (unknown) thickness of the enzyme layer. Two special cases of Eq. 4 are:

High reactivity ($\alpha\delta_e > 1$):

$$P_w^* = \alpha R \qquad \text{(Eq. 6)}$$