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BODY FLUID ANALYSIS OF A PHOSPHONIC ACID ANGIOTENSIN-CONVERTING ENZYME INHIBITOR USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND POST-COLUMN DERIVATIZATION WITH *o*-PHTHALDEHYDE

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SUMMARY

A method is described for the extraction of a phosphonic acid angiotensin-converting enzyme inhibitor from either urine or plasma, and subsequent quantitation using high-performance liquid chromatographic (HPLC) analysis and post-column *o*-phthaldehyde reagent derivatization. The compound cannot be quantitatively extracted from the body fluids, but use of a fluorinated internal standard allowed for the computation of accurate results. With the use of an internal standard, excellent precision, linearity, and recovery were obtained for analyte response in both urine and plasma. In urine a working range of 0.2–10 μg/ml was found, with a limit of detection of 0.1 μg/ml. For plasma the working range was found to be 2–500 ng/ml, and the limit of detection was established as 1 ng/ml. Due to the non-polar character of the analyte at low pH values, it was possible to use novel extraction (solid-phase C_8 column) and HPLC [poly(styrenedivinyl benzene) HPLC column] conditions to separate and quantitate the compound from plasma and urine.

INTRODUCTION

SQ 29852, I, is an angiotensin-converting enzyme (ACE) inhibitor [1], and is the first member of this class which contains a phosphonic ester functionality (Fig. 1).

The therapeutic potency of I is such that it is orally administered at very low dosage levels, resulting in extremely low body fluid concentrations. The analytical sensitivity of the weak-UV-absorbing, non-fluorescing compound therefore required enhancement through derivatization. Excellent sensitivities were obtained through post-column reaction with the *o*-phthaldehyde (OPA) reagent [2], where a fluorescent derivative was formed after reaction at the amine functionality. Mercaptopropionic acid was substituted for the conventional mercap-

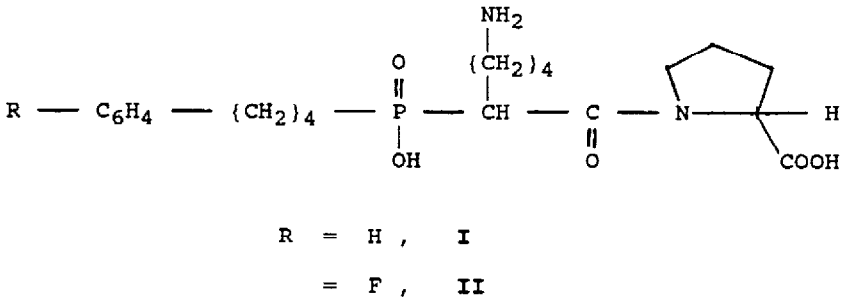

Fig. 1. Structures of compounds I and II.

toethanol in the OPA reaction, since it was reported that more stable derivatives can be thus formed [3].

It had been established that I self-associates extensively in aqueous solutions and acquires substantial non-polar character at low pH values. It was therefore possible to use novel extraction (solid-phase C_8 column) and high-performance liquid chromatographic (HPLC) [poly(styrenedivinyl benzene) HPLC column] conditions to separate and quantitate the compound from plasma and urine. The fluorinated analogue of I, identified henceforth as II (Fig. 1), was used as an internal reference since it was found to behave chemically quite similar to the analyte of interest, I.

EXPERIMENTAL

Apparatus

The HPLC system consisted of two dual-piston (Kratos Analytical, Ramsey, NJ, U.S.A.) Model 400 pumps, one of which was used to deliver the mobile phase and the other used to introduce the post-column OPA reagent. Samples were injected into the system by a Perkin-Elmer (Norwalk, CT, U.S.A.) ISS-100 autosampler. The analytical column was a 150 mm×4.1 mm Hamilton (Reno, NV, U.S.A.) PRP-1 poly(styrene divinylbenzene) column (5 μm particle size), which was heated at 40°C to improve peak resolution. The OPA reaction was carried out in a Kratos PCR-520 post-column reactor, heated at 45°C to optimize the derivatization reaction. The fluorescent product was detected by a Kratos 980 fluorescence detector, which used an excitation wavelength of 229 nm and a 418-nm emission cut-off filter. Quantitation of the HPLC results was carried out automatically on a Hewlett-Packard (Palo Alto, CA, U.S.A.) HP-3357 computer system.

The degassed mobile phase was pumped through the HPLC system at a flow-rate of 0.85 ml/min and the degassed post-column OPA reagent introduced into the system at a flow-rate of 0.45 ml/min.

Reagents

All solutions were prepared using either glass-distilled or Millipore deionized water to minimize any possible background reaction with the OPA reagent. Com-

pounds I and II were characterized materials, with assigned potencies (E.R. Squibb and Sons, Princeton, NJ, U.S.A.). A stock solution of I was prepared by dissolving 20 mg of compound I in 200 ml of methanol and a standard solution was prepared from this by diluting 100 μl to 100 ml with mobile phase. A stock solution of the internal reference, II, was prepared by dissolving 5 mg of II in 50 ml methanol and its standard solution obtained by diluting 500 μl of the stock to 100 ml with mobile phase.

HPLC mobile phase

A dilute solution of phosphoric acid was prepared by adding 1.5 ml of 85% HPLC-grade phosphoric acid to 1500 ml of chromatographic-quality water. The HPLC mobile phase was then prepared by transferring 300 ml of HPLC-grade acetonitrile to a 2-l volumetric flask and diluting to the mark with the dilute phosphoric acid solution.

Derivatization reagent

The OPA reagent was prepared by dissolving 50 mg of chromatographic-grade OPA (Pierce, Rockford, IL, U.S.A.) in 10 ml of methanol and adding 950 ml of 1.0 *M* borate buffer (pH 10.4) to which 3.3 ml of 30% Brij-35 had been added. Both the borate buffer and Brij reagent are available from Pierce in highly purified forms. After complete mixing, 1.0 ml of puriss-grade 3-mercaptopropionic acid (Fluka, Ronkonkoma, NY, U.S.A.) was added and the mixture completely mixed. The solution was refrigerated under a nitrogen headspace when not in use and discarded after one week.

Sample preparation and extraction

Sample preparation was performed immediately prior to solid-phase extraction. A sample blank was prepared by pipetting 1.0 ml of recently thawed pre-dosage plasma or urine into a polyethylene sample vial, followed by 4.0 ml of 0.1 *M* sodium acetate and 1.0 ml of the internal standard solution II. A standard sample of II was prepared by pipetting 1.0 ml of recently thawed pre-dosage plasma or urine into a polyethylene sample vial, followed by 4.0 ml of 0.1 *M* sodium acetate, 0.5 ml of the standard solution I, and 0.5 ml of the internal standard solution I. The working samples were prepared by pipetting 1.0 ml of recently thawed post-dosage plasma or urine into a polyethylene sample vial, followed by 4.0 ml of 0.1 *M* sodium acetate and 1.0 ml of the internal standard solution II.

The required number of C_8 (500 mg) solid-phase extraction columns (Analytichem International, Harbor City, CA, U.S.A.) were inserted into a vacuum manifold (Vac Elut ten-position manifold, Analytichem International), and a 10-ml syringe was attached to each. The C_8 extraction columns were activated by first washing with 5 ml of methanol and then by 5 ml of 0.01 *M* sodium acetate. After column preparation was complete, the solutions (blank, standard, or working samples) described above were transferred to the column syringes and eluted through the columns. Each vial was washed with 5 ml of 0.01 *M* sodium acetate, which was also passed through the columns. The columns were then washed with 5 ml of 0.01 *M* sodium acetate, followed by two column volumes of heptane. Com-

pounds I and II were eluted from the columns with 20 ml of methanol into a 20-ml silylated scintillation vial. The columns were then washed with 10 ml of methanol, and the combined washings evaporated at 60 °C under vacuum. The samples were taken up in 0.5 ml of methanol, and transferred to 1-ml silylated HPLC injector vials. Each scintillation vial was washed with another 0.5-ml portion of methanol and this washing combined with the previous. The sample was evaporated to dryness, taken up in 200 μl of mobile phase, and transferred to 200-μl glass inserts in appropriately labelled 1-ml autosampler vials. A sufficient number of samples were extracted so as to fill the HPLC autosampler tray and sequenced so that each series began and ended with a blank sample and four standards.

RESULTS AND DISCUSSION

Both the water solubility and octanol/water distribution coefficient of I exhibit strong dependencies upon solution pH and compound concentration. All data obtained to date are consistent with the concept that I is extensively self-associated in aqueous solution. Even though the terminal lysine amine is protonated at low pH values, internal hydrogen bonding yields a molecular entity significantly more non-polar at low pH values relative to neutral pH. These general trends allowed the use of novel extraction and HPLC conditions.

Due to these characteristics, traces of I cannot be quantitatively extracted from acidified serum/plasma or urine with any simple solvent. Total extraction is possible, however, using ethyl acetate containing at least 0.5% of dioctyl sodium sulfosuccinate, but elimination of the carrier excess is difficult. Complete recovery of I can be obtained using C_8 solid-phase extraction columns if the analyte solutions are rendered basic. Approximately 80% of the drug is recovered during the use of C_2 columns and acidic solutions. C_{18} columns have been found to absorb over 50% of I from acid and neutral solutions, while cyclohexyl columns were found to absorb approximately 60% of I from neutral and basic solutions. It was determined that the amount of column packing was quite important, and quantitative absorption of I was found to require the use of C_8 columns containing 500 mg of absorbent.

Complete elution of I from the C_8 columns was possible using methanol. Acetonitrile and acetone solvents were found to elute only 85% of absorbed I, while ethyl acetate and methyl *tert.*-butyl ether led to less than 10% elution efficiencies. It was also found that heptane and methylene chloride were totally incapable of eluting I from the C_8 columns.

It was found that the isolation of I from either plasma or serum, or through the use of C_8 columns, yielded extracts sufficiently pure for HPLC work. In the event that further purification was required, it was found that a second clean-up step using XAD-2 columns (DuPont) could be used. Compound I would quantitatively absorb on the XAD-2 resin when working with dilute ammonium hydroxide solutions and could subsequently be eluted from these columns by methanol containing triethylamine (0.7%, v/v) and glacial acetic acid (0.06%, v/v).

Under the pH conditions used for the HPLC analysis, the non-polar character

of I also permitted the use of a novel HPLC system, based on the poly(styrenedivinyl benzene) HPLC packing material. In fact, the use of silica-based columns led to severe tailing of the analyte peak, and such tailing could be eliminated only through the use of the poly(styrenedivinyl benzene) column.

At pH values below 7, the pK_3 contribution of the amine functionality of I is negligible, and consequently the group is available for derivatization by OPA. The OPA reagent was modified from that normally used in order to enhance the method sensitivity and stability of the fluorophor. The mercaptoethanol normally used in the OPA reagent was replaced by mercaptopropionic acid, since it had been reported that more stable derivatives could be thusly formed [3]. The OPA was reduced from the recommended level of 800 mg/l [2] to 50 mg/l, since it has been reported [4–6] that excess OPA destabilized the fluorescent compound. Since the OPA derivatization site of I is at a lysine terminal group, Brij 35 was retained in the OPA reagent. It has been noted that the use of Brij 35 stabilizes the OPA derivatives of lysine [7]. Finally, all reagents were thoroughly deaerated, since it has been reported that OPA derivatives are somewhat unstable in the presence of oxygen [8].

Typical chromatograms for the urinary blank (containing the internal standard, II) and urine spiked at 0.4 μg/ml with I (and also containing the internal standard) are shown in Fig. 2. A large number of peaks were noted at short retention times, but all of these eluted before 6.25 min. These peaks undoubtably represent the presence of low-molecular-mass hydrophobic amino acids, extracted along with I. No interference in the quantitation of either I or II was

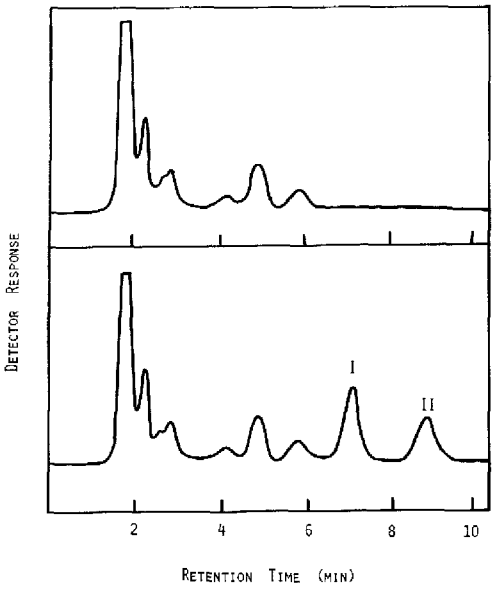

Fig. 2. Typical chromatograms obtained in the HPLC analysis of urine extracts, after post-column derivatization with OPA. The upper trace is that of a urine blank, while the lower trace represents the blank spiked with compounds I and II. Under the chromatographic conditions used to obtain these data, analyte I eluted at 7.3 min and internal standard II at 9.1 min

noted. Corresponding chromatograms for the analysis of I in plasma are shown in Fig. 3, where a much cleaner profile was obtained. The few plasma blank peaks observed immediately after the void volume posed no interference in the quantitative analysis of I.

Excellent linearity was obtained in the analysis of I in both urine and plasma. Correlation coefficients on the order of 0.9995 were obtained in calibration curves. In the urine matrix, a working range of 0.2–10 μg/ml was established, and the limit of detection was determined to be 0.1 μg/ml. The cleaner nature of the plasma matrix permitted work at much lower levels. In plasma, the working range was found to be 2–500 ng/ml, and the limit of detection was found to be 1 ng/ml.

The precision associated with the method was established through the repetitive analysis of body fluid samples spiked with I. For this study, comparison against the internal standard was not required. A blank urine aliquot was spiked with I at a concentration of 2 μg/ml, and this sample was injected onto the HPLC system in a series of multiples. An average system response of 1.88 μg/ml was obtained, with an associated standard deviation (S.D.) of 0.03, thus yielding a coefficient of variation (C.V.) of 1.6%. In a similar fashion, blank plasma was spiked with I at a concentration level of 10 ng/ml and then analyzed ten times. An average response of 8.93 ng/ml was obtained, with an S.D. of 0.48, yielding a C.V. of 5.4%. Both estimates of precision were determined to be sufficient for reproducible body fluid analysis.

Studies of compound recovery indicated that use of an internal standard would be required. The fluorinated analogue of I, compound II, was chosen for this pur-

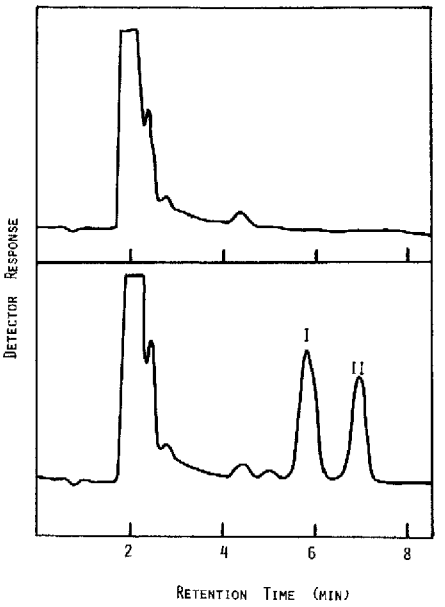

Fig. 3. Typical chromatograms obtained in the HPLC analysis of plasma extracts, after post-column derivatization with OPA. The upper trace is that of a plasma blank, while the lower trace represents the blank spiked with compounds I and II. Under the chromatographic conditions used to obtain these data, analyte I eluted at 5.9 min and internal standard II at 6.9 min.

pose by virtue of its similar chemical properties. To determine absolute and relative recovery efficiencies, blank urine and plasma aliquots were spiked with known quantities of I and split into two fractions. To one of these fractions compound II was added as an internal reference. Each fraction was extracted using the optimum body fluid method and then analyzed. The fractions containing I were quantitated directly against an external standard, and the other was quantitated against the internal standard. For the urine study, a concentration range of 0.1–10.0 μg/ml was studied. Within this range, the absolute recovery was found to be 79% (S.D. 3.7), and the relative recovery against internal standard yielded a recovery of 100% (S.D. 5.7). In plasma, concentration values of I between 2 and 500 ng/ml were studied. The absolute recovery in plasma was found to be 54% (S.D. 6.8), while the relative recovery against internal standard was obtained as 97% (S.D. 4.3). It was concluded from these studies that the use of an internal standard adequately compensates for non-quantitative extraction of I. The essentially 100% recoveries observed for both plasma and urine indicates that compounds I and II are extracted with equal efficiencies from the body fluids.

CONCLUSION

It has been shown that the phosphonic acid ACE inhibitor, I, can be extracted from either urine or plasma and quantitated using HPLC analysis and post-column OPA reagent derivatization. The compound could not be quantitatively extracted from the body fluids, but use of a fluorinated internal standard allowed the computation of accurate results. Excellent linearity was obtained for analyte response in both urine and plasma. In urine a working range of 0.2–10 μg/ml was found, with a limit of detection of 0.1 μg/ml. For plasma the working range was found to be 2–500 ng/ml, and the limit of detection was established as 1 ng/ml. The analysis in either matrix was found to exhibit good assay precision.

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