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Short communication

Analysis of perillic acid in plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple and sensitive isocratic high-performance liquid chromatographic (HPLC) method with UV detection for the quantitation of perillic acid, a major circulating metabolite of perillyl alcohol and *d*-limonene, in plasma is described. Sample preparation involved protein precipitation and subsequent transfer and dilution with 10 m*M* $NaHCO_3$. The mobile phase consisted of acetonitrile (36%) and 0.05 *M* ammonium acetate buffer pH 5.0 (64%). Separations were achieved on a C_{18} column and the effluent monitored for UV absorption at the analytes' respective UV_{max}. Separation was excellent with no interference from endogenous plasma constituents. This method was found suitable for quantifying drug concentrations in the range of 0.25 to 200.0 μg/ml using a 0.05-ml plasma sample, and was used to study the plasma pharmacokinetics of perillic acid in mice.

Keywords: Perillic acid

1. Introduction

Perillic acid, NSC-667676, (−)-(4*S*)-[2-propenyl]-1-cyclohexane-1-carboxylic acid (Fig. 1) is a potential anti-cancer compound which has been synthesized from (−)-perillaldehyde by the method of Bal et al. [1]. This compound is one of the two major circulating metabolites of D-limonene in rat [2,3] and of perillyl alcohol in dog [4].

Typical of many anti-cancer compounds under development, the solubility of perillic acid in aque-

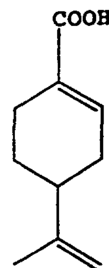

Fig. 1. Chemical structure of perillic acid.

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ous systems is very poor. Furthermore, the analytical methods available for its quantitation in biological fluids, gas chromatography and/or mass spectrometeric [4,5], are neither rugged and simple nor have wide dynamic range. The extraction techniques are cumbersome, and require multiple steps [6] or organic solvents. A sensitive and simple analytical method is therefore needed to study the pharmacokinetics of this compound. The method presented herein requires only 50 μl of sample with a single extraction but provides excellent recovery, high specificity, and retention times convenient to performing multiple analyses. This assay was applied to study the plasma pharmacokinetics of perillic acid in mice.

2. Experimental

2.1. Materials

Perillic acid was prepared in house using the procedure of Bal et al. [1]. *N*-Methyl-D-glucamine and Biochanin A used (as internal standard) were purchased from Aldrich (Milwaukee, WI, USA), and were used without further purification. All other chemicals were ACS reagent grade.

2.2. Preparation of perillic acid salt

The drug and *N*-methyl-D-glucamine were weighed to obtain a 1:1 or 1:2 mol ratio and were either dissolved in 3.0 ml of 0.1 *M* $NaHCO_3$ or dispersed in diethyl ether. These preparations were then lyophilized. Known quantities of the lyophilized products were weighed and dissolved in either distilled water or 10 m*M* $NaHCO_3$.

2.3. Chromatography

Hewlett-Packard 1050 component system was used for the chromatographic analysis, which consisted of auto sampler, isocratic pump, and variable-wavelength detector and the 3396 integrator equipped with data storage capability. Analyses were performed on a Waters Nova-Pak C_{18} (15.0×0.39 cm I.D., 4.0 μm) column preceded by RP_{18} precolumn (Millipore, Milford, MA, USA). The mobile phase was acetonitrile (ACN)–0.05 *M* ammonium acetate buffer pH 5.0 (36:64, v/v). The flow-rate was 1.1 ml/min and the detector was programed to monitor the effluent for absorbance at 217 nm from 0 to 9 min for the drug and thereafter at 261 nm for the internal standard.

2.4. Standard curve and extraction procedures

Standard curves were prepared in commercial mouse plasma or human plasma by serial dilution to give a concentration range of 0.25 to 200 μg/ml. To aliquots of 50.0 μl of plasma was added 100.0 μl of a 4.0 μg/ml Biochanin A in ACN, followed by 1.0 min of vortexing and then centrifugation for 5 min at 14 000 *g* with an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY, USA). A 100.0-μl volume of the supernatant was transferred to 250.0-μl clear borosilicate step-vial inserts in clear snap-crimp vials (Scientific Resources, Eatontown, NJ, USA), and diluted with 100.0 μl of a 10.0 m*M* $NaHCO_3$ solution. A 100.0-μl volume of this preparation was injected into the chromatographic system.

Standard curves were generated daily by plotting ratios of peak-areas of the drug to the internal standard against drug concentration. Linear least squares regression was performed using a weighting factor of $1/y_{obs}$ without inclusion of the origin [7] to determine the slope, *y*-intercept, and correlation coefficient of the best-fit line. To evaluate the pharmacokinetic disposition of the drug, 50.0-μl plasma samples were subjected to the extraction procedure as outlined above and the drug concentrations were determined using standard curve regression analyses. The pharmacokinetic samples were initially assayed in duplicate, with additional analyses performed if the duplicate determinations deviated from their average by more than 10%.

2.5. Recovery and variability studies

Aliquots of 50.0 μl of the samples used to generate the standard curves were transferred to clear microcentrifuge vials and extracted as described above. Predicted concentrations and regression parameters from standard curves of perillic acid salt solution in plasma prepared and assayed on four separate working days were used to assess the

precision, accuracy and reproducibility of the analytical method. Coefficient of variation of the mean-predicted concentration for the plasma standards provided a measure of precision. The peak areas of the perillic acid extracted from plasma and 10 m*M* $NaHCO_3$ solution were compared.

Recovery was determined as the mean (±S.D.) of four samples. Inter-day variabilities were determined for the whole standard curve range.

2.6. Animal experiments

Adult 9-week old CD2F1 mice (male and female), weight range of 28.0 to 35.3 g, were used in this study. Three ($n=3$) mice were used for each time point. To study the plasma concentration–time profile of perillic acid, the lyophilized salt of the drug was used. A 50.0 mg/ml solution of this product was prepared in distilled water and administered by a tail vein injection to each group of mice. Blood samples were taken by occular puncture at 2.0, 5.0, 15.0, 30.0, 45.0, 60.0, 90.0, 120.0, 240.0 and 480.0 min. The drug concentrations are reported as the mean of duplicate analyses. Plots of drug plasma concentration as a function of time were constructed using the geometric mean of the plasma concentrations and the mean of the time intervals for each time point. Nonlinear least squares regression analysis was performed with the computer software package Table Curve (Jandel Scientific, San Rafael, CA, USA). Exponential equations were fitted to the data set with weightings of 1, $1/y$ and $1/y^2$. The "goodness of fit" to the plasma concentration–time data was evaluated in terms of the sums of the squared deviations, correlation coefficients, the distribution pattern of the weighted residuals and visual assessment. Parameters iterated in the computer fitting were the slope and y-intercept of the regression line. Secondary pharmacokinetic parameters were calculated from these primary parameters according to model independent methods.

3. Results and discussion

Typical chromatograms of the blank plasma, plasma spiked with NSC-667676 salt solution and plasma sample obtained after intravenous injection of 50 mg/kg NSC-667676 as a salt are shown in Fig. 2. The drug and biochanin A eluted with retention times of 5.73 and 12.14 min, respectively. The UV_{max} of perillic acid and internal standard occur at 217 nm and 261 nm, respectively. The drug and the internal standard were measured at their respective UV_{max} using an HP 1050 variable wavelength detector (VWD). This assay also provided excellent extraction yields for the concentration range studied.

The short elution time for perillic acid makes viable running a large clinical study economical. Chromatographic separation was excellent, with no interfering peaks from endogenous plasma constituents. The drug concentration as a salt solution was linearly related to drug vs. internal standard area ratios over the range studied (0.25 to 200.0 μg/ml). The dynamic range of this method is linear to 200 μg/ml whereas the GC–MS method [4] is linear to only 10 μg/ml. Mean values (±S.D.) of the linear regression parameters for sixteen standard curves of perillic acid salt solution in plasma prepared and assayed over a four working day period were: slope, 0.0494±0.0094 μg/ml; y-intercept, 0.0110±0.0379; correlation coefficient, 0.9997±0.0005.

The method is rugged and the coefficients of variation of the mean predicted analyte concentrations ranged from 0.13 to 5.96% and the relative recovery from 106 to 118% (Table 1). The limits of detection and quantitation were 0.0156 and 0.125 μg/ml, respectively.

The applicability of the method was demonstrated in the study of the pharmacokinetic disposition of NSC-667676 salt solution in mice. The plasma concentration–time profile depicting the geometric mean of the observed plasma perillic acid concentrations and the line of best fit following intravenous injection of 46.8 mg/ml in mice is shown in Fig. 3. Plasma concentrations of perillic acid declined in a monoexponential fashion from approximately 200 μg/ml at 2 min to 1.5 μg/ml at 120 min, after which they were below the limit of detection of the analytical method.

The estimated area under the curve (AUC) was 3032.0 μg h^{-1} ml^{-1}, while the elimination rate constant k_{el}, was 15.4 ml h^{-1} kg^{-1}. The half-life for the line of best fit was 16.9 min. The short biological half-life suggests that frequent administration or continuous infusion will be necessary to maintain an

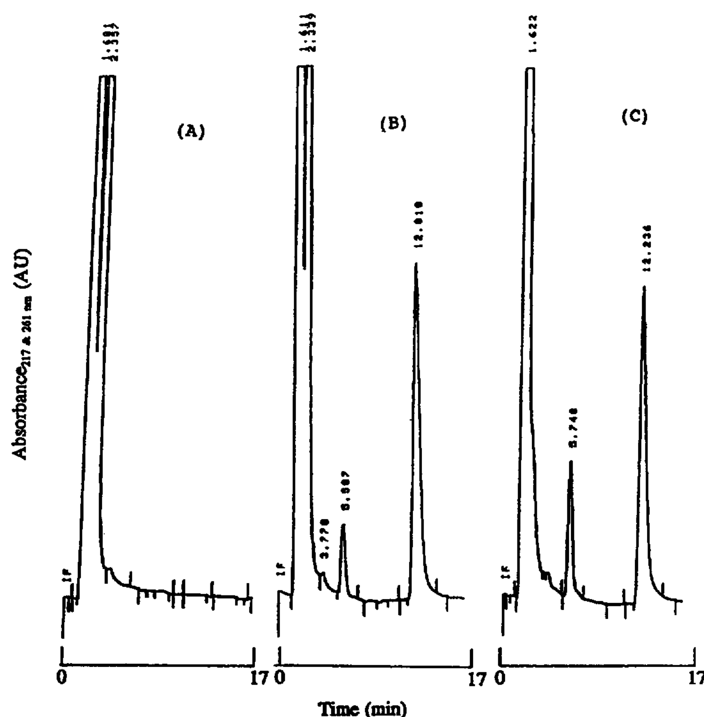

Fig. 2. Chromatograms of perillic acid as a salt in mice: (A) blank plasma; (B) blank plasma spiked with perillic acid (2.5 μg/ml) and internal standard (Biochanin A); (C) plasma sample 90 min after a 46.8 mg/kg intravenous dose of perillic acid salt solution (1 μl/g); 217 nm and 5.74 min=perillic acid; 261 nm and 12.24 min=Biochanin A.

effective concentration of perillic acid in plasma over a prolonged period.

The estimated apparent volume of distribution (V_d) was 377.0 ml kg^{-1}, which suggests that the concentration of the drug is mostly in extravascular tissues, which clearly supports prior published data by Crowell et al. [3]. The linearity of the plasma concentration versus time indicates that the distribution follows a one compartment model upon a bolus intravenous administration. In summary, the present

Table 1
Relative recovery and reproducibility of the analytical method for the quantitation of perillic acid in mouse plasma

Amount added (μg/ml)	Number of replicates	Mean amount found (μg/ml)	Relative recovery (%)	Coefficient of variation (%)
0.250	4	0.295	118	3.27
0.500	4	0.532	106	5.96
1.00	4	1.15	115	2.11
2.50	4	2.69	108	0.13
5.00	4	5.42	108	1.69
10.0	4	11.1	111	1.89
25.0	4	28.9	116	1.41
50.0	4	55.9	112	0.84
100.0	4	111.9	112	0.88
200.0	4	224.5	112	2.08

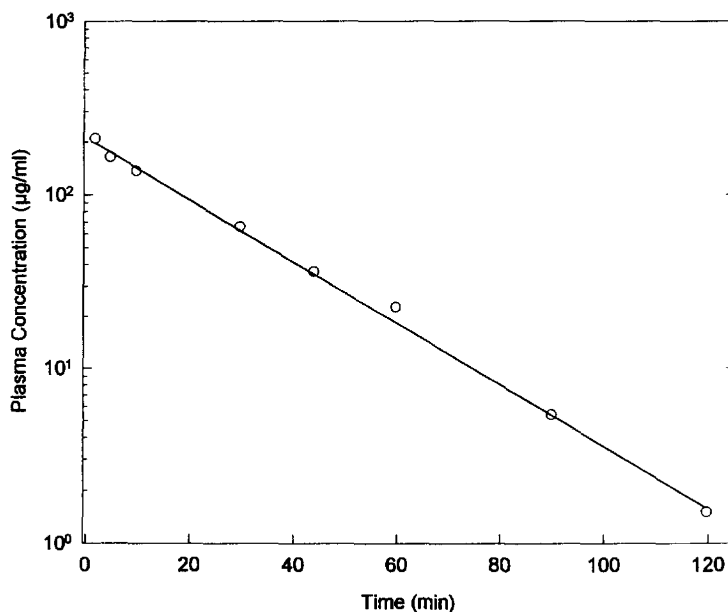

Fig. 3. Plot of mean concentration in plasma versus time, of perillic acid as a salt after bolus intravenous administration of 46.8 mg/kg of this solution to CD2F1 mice.

method is simple, sensitive and very specific and is shown by application to be suitable to study plasma pharmacokinetics of perillic acid in mice.

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