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Simultaneous determination of phenytoin, carbamazepine, and 10,11-carbamazepine epoxide in human plasma by high-performance liquid chromatography with ultraviolet detection[1]

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Abstract

The Bioanalytical Chemistry Department at the Madison facility of Covance Laboratories, has developed and validated a simple and sensitive method for the simultaneous determination of phenytoin (PHT), carbamazepine (CBZ) and 10,11-carbamazepine epoxide (CBZ-E) in human plasma by high-performance liquid chromatography with 10,11 dihydrocarbamazepine as the internal standard. Acetonitrile was added to plasma samples containing PHT, CBZ and CBZ-E to precipitate the plasma proteins. After centrifugation, the acetonitrile supernatant was transferred to a clean tube and evaporated under N_2. The dried sample extract was reconstituted in 0.4 ml of mobile phase and injected for analysis by high-performance liquid chromatography. Separation was achieved on a Spherisorb ODS2 analytical column with a mobile phase of 18:18:70 acetonitrile:methanol:potassium phosphate buffer. Detection was at 210 nm using an ultraviolet detector. The mean retention times of CBZ-E, PHT and CBZ were 5.8, 9.9 and 11.8 min, respectively. Peak height ratios were fit to a least squares linear regression algorithm with a $1/(\text{concentration})^2$ weighting. The method produces acceptable linearity, precision and accuracy to a minimum concentration of 0.050 μg ml^{-1} in human plasma. It is also simple and convenient, with no observable matrix interferences.

Keywords: Phenytoin; Carbamazepine; 10,11-Carbamazepine epoxide; Human plasma; HPLC

1. Introduction

Carbamazepine,5-H-dibenz[*b*,*f*]azepine-5-carboxamide (CBZ; Fig. 1) and Phenytoin,5,5-Diphenylimidazolidine-2,4-dione (PHT; Fig. 1) are important drugs for the treatment of epilepsy [1]. In humans, CBZ is metabolized to an active metabolite Carbamazepine-10,11-epoxide (CBZ-

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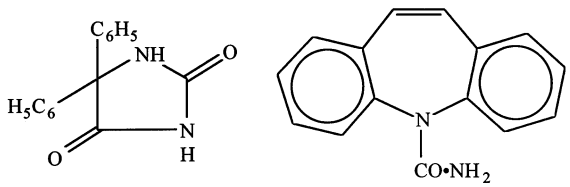

Phenytoin Carbamazepine

Fig. 1. Chemical structures of CBZ and PHT.

E) [2]. The plasma concentrations of these drugs in epileptic patients exhibit wide inter-patient variability after the administration of standard dosages [2,3]. In addition, despite the current trend of treating these patients with one drug, polytherapy is still common [4]. Polytherapy may be associated with pharmacokinetic interactions and unexpected toxicity. These interactions, which can be associated with significant changes in the plasma level of a drug, make frequent drug measurements and dosage adjustments necessary. Simultaneous multiple drug analysis is the most rapid and cost-effective approach for monitoring drug levels in epileptic patients with polytherapy.

Methods for the determination of CBZ and PHT in human plasma have been published [4–9]. Simultaneous determination of CBZ, PHT and their metabolites in human plasma has also been reported [10]. Recently Romanyshyn et. al reported a HPLC method for the simultaneous determination of CBZ, PHT, two CBZ metabolites, one PHT metabolite and three other antiepileptic drugs in human plasma [11]. Unfortunately, most of these methods use long extraction procedures. This paper describes a sensitive, specific and reproducible assay for the simultaneous determination of PHT, CBZ and CBZ-E in human plasma using a very simple one step extraction procedure.

2. Materials and method

2.1. Reagents and materials

Phenytoin and carbamazepine were purchased from Aldrich Chemical, (Milwaukee, WI). Carbamazepine 10,11-epoxide (metabolite of CBZ) and 10,11 dihydrocarbamazepine (internal standard, IS) reference materials were obtained from Alltech Associates (State College, PA). Stock

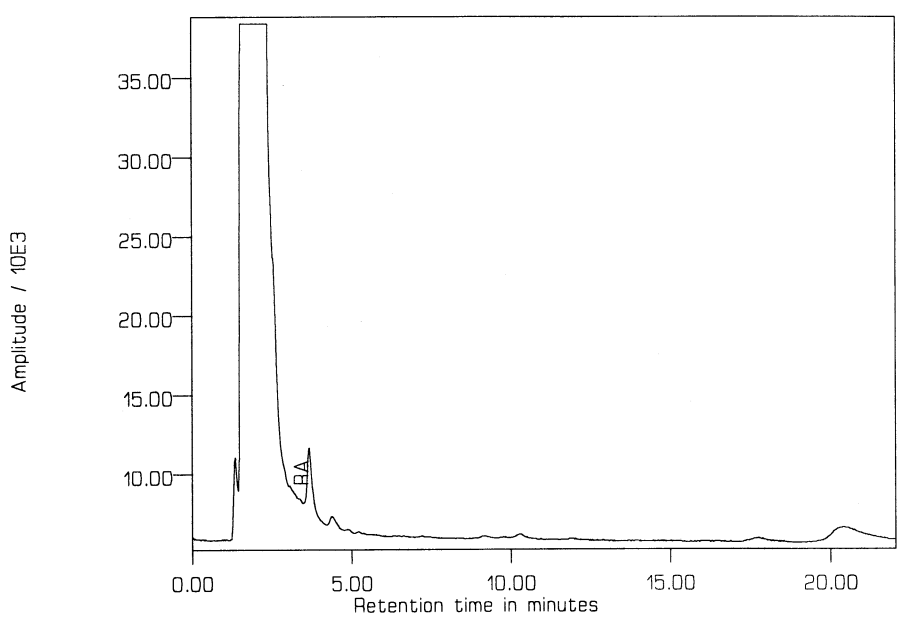

Fig. 2. Chromatogram of blank human plasma.

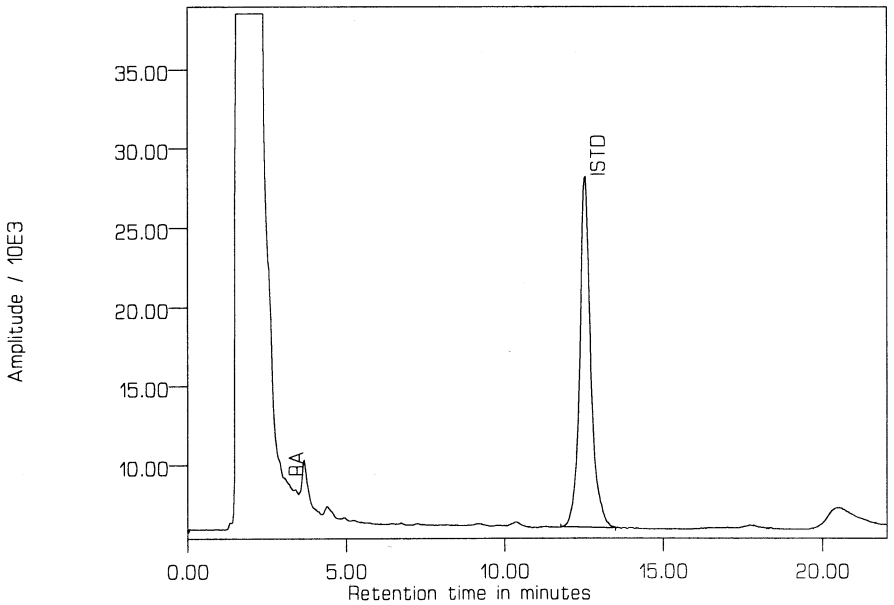

Fig. 3. Chromatogram of control zero sample.

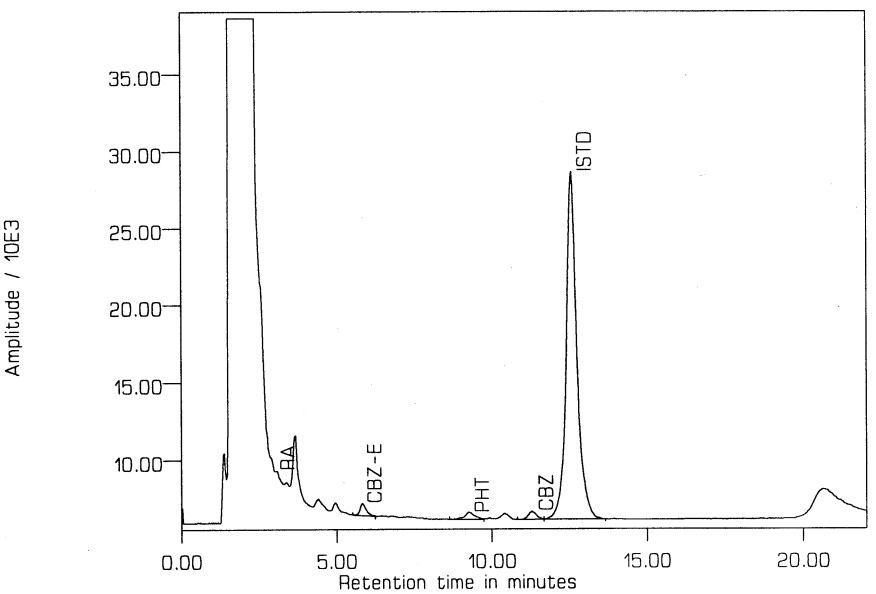

Fig. 4. Chromatogram of 0.050 μg ml^{-1} calibration standard.

standard solutions were prepared in methanol and stored at 4°C. Quality control (QC) pools (0.750, 3.00 and 20.0 μg ml^{-1} each of PHT, CBZ and CBZ-E in plasma, respectively) were prepared in glass volumetric flasks and stored at −20°C in polypropylene vials. Heparinized human plasma for preparation of the calibration standards and QC samples was purchased from Biochemed (Lil-

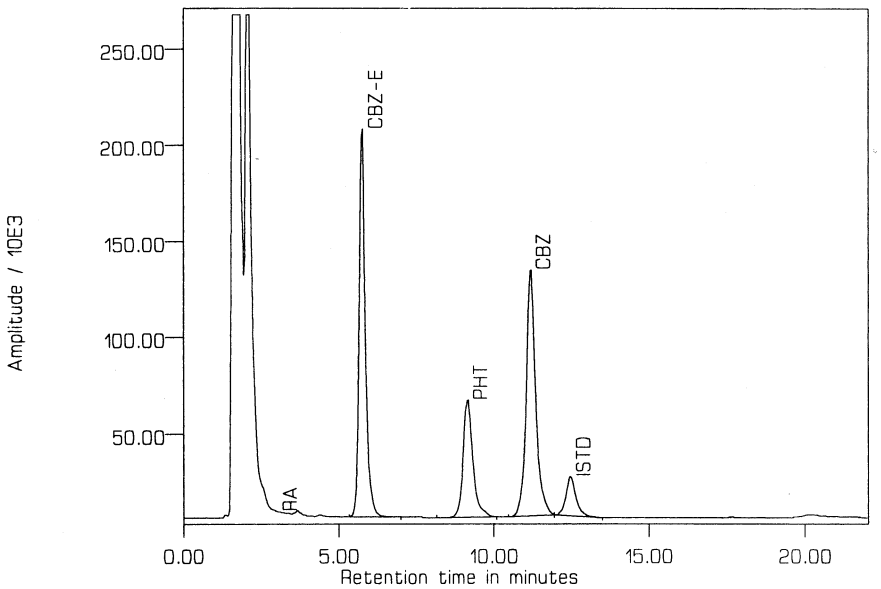

Fig. 5. Chromatogram of 20.0 μg ml^{-1} quality control.

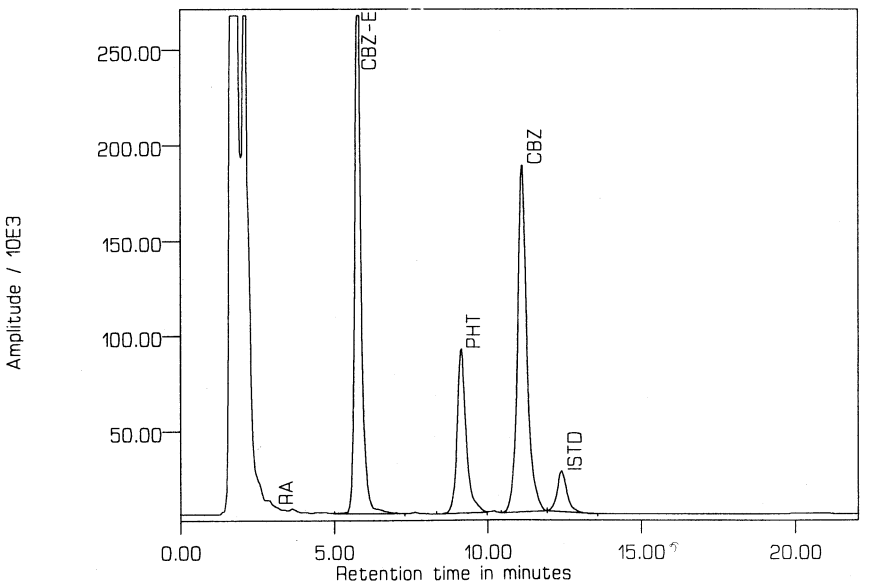

Fig. 6. Chromatogram of 25.0 μg ml^{-1} calibration standard.

burn, GA). HPLC grade methanol and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI). Other chemicals were of reagent grade and were prepared with water processed through a Mega-Pure water purification system (Corning, NY).

2.2. Apparatus and conditions

The HPLC system consisted of a Perkin–Elmer Series 10 pump (Norwalk, CT), a Perkin–Elmer ISS-200 auto-injector and an Applied Biosystems Model 785 absorbance detector with a deuterium

Table 1
Calibration curve data and statistics in human plasma of: (a) CBZ-E; (b) PHT; and (c) CBZ

(a) CBZ-E theoretical concentration (μg ml^{-1})									
Day	0.0500	0.100	0.250	0.500	1.00	2.50	7.50	15.0	25.0
1	0.0521	0.0875	0.277	0.495	1.04	2.34	7.19	14.0	27.9
	0.0525	0.0951	0.233	0.434	1.05	2.27	7.42	15.3	30.9
2	0.0522	0.0952	0.229	0.468	1.07	2.38	7.90	15.1	26.6
	0.0533	0.0940	0.236	0.442	1.11	2.40	7.88	15.6	26.1
3	0.0515	0.0963	0.242	0.469	1.07	2.34	7.85	15.3	26.0
	0.0514	0.0959	0.244	0.466	1.08	2.41	7.64	15.3	25.7
Mean	0.0522	0.0940	0.244	0.462	1.07	2.36	7.65	15.1	27.2
SD	0.0007	0.0033	0.0173	0.0218	0.0245	0.0516	0.29	0.56	1.97
RSD (%)	1.3	3.5	7.1	4.7	2.3	2.2	3.8	3.7	7.2
DMT (%)	4.3	−6.0	−2.6	−7.5	7.0	−5.7	2.0	0.7	8.8
n	6	6	6	6	6	6	6	6	6
(b) PHT theoretical concentration (μg ml^{-1})									
Day	0.0500	0.100	0.250	0.500	1.00	2.50	7.50	15.0	25.0
1	0.0499	0.105	0.228	0.468	1.03	2.41	7.49	14.5	28.6
	0.0510	0.0983	0.242	0.464	1.06	2.26	7.20	14.8	29.7
2	0.0511	0.0976	0.243	0.462	1.10	2.34	7.85	14.9	25.9
	0.0524	0.0951	0.226	0.449	1.13	2.41	7.88	15.5	25.7
3	0.0499	0.102	0.243	0.469	1.07	2.34	7.78	15.1	25.5
	0.0498	0.101	0.250	0.463	1.09	2.40	7.57	15.0	25.1
Mean	0.0507	0.100	0.239	0.463	1.08	2.36	7.63	15.0	26.8
SD	0.001	0.004	0.010	0.007	0.035	0.059	0.261	0.333	1.91
RSD (%)	2.0	3.5	4.0	1.6	3.2	2.5	3.4	2.2	7.1
DMT (%)	1.4	−0.2	−4.5	−7.5	8.0	−5.6	1.7	−0.2	7.0
n	6	6	6	6	6	6	6	6	6
(c) CBZ theoretical conventration (μg ml^{-1})									
Day	0.0500	0.100	0.250	0.500	1.00	2.50	7.50	15.0	25.0
1	0.0526	0.0940	0.230	0.467	1.03	2.45	7.63	14.8	28.7
	0.0522	0.0944	0.237	0.465	1.07	2.27	7.28	14.9	29.8
2	0.0513	0.0970	0.239	0.469	1.10	2.39	7.80	14.8	25.8
	0.0522	0.0931	0.243	0.447	1.12	2.40	7.77	15.4	25.4
3	0.0513	0.0961	0.245	0.474	1.08	2.37	7.85	15.1	25.3
	0.0513	0.0953	0.248	0.470	1.09	2.42	7.64	15.1	25.5
Mean	0.0518	0.0950	0.240	0.465	1.08	2.38	7.66	15.0	26.8
SD	0.0006	0.0014	0.0064	0.095	0.031	0.062	0.207	0.232	1.97
RSD (%)	1.1	1.5	2.7	2.0	2.8	2.6	2.7	1.5	7.4
DMT (%)	3.6	−5.0	−3.9	−6.9	8.2	−4.7	2.2	0.1	7.0
n	6	6	6	6	6	6	6	6	6

lamp (Bodman, Aston, PA), set at 210 nm (0.05 AUFS). The analytical column was a Spherisorb ODS2, 150 × 4.6 mm, 3-μm particle size (Keystone Scientific, Bellefonte, MD). The mobile phase was acetonitrile:methanol:potassium phosphate (pH 6.9; 0.05 M; 18:18:64 v/v/v) with a flow rate of 0.85 ml min^{-1}. Data collection and calculations were on a HP1000 computer with a Hewlett–Packard 3350A laboratory automation system (Palo Alto, CA).

2.3. Stock solutions

Two separate weighings each of CBZ, PHT and CBZ-E were performed. Each weighing was brought to a final volume of 10 ml with methanol, yielding two stock solutions each of CBZ, PHT and CBZ-E. One solution was used to prepare the calibration curve standards and the other was used for QC preparation. A stock solution (1.00 mg ml^{-1}) of the IS was prepared by transferring an accurately-weighed amount (10.0 mg) of the IS into a 10-ml volumetric flask and diluting to volume with methanol. The stock solution was stored in a refrigerator set to maintain 5°C.

Table 2
Curve parameters

Day	Slope	*Y*-intercept	Correlation coefficient
Calibration curve parameters of CBZ-E			
1	0.692	−0.0015	0.9968
	0.713	0.0080	0.9950
2	0.655	0.0020	0.9982
	0.635	0.0001	0.9971
3	0.676	−0.0002	0.9987
	0.661	−0.0012	0.9988
Mean	0.672	0.0012	0.9974
SD	0.03		
RSD (%)	4.1		
n	6		
Calibration curve parameters of PHT			
1	0.216	0.0065	0.9969
	0.218	0.0111	0.9955
2	0.197	0.0091	0.9980
	0.190	0.0083	0.9964
3	0.205	0.0030	0.9987
	0.199	0.0038	0.9988
Mean	0.204	0.0070	0.9974
SD	0.01		
RSD (%)	5.3		
n	6		
Calibration curve parameters of CBZ			
1	0.459	0.0066	0.9957
	0.435	0.0062	0.9927
2	0.406	0.0017	0.9976
	0.397	0.0022	0.9960
3	0.425	0.0023	0.9984
	0.412	0.0010	0.9987
Mean	0.422	0.0033	0.9965
SD	0.02		
RSD (%)	5.3		
n	6		

2.4. Extraction procedure

Calibration standards were prepared by adding 50 μl of the appropriate PHT, CBZ and CBZ-E working solutions (0.50–250 μg ml^{-1}) to 0.50 ml of blank plasma. Calibration standards, samples and controls were processed by adding 50 μl of internal standard working solution (40 μg ml^{-1}) and 1.0 ml of ACN to 0.50 ml of plasma to labeled 13 × 75 mm glass culture tubes. The samples were mixed by vortexing for 1 min on a multitube vortex mixer and centrifuged at 4000 rpm for 10 min. The organic layer was transferred to a labeled 10 ml glass conical centrifuge tube. The acetonitrile was evaporated under a nitrogen stream at 15 psi in a water bath set to a temperature of 40°C. The dried extracts were reconstituted in 400 μl mobile phase. All tubes were vortexed briefly and 50 μl aliquots of the extracted solution were injected onto the HPLC system

2.5. Data analyses

Peak height ratios (CBZ, PHT or CBZ-E/IS) were evaluated by weighted least-squares linear regression analyses, with a weighting factor of $1/(\text{concentration})^2$. The formula used to calculate 'unknown' concentrations is as follows:

$$x = \frac{y - b}{m}$$

where m is the slope, b is y − intercept, x is the concentration (μg ml^{-1}) and y is the peak height ratio.

3. Results and discussion

3.1. Chromatography

The specificity and sensitivity of the CBZ, PHT and CBZ-E assay procedure is demonstrated in Figs. 2–6. Validation data were collected from six

Table 3
Precision and accuracy of the method: results of validation studies (QCs) over a three day period with six determinations per day

Theoretical concentration (μg ml^{-1})		PHT ($n=18$)	CBZz ($n=18$)	CBZ−E ($n=18$)
0.75	Mean	0.66	0.68	0.68
	S.D.	0.04	0.05	0.05
	%RSD	6.3	6.8	6.7
	%Bias	−12.2	−9.5	−8.8
3.00	Mean	2.88	2.88	2.89
	S.D.	0.18	0.18	0.17
	%RSD	6.2	6.2	5.8
	%Bias	−4.2	−4.1	−3.7
20.0	Mean	19.4	19.7	19.3
	S.D.	1.43	1.51	1.31
	%RSD	7.4	7.7	6.8
	%Bias	−3.2	−1.5	−3.5

analytical runs conducted over 3 days. All human plasma lots used to prepare calibration standards and QC samples were free from interfering peaks in the chromatographic windows for CBZ, PHT, CBZ-E and the IS. Retention times of approximately 5.8, 9.9, 11.8 and 13.3 min were consistently observed for CBZ-E, PHT, CBZ and the IS, respectively, throughout all analytical runs.

3.2. Linearity of calibration standard curves

Calibration curve data (Table 1) and calibration curve parameters (Table 2) for CBZ, PHT and CBZ-E in human plasma demonstrate that the calibration curves were linear in the concentration range from 0.050 to 25.0 μg ml^{-1}. The correlation coefficients were greater than, or equal to, 0.993 for all curves.

3.3. Precision and accuracy

The lower limit of quantitation (LLOQ) for this assay was set at 0.050 μg ml^{-1}. At the LLOQ the relative standard deviation (RSD; $n=6$) of the mean calculated concentration was $<2.0\%$ and the deviation of the mean from theoretical (DMT; $n=6$) was $<4.3\%$. For the upper limit of quantitation (ULOQ) set at 25.0 μg ml^{-1}, the RSD ($n=6$) of the mean calculated concentration was $<7.4\%$ and the DMT was $<8.8\%$ (Table 1). Intra- and inter-day accuracy and precision for CBZ, PHT and CBZ-E was assessed by the QC samples. During the three validation runs, aliquots of each of the three QC levels were run in replicates of six. No significant bias was detected in the mean concentration of any of the QC samples ($<12.2\%$ from theoretical at 0.75 μg ml^{-1}) and all RSDs were $<7.7\%$ (Table 3). There was no effect on reported concentrations of the compounds in human plasma after subjecting QC samples to three freeze/thaw cycles. Leaving the QC samples thawed at room temperature for up to 24 h also had no effect on reported concentrations.

3.4. Absolute recovery

Absolute recoveries were determined by comparing the peak height of extracted QC samples with the peak height of recovery standards at the same nominal concentrations. The overall recoveries for CBZ, PHT, CBZ-E and the IS from human plasma were $>79\%$.

3.5. In-process stability

The in-process stability was tested by reinjecting a set of QC samples ($n=3$ at the low, mid and high levels) with freshly extracted calibration standards and QC samples on day 4 of the validation study. The process stability samples were allowed to remain at room temperature for 24 h prior to reanalysis by HPLC. No significant difference was observed for either the accuracy or precision of the results.

4. Conclusions

The analytical procedure proved to be a very simple, selective, precise and accurate method for quantitation of CBZ, PHT and CBZ-E in human plasma. This procedure also has the necessary sensitivity for routine therapeutic monitoring of these drugs and will prove useful in supporting pharmacokinetics and drug interaction studies.

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