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Simultaneous determination of physostigmine and tetrahydroaminoacridine in a transdermal permeation study by high-performance liquid chromatography

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SUMMARY

A selective and stability-indicating high-performance liquid chromatographic assay with diazepam as the internal standard was developed for simultaneously analyzing physostigmine and tetrahydroaminoacridine in skin samples, permeation diffusates and dosage form. Baseline resolution was achieved with an octadecyl column for physostigmine, its two degradation products and tetrahydroaminoacridine. Peak homogeneity of physostigmine and tetrahydroaminoacridine was confirmed. The calibration curves for both drugs in skin samples were established at 1–50 μg per 200 mg skin. Those for diffusate samples were at 10–50 ng per 50 μl for physostigmine and 30–150 ng per 50 μl for tetrahydroaminoacridine. The assay was reproducible with within-day and between-day variations of 5–6 and 4–10%, respectively. Application of the assay for in vitro transdermal permeation study was demonstrated.

INTRODUCTION

Physostigmine (PHY), an alkaloid from the Calabar bean, and tetrahydroaminoacridine (THA, 9-amino-1,2,3,4-tetrahydroacridine) exhibit central anticholinesterase activity and both have shown efficacy in alleviating the memory loss of Alzheimer's disease [1,2]. The mechanism of action of each drug appears to differ [3]. Therefore, it may be beneficial to incorporate both PHY and THA into the treatment regimen to maximize the therapeutic efficacy and to minimize side-effects by lowering individual doses of each drug.

Due to the very short half-life of PHY [4] and difficulty of Alzheimer's

disease patients to comply with the frequent dosing regimens, there is a need to develop a controlled-release dosage form. The transdermal therapeutic system is a logical approach. A specific and sensitive high-performance liquid chromatographic (HPLC) assay to analyze these drugs is a prerequisite to characterize the preformulation parameters of PHY and THA. Many reports on the HPLC assays of PHY [5–12] and THA [13–16] in biological samples have been published. However, these assays were applied to brain, liver, whole blood, plasma, serum or urine, and were unsuitable for skin tissue which requires extensive homogenization and extraction procedures. Moreover, these HPLC assays, except those developed by Whelpton and Moore [8], Somani and Khalique [9] and Brodie et al. [10], did not employ any internal standard to quantitate PHY and THA.

This paper describes a selective and stability-indicating HPLC assay, which measures PHY and THA simultaneously in skin samples and permeation diffusates after in vitro skin permeation experiments and in dosage form.

EXPERIMENTAL

Materials

PHY free base and tetrahydroaminoacridine hydrochloride (THA·HCl) were purchased from Sigma (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively. Diazepam (DZ) was supplied by Hoffman-La Roche (Nutley, NJ, U.S.A.). All reagents were of analytical or HPLC grade. Permeation penetrant solution was prepared by dissolving PHY and THA·HCl in 1 ml of propylene glycol (PG) with vortex-mixing.

Chromatography

The liquid chromatograph used (Consta-Metric I, LDC, Riviera Beach, FL, U.S.A.) was equipped with a 50-μl sample loop (Valco, Houston, TX, U.S.A.), a fixed-wavelength UV detector monitoring at 254 nm (UV-III monitor, LDC), an ODS guard column (40 μm particle size, 20 mm$\times$4 mm I.D., Valco), a Spherisorb ODS-I column (5 μm particle size, 15 cm$\times$4.6 mm I.D., Custom LC, Houston, TX, U.S.A.) and a chart recorder (Linear Instrument, Irvine, CA, U.S.A.). A variable-wavelength UV detector (Spectro Monitor III 1204D, LDC) was used for peak homogeneity and stability-indicating studies. The isocratic mobile phase was 0.01 *M* octanesulfonic acid and 1% (v/v) acetic acid in a mixture of acetonitrile–water (52:48, v/v), pH 3.5. The flow-rate was 1 ml/min.

Extraction of skin samples

PHY and THA were extracted by homogenizing the skin sample (150–200 mg) with chloroform twice, 4 ml each (Polytron, PCU-2, Brinkmann Instruments, Westbury, NY, U.S.A.). The extracts were filtered through phase sep-

aration paper. The skin sample was then alkalinized with 2 ml of 10% (w/v) sodium hydroxide solution, extracted for THA under the same condition, and the chloroform extracts were washed twice with 2 ml of water. The washed chloroform was filtered through phase separation paper, combined with previously collected chloroform and then blown dry under air. The residue was reconstituted with 1 ml of the mobile phase, filtered through a microfilter and injected into the HPLC system. The internal standard, DZ, was added before extraction for the relative recovery study as well as for actual assays and, after extraction, for the absolute recovery study.

Calibration curve

Calibration curves for skin sample were constructed in the concentration range 1–50 μg per 200 mg skin for both PHY and THA. Calibration curves for permeation diffusate were constructed in the range 10–50 ng per 50 μl for PHY and 30–150 ng per 50 μl for THA.

Validation of assay

Four sets of samples were prepared and analyzed on the same day to establish the within-day variation. The assay was repeated over an eight-month period to establish the between-day variation.

Peak homogeneity and stability-indicating studies for PHY and THA

Fresh PHY and THA aqueous solutions spiked with internal standard, DZ, were injected into the HPLC system and monitored at 254, 290 and 320 nm, respectively. Another set of PHY and THA solutions were boiled for 0.5 h and analyzed by HPLC as described above after adding internal standard. The peak-height ratios of PHY/DZ and THA/DZ at different wavelengths were compared before and after heating. The procedure was repeated as above, except that PHY and THA were subjected to both conditions of pH < 1 (with 1 ml of 1 *M* hydrochloric acid) and pH > 13 [with 1 ml of 10% (w/v) sodium hydroxide solution] and boiled for 1.25 h to establish the stability-indicating capability of the assay.

In vitro permeation study and data analysis

The procedure for permeation study was as described by Chow et al. [17]. Full-thickness human cadaver abdominal skin from a subject of 53-year-old, white male was used as the permeation barrier. Aliquots of saline phosphate buffer, 0.2 ml, were withdrawn at 0.5, 1, 3, 5, 10, 12, 24, 36, 48, 60 and 72 h from the side-arm of the receptor chamber. This volume was replaced immediately with fresh buffer at 32°C. The samples were stored at −70°C until HPLC analysis.

A cumulative amount of PHY or THA·HCl penetrated versus time profile was constructed. The time intercept of the extrapolated linear region of the

curve yielded T as lag time. The diffusion coefficient, D, normalized by the square of the thickness of the stratum corneum, was estimated as $D/d^2 = 1/6T$. The slope of the linear portion of the penetration profile, determined by linear regression, was the penetrant flux (J_x). Since $J_x = (K_m D C_s)/d = K_p C_s$, where K_m and C_s were the stratum corneum–vehicle partition coefficient and initial concentration of the penetrant, respectively, and $K_p = (K_m D)/d$, the permeability constant K_p could be calculated from $K_p = J_x/C_s$ and K_m from $K_m d = K_p/(D/d^2)$.

RESULTS AND DISCUSSION

Baseline separation of PHY, DZ and THA was achieved with retention times of 4.5, 6.0 and 7.7 min, respectively (Fig. 1). No interfering peak in the drug-free skin extract was evident. Octanesulfonic acid was used as ion-pairing agent to improve the peak shapes of PHY and THA, since these drugs were completely ionized in the mobile phase of pH 3.5 (pK_a values of PHY: 1.76 and 7.88 [18]; pK_a value of THA: 10 [19]). Carbaryl, with a retention time of 3.5 min, was initially used as the internal standard. However, its use was discon-

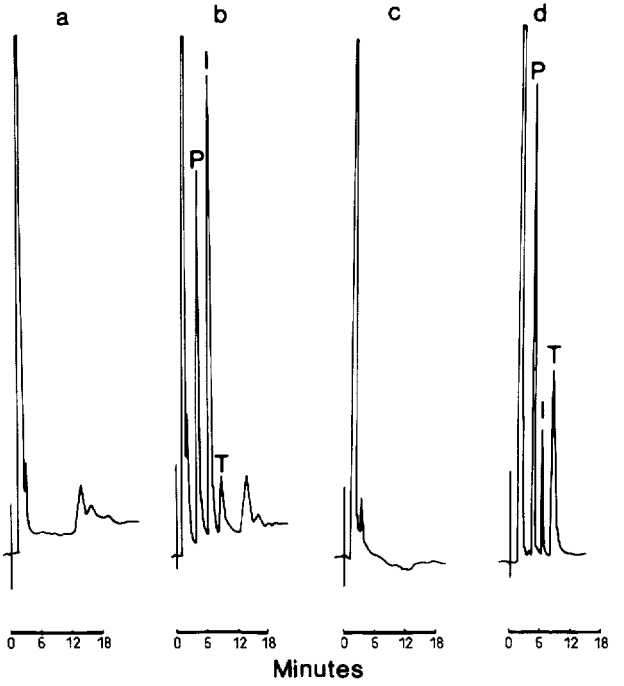

Fig. 1. Chromatograms of (a) drug-free skin extract, (b) skin sample extract with drugs spiked before homogenization and extraction, (c) control diffusate and (d) diffusate sample. Peaks: P = PHY; I = internal standard (DZ); T = THA. All monitored at 254 nm and 0.008 a.u.f.s.

TABLE I

CALIBRATION CURVES OF PHY AND THA IN SKIN SAMPLE

Numbers in parentheses are standard deviations ($n=4$).

Parameter	PHY	THA
Concentration range (μg per 200 mg skin sample)	1-50	1-50
Slope	0.0884	0.0145
Between-day variation (%)	10.2	4.17
Within-day variation (%)	5.25	5.65
Intercept	−0.0344	−0.0133
Coefficient of correlation	>0.998	>0 998
Relative recovery (%)	98.9 (11.4)	89.9 (3.82)
Absolute recovery (%)	65 4 (6.5)	57.1 (2.3)
Limit of detection (μg per 200 mg skin sample)	1	1

TABLE II

COMPARISON OF PEAK-HEIGHT RATIOS OF PHY/DZ AND THA/DZ AT DIFFERENT WAVELENGTHS

PHR_{254}, PHR_{290} and PHR_{320} are peak-height ratios recorded at 254, 290 and 320 nm, respectively. PHY degrades within seconds at pH > 13, so it was impossible to obtain its PHR with DZ.

Condition	Sample	$\frac{PHR_{254}}{PHR_{290}}$	$\frac{PHR_{254}}{PHR_{320}}$	$\frac{PHR_{290}}{PHR_{320}}$
PHY				
Water	Fresh	0.82	1.45	1.77
	Boiled	0.86	1.50	1.74
	Percentage change	5.0	3.4	2.0
pH < 1	Fresh	0.86	1.47	1 71
	Boiled	0.87	1.82	2.08
	Percentage change	1.4	19	17
THA				
Water	Fresh	0.24	0.040	0.17
	Boiled	0.25	0.038	0.15
	Percentage change	3.7	5.5	9.4
pH < 1	Fresh	0.27	0.040	0.15
	Boiled	0.26	0.041	0.16
	Percentage change	3.8	1.2	5.0
pH > 13	Fresh	0.20	0.0187	0.09
	Boiled	0.19	0.019	0.10
	Percentage change	8.4	1.0	9.5

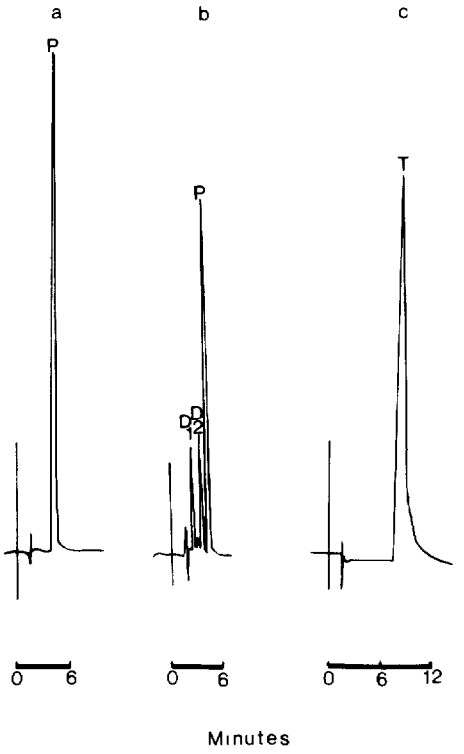

Fig. 2. Chromatograms of PHY in (a) pH < 1 after boiling and (b) pH > 13; (c) chromatogram of THA in pH > 13 after boiling. Peaks: D1 and D2 = degradation products of PHY; P = PHY; T = THA. All monitored at 254 nm and 0.064 a.u.f.s.

tinued due to interference with a degradation product of PHY. DZ was selected as the internal standard, because it showed no interference to degradation products of PHY and yielded high relative recovery with both PHY and THA.

The assay of skin samples was reproducible. The within-day variations of the slopes of calibration curves for PHY and THA were less than 6%, whereas the between-day variations for PHY and THA ranged from 4 to 10% (Table I). The detection limits of the assay for both drugs were 1 μg per 200 mg skin sample at a signal-to-noise ratio of 4. Linear response was obtained for both drugs over the range 1–50 μg per 200 mg skin sample. Relative and absolute recoveries were 98.9 and 65.4% for PHY and 89.9 and 57.1% for THA, respectively. Extraction of THA with chloroform required alkalinization since THA was highly ionized at neutral pH. The low absolute recoveries for both drugs might be due to the homogenizing procedure and emulsion formation after alkalinization.

The purity of standard PHY, DZ and THA·HCl was verified by the single peak on the chromatogram after employing different mobile phases and columns (C_8 and C_{18}). The proof of peak homogeneity for PHY and THA under

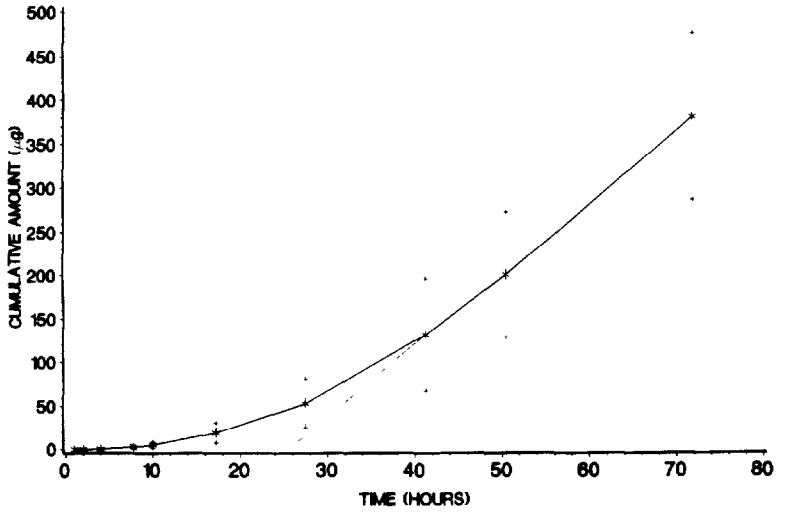

Fig. 3. Plot of cumulative amount penetrated versus time for PHY through human skin.

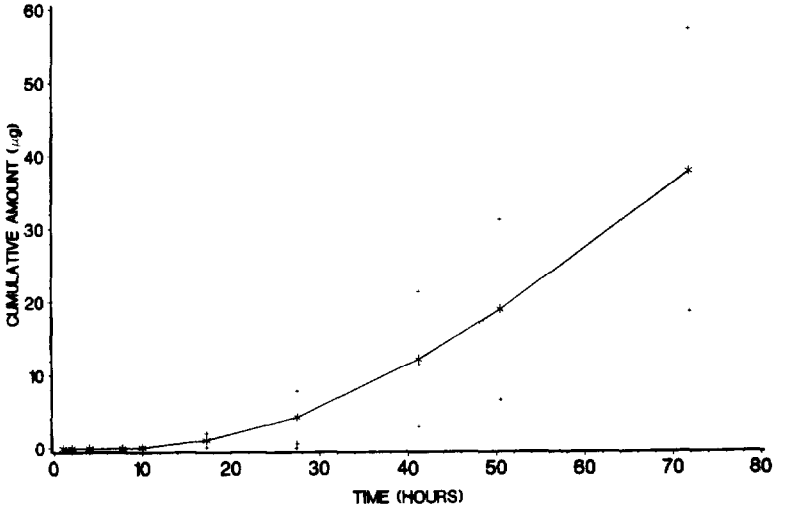

Fig. 4. Plot of cumulative amount penetrated versus time for THA·HCl through human skin.

different conditions was confirmed (Table II). The change of peak-height ratio of PHY/DZ before and after heating at different wavelengths was less than 5% under all conditions, except at pH < 1 (17–19%). This high percentage difference might be due to the low absorbance of PHY at 320 nm. Peak-height ratio change of THA/DZ before and after heating at different wavelengths was less than 10% under all conditions. Therefore, the presence of any coeluting compound at the retention times of PHY and THA was unlikely.

TABLE III

TRANSDERMAL PERMEATION PARAMETERS OF PHY AND THA·HCl

Parameter	PHY	THA·HCl
C_s (mg/ml propylene glycol)	150.0	80.3
Extent of permeation		
Diffusate (μg)	382.0	37.9
(percentage of applied dose)	1.63	0.59
Skin (mg)	0.921	0.371
(percentage of applied dose)	3.92	5.78
T (h)	26.1	27.4
J_x (μg/cm^2 per h)	13.16	1.28
K_p ($\times 10^{-5}$ cm/h)	8.76	1.60
D/d^2 ($\times 10^{-3}$ h^{-1})	7.16	6.55
$K_m d$ ($\times 10^{-3}$ cm)	13.81	2.51

As reported previously [20], PHY was extensively degraded at pH > 13 within seconds without heating (Fig. 2). The degradation products of PHY, i.e. D1 and D2, at pH > 13 had retention times of 2.7 and 3.6 min, respectively. In water, PHY was degraded upon heating yielding degradation products with the same retention times as those from pH > 13. No degradation product was observed for PHY at pH < 1 and for THA at pH < 1 and pH > 13, even upon boiling for over 1 h. THA in water at pH < 1 and pH > 13 after boiling gave equivalent chromatograms. No extra degradation peak of PHY and THA under these conditions was observed when the acetonitrile content in the mobile phase was lowered to 45% (v/v). This further confirmed the peak homogeneity and stability-indicating capability of the PHY and THA assay.

The developed assay was applied to the in vitro simultaneous permeation study of PHY and THA·HCl. The control diffusate was free of interfering peaks with those of PHY, DZ and THA (Fig. 1). Cumulative penetration profiles of PHY and THA·HCl through human skin given in Figs. 3 and 4 showed that the penetrant flux was 13.2 and 1.28 μg/cm^2 per h for PHY and THA·HCl, respectively. The estimated lag time was 26.1 and 27.4 for PHY and THA·HCl, respectively. Other transdermal permeation parameters of PHY and THA·HCl are listed in Table III.

This work is the first study of a selective and stability-indicating HPLC assay allowing the simultaneous monitoring of PHY and THA. The assay is sensitive, reproducible and suitable for analyzing biological samples from in vitro transdermal permeation studies. Potential applications of this assay include the monitoring of PHY and THA in both conventional and controlled-release dosage forms and the permeation evaluation of both drugs under the influence of permeation enhancers.

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