Simultaneous Determination of Six Analytes by HPLC-UV for High Throughput Analysis in Permeability Assessment

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Abstract

A methodology for the simultaneous determination of six control analytes, including carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin, was developed and validated utilizing reversed-phase high-performance liquid chromatography with ultraviolet detection for high throughput analysis for permeability assessment. The analytes were separated on Agilent Zorbax SB-C18 (50 × 4.6 mm I.D., 5 µm) with a gradient mobile phase consisting of water (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid) and acetonitrile (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid). The flow rate was 2.0 mL/min and the eluent was monitored at 280 nm. A linear response was found for all six analytes over a broad concentration range (1.00–200 µM). The correlation coefficient for each analyte was greater than 0.999. The limit of detection and limit of quantitation were 0.03 and 0.10 µM, 0.10 and 0.30 µM, 0.05 and 0.15 µM, 0.03 and 0.10 µM, 0.05 and 0.15 µM, 0.10 and 0.30 µM for carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin, respectively. The optimized method was further successfully applied to high throughput analysis for parallel artificial permeability assay.

Introduction

Various cell-based in vitro methods for the assessment of permeability have been developed (1–7) in order to predict drug transport properties in humans. However, traditional methods are usually time-consuming, labor-intensive, relatively expensive, and limited in throughput. As a result, many new methods, which are fast and cost-efficient, have been developed to estimate drug permeability in early drug discovery. The parallel artificial permeability assay (PAMPA) was developed as an alternative for low and high throughput Caco-2 assays for the prediction of blood brain barrier permeability (8). PAMPA measures the permeability of an immobilized lipid membrane and can efficiently process many analytes in parallel via automated technology. Together with the rapid development of combinatorial chemistry and the increasing number of analytes having poor aqueous solubility, sample analysis by PAMPA studies has become the 'bottleneck' of this drug screening assay. Therefore, an essential aspect of the drug discovery process is to dramatically increase the throughput of sample analysis for this assay.

In the in vitro PAMPA tests, it is very important to investigate the permeability of several control samples including carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin, etc., in order to assess the integrity of membrane and the validity of the resulting data. As a result, a prerequisite for the present PAMPA study was the development and validation of a novel analytical method to simultaneously determine control compounds in order to increase the throughput, sensitivity and specificity for the quantitation of compound levels in the Discovery PAMPA assay.

High-performance liquid chromatography (HPLC) coupled with mass spectrometric (MS) detection is a powerful method to achieve good assay performance. Unfortunately, LC–MS–MS is not widely available in average laboratories. Although the individual analysis of carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin have been broadly reported (9–14), no method has been established for the simultaneous determination of them all together.

Herein, we report a reliable HPLC-UV method which was developed, optimized and validated for the simultaneous determination of carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin in a single injection.

Experimental

Chemical reagents and materials

Reference standards of carbamazepine (100.0%) and methotrexate (99.6%) were provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Reference standards of desipramine hydrochloride (98.0%), propranolol hydrochloride (99.5%), guanabenz acetate

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(100.0%), and warfarin (99.9%) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Acetonitrile and isopropyl alcohol of HPLC grade were provided by Tedia Company Inc. (Fairfield, OH). Dioleoylphosphatidylcholine, heptafluorobutyric acid, phosphoric acid, and ethanol purchased from Beijing Chemical Reagents Co. (Beijing, China) were all analytical grade reagents. Distilled water, prepared by a Milli-Q water purification system (Millipore, Molsheim, France), was used throughout the study. All solutions prepared for HPLC analysis were passed through a 0.45 µm filter before use. Filter plates (96-well) were purchased from Millipore, Inc. (Bedford, MA).

Instrumentation

Chromatographic separations were performed on a Thermo Finnigan Surveyor HPLC instrument equipped with an ultraviolet detector (Thermo Finnigan, San Jose, CA). Data integration was performed by using X-calibur data software (Thermo Finnigan, San Jose, CA). Injections were made using a 10 µL loop.

Chromatographic conditions

The HPLC separation was performed using Agilent Zorbax SB-C18 (50 × 4.6 mm i.d., 5 µm) and a Shim-pack GVP-ODS C18 guard column (10 × 4.6 mm i.d, 5 µm). The gradient mobile phase, consisting of water (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid, solvent A) and acetonitrile (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid, solvent B), was delivered at a flow-rate of 2.0 mL/min. The gradient condition of the mobile phase was: 0 min 95% solvent A, 0.5 min 95% solvent A, 4.5 min 5% solvent A, 4.6 min 95% solvent A, and 6 min 95% solvent A. Prior to use, the mobile phase was filtered through 0.45-µm Millipore membrane filter and degassed by sonication in an ultrasonic bath. Detection was set at 280 nm and the column temperature was maintained at 20°C.

Standard solutions and calibration curves

Stock solutions of carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin were prepared in 5% DMSO (v/v) Phosphate Buffer Solution (PBS, containing 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM K2HPO4, pH 7.4) at the concentration of 5.0 mM, taking into account the purity of the standards. Then drug-containing donor solutions of six compounds (final concentration: 100 µM) were prepared from these stock solutions using 5% DMSO (v/v) PBS as diluent. These solutions were stored at 4°C in the dark before use. For quantitative analysis, matrix matched calibration standards were prepared in triplicate at a series of concentrations (1.00–200.0 mM) for these six compounds.

PAMPA assay and sample collection

The parallel artificial permeability assay was performed in a 96-well plate similar to that described in the literatures (2,4,8). All compounds were tested in triplicate. The membranes of a 96-well filter plate (Millipore, Bedford, MA) were coated with 5 µL of a 1.0% (w/v) dodecane solution of dioleoylphosphatidylcholine. Then 150 µL of drug-containing donor solutions (100 µM, dissolved in PBS containing 5% DMSO) was immediately added into each well of the donor plate. A 300 µL aliquot of buffer (5% DMSO in PBS, pH 7.4) was added into each well of the PTFE acceptor plate. Then the drug-filled donor plate was placed into the acceptor plate, making sure the bottom of the membrane is in contact with the buffer. After 16 h incubation at room temperature, aliquots of the donor and bottom samples were appropriately pooled and then determined by the developed HPLC-UV method. Effective permeability value (Pe) was calculated using the equation below:

$$P_e = -V_d \times V_a/[(V_d + V_a) \times A \times t] \times \ln (1 - C_{acceptor}/C_{equilibrium})$$

where V_d = volume of donor well, V_a = volume in acceptor well, A = filter area, t = permeation time, $C_{acceptor}$ = concentration in acceptor well, and $C_{equilibrium}$ = the resulting concentration if the donor and acceptor solution were simply combined.

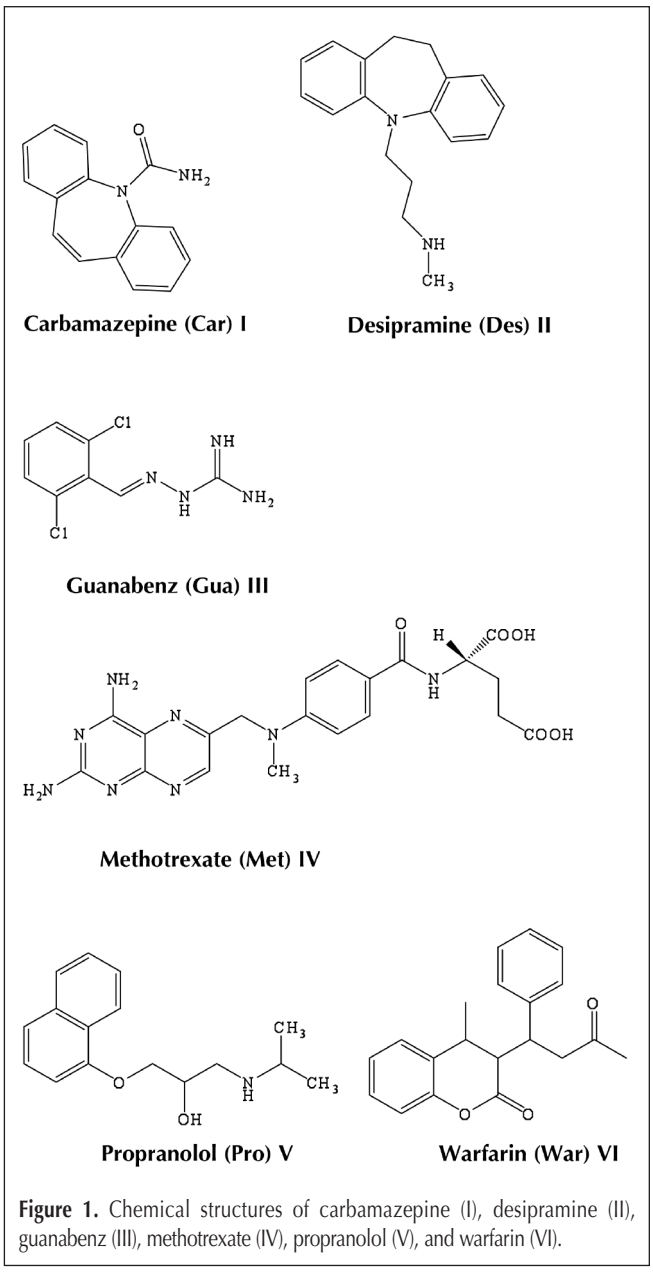

Figure 1. Chemical structures of carbamazepine (I), desipramine (II), guanabenz (III), methotrexate (IV), propranolol (V), and warfarin (VI).

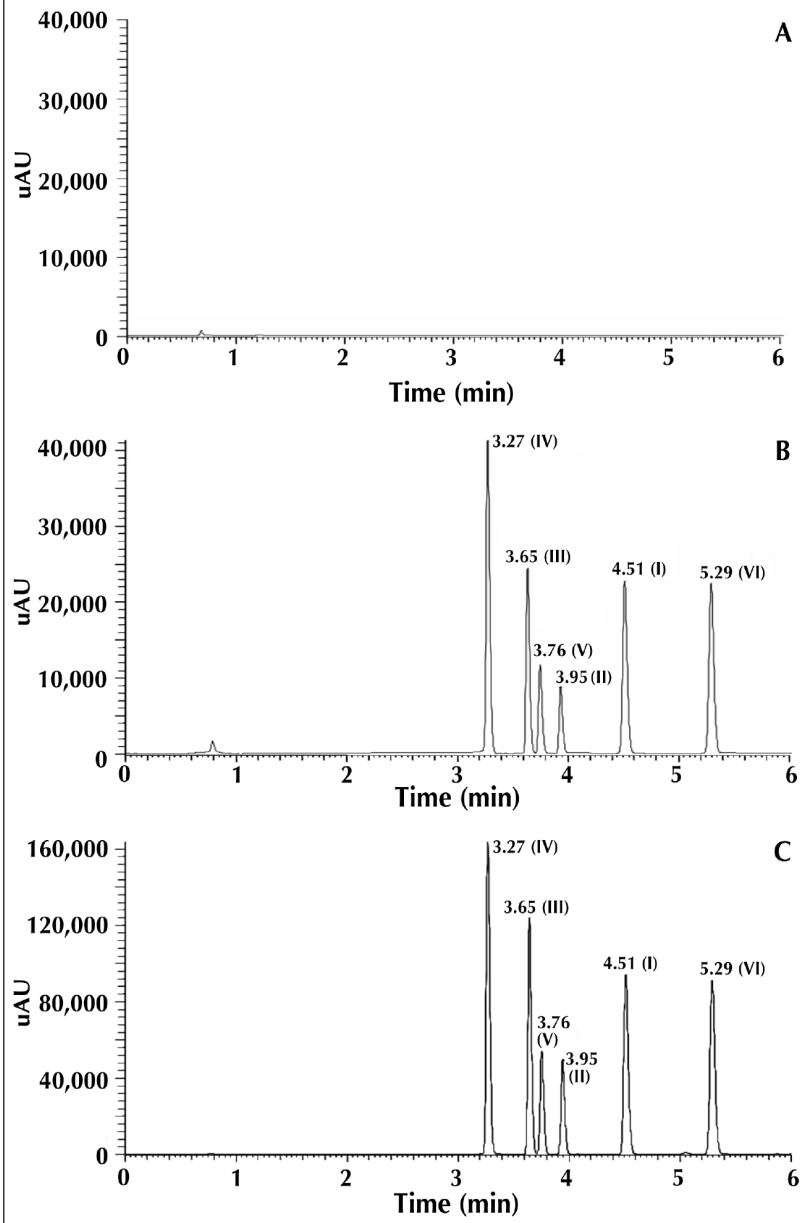

Figure 2. Typical chromatograms of six control compounds determined by HPLC-UV. (A) Blank matrix; (B) standard solution spiked with carbamazepine (9.93 µM), propranolol (10.17 µM), and warfarin (10.28 µM); (C) a real pooled PAMPA sample: (I) carbamazepine, (II) desipramine, (III) guanabenz, (IV) methotrexate, (V) propranolol, and (VI) warfarin.

Table I. Stability for the Quantitative Determination of Six Compounds by HPLC-UV (*n* = 5)

Time (h)	CAR	DES	GUA	MET	PRO	WAR
0	9.93	9.81	11.14	10.38	10.17	10.28
2	10.01	9.91	11.48	10.98	10.23	10.38
5	9.98	9.8	11.09	10.09	10.19	10.47
12	9.79	9.73	11.23	10.72	10.10	10.03
24	9.90	9.75	10.54	10.78	10.03	10.19
Mean	9.92	9.80	11.10	10.59	10.14	10.27
RSD (%)	0.86	0.71	3.11	3.34	0.78	1.66

Results and Discussion

Optimization of the HPLC conditions

During method development, top priority was given to the complete separation of the six analytes of interest from each other. These six compounds have different structures (shown in Figure 1) and different physical-chemical properties. Typical chromatograms corresponding to a blank matrix, a standard mixture of the selected six control compounds and a real pooled PAMPA sample, using UV detection, are shown in Figure 2.

The mobile phase was chosen after several trials with acetonitrile, methanol, isopropyl alcohol, and water in various proportions. In addition different types of acid modifiers were added to adjust the pH value of the mobile phase. A mobile phase consisting of water (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid, solvent A) and acetonitrile (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid, solvent B) was finally selected, which produced optimal separation, high sensitivity, and good peak shape. Addition of 0.01% heptafluorobutyric acid could act as an ion pair reagent as well to improve the retention behavior of several compounds. Good sensitivity was confirmed using UV detection at 280 nm for all of the analytes of interest.

According the current optimized HPLC conditions, the resolution values for the six compounds were all greater than 1.5. By using the proposed chromatographic conditions, the analytes of interest could be well separated from each other within 6 min. The retention times of carbamazepine, desipramine, guanabenz, methotrexate, propranolol and warfarin were 4.51 min, 3.95 min, 3.65 min, 3.27 min, 3.76 min, and 5.29 min, respectively.

Calibration and method validation

The developed HPLC method was validated by evaluating different validation parameters such as stability, linearity, limit of detection (LOD), and limit of quantitation (LOQ), precision, and accuracy. For qualitative purposes, the method was further evaluated by taking into account the precision of the retention times of the analytes.

The stability was examined over a period of 24 h using standard solutions of six compounds indicating a relative standard deviation of 0.86% for carbamazepine, 0.71% for desipramine, 3.11% for guanabenz, 3.34% for methotrexate, 0.78% for propranolol, and 1.66% for warfarin, respectively (data shown in Table I).

Six standard solutions, at a series of concentrations (1.00–200 µM), were prepared and analyzed. The calibration curves for individual compounds were created using Excel software. The peak area values (expressed in µAU.s) were plotted as average values of

duplicate injections. The results of the calibration results are summarized in Table II, which showed good linearity ($r > 0.999$) for all the compounds in the concentration range tested (1.00–200 µM).

The LOD and LOQ were separately determined at a signal-to-noise ratio (S/N) of 3 and 10. The LOD and LOQ were experimentally verified by diluting known concentrations of six compounds until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations. The LOD and LOQ were 0.03 and 0.10 µM, 0.10 and 0.30 µM, 0.05 and 0.15 µM, 0.03 and 0.10 µM, 0.05 and 0.15 µM, and 0.10 and 0.30 µM for carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin, respectively. Under the present LOQ, the concentrations of six compounds could be determined in the donor and acceptor samples, which was sensitive enough to investigate the concentration of six compounds.

The intra- and inter-day precisions [expressed as the relative standard deviation (RSD)] and accuracy [expressed as the relative error (RE)] for the six analytes were determined by the spiked samples with the standard solutions of the six compounds (n = 6), consecutively, using the analytical method above. The intra- and inter-day precisions were better than 2.24% and 3.78%, 2.85% and 2.89%, 1.98% and 4.30%, 2.68% and 4.35%, 1.79% and 4.46%, 2.70% and 3.10% for carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin, respectively. The accuracies were calculated to be 1.31%, –0.51%, –1.53%, –0.39%, 1.97% and 1.26% for these six compounds, respectively. The results, calculated with the one-way ANOVA, indicated that the values were within the acceptable range. These results are summarized in Table III.

Table II. Calibration, LOD, and LOQ data of Six Compounds Determined by HPLC-UV

Comp.	Linearity range (µM)	Calibration equation*	LOD (µM)	LOQ (µM)	Correlation factor (r)
CAR	0.993–198.6	y = 3316.9x – 22.409	0.03	0.10	1.0000
DES	0.981–196.2	y = 1412.5x – 21.974	0.10	0.30	0.9999
GUA	1.114–222.8	y = 3321.5x – 5.5086	0.05	0.15	0.9999
MET	1.038–207.6	y = 4644.2x + 21.878	0.03	0.10	1.0000
PRO	1.017–203.4	y = 1494.9x + 12.353	0.05	0.15	1.0000
WAR	1.028–205.6	y = 3567.3x – 18.436	0.10	0.30	0.9999

* Seven data points (n = 3); x: the conc. of each compound (µM); y: the peak area.

Table III. Intra- and Inter-day Precision and Accuracy for the Quantitative Determination of Six Compounds by HPLC-UV (n = 6)

		Intra-day precision		Inter-day precision		
Comp.	Nominal conc. (µM)	Detected conc. (µM)	RSD (%)	Detected conc. (µM)	RSD (%)	RE (%)
CAR	9.93	10.06	2.24	10.22	3.78	1.31
DES	9.81	9.76	2.85	9.71	2.89	–0.51
GUA	11.14	10.97	1.98	10.94	4.30	–1.53
MET	10.38	10.34	2.68	10.45	4.35	–0.39
PRO	10.17	10.37	1.79	10.03	4.46	1.97
WAR	10.28	10.41	2.70	10.72	3.10	1.26

Table IV. Comparison of Observed PAMPA Results with the Corresponding Literature Data

Test compound	Observed Value	Literature Value
MET	–7.81 ± 0.05	–7.20 ± 0.10
GUA	–5.47 ± 0.01	NA*
PRO	–4.93 ± 0.02	–5.00 ± 0.10
DES	–5.17 ± 0.02	NA
CAR	–5.00 ± 0.05	–5.20 ± 0.00
WAR	–6.32 ± 0.02	–6.00 ± 0.00

*NA: Not applicable.

Permeability of six important control compounds in the PAMPA assay

According to the equation listed in section "2.5. PAMPA assay and sample collection", the Log P_e values of six control compounds were calculated as follows: –5.11 ± 0.01 for carbamazepine, –5.17 ± 0.02 for desipramine, –5.47 ± 0.01 for guanabenz, –7.30 ± 0.04 for methotrexate, –4.95 ± 0.03 for propranolol, and –6.03 ± 0.03 for warfarin, respectively. Table IV shows the comparison of the above results with the corresponding data reported in literature (1–8).

Conclusion

The present study describes a novel high throughput HPLC-UV methodology for quantitatively determining the permeability of six compounds simultaneously. This method was tested and validated by using carbamazepine, desipramine, guanabenz, methotrexate, propranolol, and warfarin. The use of an Agilent Zorbax SB-C18 column and a gradient mobile phase of acetonitrile (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid) and water (containing 1% isopropyl alcohol and 0.01% heptafluorobutyric acid) enables good separation of six analytes of interest. The method is rapid and sensitive for the analysis of carbamazepine, desipramine, guanabenz, methotrexate, propranolol and warfarin with LOQ of 0.10 µM, 0.30 µM, 0.15 µM, 0.10 µM, 0.15 µM, and 0.30 µM, respectively. Excellent levels of accuracy and precision were obtained for six analytes of interest. The validated method was successfully applied to the analysis of six important control compounds for high throughput analysis applied to permeability assessment.

References

1. P. Artursson, K. Palm, and K. Luthman. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **46:** 27–43 (2001).

2. M. Kansy, F. Senner, and K. Gubernator. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. *J Med. Chem.* **41:** 1007–1010 (1998).
3. F. Wohnsland and B. Faller. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *J. Med. Chem.* **44:** 923–930 (2001).
4. A. Avdeef, M. Strafford, E. Block, M.P. Balogh, W. Chambliss, and I. Khan. Drug absorption in vitro model: filter-immobilized artificial membranes. 2. Studies of the permeability properties of lactones in Piper methysticum Forst. *Eur. J. Pharm. Sci.* **14:** 271–280 (2001).
5. A. Seelig, R. Gottschlich, and R.M. Devant. A method to determine the ability of drugs to diffuse through the blood-brain barrier. *Proc. Natl. Acad. Sci. USA.* **91:** 68–72 (1994).
6. K.W. Otis, M.L. Avery, S.M. Broward-Partin, D.K. Hansen, H.W. Behlow Jr, D.O. Scott, and T.N. Thompson. Evaluation of the BBMEC model for screening the CNS permeability of drugs. *J. Pharmacol. Toxicol. Methods* **45:** 71–77 (2001).
7. J. Mensch, M. Noppe, J. Adriaensen, A. Melis, C. Mackie, P. Augustijns, and M.E. Brewster. Novel generic UPLC/MS/MS method for high throughput analysis applied to permeability assessment in early drug discovery. *J. Chromatogr B.* **847:** 182–187 (2007).
8. L. Di, E.H. Kerns, K. Fan, O.J. McConnell, and G.T. Carter. High throughput artificial membrane permeability assay for blood-brain barrier. *Eur. J. Med. Chem.* **38:** 223–232 (2003).
9. M. Gupta, K. Kohli, D. Kumar, and Y.K. Gupta. A reverse phase high performance liquid chromatography method for simultaneous estimation of melatonin, carbamazepine epoxide and carbamazepine simultaneously in serum. *Indian J. Physiol. Pharmacol.* **50:** 427–430 (2006).
10. A.G. Chen, Y.K. Wing, H. Chiu, S. Lee, C.N. Chen, and K. Chan. Simultaneous determination of imipramine, desipramine and their 2- and 10-hydroxylated metabolites in human plasma and urine by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **693:** 153–158 (1997).
11. L. Vio, M.G. Mamolo, and G. Furlan. Quantitative high pressure liquid chromatographic determination of guanabenz and mephruside in pharmaceutical formulations. *Farmaco.* **43(1):** 27–36 (1988).
12. L.D. Liang, W. Wong, and H.M. Burt. Pharmacokinetic study of methotrexate following intra-articular injection of methotrexate loaded poly(L-lactic acid) microspheres in rabbits. *J. Pharm. Sci.* **94:** 1204–1215 (2005).
13. R. Panchagnula, T. Bansal, M.V. Varma, and C.L. Kaul. Reversed-phase liquid chromatography with ultraviolet detection for simultaneous quantitation of indinavir and propranolol from ex-vivo rat intestinal permeability studies. *J. Chromatogr. B* **806:** 277–282 (2004).
14. A. Osman, K. Arbring, and T.L. Lindahl. A new high-performance liquid chromatographic method for determination of warfarin enantiomers. *J. Chromatogr B* **826:** 75–80 (2005).

Manuscript received July 17, 2008;
revision received December 31, 2009.