AAPS PharmSciTech, Vol. 13, No. 2, June 2012 (© 2012)
DOI: 10.1208/s12249-012-9780-9

Research Article

Single-Injection HPLC Method for Rapid Analysis of a Combination Drug Delivery System

Robert M. Tucker,[1] Benjamin W. Parcher,[1] Ella F. Jones,[2] and Tejal A. Desai[1,3]

Received 12 January 2012; accepted 28 March 2012; published online 26 April 2012

Abstract. Developing combination drug delivery systems (CDDS) is a challenging but necessary task to meet the needs of complex therapy regimes for patients. As the number of multi-drug regimens being administered increases, so does the difficulty of characterizing the CDDS as a whole. We present a single-step method for quantifying three model therapeutics released from a model hydrogel scaffold using high-performance liquid chromatography (HPLC). Poly(ethylene glycol) dimethacrylate (PEGDMA) hydrogel tablets were fabricated via photoinitiated crosslinking and subsequently loaded with model active pharmaceutical ingredients (APIs), namely, porcine insulin (PI), fluorescein isothiocyanate-labeled bovine serum albumin (FBSA), prednisone (PSE), or a combination of all three. The hydrogel tablets were placed into release chambers and sampled over 21 days, and APIs were quantified using the method described herein. Six compounds were isolated and quantified in total. Release kinetics based on chemical properties of the APIs did not give systematic relationships; however, PSE was found to have improved device loading *versus* PI and FBSA. Rapid analysis of three model APIs released from a PEGDMA CDDS was achieved with a direct, single-injection HPLC method. Development of CDDS platforms is posited to benefit from such analytical approaches, potentially affording innovative solutions to complex disease states.

KEY WORDS: combination drug delivery systems; combination therapy; controlled release; HPLC; hydrogel; PEGDMA; protein therapeutic.

INTRODUCTION

In the field of multi-drug therapy regimens, single administration of combination drug therapy has been a staple approach to both enhance therapeutic synergy and patient compliance for improved treatment outcomes. In practice, combination therapy has been routinely prescribed as a single, non-controlled dose via conventional administration routes. Categorically, the most recognized example of combination therapy is the oral dosage form of two small molecules. These include marketed products such as Dyazide (triamterene and hydrochlorothiazide for hypertension), Augmentin (amoxicillin and clavulanic acid as an antibiotic), and Sinemet (carbidopa and levodopa for Parkinson's dopamine replacement) (1). While these precedents have shown increased efficacy, enhanced patient compliance, and improved outcomes relative to their respective monotherapies, the development of more sophisticated combination therapies remains an elusive goal for researchers and clinicians alike.

Combination Drug Delivery Systems

Drug delivery systems are commonly used for the delivery of single therapeutic agents, but further development to deliver "cocktails" of drugs is needed (2). Two horizons for combination drug delivery systems (CDDSs) are to independently control release of the given active pharmaceutical ingredients (APIs) and the capacity to concurrently administer both small and macromolecule therapeutics. Examples of disease states that demand a combined, controlled dose of a small molecule and protein therapeutic include melanoma, leukemia, rheumatoid arthritis, multiple sclerosis, anemia, and hepatitis C (3–8). Thus, an emerging class of CDDSs with such capability is envisioned to expand the therapeutic efficacy of combination therapy as a whole.

Robert M. Tucker and Benjamin W. Parcher contributed equally to this work.

[1] Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, 1700 4th St, Byers Hall 204, Box 2520, San Francisco, California 94158, USA.
[2] Department of Radiology and Biomedical Imaging, Center for Molecular and Functional Imaging, University of California, San Francisco, San Francisco, California 94158, USA.
[3] To whom correspondence should be addressed. (e-mail: tejal.desai@ucsf.edu)

ABBREVIATIONS: CDDS, Combination drug delivery system; HPLC, High-performance liquid chromatography; API, Active pharmaceutical ingredient; PEGDMA, Poly(ethylene glycol) dimethacrylate; DMPA, 2,2-Dimethoxy-2-phenylacetophenone; VPY, 1-Vinyl-2-pyrrolidinone; FBSA, Fluorescein isothiocyanate bovine serum albumin; PI, Porcine insulin; PSE, Prednisone; RT, Retention time.

An implicit consequence for escalating the complexity of drug delivery systems and the number of administered APIs is the analytical challenge of quantifying delivery rates of multiple therapeutics from a device or delivery system. While single-component systems can be analyzed with routine, single-pass methods such as spectrophotometry and protein detection kits, multi-component systems require more intricate study. Recent interesting work into the development and characterization of CDDSs has readily exemplified such a heightened need (9–14). Of the analysis strategies considered in meeting this challenge, we selected analytical high-performance liquid chromatography (HPLC) for isolation of the model APIs and quantification by UV absorbance. Although HPLC is routinely used in such characterization settings, our goal for a single-injection method was nonetheless compounded by the varying properties of the selected APIs and additional interference with the CDDS components themselves (Table I).

CDDS Components

Poly(ethylene glycol) (PEG) was selected as the material for the CDDS device due to its known advantages of low immunogenicity, high biocompatibility, ease of functionalization, and FDA approval status (13,15–17). Specifically, we employed the bifunctionalized poly(ethylene glycol) dimethacrylate (PEGDMA) monomer in our CDDS fabrication (Fig. 1a). Previous work has demonstrated its utility in the fabrication of three-dimensional, microtopographical devices (18,19). Polymerization of the dimethacrylate groups was afforded by photoinitiated crosslinking with 2,2-dimethoxy-2-phenylacetophenone (DMPA) as a free radical source (Fig. 1b, left). A third reagent in the fabrication process was 1-vinyl-2-pyrrolidinone (VPY), utilized as a stabilizer and interfacial solvent for DMPA (Fig. 1b, right). All three of these components have overlapping spectrophotometric absorbance ranges and interfere with commonly used quantification techniques (Table I). For example, VPY is a reducing agent and interferes with many colorimetric detection assays, and the methacrylate group of the PEGDMA interferes with most spectroscopic quantification techniques due to its high UV absorbance between 200 and 250 nm. Additionally, these system components have a considerable breadth of hexane–water partitioning, making liquid–liquid extraction prior to analysis difficult. The applicable HPLC method we hoped to achieve would be required to isolate all of these CDDS components, while resolving each model API to an independent retention time (RT).

Model APIs

Selection of model APIs was based on the complexity previously cited as an emerging horizon for combination therapy. We aspired to track both small molecule and protein therapeutics in a combination release assay from solid PEGDMA delivery constructs (20). Additionally, we included a second protein therapeutic for three model APIs in all: porcine insulin (PI), fluorescein isothiocyanate bovine serum albumin (FBSA) and prednisone (PSE). Moreover, the APIs investigated in this work were chosen for their clinical relevance, to explore the effects of construct loading, and to deliver APIs of varying sizes and hydrophobicities. The specific combination of APIs to be delivered in the aforementioned model could be varied depending on the desired application.

PSE, the small molecule, was chosen for its regular application as an immunosuppressant with other protein immunomodulators, such as interferon alpha variants (21). Two macromolecules were included to investigate the relative differences that molecular weight and hydrophobicity imparted on release behavior from the PEGDMA CDDS of our study. FBSA and PI with molecular weights of 66 and 5.8 kDa, respectively, had hydrophobicities of approximately −0.395 (FBSA) and 0.218 (PI). This roughly corresponds to a tenfold difference in molecular weight and fourfold difference in hydrophobicities (22). After the hydrogel tablets were crosslinked, they were placed into “loading solutions” containing high concentrations of the APIs and loaded over 72 h.

MATERIALS AND METHODS

Materials

PEGDMA (M_N=750), fluorescein isothiocyanate bovine serum album (FBSA or FITC-BSA), 1-vinyl-2-pyrrolidinone (VPY), 2,2-dimethoxy-2-phenylacetophenone (DMPA) of ACS

Table I. Sensitivity Limits and Quantification Strategies for a Model FITC-labeled Protein

			Simultaneous quantification of other reagents present?			
Analytical technique	Reference	Linear detection range (μg/mL)	PEGDMA	Second protein	Small molecule	Photoinitiator components
Fluorescence spectrophotometry	(23)	0.02–2.2	No[a]	No[a]	No[a]	No[a]
Ultraviolet spectroscopy	(24)	0.08–8,000	No	No	No	No
Bicinchoninic acid assay (BCA)	(25)	20–2,000	No	No[a]	No	No
Micro bicinchoninic acid assay (μBCA)	(25)	2–40	No	No[a]	No	No
Bradford assay	(26)	1–1,400	No	No[a]	No	No
Gel electrophoresis	(27)	0.1–10	Yes	Yes	No	No
UV high-performance liquid chromatography	(~)	1–500	Yes	Yes	Yes	Yes

The left column lists commonly used techniques for quantitative analysis, while the right columns list the detection limits and compatibility with other components in a CDDS design

PEGDMA poly(ethylene glycol) dimethacrylate, ~ indicates the presented work

[a] Reagents cause interfering background signals in the technique without prior isolation

grade, PSE, and insulin (PI, porcine pancreas, lyophilized powder) were all obtained from Sigma, USA, and used without further purification.

Standard Solutions

Phosphate-buffered saline (PBS) was purchased from VWR at a concentration of 10× and diluted tenfold to a final concentration of 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer). The photoinitiator solution was made by dissolving 2,2-dimethoxy-2-phenylacetophenone into 1-vinyl-2-pyrrolidinone to make a 100 mg/mL solution. Standards of PEGDMA, FBSA, VPY, and DMPA were prepared in ranges of 0.1–500 μg/mL in PBS.

HPLC Equipment

A LaChrom Elite® high-performance liquid chromatography system (Hitachi; USA) equipped with an L-2455 diode array detector was utilized for continuous absorbance measurement from 190 to 400 nm during analysis. Stationary phase was afforded with a Luna 5 μm C18(2) 100A, 250× 4.6 mm (Phenomenex Inc.; Torrance, CA), enclosed in a L-2300 column oven equilibrated at 25°C. HPLC grade water (product #JT4218-3), acetonitrile (product #EM-AX0145-1), isopropanol (product #EMD-PX1835-5), and trifluoroacetic acid (TFA, product #EM-TX1276-0004) were purchased from VWR and premixed into three solvent reservoirs prior to use. Solvent A was 100 % water, solvent B was 100 % acetonitrile, and solvent C was a 2:1 (*v*/*v*) mixture of isopropanol and acetonitrile. TFA was added to solvents A and B at 0.1 % (*v*/*v*) and solvent B at 0.08 % (*v*/*v*). The mobile phase eluded at 1 mL/min beginning at 70 % solvent A and 30 % solvent B, increasing to 90 % solvent B and 10 % solvent A from 0 to 21 min. From 21.1 to 30 min, a 100 % solvent C phase was utilized to detect any remaining proteinaceous material adhering to the small-pore column. Finally, the column was re-equilibrated to its starting composition of 70 % solvent A and 30 % solvent B from 30.1 to 35 min.

Hydrogel Tablet Fabrication

PEGDMA tablets were prepared as follows: Hydrogel tablets were made by pipetting 200 μL of a pre-polymer solution containing 50 % PEGDMA, 42 % PBS, and 8 % photoinitiator (*v*/*v*) into wells of a 48-well plate. The pre-polymer solution was cross-linked for 5 min at 99 J and 365 nm in a UV-box (UVITEC, CL508-BL, UK) and then placed in a vacuum oven for 60 min at 50°C, forming hydrogel tablets. After dehydrating, the tablets were removed from their wells and placed into a loading buffer. After 96 h of loading at 23°C, the tablets were rinsed with PBS, and the wash was collected and tested for API presence. After rinsing, the tablets were placed into 3 mL of elution buffer (fresh PBS) in 5 mL microfuge tubes and shaken at 37°C. Every 72 h, the full volume of liquid was removed from the tubes, analyzed using HPLC, and replaced with 3 mL of fresh PBS.

RESULTS AND DISCUSSION

Characterization of the release kinetics proved challenging due to multiple factors, particularly interactions between the hydrogel components and the APIs. LogP values are routinely used to characterize the relative hydrophobicities of analytes. The octanol–water partition coefficient of the components, logP values, resulted in 7.8× 10^8-fold range of partitioning. Separation of PEGDMA (-4.8_{logp}) from DMPA ($+3.068_{logp}$) was readily obtained with isocratic water/acetonitrile mobile phases. However, isolating FBSA (-0.395_{logp}) from VPY (-0.179_{logp}) proved more difficult.

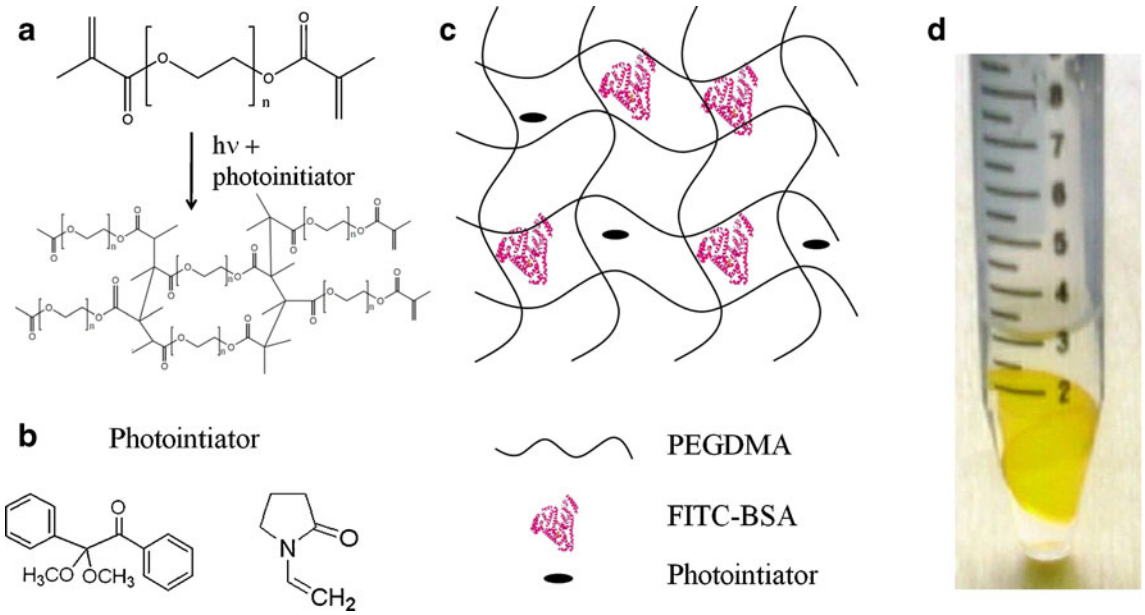
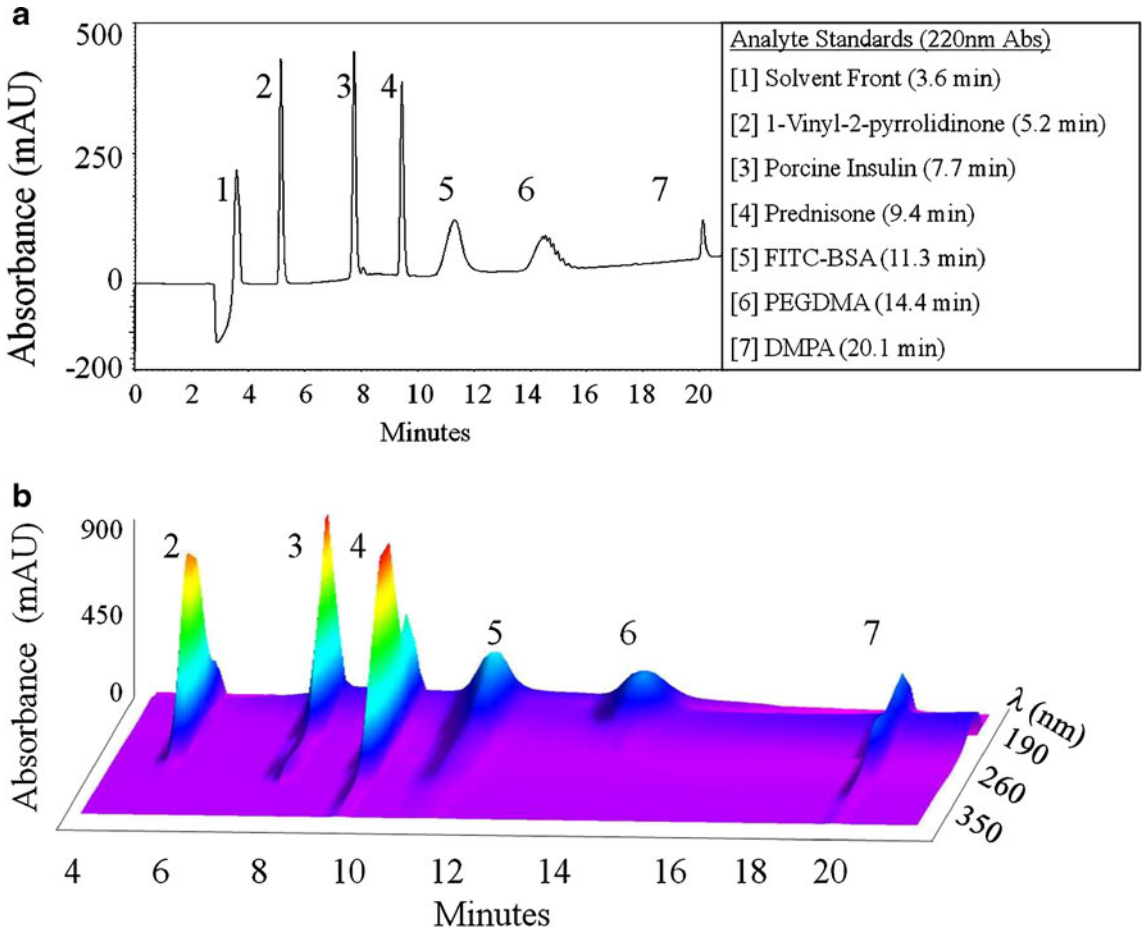


Fig. 1. Hydrogel components and structure. **a** PEGDMA is UV-irradiated in the presence of a photoinitiator resulting in a cross-linked polymer network. **b** Photoinitiator components: (*left*) DMPA and (*right*) VPY. **c** Schematic of the protein and small molecule-loaded hydrogel and **d** image of a FBSA-loaded hydrogel tablet eluting into buffer



c	Molecular Weight (kDa)	log(P)	Retention Time (min)	Acetonitrile in Mobile Phase (%)
1-vinyl-2-pyrrolidinone	0.11	-0.179	5.2	45.6
Porcine Insulin	5.8	0.218**	7.7	53.1
Prednisone	0.4	1.46	9.4	58.2
FITC-BSA	66	-0.395	11.3	64.5
PEGDMA	32*	-4.8	14.4	73.2
DMPA	0.26	3.068	20.1	90

Fig. 2. **a** Chromatograph of all components at λ=220 nm with retention times, **b** three-dimensional plot of APIs and hydrogel components across UV wavelengths, and **c** chemical properties of the CDDS components. (*) Mw for PEGDMA. (**) Based on Kyte and Doolite hydrophobicity estimate

The separation efficacy of assorted components of the hydrogel system is displayed in Fig. 2a. The peaks in time order were: (1) solvent front (RT=3.6 min), (2) VPY (RT=5.2 min), (3) PI (RT=7.7 min), (4) PSE (RT=9.4 min), (5) FBSA (RT=11.3 min), (6) PEGDMA (RT=14.4 min), and (7) DMPA (RT=20.1 min). The retention times shown with all components present were in agreement with retention times of components run individually. The three-dimensional plot (Fig. 2b) exhibits baseline resolution for each peak across all wavelength absorbances. While each component has well-resolved peaks to the baseline at the 220 nm channel, three-dimensional plotting aids in corroborating entity identification. The chemical properties, retention times, and mobile phase composition at RT are shown in Fig. 2c. The sensitivity limits and linear calibration ranges of the method are plotted in Fig. 3. The maximum linear detection rages (with units of micrograms per milliliter) are comparable to many commonly used assays and are 2.5–500 for PEGDMA, 1–500 for FBSA, 1–200 for PI, 0.5–200 for PSE, 0.25–10 for VPY, and 1–250 for DMPA.

As a platform analytical technique, HPLC is readily capable of separating a diverse set of molecules in given parallel steps and allows for collection of individual components for further analysis. However, the high number of molecular entities within the system significantly increased the difficulty of developing a viable method. A variety of

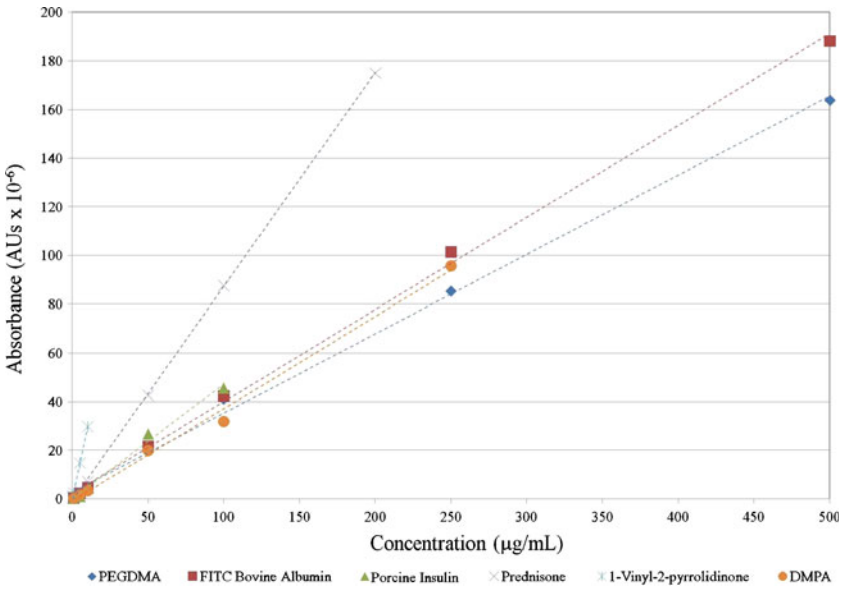


Fig. 3. Linear detection ranges. The sensitivity limits and maximum linear concentration of the analytes were (microgram-per-milliliter units): PEGDMA (*blue diamonds*, 2.5–500), FBSA (*red squares*, 1–500), PI (*green triangles*, 1–200), PSE (*purple Xs*, 0.5–200), VPY (*blue pluses*, 0.25–10), and DMPA (*orange circles*, 1–250)

HPLC columns and methods were attempted, including size exclusion, but ultimately, the long carbon chain and small pore size proved most adept. The developed method effectively separated all six components, and furthermore, additional entities can be isolated with modifications of mobile phase composition. The release kinetics of the APIs are displayed in Fig. 4 and were fit to equations of mass released=100 %×(1-exp($t/t_{1/2}$)) for each respective API. The delivery half-lives and total mass eluted for porcine insulin, FBSA, and prednisone are 0.1, 1.7, and 3.6 days and 11.9, 13.9, and 64.8 µg, respectively. No apparent trend in release rate *versus* molecular weight was seen; however, PSE was found to have improved device loading *versus* PI and FBSA. Ultimately, the hydrogel itself is envisioned to tune the release of therapeutics with rapid feedback from our single-pass technique.

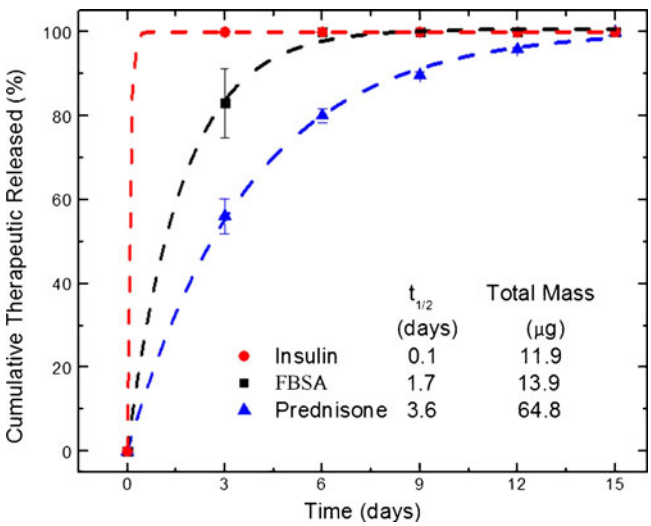


Fig. 4. Release profiles of the model APIs with exponential time constants ($t_{1/2}$) and total mass released

As a model CDDS platform, the studied compounds provide a general approach for analysis of a photo-crosslinkable PEGDMA device. Substituting other model APIs into each compound class would provide a breadth of tunable prototypes for controlled release, biodegradation, and morphological effect studies. In a similar fashion, the analysis method can be tuned for these substitutions with varying percentages of mobile phase during chromatography. The method presented utilized a UV detector to identify the isolated components which allowed for unique fingerprinting of the APIs and wavelength selection to achieve optimum detection sensitivity. However, the UV detector can be replaced or used in tandem with a refractive index detector to characterize systems and APIs without chromophores.

CONCLUSION

The need for a single-pass, quantitative analysis of multi-agent APIs for combination therapies is a growing demand in the field of drug delivery systems. As the number of emerging scaffolds and therapeutics increases, so does the need to develop efficient characterization techniques to aid in CDDS development. The method presented exemplifies an approach to this challenge, providing an efficient separative and quantitative means to analyze a PEG-based delivery device and kit of model therapeutics. Further versatility was achieved by resolving device components used in scaffold fabrication which is important to the clinical application of the CDDS. Total characterization in a single-injection HPLC technique presents a valuable and productive resource for affording innovative solutions in CDDS evolution.

ACKNOWLEDGMENTS

The authors thank Drs. Christopher Drake, Hariharasudhan Chirra, Vincent Venditto, and Barbara Des Rochers for their careful review and discussion of the manuscript. This

work was partially supported by grants from NIH HL090523, NSF EEC-0914790, and the California Institute for Regenerative Medicine.

REFERENCES

1. Health NIo. U.S. National Library of Medicine. 2008 September 1, 2008.
2. Greco F, Vicent MJ. Combination therapy: opportunities and challenges for polymer-drug conjugates as anticancer nanomedicines. Adv Drug Deliv Rev. 2009;61(13):1203–13.
3. Yamanaka K, Nakahara T, Yamauchi T, Kita A, Takeuchi M, Kiyonaga F, *et al.* Antitumor activity of YM155, a selective small-molecule survivin suppressant, alone and in combination with docetaxel in human malignant melanoma models. Clin Cancer Res. 2011;7:2011.
4. Tang W, Lu Y, Tian Q-Y, Zhang Y, Guo F-J, Liu G-Y, *et al.* The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice. Science. 2011;10:2011.
5. Abou-Nassar K, Brown J. Novel agents for the treatment of chronic lymphocytic leukemia. Clin Adv Hematol Oncol. 2010;8 (12):886–95.
6. Smith DR, Weinstock-Guttman B, Cohen JA, Wei X, Gutmann C, Bakshi R, *et al.* A randomized blinded trial of combination therapy with cyclophosphamide in patients with active multiple sclerosis on interferon beta. Mult Scler. 2005;11(5):573–82.
7. Landewé RBM, Boers M, Verhoeven AC, Westhovens R, van de Laar MAFJ, Markusse HM, *et al.* COBRA combination therapy in patients with early rheumatoid arthritis: long-term structural benefits of a brief intervention. Arthritis Rheum. 2002;46 (2):347–56.
8. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med. 1998;339(21):1485–92.
9. Lin C-C, Anseth K. PEG hydrogels for the controlled release of biomolecules in regenerative medicine. Pharm Res. 2009;26 (3):631–43.
10. Falco E, Wang M, Thompson J, Chetta J, Yoon D, Li E, *et al.* Porous EH and EH-PEG scaffolds as gene delivery vehicles to skeletal muscle. Pharm Res. 2011;28(6):1306–16.
11. Falco EE, Roth JS, Fisher JP. EH networks as a scaffold for skeletal muscle regeneration in abdominal wall hernia repair. J Surg Res. 2008;149(1):76–83.
12. Oss-Ronen L, Seliktar D. Polymer-conjugated albumin and fibrinogen composite hydrogels as cell scaffolds designed for affinity-based drug delivery. Acta Biomater. 2011;7(1):163–70.
13. Zustiak SP, Boukari H, Leach JB. Solute diffusion and interactions in cross-linked poly(ethylene glycol) hydrogels studied by fluorescence correlation spectroscopy. Soft Matter. 2010;6 (15):3609–18 [Article].
14. Zustiak SP, Leach JB. Characterization of protein release from hydrolytically degradable poly(ethylene glycol) hydrogels. Biotechnol Bioeng. 2011;108(1):197–206.
15. Bae M, Gemeinhart RA, Divan R, Suthar KJ, Mancini DC. Fabrication of poly(ethylene glycol) hydrogel structures for pharmaceutical applications using electron beam and optical lithography. J Vac Sci Technol B. 2010;28(6):C6P24–9 [Proceedings paper].
16. De Silva DA, Hettiarachchi BU, Nayanajith LDC, Milani MDY, Motha JTS. Development of a PVP/kappa-carrageenan/PEG hydrogel dressing for wound healing applications in Sri Lanka. J Natl Sci Found Sri Lanka. 2011;39(1):25–33 [Article].
17. Leach JB, Schmidt CE. Characterization of protein release from photocrosslinkable hyaluronic acid-polyethylene glycol hydrogel tissue engineering scaffolds. Biomaterials. 2005;26(2):125–35 [Article].
18. Ayala P, Lopez JI, Desai TA. Microtopographical cues in 3D attenuate fibrotic phenotype and extracellular matrix deposition: implications for tissue regeneration. Tissue Eng A. 2010;16 (8):2519–27.
19. Scott RA, Peppas NA. Highly crosslinked, PEG-containing copolymers for sustained solute delivery. Biomaterials. 1999;20 (15):1371–80 [Article].
20. Liu B, Lee K-W, Li H, Ma L, Lin GL, Chandraratna RAS, *et al.* Combination therapy of insulin-like growth factor binding protein-3 and retinoid X receptor ligands synergize on prostate cancer cell apoptosis *in vitro* and *in vivo*. Clin Cancer Res. 2005;11 (13):4851–6.
21. Vilar Gómez EGOB, Arús Soler E, Ruenes Domech C, Dávila González Y. Sequential combination therapy with prednisone, lamivudine and interferon alfa-2b for HBeAg-positive chronic hepatitis B. Gastroenterol Hepatol. 2006;29(9):534–41.
22. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol. 1982;157 (1):105–32.
23. Chen HQ, Wang L, Wang LY, Wang GF, Li L, Xu FG. Preparation of novel composite nanoclusters and their application in the ultrasensitive detection of proteins. Anal Chim Acta. 2004;521 (1):9–15 [Article].
24. Leca-Bouvier B, Blum LJ. Biosensors for protein detection: a review. Anal Lett. 2005;38(10):1491–517.
25. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, *et al.* Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150(1):76–85 [Article].
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72 (1–2):248–54.
27. Kurfurst MM. Detection and molecular-weight determination of polyethylene glycol-modified hirudin by staining after sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. Anal Biochem. 1992;200(2):244–8 [Article].