ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 209–215

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Rapid microtiter plate assay for determination of inulin in human plasma and dialysate

Thomas D. Nolin [a], Ivan V. Colaizzi [a], Paul M. Palevsky [b,c], Gary R. Matzke [a], Reginald F. Frye [a,*]

[a] *Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[b] *Renal-Electrolyte Division, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[c] *VA Pittsburgh Healthcare System, Pittsburgh, PA 15240, USA*

Received 1 March 2001; accepted 14 September 2001

Abstract

A rapid, sensitive, and reproducible microtiter plate assay for the determination of inulin in human plasma, dialysate, and phosphate-buffered saline (PBS) was developed. Plasma or PBS samples (100 μl aliquots) were prepared by the addition of indole-3-acetic acid (150 μl) and HCl (3 ml) and then briefly vortex-mixed. Samples were then incubated in a 60 °C water bath for 20 min, cooled in a room temperature water bath for 40 min, then diluted with deionized, distilled water (DDW; 3 ml) and again vortex-mixed. Finally, an aliquot (200 μl) of each sample was transferred to a 96-well microtiter plate and read spectrophotometrically at 490 nm. Dialysate samples were processed in a similar manner, but required an initial enzymatic step in order to remove dextrose and minimize assay interference. Samples (100 μl aliquots) were prepared by the addition of glucose oxidase/catalase solution (100 μl), briefly vortex mixed, and then incubated in a 37 °C water bath for 60 min, samples were then reacted with indole-3-acetic acid as before. Calibration curves were linear over the concentration range of 0.5–4 mg/ml or 0.025–0.4 mg/ml for plasma or PBS and dialysate, respectively; correlation coefficients (r^2) were >0.99. The intra- and inter-day coefficients of variation in plasma, PBS, and dialysate were $<15\%$. This method is well suited for the rapid analysis of large numbers of samples and is currently being used for in vitro investigations of solute removal by hemodialysis.

Keywords: Microtiter plate; Spectrophotometry; Colorimetric assay; Inulin; Hemodialysis

1. Introduction

Inulin is a polyfructosan that has most commonly been used to determine glomerular filtration rate but is also useful for the determination of solute removal by various dialytic modalities that are utilized both clinically and experimentally [1]. Inulin has a molecular weight of 5200 Da,

* Corresponding author. Present address: Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, 807 Salk Hall, Pittsburgh, PA 15261, USA. Tel.: +1-412-624-4683; fax: +1-412-383-7426.
E-mail address: rfrye+@pitt.edu (R.F. Frye).

0731-7085/02/$ - see front matter
PII: S0731-7085(01)00643-4

making it useful experimentally as a surrogate marker for the removal of 'middle-molecules' (i.e. compounds in the molecular weight range of ~500–5000 Da) [1,2]. Notably, the relative efficiency of removal of middle-molecules has been associated with mortality in hemodialysis patients [3].

There are more than 100 hemodialysis filters available in the US that differ with respect to membrane composition (e.g. polysulfone, polymethylmethacrylate, etc.) and membrane surface area [4]. While hemodialysis filters are generally characterized for removal of standard compounds such as creatinine (MW = 113), blood urea nitrogen (MW = 60), and vitamin B_{12} (MW = 1355), there is little to no information available for most of the filters regarding their relative efficiency in the removal of other important endogenous (e.g. β2-microglobulin) or exogenous (e.g. drugs) compounds. An additional aspect is that in many hemodialysis centers, filters are reused (as a cost-saving measure) after being processed by methods that use heat or chemicals (e.g. formaldehyde or bleach). The reprocessing methods have been shown to affect dialyzer performance and the clearance or removal of drugs or other compounds; solute removal may be differentially affected based on molecular weight [5]. Thus, several groups have proposed that in vitro and ex vivo hemodialysis techniques be used to investigate common clinical hemodialysis issues, including dialyzer compatibility [6], solute clearance characteristics [7], hemolysis [8], and the effects of filter reprocessing [1]. An important advantage is that these in vitro evaluations can be done without the need to expose patients to unneeded drugs or chemicals. Also, these studies can allow for more rigorous evaluations of factors (e.g. dialysis and blood flow rates) that impact dialyzer performance under carefully controlled conditions. The rigorous nature of these studies requires an efficient method of analysis due to the large numbers of samples that are generated.

Many of the methods for quantifying inulin that have been reported are colorimetric assays that have been criticized for being inaccurate and also dangerous due to the use of caustic agents [9]. Enzymatic methods for the determination of inulin have also been reported [10–15], but these methods require specialized and costly equipment [14]. High-performance liquid chromatographic (HPLC) methods have been described [16,17], but these methods also require specialized and costly laboratory equipment, and more importantly require several minutes per sample for analysis.

Due to their cost, time, and/or labor intensive nature, it would be impractical to use any of the aforementioned analytical methods for the quantitation of inulin in a research setting in which large numbers of samples are being generated. The ideal method would be inexpensive and have a rapid turn-around time while not sacrificing accuracy and reproducibility. Thus, we describe a sensitive, reproducible, and efficient method for determining inulin in plasma, phosphate-buffered saline (PBS), and dialysate.

2. Experimental

2.1. Materials

Inulin 100 mg/ml in NaCl injection, USP, was obtained from the Cypros Pharmaceutical Corporation (Carlsbad, CA, USA). Indole 3-acetic acid, glucose oxidase, and catalase were purchased from Sigma (St. Louis, MO, USA). Hydrochloric acid (HCl) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized, distilled water (DDW) was acquired from a Barnstead Nanopure purification system (Barnstead, Boston, MA, USA). Human plasma was obtained from the Central Blood Bank, Pittsburgh, PA. PBS was used as a simulated protein-free blood solution. Polysulfone dialyzers (F40; 0.65 m^2; $K_{uf} = 20$ ml/h mmHg; Fresenius Inc, Walnut Creek, CA), standard hemodialysis blood tubing sets (8 mm ID; Readyset, Medisystems Corp., Seattle, WA), and Nephrosol RS-225 acetate concentrate (Minntech, Minneapolis, MN, USA) were used in the in vitro dialysis experiments. Flat bottom, polystyrene, 96-well microplates (Costar) were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Equipment

In vitro hemodialysis was performed using a Baxter 550 hemodialysis controller (Baxter, Deerfield, IL). Dialysate was prepared from concentrate in a 1:34 dilution using a Baxter Extra Pure reverse osmosis system (Baxter). The optical density of all samples was measured utilizing a Victor^2 1420 multilabel counter (Wallac, Turku, Finland) set at 490 nm.

2.3. Preparation of stock solutions and spiked standards

Stock inulin solutions of 1.0 and 10.0 mg/ml were made in DDW. Standards and quality control (QC) samples were made by the addition of the specified quantity of stock inulin solution to drug free plasma, PBS, or acetate concentrate diluted 1:34. A stock solution of 50 mM indole 3-acetic acid was prepared by dissolving 493 mg of sample in DDW and brought up to a volume of 50 ml. A stock enzyme solution of glucose oxidase 100 U/ml and catalase 1000 U/ml was also prepared. About 105.3 mg glucose oxidase (47.5 U/mg solid) and 4.67 mg catalase (10 700 U/mg solid) were dissolved in K_2HPO_4 (0.5 M, pH 7.0) and brought up to a volume of 50 ml. Stock solutions were stored at 4 °C, and standards and QC samples were made daily.

2.4. Procedure for determination of inulin

Aliquots (100 μl) of plasma (diluted 1:10) or PBS samples were added to disposable glass tubes (16 × 125), followed by the addition of indole-3-acetic acid (50 mM; 150 μl) and HCl (12.1 N; 3 ml) and then briefly vortex-mixed. Samples were then incubated in a 60 °C water bath for 20 min. After cooling in a room temperature water bath for 40 min, samples were diluted with DDW (3 ml) and again briefly vortex-mixed. Lastly, an aliquot (200 μl) of each sample was transferred to a 96-well microtiter plate and read spectrophotometrically at 490 nm.

Processing of dialysate samples was similar to plasma and PBS, but also required an initial enzymatic incubation step in order to remove dextrose and eliminate interference with the assay. The dextrose concentration in dialysate is 200 mg/dl; the concentration in human plasma is typically half, about 100 mg/dl. Samples (100 μl aliquots) were prepared by the addition of a solution of glucose oxidase 100 U/ml and catalase 1000 U/ml (100 μl), briefly vortex mixed, then incubated in a 37 °C water bath for 60 min. After removal from the bath, indole-3-acetic acid (50 mM; 150 μl) and HCl (12.1 N; 3 ml) were added, briefly vortex-mixed, incubated at 60 °C for 20 min, then cooled at room temperature for 40 min. Dialysate was not diluted. Lastly, 200 μl aliquots of each sample were transferred to a 96-well microtiter plate and read spectrophotometrically at 490 nm.

2.5. Linearity

Calibration curves were constructed using six standard concentrations of inulin in plasma, PBS, and dialysate and were run in duplicate. Curves were obtained daily for 3 days. Standard concentration ranges in plasma and PBS were 0.5–4.0 mg/ml inulin. The standard concentration range in acetate dialysate was 0.025–0.4 mg/ml inulin. Individual standard concentrations in plasma, PBS, and dialysate are shown in Tables 1–3, respectively.

2.6. Precision and accuracy

The precision and accuracy of the assay were determined based on analysis of plasma, PBS, and dialysate QC samples. QC sample concentrations for inulin were 0.75, 2.0, and 3.5 mg/ml in plasma and PBS. QC concentrations in dialysate were 0.03, 0.15, and 0.35 mg/ml. Six replicate QC samples at each concentration were analyzed on 2 consecutive days, followed by analysis of 12 replicate QC samples at each concentration on the third day, after which intra- and inter-day means, standard deviations (S.D.), and relative standard deviations (R.S.D.) were calculated. QC samples were subjected to three freeze-thaw cycles (-80 °C to room temperature) to evaluate inulin stability.

2.7. In vitro dialysis procedure

In vitro dialysis was performed in a manner that simulates the procedure used in vivo. Six liters of simulated plasma was prepared with phosphate buffered saline and spiked with inulin to a concentration of 3 mg/ml. Dialysis was performed continuously for 45 min with three discrete 15 min dialysate collections. The simulated plasma flow rate was maintained at 300 ml/min and the ultrafiltration rate was maintained at 0 l/h throughout. Dialysate flow rate was maintained at 500 ml/min. The volume of dialysate effluent generated during each 15 min collection interval was measured. Samples were drawn simultaneously from the arterial and venous sides of the dialyzer at the beginning and end of each collection interval, and a sample of effluent was obtained from each dialysate collection.

2.8. Calculations

Dialytic clearance was determined by two methods. Eq. (1) depicts calculation of the extraction or clearance of inulin from plasma or simulated plasma (i.e. PBS; CL_P):

$$CL_P = Q\left[\frac{C_A - C_V}{C_A}\right] \quad (1)$$

where, Q is the flow of simulated plasma, C_A is the concentration of simulated plasma in the arterial side (i.e. entering the dialyzer) and C_V is the concentration of simulated plasma in the venous side (i.e. exiting the dialyzer). Clearance was also determined by calculating the amount recovered in dialysate (CL_D; Eq. (2)):

$$CL_D = \frac{V_D C_D}{AUC} \quad (2)$$

where, V_D is the volume of dialysate collected during the interval, C_D is the concentration of dialysate, and AUC is the area under the plasma or simulated plasma concentration–time curve of the intervals during the hemodialysis procedure.

3. Results and discussion

The method we report demonstrates the successful adaptation of similar colorimetric and enzymatic methods to a microtiter plate format, which substantially improves sample throughput. Calibration curves generated using linear least-

Table 1
Intra- and inter-day precision and accuracy in plasma

	Concentration (mg/ml)		%R.S.D.	%Bias
	Added	Observed (mean ± S.D.)		
Intra-assay reproducibility[a]				
Quality controls	0.75	0.72 ± 0.03	4.0	−4.6
	2	2.04 ± 0.03	1.7	2.0
	3.5	3.55 ± 0.09	2.4	1.5
Inter-assay reproducibility[b]				
Quality controls	0.75	0.72 ± 0.03	4.0	−4.1
	2	2.10 ± 0.08	3.9	4.9
	3.5	3.60 ± 0.12	3.2	2.7
Standards	0.5	0.55 ± 0.02	4.2	9.5
	0.75	0.76 ± 0.01	1.9	1.9
	1	0.97 ± 0.03	3.2	−2.9
	2	1.93 ± 0.05	2.6	−3.6
	3	2.85 ± 0.14	4.9	−4.9
	4	3.54 ± 0.13	3.8	−11.5

[a] Six to twelve QC samples per concentration.
[b] Six to twelve QC samples or two standards per day per concentration for 3 days.

Table 2
Intra- and inter-day precision and accuracy in PBS

	Concentration (mg/ml)		%R.S.D.	%Bias
	Added	Observed (mean ± S.D.)		
Inter-assay reproducibility[a]				
Quality controls	0.75	0.78 ± 0.04	4.8	3.4
	2	2.09 ± 0.04	1.9	4.5
	3.5	3.56 ± 0.08	2.4	1.6
Inter-assay reproducibility[b]				
Quality controls	0.75	0.76 ± 0.04	4.7	1.4
	2	2.13 ± 0.07	3.3	6.4
	3.5	3.58 ± 0.10	2.7	2.3
Standards	0.5	0.49 ± 0.03	5.2	−1.6
	0.75	0.73 ± 0.03	4.3	−3.0
	1	0.95 ± 0.04	4.7	−4.8
	2	1.92 ± 0.03	1.5	−4.2
	3	2.77 ± 0.08	2.8	−7.5
	4	3.52 ± 0.11	3.1	−12.1

[a] Six to twelve QC samples per concentration.
[b] Six to twelve QC samples or two standards per day per concentration for 3 days.

squares regression were linear over the concentration ranges evaluated in plasma, PBS, and dialysate with all correlation coefficients (r^2) > 0.992. The intra- and inter-day %R.S.D. in plasma (Table 1), PBS (Table 2), and dialysate (Table 3) ranged from 17.6 to 1.5%. The intra- and inter-day% bias ranged from 9.5 to −12.1%. Inulin samples were stable through three freeze-thaw cycles.

Most of the methods for the determination of inulin reported during the last 50 years are colorimetric assays that require hydrolysis of inulin in concentrated acid to form fructose [9]. Fructose is then reacted with compounds such as anthrone, resorcinol, cysteine/tryptophan, diphenylamine, or indole-3-acetic acid to yield a colored product that is measured spectrophotometrically [14,16]. Many of these assays are time and labor intensive, and prone to interference from substances such as endogenous glucose [9]. Simplified methods for the determination of inulin have also been reported. Some of these methods utilize enzymes such as inulinase rather than acids to hydrolyze inulin to fructose [10–15]. These enzymatic methods are more sensitive than classical colorimetric assays, avoid the use of caustic reagents and may be automated [14]. The latter is not easily done and requires the use of costly and specialized laboratory equipment, however. HPLC methods have been developed that essentially eliminate glucose interference, exhibit improved sensitivity, and may be automated [16,17]. However, they also are time consuming due to the fact that samples must be analyzed individually with run-times of at least 8 min each [16]. In contrast, the method we report allows for the analysis of nearly 80 samples (40 if done in duplicate) with standards and QCs in less than 3 h.

A potential limitation of our method is that carbohydrates, including endogenous glucose, could interfere and lead to erroneous plasma results if the enzymatic step is not performed. Although the concentration of glucose present in normal human plasma (e.g. ∼100 mg/dl) did not affect assay performance, it is conceivable that plasma samples obtained from diabetic patients, in whom glucose concentrations can rise > 200 mg/dl, may interfere. Samples from hyperglycemic patients should be processed using the additional enzymatic step required with dialysate samples.

3.1. Application to hemodialysis clearance studies

Inulin can be used as a surrogate marker for the removal of 'middle-molecules' [2]. Our laboratory is currently investigating factors that affect solute removal by hemodialyzers by evaluating the clearance of multiple prototypical substrates including inulin. Depending on the nature of the study, these in vitro dialysis experiments can be conducted using whole blood (expired blood obtained from the local blood bank) or a simulated matrix consisting of PBS with or without the plasma protein albumin. Thus, we have validated the assay in both plasma and PBS. Data generated from a single in vitro dialysis experiment in which clearance was calculated from the 'blood side' (Eq. (1)) and 'dialysate side' (Eq. (2)) are shown in Table 4. The inulin clearance values obtained are consistent with values previously reported [1].

Although several accurate methods for the determination of inulin have been reported, their utility for measuring inulin clinically in large numbers of samples is questionable due to their time and labor intensive nature. Our method is well suited for the analysis of large numbers of samples. Numerous samples are processed and subsequently measured in 96-well microtiter plates simultaneously. The benefits of using 96-well plates have been described previously [18]. Up to 96 plasma and/or PBS samples (including standards and QCs) are processed and measured in under 2 h with our method. Dialysate samples are processed and measured in under 3 h due to the additional enzymatic incubation step required.

This assay may also be useful for QC in the manufacturing or monitoring of dialyzers or ultrafiltration membranes. Since inulin is a surrogate marker for middle molecules, it could provide a useful functional index of middle molecule clearance by newly manufactured membranes. Similarly and perhaps more importantly, the assay could be applied in clinical hemodialysis units, where most patients undergo hemodialysis with reused dialyzers, to confirm performance characteristics of the dialyzers and assure patients are receiving optimal dialytic therapy.

In conclusion, the method described here is a rapid, sensitive, and reproducible assay for the determination of inulin in plasma, PBS, and dialysate with significant advantages over previously described methods. It is currently being used in investigations of dialytic solute removal.

Table 3
Intra- and inter-day precision and accuracy in dialysate

	Concentration (mg/ml)		%R.S.D.	%Bias
	Added	Observed (mean ± S.D.)		
Intra-assay reproducibility[a]				
Quality controls	0.03	0.033 ± 0.004	11.4	9.3
	0.15	0.163 ± 0.006	3.9	8.5
	0.35	0.370 ± 0.021	5.7	5.6
Inter-assay reproducibility[b]				
Quality controls	0.03	0.030 ± 0.004	14.2	1.4
	0.15	0.162 ± 0.010	6.4	7.8
	0.35	0.364 ± 0.020	5.4	4.0
Standards	0.025	0.025 ± 0.004	17.6	−1.8
	0.05	0.050 ± 0.004	8.1	0.4
	0.1	0.101 ± 0.003	2.8	1.2
	0.2	0.198 ± 0.010	4.9	−1.2
	0.3	0.302 ± 0.012	3.8	0.6
	0.4	0.400 ± 0.014	3.6	−0.1

[a] Six to twelve QC samples per concentration.
[b] Six to twelve QC samples or two standards per day per concentration for 3 days.

Table 4
Dialytic clearance of inulin from simulated plasma

Arterial-venous pair method (Eq. (1))					
Time (min)	Q (ml/min)	C_A (mg/ml)	C_V (mg/ml)	CL_P (ml/min)	Clearance (Mean ± S.D.)
0	300	2.795			
15	300	2.666	2.271	44.4	
30	300	2.271	2.038	30.8	
45	300	2.025	1.771	37.6	37.6 ± 6.8
Amount recovered method (Eq. (2))					
Time (min)	V_D (ml)	C_D (mg/ml)	AUC (mg min/ml)	CL_D (ml/min)	
0–15	8265	0.179	41.0	36.1	
15–30	8005	0.162	37.3	34.9	
30–45	7950	0.147	37.0	31.6	34.2 ± 2.4

Acknowledgements

This work was supported by the Department of Veterans Affairs Stars and Stripes Healthcare Network CPPF, the Dialysis Clinics, Inc., Paul Teschan Research Fund (Project # 1098-6), and the University of Pittsburgh Pharmacy Associates. The technical assistance of Cheryl Galloway is gratefully acknowledged.

References

[1] M.K. Scott, B.A. Mueller, K.M. Sowinski, Pharmacotherapy 19 (1999) 1042–1049.
[2] M.K. Scott, B.A. Mueller, K.M. Sowinski, W.R. Clark, Am. J. Kidney Dis. 33 (1999) 87–96.
[3] J.K. Leypoldt, A.K. Cheung, C.E. Carroll, D.C. Stannard, B.J. Pereira, L.Y. Agodoa, F.K. Port, Am. J. Kidney Dis. 33 (1999) 349–355.
[4] J.C. Van Stone, in: J.T. Daugirdas, T.S. Ing (Eds.), Handbook of Dialysis, Little, Brown and Company, Boston, 1994, pp. 30–52 Chapter 3.
[5] J.K. Leypoldt, A.K. Cheung, R.B. Deeter, Am. J. Kidney Dis. 32 (1998) 295–301.
[6] J. Himmelfarb, E. McMonagle, D. Holbrook, C. Toth, J. Lab. Clin. Med. 126 (1995) 392–400.
[7] G.R. Matzke, T.D. Nolin, R.F. Frye, D.A. Baritot, P.M. Palevsky, J. Am. Soc. Nephrol. 10 (1999) 193A–194A.
[8] M.C. Yang, C.C. Lin, ASAIO J. 46 (2000) 293–297.
[9] W.H. Waugh, Clin. Chem. 23 (1977) 639–645.
[10] C.P. Degenaar, L.A. Frenken, J.P. von Hooff, Clin. Chem. 33 (1987) 1070–1071.
[11] J. Delanghe, J. Bellon, M. De Buyzere, G. Van Daele, G. Leroux-Roels, Clin. Chem. 37 (1991) 2017–2018.
[12] H.F. Kuehnle, K. von Dahl, F.H. Schmidt, Nephron 62 (1992) 104–107.
[13] O. Sugita, Y. Tomiyama, T. Matsuto, M. Okada, F. Gejyo, M. Arakawa, S. Takahashi, Y. Watazu, N. Kaneda, Ann. Clin. Biochem. 32 (Pt 6) (1995) 561–565.
[14] A.L. Summerfield, G.L. Hortin, C.H. Smith, T.R. Wilhite, M. Landt, Clin. Chem. 39 (1993) 2333–2337.
[15] E.C. Wong, T.R. Wilhite, R. Miller, C.H. Smith, M. Landt, Clin. Chem. 40 (1994) 1788–1789.
[16] R. Dall'Amico, G. Montini, L. Pisanello, G. Piovesan, S. Bottaro, A.T. Cracco, G. Zacchello, F. Zacchello, J. Chromatogr. B Biomed. Appl. 672 (1995) 155–159.
[17] T.I. Ruo, Z. Wang, M.S. Dordal, A.J. Atkinson Jr, Clin. Chim. Acta 204 (1991) 217–222.
[18] R. Oehler, B. Hefel, E. Roth, Anal. Biochem. 241 (1996) 269–271.