ELSEVIER

Journal of Chromatography A, 743 (1996) 195–206

JOURNAL OF CHROMATOGRAPHY A

Enzyme-linked immunosorbent assays in a chromatographic format

Margaret A. Johns*, Laura K. Rosengarten, Martha Jackson, Fred E. Regnier

PerSeptive Biosystems, Inc., Framingham, MA 01701, USA

Abstract

This paper describes the execution of enzyme-linked immunosorbent assays (ELISA), (i) in immunosorbent columns with antibodies immobilized on porous particles, (ii) where samples and reagents are metered by valves and syringe pumps, (iii) samples, reagents, substrate, and wash buffers are transported into or through the system by high pressure liquid chromatography pumps, and (iv) enzyme reaction product is detected by absorbance. The normal protocol used for ELISA was modified in that antigen was complexed with enzyme conjugated antibody in the autosampler and an aliquot introduced into the system where it was transported to the immunosorbent and captured. Substrate was subsequently pumped into the immunosorbent bed and the product swept to an absorbance detector for quantitation.

Keywords: Enzyme-linked immunosorbent assays; Immunoassays; Sorbents; Hormones

1. Introduction

It has been shown that an enzyme-linked immunosorbent assay (ELISA) [1] may be carried out in open tubular chromatography columns in which the walls of the fused-silica capillary column are derivatized with antibodies directed against the antigen [2]. One advantage of this system is that antigen can be concentrated from samples many times the volume of the column by capture at the capillary walls. A second advantage is that the liquid volume of capillary systems is so small that product formed during the enzyme amplification phase of the assay is released into a very much smaller volume of liquid than in a conventional microtiter well ELISA [3]. Liquid volumes of capillary ELISA systems are generally 3–4 orders of magnitude smaller than a microtiter well ELISA. Since the rate of product formation is independent of system volume, this means that product concentration will rise to detectable levels much faster in capillary ELISA. A third advantage is that the surface area to volume ratio of capillaries is larger than that of titer wells. This means that more capture antibody per unit volume of solution may be used with capillary systems, accelerating the rate of antigen capture. Finally, a fourth advantage is that diffusion distances to the capture antibody are much smaller in capillaries than microtiter wells. Microtiter wells are generally 0.5–1.0 cm in diameter whereas capillaries are 100 μm or less. Taken together, these advantages allow lower detection limits with immunoglobin G of 50 fg, i.e. $3\cdot10^{-19}$ mol, in 10–20 min with the capillary ELISA system. It is estimated that antigen concentration in capillary columns is approximately 10^{-12} *M* [2].

Although capillary ELISA is superior to the conventional approach for the reasons given above, it still has limitations. One is that diffusion distances to the capture antibody are still sufficiently large that a few minutes are required for antigen to diffuse to the capillary wall. The same is true with capture of the second antibody. A second problem is that higher concentrations of enzyme conjugated second antibody must be used to accelerate binding of the

*Corresponding author.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
PII S0021-9673(96)00370-6

second antibody. A third problem, common to all ELISA systems, is that the binding constant, and therefore the capture rate constant, of the enzyme conjugated second antibody can be low. To overcome this problem, higher concentrations of second antibody are often used to achieve binding in a shorter time. This causes "non-specific binding" of the enzyme-labeled second antibody to increase with concomitant increases in background. Non-specific binding is a significant issue because the lower detection limit of ELISA is generally determined by background arising from non-specifically bound enzyme.

The objective of these studies was to examine systems of still higher surface area to volume ratio and smaller diffusion distances. The system chosen for study was a column packed with perfusible chromatography particles in which capture antibodies had been immobilized on the surface of support particles. It would be anticipated that these higher surface area systems would have even greater non-specific binding problems. This issue was dealt with by altering the format of the ELISA. Antigen was complexed with enzyme labeled second antibody external to the column and subsequently captured in the immunosorbent column where the enzyme amplification and detection portions of the assay were executed. This allows lower concentrations of the second antibody to be used, longer equilibration time, and a homogeneous antigen:antibody complexation reaction.

2. Materials and methods

2.1. Reagents

Ortho-phenylenediamine tablets were purchased from Sigma (St. Louis, MO, USA). Human serum was supplied by Scantibodies Laboratory (Santee, CA, USA). The Medix Ultrasensitve Thyroid Stimulating Hormone (U-TSH) Kit KIF4095 and the Total Human Chorionic Gonadotropin (hCG) Enzyme Immunoassay Test Kit KIF4011 were obtained from Medix Biotech (San Carlos, CA, USA). Murine anti-hCG antibody conjugated to horseradish peroxidase (HRP), anti-hCG murine monoclonal antibody from clone M94138, recombinant TSH and anti-TSH murine monoclonal antibody from clone M94206 were purchased from Fitzgerald Industries International (Concord, MA, USA). HRP conjugated anti-TSH antibody was obtained from the Enzymun-Test TSH (Boehringer Mannheim, Indianapolis, IN, USA). Sheep antibody to fluorescein isothiocyanate (FITC) was provided by PerSeptive Diagnostics (Cambridge, MA, USA). The Poros AL and Poros B/A materials were supplied by PerSeptive Biosystems (Framingham, MA, USA).

2.2. Instrumentation

Assays were run on an Integral Micro-Analytical Workstation (PerSeptive Biosystems) using mini-cartridge immunosorbent columns (20×1 mm).

2.3. TSH assay

The solid-phase antibody, murine monoclonal clone M94206 (Fitzgerald Industries International) was biotinylated by standard methods and coupled to a Poros B/A cartridge (20×1 mm). The HRP conjugated antibody (from the Enzymun-Test TSH, Boehringer Mannheim) was diluted 1:10 or 1:20 (≥0.5 IU/ml peroxidase activity) in running buffer for use. Recombinant TSH (Medix Biotech) was used as standard. Other reagents were:

Running Buffer:	10 m*M* phosphate buffered saline pH 7.2 with 1% bovine serum albumin (BSA) and 0.1% Zwittergent 3-12
Substrate:	Ortho-phenylenediamine tablets (Sigma)
Substrate diluent:	25 m*M* citric acid+50 m*M* Na_2HPO_4 pH 5.0 with 0.02% sodium perborate
Regeneration reagent:	12 m*M* HCl pH 1.9 with 150 m*M* NaCl and 0.1% Zwittergent 3-12

Standards and controls were made in lipid stripped TSH free pooled human serum (Scantibodies Laboratory) and frozen in 100 μl aliquots for storage. Human specimens covering hypo-, normal and hyperthyroid ranges were purchased from a local reference laboratory. Assay results were compared to those from the Medix Ultrasensitve TSH (U-TSH) Kit KIF4095 (Medix Biotech). Samples, controls and standards were premixed with HRP labeled antibody at a 2:1 sample to antibody ratio and preincubated

for 10 min before injection of 50 μl onto the cartridge.

2.4. hCG assay

This assay used a universal cartridge format with sheep anti-FITC covalently linked to Poros AL as the solid-phase. Anti hCG murine monoclonal clone M94138 (Fitzgerald Industries) was labeled with FITC at a ratio of 3 FITC:1 antibody. Another murine anti hCG antibody conjugated to HRP (Fitzgerald Industries) was used as detector antibody. Other reagents were the same as for the TSH assay except that the running buffer BSA concentration was lowered to 0.1%. Standards and controls were made in lipid stripped hCG free pooled human serum (Scantibodies Laboratory) and frozen in 100 μl aliquots for storage before use. For correlation assays, results were compared to the Total hCG Enzyme Immunoassay Test Kit KIF4011 (Medix Biotech). Samples, controls and standards were premixed with both antibodies at a 1:1:1 ratio and preincubated for 1 h before injection of 100 μl onto the anti-FITC cartridge.

3. Results

Human thyroid stimulating hormone (TSH) and human chorionic gonadotropin (hCG) are both present at low concentrations in serum and are measured in clinical laboratories by immunoassays. These analytes were chosen for this study because they are either found in low concentration in serum or vary widely in concentration and commercial kits are available which can be used for comparison with the chromatographic ELISA method being examined here.

3.1. Assay format

Automation of immunological assays is frequently achieved in a robotic system where reagents are pipetted into a large number of incubation vessels in parallel, individual reagent and washing steps generally involve incubation, and final measurements of concentration are generally achieved in the incubation vessel [4]. After a single use, the reaction vessel with antibody is discarded. In contrast, assays in this study were achieved in a serial processing mode in which (i) the immunosorbent cartridge is recycled after each use, (ii) reagents and washing buffers are continuously pumped into the immunosorbent cartridge, i.e. when reagents are not being added the system defaults to a flow of washing buffer, (iii) reagents are metered with valves in the flow train, (iv) reaction time is controlled with flow-rate through the immunosorbent bed, (v) antigen is complexed with the enzyme conjugated antibody external to the immunosorbent and (vi) reaction product from the ELISA is transported to an external detector for quantitation.

Reagents were delivered to the reagent valves through two syringes controlled by the system computer. This mode of addition was found to be more precise and faster than using the high pressure system pumps. This configuration of the instrument allows rapid delivery of substrate and regeneration reagent to the cartridge during an assay while running buffer is being pumped through one of the instrument's two pumps (plumbing diagram Fig. 1). Samples or standards were premixed with antibody and/or antibody–enzyme conjugates and incubated for the required time in the autosampler before injection. Injection volume varied from 50 to 100 μl depending on the assay. The method consisted of a loading step at 0.1 ml/minute followed by washing with running buffer at 1.5 ml/minute to remove unbound reagents; addition of 0.5 ml substrate from syringe 1 at 0.1 ml/minute; elution of product until absorbance was near maximum and regeneration of the cartridge with 12 m*M* HCl+150 m*M* NaCl delivered from syringe 2. Product peak absorbance was measured at a predetermined time and standard curves were generated using Microcal Origin software and data analyzed in Excel.

3.2. TSH assay

3.2.1. Assay range and standard curve

Standards were prepared in lipid stripped TSH free pooled human serum at levels of 0, 0.1, 0.5, 1, 3, 12 and 40 μIU/ml. The assay range, concentration of reagents and run timing were optimized on the Integral using 1×20 mm cartridges with antibody to TSH bound in excess by varying dilution of labeled

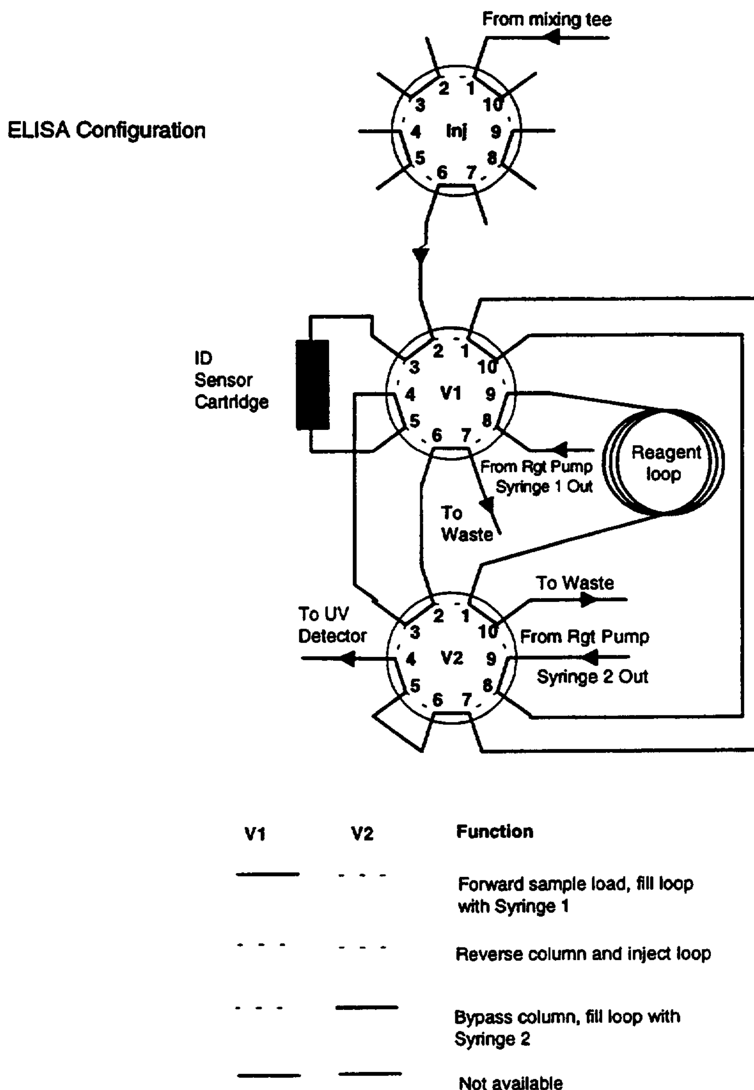

Fig. 1. The plumbing configuration for the Integral Micro-Analytical Workstation in the ELISA format: sample is injected into port 1 of the injection valve by the autosampler; mobile phase is pumped through the system moving sample onto the cartridge; substrate is delivered by syringe pump 1 into the reagent loop; the mobile phase moves substrate onto the cartridge in reverse; acid elution reagent is pumped by syringe pump 2 into the reagent loop and the solvent pump moves acid into the cartridge to remove the immune complex and allow for regeneration of the cartridge.

antibody, ratios of standard to antibody, preincubation times and assay run time. In the final format, 100 μl of each standard was combined with 50 μl of HRP labeled antibody to TSH. The mixture was preincubated at room temperature for 10 min and then 50 μl was injected onto the cartridge where -Antibody:Antigen:Antibody–Enzyme complex was formed. Ortho-phenylenediamine substrate was introduced from the substrate loop into the flowing mobile phase and moved slowly into the cartridge containing captured immunological complex. After 3 min of substrate addition, the column was regenerated for the next run by addition of acidic mobile phase to dissociate the immunological complex. Assay time including regeneration was 8.6 min. Fig. 2 shows typical chromatograms at different con-

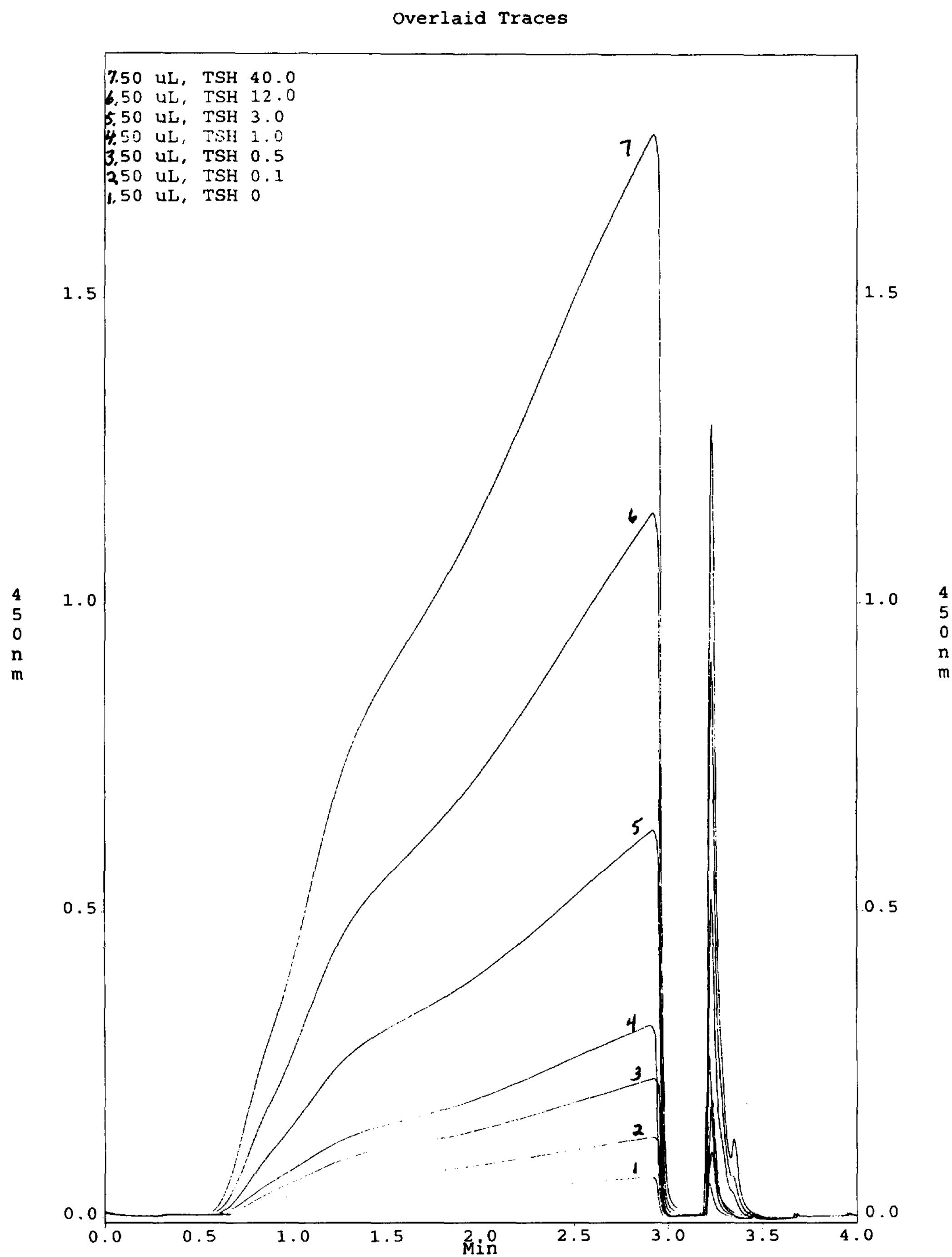

Fig. 2. Typical TSH chromatograms: these chromatograms represent a standard curve assay. Substrate is pumped through the cartridge at 0.1 ml/min for 3 min; the assay is then stopped and the absorbance read at 2.8 min into substrate addition.

centrations of ligand. The 450 nm absorbance at 2.8 min into substrate addition was used to generate a standard curve (Fig. 3) encompassing the clinically relevant assay range from 0 to 40 μIU/ml or 0 to $2.9 \cdot 10^{-10}$ *M*. Clinical specimens obtained from a local laboratory were assayed, values were calculated from the standard curve using four parameter fit and results compared to those obtained by the clinical laboratory. Although the assays were run several days after the original results were generated, correlation was >0.96 which indicated feasibility for a performance study.

3.2.2. Proof of performance studies

Clinical ELISAs must meet certain standards of performance in the areas of precision, reproducibility, sensitivity and correlation to already accepted methods. A limited performance study was done for this chromatographic based ELISA. Controls were prepared at low, medium and high levels by diluting recombinant TSH in lipid stripped TSH free pooled human serum. Controls were frozen in aliquots for use throughout the study.

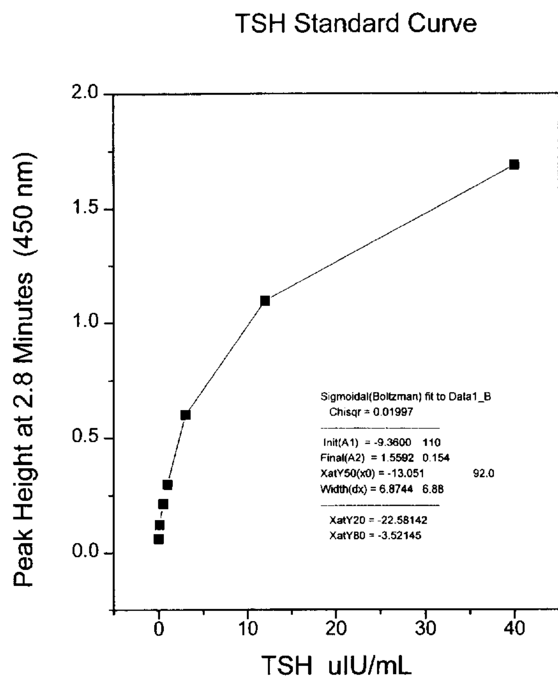

Fig. 3. TSH standard curve: the curve was constructed using Microcal Origin software from the 450 nm absorbance values at 2.8 min into substrate addition. The curve parameters were used to determine the values of specimens and controls.

Table 1
TSH assay sensitivity

Mean of 20 zero replicates	0.0847 absorbance units
S.D. of 20 zero replicates	0.0054 absorbance units
R.S.D.	6.35%
Mean + 2 S.D.	0.06 μIU/ml or $4.3 \cdot 10^{-13}$ *M*

Assay sensitivity was determined by running twenty replicates of the zero standard along with duplicates of the whole standard curve. The mean and standard deviation of the twenty replicates was determined and the value of the mean plus two standard deviations was read from the standard curve. The results are shown in Table 1. The assay sensitivity was calculated to be 0.06 μIU/ml or $4.3 \cdot 10^{-13}$ *M*.

Intraassay precision was determined by running ten replicates of the three controls and two patient specimens using the same set of reagents and cartridge on the same day with the standard curve run in duplicate. The standard values were averaged and a standard curve constructed. The replicates of each sample were calculated from this curve and the mean, standard deviation and relative standard deviation (R.S.D.) determined. Table 2 presents the values obtained. R.S.D. ranged from 3.7 to 9.5%, well within the range of microtiter plate assays.

The three controls and standards were run in duplicate in assays on different days to access interassay precision. The values obtained on each day were averaged and this average combined with those from all runs to determine the mean, standard deviation and R.S.D. for day to day assay precision.

Table 2
TSH intraassay precision

	Low Control	Medium Control	High Control
Mean (μIU/ml)	1.10	4.63	13.05
S.D.	0.01	0.32	0.49
R.S.D. (%)	9.24	6.80	3.78
n	10	10	10
	Patient 5641	Patient 0070	
Mean (μIU/ml)	11.50	0.80	
S.D.	0.44	0.06	
R.S.D.	3.79	8.06	
n	10	10	

Table 3
TSH interassay precision

	Low Control	Medium Control	High Control
Mean (μIU/ml)	2.57	5.71	15.39
S.D.	0.17	0.60	1.21
R.S.D.	6.80	10.50	7.90
n	6	6	4

The values, presented in Table 3, range from 6.8 to 10.5% R.S.D..

Dilution linearity was assessed when specimens were diluted. In this case, three hypothyroid specimens were diluted at four levels within the assay range and run. The value of the lowest dilution of each specimen was considered 100% of expected and compared to the recoveries of the higher dilutions. Recovery was within acceptable levels (Table 4).

Fifty specimens including hypo-, hyper and euthyroid levels were obtained from a local clinical laboratory and used to examine correlation of the chromatographic method to another method. All samples were passed through a 0.22 μ filter before use. Samples were run by the chromatographic ELISA and in the U-TSH Kit (Medix Biotech) on the same day. A standard curve and controls in duplicate were included each day. If results between the two assays did not agree for a given specimen, that specimen was rerun in both assays. Repeat runs on two specimens showed that the chromatographic method was correct and operator error was probably responsible for the first results of the manual method. Fig. 4 shows that correlation between the 2 methods was 0.99 with a slope of 1.13. The four out of range hypothyroid values show greater variance between the two assays probably because the samples had to be diluted into the assay range and correction results in magnification of error.

3.3. *hCG assay*

3.3.1. *Assay range and standard curve*

Standards were prepared in hCG free lipid stripped human serum at levels of 0, 5, 50, 200, 400 and 800 mIU/ml in order to cover the range of commercial assays. The assay was optimized by the same methods as the TSH assay except that the 1×20 mm cartridge contained Poros aldehyde covalently linked to sheep anti-FITC affinity purified antibody. This cartridge can be used for any assay if one antibody is labeled with FITC and the other with signal generator. The preincubation mixture consisted of FITC labeled antibody to hCG, recombinant hCG standard and HRP labeled antibody to hCG in a 1:1:1 ratio. Incubation time was 1 hour at 37°C. Assay conditions were the same as TSH conditions except injection size was 100 μl increasing the assay time slightly (8.85 min). The chromatographic data was analyzed by the same methods as for TSH. Fig. 5 shows a typical standard curve for hCG encompassing the assay range from 0 to 800 mIU/ml (0 to 66.6 ng/ml). To show feasibility several samples were tested including males and both non-pregnant and pregnant females at all levels of gestation and results compared to a commercial laboratory. Correlation at levels was >0.9 even on very high speci-

Table 4
TSH dilution linearity study

Sample	Dilution	Expected μIU/ml	Observed μIU/ml	% Recovery	Mean Recovery
32	2		10.2	100.0	
	4	5.1	4.9	96.1	89.2
	10	2.04	1.9	93.1	
	20	1.02	0.8	78.4	
43	2		6.3	100.0	
	4	3.15	2.0	63.4	87.3
	10	1.26	1.3	103.2	
	20	0.63	0.6	95.2	
33	4		18.5	100.0	
	10	7.4	8.0	108.1	102.7
	20	3.7	3.6	97.3	

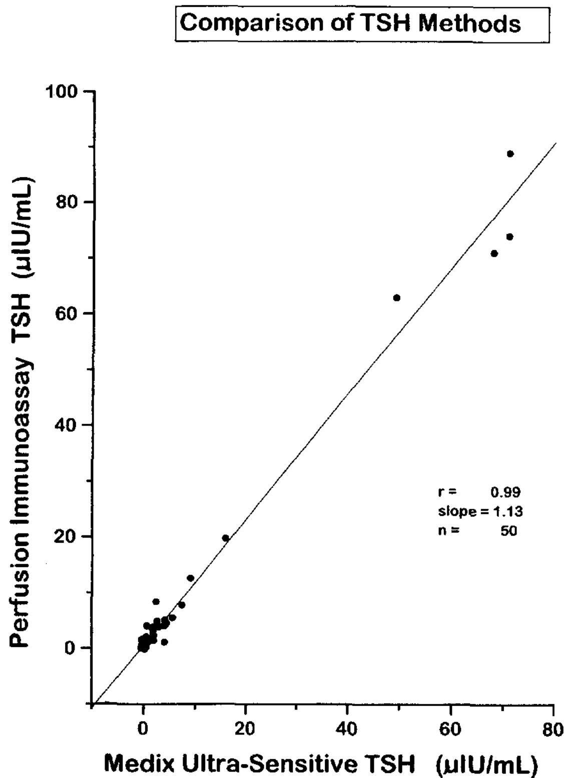

Fig. 4. Comparison of TSH methods: specimens obtained from a clinical laboratory were run in both assays on the same day. The standard curve parameters for each assay run were used to calculate the Perfusion Immunoassay values and the kit was run according to the package insert. The two assays performed equally well.

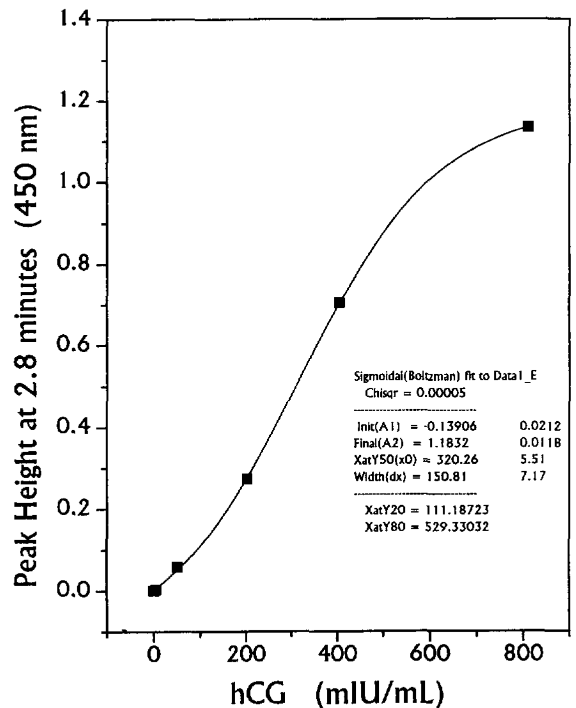

Fig. 5. hCG standard curve: the curve was constructed from the absorbance values at 2.8 min using Microcal Origin software. The curve parameters were used to determine the values of specimens and controls.

mens which required substantial dilution (results not shown).

3.3.2. *Proof of performance studies*

Again proof of performance was assessed by examining assay sensitivity, sample carry-over, intra-assay precision, interassay precision, dilution linearity and correlation to another method. A limited performance study was also done for this assay. Controls were prepared at low, medium and high levels on the standard curve by diluting recombinant hCG in lipid stripped hCG free human serum. These controls were frozen in aliquots and used throughout the study.

Assay sensitivity was determined in the same manner as TSH. The result shown in Table 5 was 2 mIU/ml (160 pg/ml or $5 \cdot 10^{-12}$ *M*).

A major problem with automated assays for hCG is sample carry-over from a high specimen to a low or negative specimen running after it. Fig. 6 shows that this assay did not have this problem. Replicates of a low specimen were run interspersed with replicates of an undiluted high specimen. The high specimen (33,500 mIU/ml) ran at the top of the assay range with a R.S.D. of 1.1% while the low specimen (22 mIU/ml) ran consistently low with a

Table 5
hCG assay sensitivity

Mean of 20 zero replicates	0.0641 absorbance units
S.D. of 20 zero replicates	0.0028 absorbance units
R.S.D.	4.3%
Mean + 2 S.D.	2 mIU/ml, 160 pg/ml, $5 \cdot 10^{-12}$ *M*

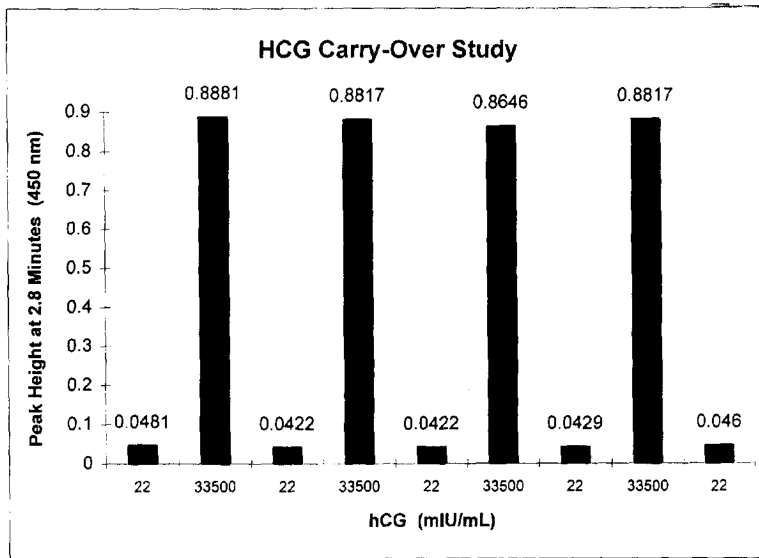

Fig. 6. hCG sample carry-over study: injections of a low specimen were interspersed with injections of an undiluted high specimen to show the lack of carry-over from one sample to another. This allows the accurate quantitation of negative or low specimens which might follow a high one in an automated system.

R.S.D. of <6%. This data was confirmed with a specimen obtained later which contained 500 000 mIU/ml and also ran at the top of the assay range demonstrating no high dose hook effect.

Intra-assay precision was examined by running twenty replicates of each control in a single assay day using one cartridge and set of reagents with the standard curve in duplicate. The mean, standard deviation and % R.S.D. for each control are presented in Table 6. Precision ranged from 2.3% at the lower end to 5.7% at the upper end.

Interassay precision was determined by running ten assays of each control in duplicate on separate occasions with a standard curve in duplicate. The precision data ranged from 7.7 to 8.8% R.S.D. as shown in Table 6.

Because hCG concentration varies over a wide range, a large number of specimens must be diluted into the assay range for quantitation. Dilution linearity was assessed using three specimens diluted within the range of the assay. Recovery was found to be linear at all dilutions (Table 7).

Correlation to another method was achieved by comparing the chromatographically based ELISA format to a commercial assay kit, the Medix Biotech Total HCG Enzyme Immunoassay Test Kit KIF4011. Fifty-one specimens including sera from males, non-pregnant females and females at all stages of pregnancy were examined. A standard curve and controls in duplicate were run with each set of specimens. Many samples had to be diluted into the assay range for quantitation. Two specimens needed to be repeated because results of the two methods disagreed. On repeat the results matched. One was caused by a bad injection and the other by the manual method.

Table 6
hCG intraassay and interassay precision

hCH Intraassay Precision

	Low Control	Medium Control	High Control
Mean (mIU/ml)	182.9	426.2	674.5
S.D.	4.2	13.7	38.1
R.S.D. (%)	2.3	3.2	5.7
n	20	20	20

hCG Interassay Precision

	Low Control	Medium Control	High Control
Mean (mIU/ml)	166.5	392.1	624.4
S.D.	13.2	30.1	54.9
R.S.D.	8	7.7	8.8
n	9	10	10

Table 7
hCG dilution linearity study

Sample	Dilution	Expected mIU/ml	Observed mIU/ml	% Recovery	Mean Recovery
7	20		922	100.0	
	30	615	582	94.6	98.3
	40	461	501	108.7	
	50	369	338	91.6	
3	undiluted		550	100.0	
	1.5	366	399	109.0	104
	2	275	278	101.1	
	4	138	129	93.8	
	8	69	77	111.9	
9	4		466	100.0	
	6	311	355	114.1	106.5
	8	233	222	95.3	
	10	186	205	110.2	

Fig. 7 shows that correlation between the 2 methods was 0.99 with a slope of 0.88.

4. Discussion

Data presented show the feasibility of doing enzyme immunoassays using packed immunosorbent beds on a microanalytical high-performance liquid chromatography system. The ability to add reagents through valves is a viable alternative to manual or robotic pipetting used in microtiter well systems. Reagent transport and mixing is also seen to be very efficient. The autosampler can dilute, pre-mix reagents and incubate for the required time before injection of sample allowing a "walk-away" format. Two clinically relevant assays, TSH and hCG have been demonstrated to yield results equal to or better than kits approved for clinical use. The method reuses the same solid-phase reducing antibody expense. Cartridges can be used at least 1000 times over a minimum period of 6 months if stored at 4°C in buffer containing 0.01% azide.

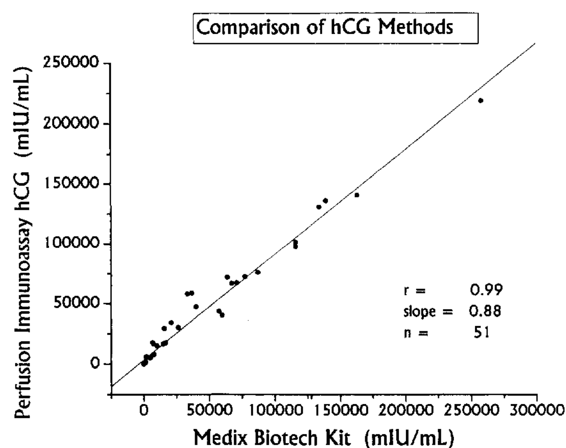

Fig. 7. Comparison of hCG methods: specimens obtained from a clinical laboratory were run in both assays on the same day. The two assays showed good correlation even for specimens needing dilution.

Precision can be much better than that obtained with microtiter plate assays. The TSH assay was sensitive to 0.06 μIU/ml with a R.S.D. of 6.35% while several kits claim this ultrasensitive range with 20% C.V.. The hCG assay shows very tight intra- and interassay precision from a low of 2.3% to a high of 8.8%. This assay also shows a remarkable lack of carry-over from high to low specimens, a problem with many automated systems caused by inadequate washing. Regeneration with mild acid desorbs both specifically and non-specifically adsorbed species, preventing build-up of antigen and other serum proteins which might interfere with assay sensitivity. It may be concluded that the chromatographically based ELISA method is comparable or superior to standard ELISA clinical assay kits in terms of cost, precision, and sensitivity.

The chromatographic ELISA approach has several other advantages. One is that a single, high sensitivi-

ty immunological assay may be achieved in much less time. This is due to a reduction of diffusion distances and reaction volumes noted in the Section 1. The speed with which reagent addition, mixing and washing may be achieved in the chromatographic format also contributes to this time reduction. A second advantage to the particular format used in this study was that background could be reduced by using less enzyme conjugated antibody. Still another advantage is that higher precision is achieved in (i) sample and reagent metering, (ii) analyte, reagent and reaction product transport, (iii) reaction times and (iv) the detection system of liquid chromatography instrumentation. This is almost certainly responsible for the greater precision achieved with this system. A fourth advantage is that sample volumes greater than the volume of the incubation chamber may be processed. This allows analyte from a large volume to be concentrated in the assay chamber. Finally, reuse of the antibody cartridge reduces the possibility of variability in antibody immobilization and concentration between sample wells.

Throughput (T_p in samples/h) in a chromatographically based serial processing system is $T_p = 1/T_{cy}$, where T_{cy} is the cycle time in hrs. A series of operations occur in these serial processing assays which include all washing steps (T_{w1}, T_{w2}, T_{w3}, and T_{w4}), sample addition (T_{sad}), substrate addition (T_{sub}), substrate incubation (T_{inc}), product transport (T_{pdt}) to the detector and column regeneration (T_{reg}). These steps may be divided into instrumentation (T_{in}) and chemical (T_{ch}) activities.

$$T_{in} = T_{w1} + T_{w2} + T_{w3} + T_{w4} + T_{sad} + T_{sub} + T_{pdt} \quad (1)$$

$$T_{ch} = T_{inc} + T_{reg} \quad (2)$$

In serial processing assays, there is only one reaction vessel and the instrument can only perform one operation at a time, i.e., events must occur in sequential order. This means that

$$T_{cy} = T_{ch} + T_{in} \quad (3)$$

A major focus of this paper was to reduce these times. It has been shown that convective transport in perfusible particles, such as the Poros particles used in these assays, allow intraparticle transport of analytes even when liquid is pumped through columns in 500 ms [5,6]. Rapid transport of analyte and reagents within the bed of perfusible particles used in these studies allowed the time required for most of the steps in T_{in} to be reduced to 1–10 s. The rate limiting step in the serial assays described in this paper was the time required for product amplification (T_{inc}), particularly at low antigen concentration. In ultrahigh sensitivity assays, $T_{inc} \geq$ the sum of other time elements in the assay.

The disadvantage of this serial processing approach is that it can be of lower throughput than parallel processing systems. In contrast to serial processing assays, multiple reaction vessels and multiple robotic fluidic units for reagent dispensing, washing and substrate addition are used in parallel processing assays. The format in a microtiter well system is to have eight parallel channels with twelve wells in each channel. Assuming that all wells on the plate are used for a different assay and the sum of incubation times (T_{tinc}) for antigen binding, second antibody binding and substrate incubation is much larger than the sum of the time required for robotic additions (T_{rob}) and detection (T_{det}), then

$$T_{tinc} \cong T_{tinc} + T_{rob} + T_{det} \quad (4)$$

and

$$T_p = 96/T_{tinc} \quad (5)$$

In practice, some number (n) of the 96 wells on a plate are used for standards. This reduces throughput to

$$T_p = (96 - n)/T_{tinc} \quad (6)$$

Even higher throughput may be obtained by using multiple 96 well plates. When large numbers of plates are used the robotics become the rate limiting phase of the system. Again assuming eight sets of robotic fluid handling units, throughput becomes a function of the time required by the robotics to service and detect product in each sample well, i.e.,

$$T_p = 8/(T_{rob} + T_{det}) \quad (7)$$

The two major advantages of the parallel processing system are that in any single channel the instrument may be servicing other sample wells in

the channel during incubation steps and multiple instrument operations may be occurring at once. Theoretically, one could also run multiple serial processing systems, but this is impractical both economically and in terms of complexity.

5. Conclusions

It may be concluded from these studies that analytes ranging down to $4 \cdot 10^{-13}$ *M* may be determined with high precision in the packed column ELISA format. This is within the range of the most sensitive clinical assays available today. Comparing the results of this study to published data on capillary based chromatographic ELISA, the packed bed system is superior in terms of assay time and detection limits. From this it may be concluded that the advantage of packed beds is that the assay may be achieved faster because (i) diffusion distances are in the μm range and (ii) high surface area porous particles accelerate the rate of antigen binding by allowing more antibody to be immobilized per unit volume of incubation chamber. Finally, it may be concluded that using the columns, valves, pumps and detectors of modern, multivalved liquid chromatography instrumentation provides a viable alternative to automated, high sensitivity immunological assays that is superior in assay cycle time and precision to any other immunological assay.

References

[1] E. Engvall and P. Perlman, Immunochemistry, 8 (1971) 871.
[2] M. De Frutos, S. Paliwal and F. Regnier, Anal. Chem., 37 (1993) 345.
[3] B. Harmon, D. Patterson and F. Regnier, Trends Anal. Chem., 11 (1995) 139.
[4] D.W. Chan, Program of the Clinical Ligand Assay Society (1995) 33.
[5] F.E. Regnier, Nature, 350 (1991) 634.
[6] S. Fulton, N. Afeyan, N.F. Gordon and F.E. Regnier, J. Chromatogr., 547 (1991) 452.