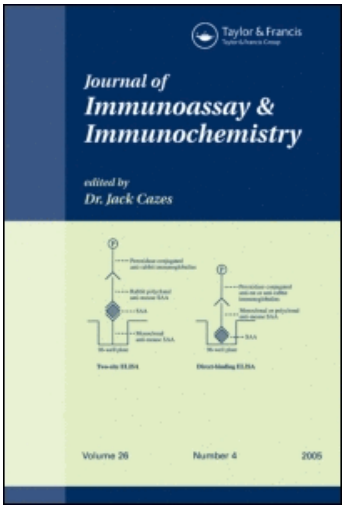

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Application of Evidence Investigator™ for the Simultaneous Measurement of Soluble Adhesion Molecules: L-, P-, E- Selectins, VCAM-1, ICAM-1 in a Biochip Platform

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Application of Evidence Investigator™ for the Simultaneous Measurement of Soluble Adhesion Molecules: L-, P-, E- Selectins, VCAM-1, ICAM-1 in a Biochip Platform

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Abstract: The semiautomated Evidence Investigator™ has been applied to the simultaneous specific measurement of soluble adhesion molecules: L-, P-, E- selectins, VCAM-1, ICAM-1 using a reduced volume of sample. The biochip is the solid support and vessel where the sandwich immunoassay takes place. Signal detection, imaging, data processing, and storage are fully automated. Calibration curves are generated simultaneously for each analyte, with automatic validation against supplied calibration data. These curves are used for the calculation of the concentrations in multi-analyte controls and human serum samples. Data from the evaluation parameters assessed indicate suitability of the Evidence Investigator™ system for the application.

Keywords: Evidence investigator, Soluble adhesion molecules, Selectins, Biochip platform

INTRODUCTION

In the late 1980s, the concept of assays based on ligand binding on microarrays was introduced by Ekins et al.[1–3] Panels of microarrays can increase the capacity of detection and quantification of biomarkers, as analysis of several biomarkers can be performed at the same time with a single patient

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sample. The development of stable, reproducible protein-biochip microarray technology and the automation of biochip-based immunoassays in a fully automated high-throughput Evidence® system have been reported.[4] This technology uses a chemically activated biochip (9 mm^2) as solid support, in which multiple specific protein ligands are precisely dispensed, immobilized, and stabilized in predefined x, y coordinates, creating ordered discrete test regions (DTRs) forming arrays. The biochip is then used as the reaction platform to perform competitive or sandwich immunoassays. Nine biochips are held in a carrier and are automatically processed during the immunoassay steps. The supercooled charged couple device (CCD) camera in the analyser simultaneously detects the light signals emitted by the DTRs of the array and the dedicated software processes, quantifies, validates, and archives the multiple data. Thorough quality control monitoring is implemented through every single step, from the fabrication of the biochips, the immunoassay reactions, signal detection, and processing, to the management of data in the system software.[5] Using the same technology, the semiautomated Evidence Investigator™ system is designed for research and clinical applications, with lower sample throughput. The immunoassay steps are manually performed, but incubation conditions are controlled by means of a custom thermoshaker. Reagents, multi-analyte calibrators, and multi-analyte controls information is entered with the aid of a handheld barcode scanner. The detection and software units are equivalent to the fully automated Evidence® system. The use of biochip array platforms offers benefits in sample analysis, enabling assay miniaturization, and simultaneous measurement of multiple analytes using reduced sample and reagent volumes. Furthermore, they facilitate more cost-effective and comprehensive approaches to the study of pathological conditions. This technology paves the way to the development of flexible ranges of biochip-array multiplexed assays. The advantages that this technology can offer is illustrated here with the application of Evidence Investigator™ for simultaneous detection of soluble forms of adhesion molecules: L-, P-, E- selectins, vascular cell adhesion molecule-1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1).

Adhesion molecules are complex membrane proteins and have been classified into four major families: Immunoglobulin (Ig) like superfamily cell adhesion molecules (CAMs), integrins, cadherins, and selectins. VCAM-1 and ICAM-1 are members of the Ig-like cell adhesion molecules superfamily. Adhesion molecules are involved in a wide range of physiological processes.[6–8] L-, P-, and E- selectins, ICAM-1, and VCAM-1 are implicated in the interactions between leukocytes and the endothelium during the inflammatory process in what is called the "adhesion cascade". Throughout this process, L-, P-, and E- selectins act in cooperation with ICAM-1, VCAM-1, and leukocyte integrins. Each member of the adhesion molecule superfamilies regulates a particular step in the adhesion cascade. Selectins facilitate tethering of leukocytes over the endothelium by means of weak low affinity bonds; activated integrins form strong bonds and immobilize the tethered leucocytes;

ICAM-1 and VCAM-1 help to create stronger bonds and facilitate the extravasation of leukocytes.[9–15] The adhesion molecules can be enzymatically cleaved from the cell surface and exist in soluble form in circulation;[7,8,16,17] they are found in healthy persons and can be used for non-invasive quantification. Numerous studies have reported changes in the levels of the soluble forms in body fluids in different disease states, such as peripheral artery disease and ischaemic heart disease,[18,19] stroke,[20,21] allergy,[22] sepsis,[23] HIV,[24,25] diabetes,[26,27] asthma,[28–30] autoimmune diseases,[31–36] and cancer.[37–40]

However, the concentrations of each of these molecules in body fluids had to be determined separately. As these molecules act in cooperation, the simultaneous measurement of soluble forms, at a single point in time, with only a reduced volume of patient sample, could provide new insights into the investigation of their (patho)physiological implications, the monitoring of recovery, and in the targeting of preventive therapies. The clinical relevance of the quantitative analysis of adhesion molecules is enhanced by the availability of reproducible diagnostic test assays in multi-analyte format. The Evidence Investigator™ system can meet these requirements and this study reports data of the simultaneous measurement of soluble L-, P-, and E-selectins, VCAM-1, and ICAM-1 in a reproducible biochip array-platform. This compact semi-automated device, designed for research and clinical applications, combines controlled manual immunoassay steps with equivalent detection and software capabilities as a fully automated analyser without detriment in the quality of the results. The system makes possible the multiplexed analysis in real–time, with a small volume of patient sample. This technology could be an useful tool in the investigation of physiological and pathological processes in which these molecules are involved, especially in research and clinical laboratories, where the sample volume availability is of prime importance.

EXPERIMENTAL

Instrument System

The Evidence Investigator™ (Randox Laboratories, Crumlin, Northern Ireland) is a bench top semiautomated instrument, dedicated to the imaging capture and analysis of Randox biochip array assays. The immunoassay steps of the analysis are manual, and are followed by a set of fully automated operations once the biochips are inserted into the instrument:

Manual Immunoassay Steps

Evidence Investigator adhesion molecules kit (catalogue n^0 EV3519, Randox Laboratories, Crumlin, Northern Ireland) was used for the simultaneous

measurement of human adhesion molecules L-, P-, and E-selectin, VCAM-1, and ICAM-1.

The methodology of the assay is a sandwich immunoassay format. The adhesion molecules present in the sample are captured by their respective specific antibodies which are bound to the biochip; an enzyme labelled multi-conjugate is used as detector. The chemiluminescent signal output is then directly proportional to the concentration of adhesion molecules in the sample.

The kit contained ready-to-use, bar-coded reagents: multi-analyte calibrators complete with parameter details, multi-analyte conjugate, assay reagent, signal reagent, wash buffer, and adhesion molecules biochips. Multi-analyte controls, complete with parameter details (Cat n^0 EV3569, Randox Laboratories, Crumlin, Northern Ireland) were also used.

A handheld barcode scanner identified calibrators and controls and automatically input the details in the system database. This information was used to analyse and validate calibration results, and as quality control (QC) material.

The adhesion molecules biochips represented the activated solid substrate of 9 mm^2 containing immobilized antibodies specific to the adhesion molecules, in defined discrete test regions (DTRs), in an ordered array arrangement. The biochips were supplied in individually labelled carriers containing 3×3 biochips, which is equivalent to 9 reaction wells per carrier, where samples and reagents were added to perform the assay. The carriers were supplied with a handle to facilitate manipulation and prevent human contact with the biochips during the assay procedures. A carrier handling tray was provided with the system, which allowed the simultaneous handling of 6 carriers (54 biochips). A protruding part in the biochip carrier held it to the handling tray and to the detection system in the instrument. Kinetics of the assay were controlled by incubating the biochips in a custom thermoshaker unit provided with the system.

Automated Operations

After introduction of the biochip carrier in a light-tight compartment in the image station, the chemiluminescent reactions produced at the different discrete test regions (DTRs) on the surface of the nine biochips contained in a carrier were simultaneously detected and recorded by a cooled charge coupled device (CCD) camera in the Evidence Investigator™. The CCD camera has a sensor that converts incident photons produced in the chemiluminescent reaction into electrons; the light output generated is quantified by the CCD camera. Image processing, quantification, and validation were carried out by instrument specific software. Images and numerical data were automatically stored. The system software presented the added facility of checking the performance of manual steps (washing, absence of conjugate, absence of signal reagent).

The experimental procedures were performed following manufacturer instructions and are schematised in Figure 1.

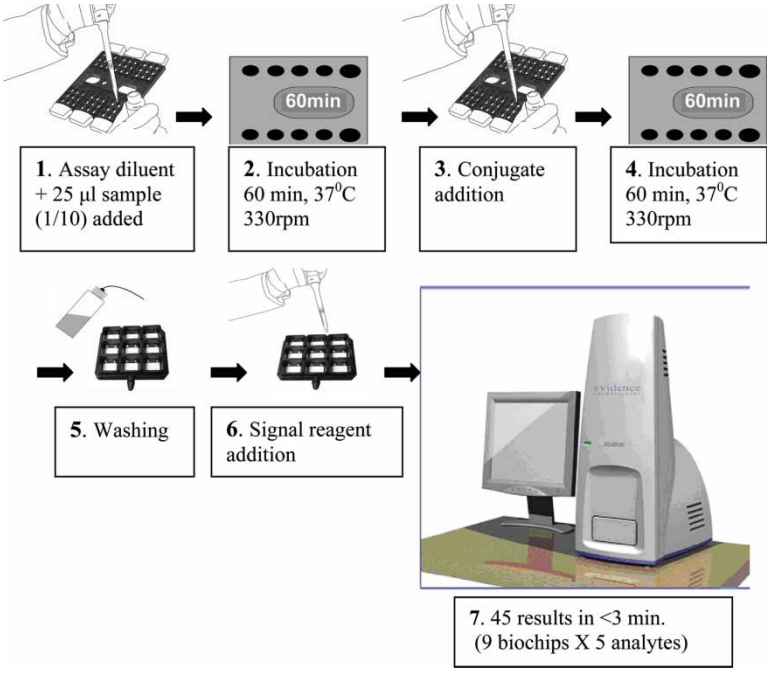

Figure 1. Experimental procedures for adhesion molecules biochip immunoassay in Evidence Investigator™. Incubation steps were performed in the custom thermoshaker unit provided with the system.

Evaluation Parameters

In house standard operating procedures were applied:

Precision

Intra-assay precision was determined from the results of 20 replicates of each of three levels of multi-analyte controls within the same run. Inter-assay precision was determined from 2 replicates of each level of multi-analyte controls over 10 separate runs.

Sensitivity

The theoretical sensitivity was determined from multiple replicates of the zero calibrator ($n = 20$) and calculated from the mean + 2SD; it is the lowest concentration that can be distinguished from 0 with a 95% confidence. Functional sensitivity was determined from multiple replicates of samples with known

concentrations (n = 5) and is defined as the lowest mean concentration at which the imprecision is <20% and the recovery is within 80–120%.

Recovery

Low and high level serum samples were mixed in various ratios to generate three different levels for each of the adhesion molecules; the recovery from these levels was determined.

Interfering Substances

Procedures were based upon the National Committee for Clinical Laboratory Standards (NCCLS) guideline EP7-A. Several substances with the potential for interfering with the assay, such as hemoglobin, bilirubin, triglycerides, lipids, and human IgG were individually added to control samples at the equivalent concentrations of 10 g/L, 1.5 g/L, 15 g/L, 20 g/L, and 10 mg/mL. Their effect upon L-, P-, and E- selectins, VCAM-1, and ICAM-1 values was determined. The spiked control samples were assayed neat.

Specificity

Control samples, were supplemented with L (3,000 ng/mL), P (700 ng/mL), and E-selectin (125 ng/mL), VCAM-1 (2,000 ng/mL), and ICAM-1 (500 ng/mL), ICAM-2 (10,000 ng/mL), ICAM-3 (3,600 ng/mL), NCAM (7,000 ng/mL), MCAM (7,000 ng/mL), and PECAM (100 ng/mL). Each analyte was added individually to determine that they were not crossreacting in the panel. The spiked control samples were assayed neat.

Sample Reference Ranges for Evidence Investigator™

Serum samples from apparently normal males (n = 20) and females (n = 20) were assessed and the reference ranges for each analyte were established for the system.

Correlation Studies

For correlation studies, a quantitative enzyme linked immunoabsorbent assay (ELISA, from R&D Systems, Abingdon, UK) was used in accordance with the manufacturer's instructions. This system was run in parallel with Evidence Investigator™ system with 40 serum samples.

Data from the two procedures were plotted against each other and the correlation coefficients were determined by linear regression analysis.

RESULTS

Simultaneous Measurement of Adhesion Molecules: L-, P-, E-Selectins, VCAM-1, ICAM-1 in the Biochip Platform

The capability of the CCD camera in Evidence Investigator™ to measure, simultaneously, the analytes by detecting and recording the light intensities emitted by all the discrete test regions of each biochip in the carrier (9 biochips) is illustrated for the level 9 adhesion molecule multi-analyte calibrator (Figure 2). Light intensities were expressed as relative light units (RLUs).

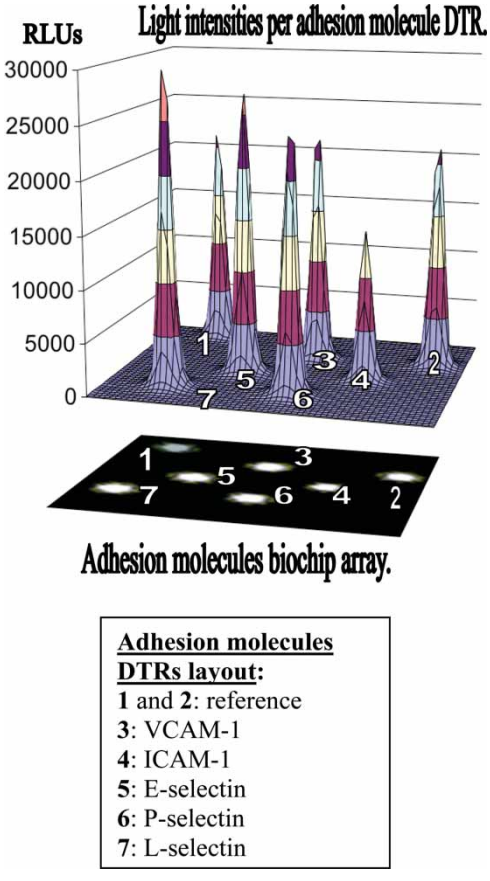

Figure 2. Simultaneous records of relative light units (RLUs) in Evidence Investigator™ CCD. Light signal was emitted by each of the discrete test regions (DTRs) for level 9 adhesion molecules multi-analyte calibrator in the biochip array platform.

For the generation of calibration curves, one carrier (9 biochips) accommodates nine multi-analyte calibrator levels for the five adhesion molecules. Evidence Investigator™ generated, simultaneously, nine-point calibration curves for each analyte tested. The software automatically validated each calibration against the calibration data supplied. The target and current curve fit correlation coefficients were displayed in the screen of the instrument for each of the adhesion molecules (Figure 3). Current valid calibration curve details were stored in the software, as illustrated for P-selectin (Figure 4), and were then used to calculate adhesion molecules concentrations in controls and samples.

Precision and Sensitivity Data in Adhesion Molecules Biochip Array Platform

Intra-assay precision was determined from the results of 20 replicates within the same run and the results are presented in Table 1. Inter-assay precision was determined from 2 replicates of the same samples over 10 separate runs and the results are presented in Table 2. Inter- and intra-assay CVs for all the analytes were typically $\leq 10\%$ for levels 1, 2, and 3.

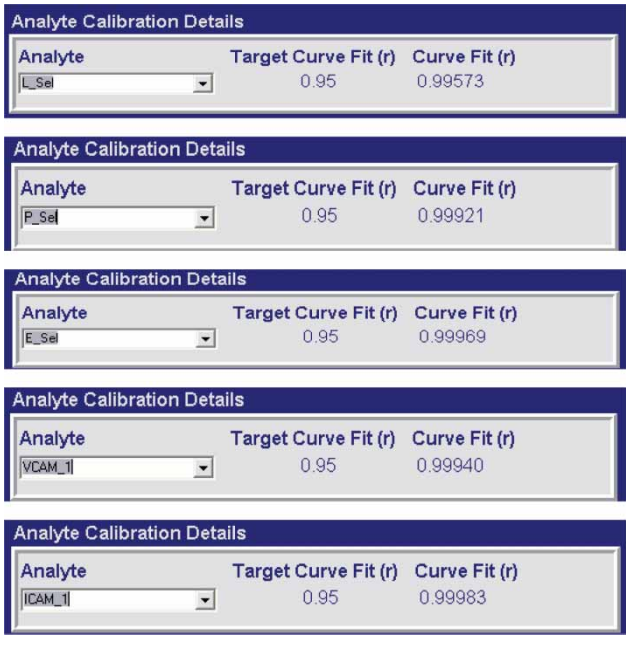

Figure 3. Calibration curve fit results for five adhesion molecules generated simultaneously in the biochip platform as shown in the Evidence Investigator™ system.

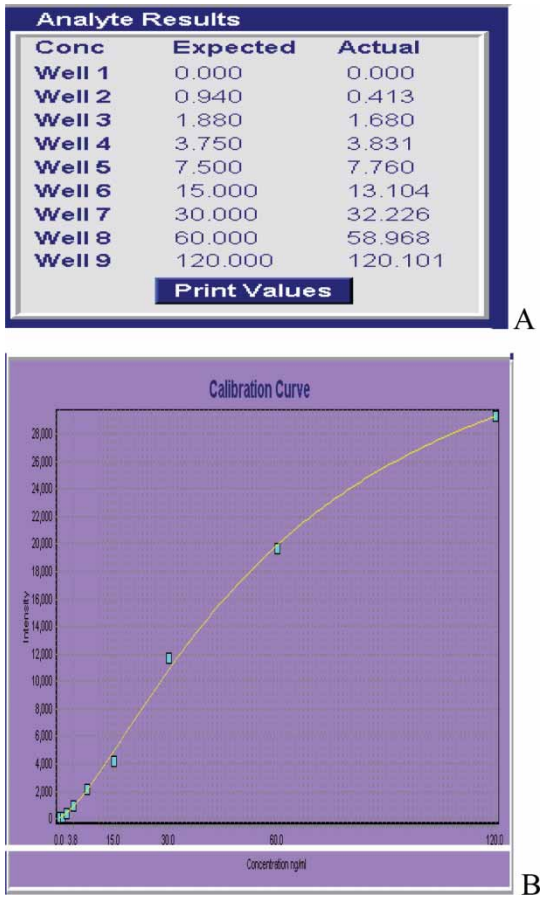

Figure 4. Example of calibration data details displayed for P-selectin. A, the expected values from the parameters details provided and the current values were shown. B, current calibration graph. Concentrations in ng/ml without dilution factor correction × 10.

Table 1. Intra-assay precision of adhesion molecules biochip platform for each of the simultaneously measured analytes, n = 20

	Multi-analyte control 1		Multi-analyte control 2		Multi-analyte control 3	
Analyte	Mean ng/ml	%CV	Mean ng/ml	%CV	Mean ng/ml	%CV
L-selectin	54.4	8.2	72.2	6.7	117.5	6.2
P-selectin	10	5.1	19.7	4.4	31.3	7
E-selectin	1.4	9.1	2.8	6.4	5.0	5.6
VCAM-1	83.9	6.9	109.3	7.6	156.7	8.6
ICAM-1	12.5	8	25.7	3.9	48.8	6

Table 2. Inter-assay precision of adhesion molecules biochip platform for each of the simultaneously measured analytes, n = 20

	Multi-analyte control 1		Multi-analyte control 2		Multi-analyte control 3	
Analyte	Mean ng/ml	%CV	Mean ng/ml	%CV	Mean ng/ml	%CV
L-selectin	56.1	8.7	75.8	7.7	137.4	13.4
P-selectin	10.4	5.7	19.7	4.8	34.4	7.2
E-selectin	1.4	7.5	2.9	8	5.4	8.6
VCAM-1	81.1	5.9	100.4	9.6	191.0	7.5
ICAM-1	13.2	3.5	25	7.9	52.3	8.3

Sensitivity of the multiplexed L-, P-, and E- selectins, VCAM-1, and ICAM-1 assay are presented in Table 3. The concentration detected in both theoretical and functional sensitivity for neat sample was always significantly lower than the minimum concentration in the reference range established for the system from apparently normal males and females.

Recovery

Low and high level serum samples were mixed in various ratios to give three levels, 1, 2 and 3, and the recovery values were >90% for all the analytes as shown in Table 4.

Interfering Substances

None of the substances tested at high levels, such as hemoglobin, bilirubin, triglycerides, lipids, and human IgG significantly changed the values of L-, P-, and E- selectins, VCAM-1, and ICAM-1.

Table 3. Theoretical and functional sensitivity data for neat sample with the adhesion molecules biochip array. The assay range was determined by the highest calibrator concentration × dilution factor of 10

	Sensitivity (ng/mL)		Assay range (ng/mL)	Reference range in serum ±1SD (ng/mL)	
Analyte	Theoretical	Functional		Male n = 20	Female n = 20
L-selectin	7	37.6	0–3500	1126.8–1579.9	859.7–1263
P-selectin	3	17.6	0–1200	99.4–153.7	49.5–106.5
E-selectin	3	3.2	0–250	19.2–43.7	8.3–30.7
VCAM-1	6	12.5	0–3300	468.6–634	333.8–554.9
ICAM-1	4	12.4	0–1000	253.2–603	134.7–575.3

Table 4. Recovery data for adhesion molecules biochip array for neat serum samples

	L-selectin	P-selectin	E-selectin	VCAM-1	ICAM-1
Level 1					
Concentration ng/ml	1301.5	249.9	52.8	681.1	196.2
%Recovery	114.8	105.5	100.4	110.9	100.9
Level 2					
Concentration ng/ml	1459.9	319.7	71.8	876.8	243.6
%Recovery	96.4	104.7	94.4	103.7	97
Level 3					
Concentration ng/ml	1618.4	389.4	90.7	1072.4	290.9
%Recovery	113.2	103.8	102.0	100.1	100.9

Specificity of Adhesion Molecules Biochip Platform

Specificity results showed no cross reactivity between L-, P-, and E- selectins, VCAM-1, ICAM-1, ICAM-2, ICAM-3, NCAM, MCAM and PECAM in the multianalyte biochip array platform.

Correlations

A comparison of the adhesion molecules biochip platform and a commercial ELISA, based on the analysis of 40 serum samples showed the following correlation coefficients: for L-selectin $R = 0.91$, for E-selectin $R = 0.75$, and for P-selectin $R = 0.93$, for VCAM-1 $R = 0.7$, for ICAM-1 $R = 0.73$ (Figure 5).

DISCUSSION

Our study shows, for the first time to our knowledge, the multiplexed detection of five adhesion molecules: L-, P-, and E- selectins, VCAM-1, and ICAM-1, in a biochip array platform.

Using the same technology, and by applying novel biochip fabrication procedures, Fitzgerald SP et al.[4] provided evidence of the possibility of production of stable and reproducible biochips which generate, simultaneously, quantitative results for multiple analytes in a fully automated Evidence® system. Moreover, an application for the simultaneous detection of 12 cytokines has been reported for the diagnosis of coronavirus-associated SARS using the same system.[41]

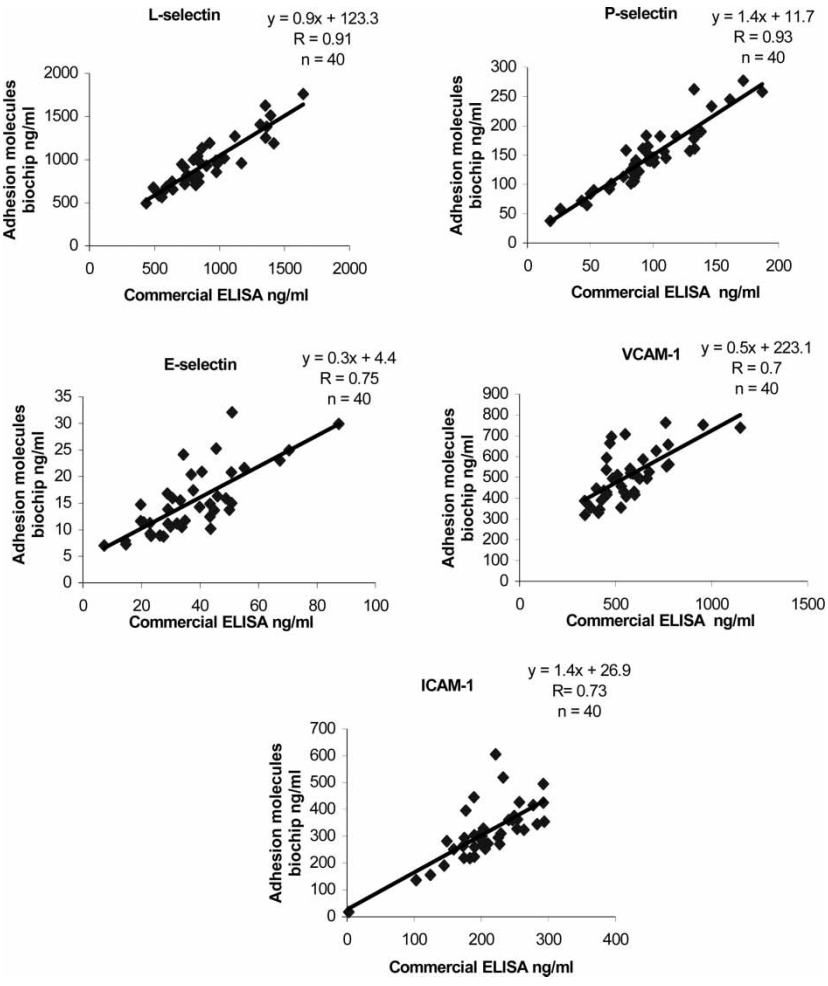

Figure 5. Comparison of the adhesion molecules biochip platform and a commercial ELISA. No recognised international reference material is available for these analytes.

The adhesion molecules biochip array platform that we present here allowed the simultaneous measurement of soluble L-, P-, and E- selectins, VCAM-1, and ICAM-1, with a reduced sample volume in the semiautomated Evidence Investigator^{TM}. Evaluation parameters indicated good performance of the system; the system was precise, both within run (intra-assay) and between run (inter-assay) imprecision typically exhibited coefficients of variation $\leq$10%, both theoretical and functional sensitivity for neat sample were consistently much lower than the minimum value for the reference range, and the recovery in three different levels in serum was $>$90% for each of the five adhesion molecules studied. The assay was specific for each

analyte, the measurements were not affected by elevated levels of common potential interferents which may be present in patient samples. Reproducibility of the results was aided by keeping controlled incubation conditions (i.e., agitation, temperature) with the custom thermoshaker unit.

The agreement between adhesion molecules array biochip and a commercial ELISA was good for soluble L- and P-selectins $R = 0.91$, 0.93, respectively. For soluble: E-selectin, VCAM-1, and ICAM-1, correlation coefficients were $R = 0.75$, 0.70, and 0.73, respectively; this could be attributable to differences in the antibodies used in the two immunoassay systems. Up to date, there is a lack of recognised reference material available for these molecules. In the present study, internal QC material was used, and the Evidence Investigator™ system was able to automatically validate the current calibration curves prior to control/sample analysis. In addition, the system enabled the automatic detection of possible experimental factors that could affect the results.

Evidence Investigator™ simultaneously generated 225 quantitative results (5 assays $\times$ 45 samples) in less than 3 h, by using ready-to-use reagents and reduced sample volumes. The combination of controlled manual immunoassay steps with automated detection and software capabilities allowed automatic data processing and archiving. The same biochip array platform can be used in a fully automated Evidence® analyser with a corresponding increase in the sample throughput.

The accessibility of this non-invasive, cost effective, semi-automated, miniaturized, multiplexed biochip-based immunoassay system, that produces reproducible quantitative results in real-time, for five soluble adhesion molecules per small amount of sample, may enhance the clinical utility of their measurement in future research and clinical studies, where the volume of sample available has to be taken into consideration. The adhesion molecules biochip array may be considered as a new tool to facilitate a novel approach in the understanding of the effect of soluble adhesion molecules in disease conditions, the monitoring of the recovery, and the effect of targeted therapies.

CONCLUSION

As the adhesion molecules act in cooperation, the simultaneous quantitative determination of the soluble forms at a single point in time, with a reduced volume of sample, can provide new insights into their clinical significance. This approach is feasible with the biochip array platform for use in the compact semiautomated Evidence Investigator™. This device, designed for research and clinical applications, combines controlled manual immunoassay steps with equivalent detection and software capabilities as a fully automated Evidence® analyser without detriment to the quality of the results. The suitability of the system for the simultaneous measurement of soluble adhesion

molecules L-, P-, and E- selectins, VCAM-1 and ICAM-1 is indicated by the evaluation parameters which show that the system is sensitive, precise, with recovery rates >90% in samples.

ABBREVIATIONS

VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; CCD, charge coupled device; DTR, discrete test region; RLU, relative light unit.

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