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Measurement of α -Fetoprotein, Carcinoembryonic Antigen and Prostate-Specific Antigen in Serum and Heparinised Plasma by Enzyme Immunoassay on the Fully Automated Serono SR1TM Analyzer

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Summary: This paper describes the new enzyme immunoassays for the measurement of α -fetoprotein (AFP), carcinoembryonic antigen (CEA) and prostate-specific antigen (PSA) in serum and heparinised plasma as developed for the fully automated Serono SR1TM analyzer. Each assay incorporates two monoclonal antibodies in an immunoenzymetric sandwich format using alkaline phosphatase as the label and magnetic solid phase separation. All processing steps are performed by the SR1.

The assays have good analytical performance with respect to detection limits (typically 0.24 μ g/l, 1.0 μ g/l and 0.1 μ g/l respectively for AFP, CEA and PSA), precision (within and between run CVs less than 7 and 12% respectively) and recovery of added analyte ($100 \pm 10\%$). Regression analysis of linearity on dilution gives correlation coefficients (r) of 0.9984–1.0000. Tumour marker concentrations measured with these assays are in good agreement with concentrations determined by established immunoassays ($r > 0.96$). Values measured in samples of healthy probands and samples of patients with malignant and non-malignant diseases are as would be expected for clinically valid assays.

The study demonstrates that the measurement of AFP, CEA and PSA in serum and heparinised plasma on the fully automated SR1 analyzer is suitable for the clinical diagnosis, monitoring and management of certain cancers and, in the case of AFP, for prenatal screening of maternal serum.

Introduction

α -Fetoprotein (AFP), carcinoembryonic antigen (CEA) and prostate-specific antigen (PSA) are accepted tumour markers used in the diagnosis, management and monitoring of certain cancers (1–3). Maternal serum AFP concentrations have also been used in the detection of fetal abnormalities (4). Various manual, semi-automated and more recently automated immunoassay methods have been described for the measurement of AFP, CEA and PSA (5–7).

Turn-around time, staff usage and assay costs can be reduced with automated immunoassays. With the continued growing use of immunoassays, the move towards automated systems is becoming more desirable. It is with this in mind that we present a summary of the tumour marker assays for the measurement of AFP, CEA and PSA in serum and heparinised plasma on the fully automated SR1 analyzer.

Materials and Methods

Assay specific reagents were provided by Serono Diagnostics S. A., Coinsins, Switzerland.

Patient samples obtained from UK hospital laboratories were stored frozen at -20°C until they were assayed.

¹) Enzyme:

Alkaline phosphatase (phosphoric monoester phosphohydrolase [alkaline optimum]: EC 3.1.3.1).

Commercial control sera (Lyphochek ECS Controls 1, 2 and 3 Lot No. 90000) were purchased from Biorad Laboratories, Herts, UK.

Other immunoassays were obtained from: Abbott Diagnostics Division, Chicago, Illinois, USA; Hybritech Europe S.A., Liège, Belgium.

Assay procedures

All AFP, CEA and PSA determinations were performed on the SR1 analyzer. For each of the assays two monoclonal antibodies are used in an immunoenzymetric assay system which incorporates magnetic solid phase separation. All incubation times and reagent volumes are determined by an assay specific software protocol.

The principle of the procedure for all of the assays is identical. Fixed amounts of fluorescein-labelled anti-(AFP, CEA or PSA) mouse monoclonal antibody and a calf intestinal alkaline phosphatase-labelled anti-(AFP, CEA or PSA) monoclonal antibody are added to sample, standard or control. The reactants are incubated at 37 °C. During the incubation the fluorescein-labelled anti-(AFP, CEA or PSA) and the alkaline phosphatase-labelled anti-(AFP, CEA or PSA) monoclonal antibodies bind to discrete sites on the AFP, CEA or PSA molecule forming a sandwich. At the end of the incubation period anti-fluorescein coupled to a magnetic solid phase is added in excess. This rapidly and specifically binds to the AFP, CEA or PSA-antibody complexes and is sedimented in a magnetic field. After aspirating the liquid phase and washing the solid phase, a solution of the enzyme substrate (phenolphthalein monophosphate) is added and the mixture incubated at 37 °C. After incubation the enzyme reaction is stopped by the addition of an alkaline buffer (pH 10.6) and the intensity of the colour developed is measured photometrically. The intensity of the colour developed is directly proportional, within the working range of the assay, to the concentration of AFP, CEA or PSA in the sample. The concentration of AFP, CEA or PSA in a patient sample or control is then determined by interpolation from a stored standard curve.

Detection limit

The detection limit for each assay was defined as the concentration of analyte equivalent to the mean absorbance of the zero standard plus two standard deviations based on twenty replicates of the zero analyte standard.

Precision

Within-assay precision was determined by replication ($n = 20$) of three concentrations of control sera. To assess between-assay precision, three concentrations of control sera were assayed in duplicate on at least forty occasions over a period of five weeks using two lots of reagents and at least five SR1 analyzers.

Recovery

The recovery of each assay was determined by adding known quantities of AFP, CEA or PSA to serum samples containing low endogenous analyte concentrations, assaying the serum samples and determining the recovery of the amount added. International reference preparations were used to prepare spikes for AFP (IRP 72/225) and CEA (IRP 73/601). Since there is no available reference preparation for PSA, the source of added PSA was PSA-containing serum.

Dilution

Five serum samples containing elevated concentrations of AFP, CEA or PSA within the respective assay ranges, were diluted 1 in 2, 1 in 4, 1 in 8, 1 in 16 and 1 in 32 in the appropriate assay diluent. The samples and their dilutions were then assayed to determine linearity on dilution. Results were interpreted by linear regression analysis of observed concentrations versus expected concentration.

Interference

Assay interference was assessed by measuring the apparent response of each assay to various concentrations of potential interfering substances spiked into normal human serum. The basal serum and the spiked serum samples were then assayed.

Hook effect (8)

Very high antigen concentrations will produce anomalously low results in simultaneous sandwich immunoassays (the "hook effect"). The hook effect for each assay was evaluated by analysing samples with elevated concentrations prepared by spiking patients' sera with AFP, CEA or PSA respectively.

Correlation with other immunoassays

The SR1 AFP, CEA and PSA assays were compared with the following immunoassays:

Abbott Diagnostics, IMx™ (AFP and CEA)

Hybritech, Tandem™-E (PSA)

The competitor's assay values were plotted on the y-axis.

Expected values

For each assay, values were determined in a population of apparently healthy blood donors, from patients with carcinomas (treated and untreated), patients with non-malignant disease and, for AFP only, from pregnant women (gestational weeks 15 to 19 inclusive).

Results

Assay protocols

Table 1 summarises each of the assay protocols for AFP, CEA and PSA. After manual pipetting of sample, all further reagent addition and incubation steps are performed by the SR1. The time to first result for the assays is approximately one hour and thereafter two results are obtained every two minutes. Results are interpolated automatically from a previously run stored standard curve (stable for up to three months).

Detection limit

The detection limits determined on at least four occasions for AFP, CEA and PSA were typically 0.24 µg/l, 1.0 µg/l and 0.1 µg/l respectively.

Tab. 1. SR1 α -fetoprotein, carcinoembryonic antigen and prostate-specific antigen assay protocols.

	Analyte		
	α -Fetoprotein	Carcinoembryonic antigen	Prostate-specific antigen
	Assay type		
	IEMA, sandwich	IEMA, sandwich	IEMA, sandwich
<i>Reagent volumes (μl)</i>			
Sample	50	100	100
Anti AFP, CEA or PSA-Alkaline phosphatase	150	150	150
Anti AFP, CEA or PSA-FITC	150	150	150
Separation reagent	200	200	200
Wash	2 $\times$ 750	2 $\times$ 750	2 $\times$ 750
Substrate	240	270	270
Stop	800	900	900
<i>Assay incubation times (min)</i>			
Immunoincubation	12	24	18
Separation	6	6	6
Substrate	12	18	18
Time to first result (min)	46	64	58
Tests per hour (batch mode)	60	60	60
Stored standard curve stability (months)	3	3	3
Assay range (μ g/l)	0.24–605	1.0–167	0.1–100
Detection limit (μ g/l)	0.24	1.0	0.1
Hook effect in excess of (μ g/l)	12100	26667	30000

Precision

The SR1 AFP, CEA and PSA assays proved reliable and reproducible as shown by the precision data in table 2. The coefficients of variation (CV) obtained for all three assays for within-assay precision ($n = 20$) were less than 7% for all control concentrations; between-assay precision was less than 12%.

Recovery

Recovery data are summarised in table 3. Recovery of added AFP, CEA or PSA at each concentration for all assays ranged from 92.2%–104.2%, with an overall mean percentage recovery of 98.8%, 101.4% and 96.7% for AFP, CEA and PSA respectively.

Tab. 2. Within- and between-assay precision using control sera.

Analyte	Within-assay precision*			Between-assay precision**		
	Mean (μ g/l)	SD (μ g/l)	CV (%)	Mean (μ g/l)	SD (μ g/l)	CV (%)
α -Fetoprotein	27.1	1.05	3.9	32.7	1.69	5.2
	116.5	5.64	4.8	142.8	10.56	7.4
	238.0	15.77	6.6	290.4	22.94	7.9
Carcinoembryonic antigen	4.3	0.30	6.9	4.3	0.50	11.9
	18.0	1.03	5.7	18.2	1.60	8.9
	35.3	2.07	5.9	36.1	3.40	9.6
Prostate-specific antigen	1.27	0.05	4.0	1.17	0.12	10.0
	3.30	0.18	5.5	3.28	0.26	8.0
	27.31	1.29	4.7	26.28	1.53	5.8

* Three concentrations of control sera were measured ($n = 20$) in one assay.

** Three concentrations of control sera were measured in duplicate over five weeks using two lots of reagents on at least five SR1 analyzers ($n > 40$ assays).

Tab. 3. Analytical recovery.

α -Fetoprotein added ($\mu\text{g/l}$)*	Number of samples	Mean recovery (%)	SD
33.6	5	98.2	4.9
69.5	5	95.3	4.4
137.3	5	97.6	5.4
313.8	5	104.2	6.4
Overall	20	98.8	6.3

Carcinoembryonic antigen added ($\mu\text{g/l}$)**	Number of samples	Mean recovery (%)	SD
8.53	5	99.3	9.1
18.91	5	101.5	5.4
37.20	5	103.4	5.9
Overall	15	101.4	8.3

Prostate-specific antigen ($\mu\text{g/l}$ ***)	Number of samples	Mean recovery (%)	SD
1.88	5	103.9	4.6
3.21	5	98.2	3.7
6.31	5	92.2	2.2
13.00	5	92.5	4.7
26.34	5	96.4	3.3
Overall	25	96.7	3.7

* The α -fetoprotein source was IRP 72/225.

** The carcinoembryonic antigen source was IRP 73/601.

*** The prostate-specific antigen source was serum, no IRP is currently available.

Dilution

The linearity on dilution was determined by multiple dilution of five samples for each assay. The observed concentration was plotted against the expected concentration and the regression equation determined for each sample. The regression analysis data for each assay is summarised in table 4. The correlation coefficients for all assays varied between 0.9968–1.0000.

Interference

Interference data are summarised in table 5. No interference was demonstrated with any of the potential interfering substances tested.

Hook effect

No high-dose hook effect was observed for the AFP, CEA and PSA assays with concentrations up to 12 100 $\mu\text{g/l}$, 26 667 $\mu\text{g/l}$ and 30 000 $\mu\text{g/l}$ respectively.

Tab. 4. Linearity on dilution.

Regression analysis: observed vs expected				
Analyte	Sample	r	Slope	y-Intercept
α -Fetoprotein	1	0.9999	1.00	−0.93
	2	1.0000	1.00	−0.58
	3	0.9999	0.99	2.42
	4	1.0000	0.99	−1.78
	5	0.9998	1.00	−2.21
Carcino-embryonic antigen	1	0.9988	1.01	0.45
	2	0.9968	0.99	−2.02
	3	0.9992	1.01	0.13
	4	0.9997	1.01	−0.21
	5	0.9999	1.01	−0.44
Prostate-specific antigen	1	0.9998	1.00	−0.12
	2	0.9991	1.01	−1.91
	3	0.9998	1.00	−0.19
	4	0.9999	0.99	0.90
	5	0.9999	1.01	−0.89

The samples used for each assay had undiluted α -fetoprotein, carcinoembryonic antigen and prostate-specific antigen concentrations within the respective assay ranges.

Tab. 5. Assay interference: No cross reaction was detectable with the following drug concentrations.

Drug	Concentration (mg/l)
Cyclophosphamide	250
Doxorubicin hydrochloride	7
Methotrexate	10
Megestrol acetate	40
Vinblastine	5
Vincristine	5
Bleomycin	10
Cisplatin	60
Actinomycin	5
Fluoro-uracil*	5
Prostatic acid phosphatase**	1
Diethylstilbestrol**	1
Estramustine phosphate**	80

* α -Fetoprotein only.

** Prostate-specific antigen only.

Correlation with other immunoassays

Figure 1 shows comparisons of AFP, CEA and PSA results with established immunoassays. In general a good correlation was obtained between the SR1 assays and the other products.

Expected values

The distribution of values determined for AFP, CEA and PSA in normal blood donors and a variety of malignant and non-malignant disease states are summarised in tables 6a, b and c. They are in good agreement with values obtained with other methods

(9–11). Median maternal serum AFP concentrations in healthy, singleton pregnancies ($n = 100$ for gestational weeks 15 to 18, $n = 90$ for gestational week 19) were 41.1, 47.6, 53.8, 62.9 and 75.3 μg/l for 15, 16, 17, 18 and 19 weeks of gestation respectively. Maternal serum AFP in pregnancies of six anencephalics ranged from 179 to 672 μg/l. A small number of patients with benign prostatic hyperplasia had serum PSA concentrations in excess of 10 μg/l (tab. 6c),

this is in accordance with published data (3). SR1 PSA concentrations from prostatectomised men were compared with values obtained using the Hybritech Tandem™-R PSA assay (tab. 7). The SR1 PSA results compare well with the Hybritech Tandem-R PSA concentrations on each sample. However samples 4–7 do not have undetectable PSA concentrations. This is indicative of residual PSA secreting tissue in these patients.

Tab. 6. Distribution of results in malignant and non-malignant disease states for (a) SR1 α -fetoprotein, (b) SR1 carcinoembryonic antigen and (c) SR1 prostate-specific antigen.

a	n	Frequency of α -fetoprotein concentration (%)				
		0–12.1 $\mu\text{g/l}$	12.2–24.2 $\mu\text{g/l}$	24.3–121 $\mu\text{g/l}$	121.1–605 $\mu\text{g/l}$	> 605 $\mu\text{g/l}$
Blood donors	200	94	4.5	1.5	0	0
Carcinoma*						
Liver/gall bladder	10	80	20	0	0	0
Ovarian	10	90	10	0	0	0
Gastrointestinal	20	90	5	5	0	0
Pulmonary	10	100	0	0	0	0
Breast	20	95	5	0	0	0
Testicular						
Seminoma	42	100	0	0	0	0
Non-seminoma	49	42.9	14.3	22.4	8.2	12.2
Non-malignant disease						
Liver/gall bladder	50	90	6	2	2	0
Genitourinary	25	88	12	0	0	0
Gastrointestinal	24	100	0	0	0	0
Cardiopulmonary	25	92	8	0	0	0

* Includes treated and untreated patients.

b	n	Frequency of carcinoembryonic antigen concentration (%)			
		0–3.09 $\mu\text{g/l}$	3.10–5.09 $\mu\text{g/l}$	5.10–10.09 $\mu\text{g/l}$	> 10.10 $\mu\text{g/l}$
Blood donors					
Non-smokers	99	89.9	10.1	0	0
Smokers	200	71.5	20.5	8	0
Carcinoma*					
Colorectal	50	52	16	0	32
Breast	50	44	18	8	30
Ovarian	10	70	10	10	10
Pulmonary	10	70	0	20	10
Genitourinary	10	70	0	20	10
Blood/Lymph	10	70	20	10	0
Other	10	70	0	10	20
Non-malignant disease					
Liver/gall bladder	25	76	16	4	4
Genitourinary	25	84	12	0	4
Gastrointestinal	25	80	8	4	8
Cardio-pulmonary	25	88	8	4	0
Other	50	82	12	2	4

* Includes treated and untreated patients.

Discussion

The SR1 tumour marker assays fulfil the following analytical criteria:

- (i) suitable ranges and detection limits for clinical applications.
- (ii) robust and reproducible: the precision of the assays is good with the CV for within-assay precision less than 8% and between-assay precision less than 12%.
- (iii) excellent recovery and linearity on dilution.
- (iv) no interference from potential interfering substances.
- (v) the hook capacity for each assay is well in excess of concentrations encountered usually with clinical patient samples.
- (vi) correlation data shows good agreement with other existing immunoassays.

Tab. 6. (continued)

c	n	Frequency of prostate-specific antigen concentration (%)				
		0—4.00 µg/l	4.01—10.00 µg/l	10.01—20.00 µg/l	20.01—40.00 µg/l	> 40.00 µg/l
Blood donors						
Male <40 years	100	100	0	0	0	0
Male >40 years	100	93	6	0	1	0
Male >60 years	50	90	10	0	0	0
Total male	200	96.5	3	0	0.5	0
Female	100	100	0	0	0	0
Malignant disease*						
Prostate						
Stage A	20	55	25	15	0	5
Stage B	20	40	10	20	20	10
Stage C	20	35	15	25	10	15
Stage D	20	0	5	0	20	75
Gastrointestinal	10	80	10	10	0	0
Pulmonary	10	90	0	10	0	0
Breast	10	100	0	0	0	0
Genitourinary	10	90	10	0	0	0
Renal	2	100	0	0	0	0
Other	10	100	0	0	0	0
Non-malignant disease						
Benign prostatic hyperplasia	50	82	14	4	0	0
Genitourinary	50	94	2	0	0	4
Other	34	91	0	3	0	6

* Includes treated and untreated patients.

Tab. 7. Prostatic carcinoma-SR1 prostate-specific antigen concentrations after radical prostatectomy.

Sample	Age	SR1 PSA (µg/l)	Tandem-R PSA (µg/l)
1	71	<0.1	<0.1
2	65	<0.1	<0.1
3	71	<0.1	<0.1
4	81	1.7	1.9
5	69	1.8	2.3
6	69	1.9	1.8
7	76	2.4	2.6

The spread of results obtained for the SR1 AFP, CEA and PSA assays in healthy blood donors and various types of malignant and non-malignant diseases are distributed as would be expected for clinically valid assays.

In pregnancy, the expected relationship between gestational week and maternal serum median AFP values was confirmed. In cases of anencephaly SR1 AFP maternal serum concentrations were markedly higher than in healthy pregnancies. This has been reported in the literature (12). Smokers gave higher CEA values than non-smokers, which concurs with published results (13). In addition, male blood donors gave higher CEA values than female blood donors and blood donors > 45 years gave higher CEA values than blood donors < 45 years. The difference with increas-

ing age has been described in the scientific literature (14).

The median PSA value for male blood donors increased with age and female blood donors gave a lower median PSA value than all male blood donors. The median female PSA value of < 0.1 µg/l, a reflection of the clinical detection limit of the assay, compared well with the lowest detectable value calculated by the standard method (i. e. precision of 20 replicates of the zero standard). In addition the SR1 PSA concentrations from prostatectomised men were generally very low and compared well with results obtained using the Hybritech Tandem-R PSA assay (15).

In conclusion, these results demonstrate the ability of the SR1 AFP, CEA and PSA assays to contribute towards accurate clinical diagnosis and monitoring of certain cancers and, for AFP, of fetal abnormalities. In addition, the SR1 offers the advantages of non-isotopic, walk-to walk-away automation, easy handling, short turn around times and random access and batch modes of operation using stored standard curve calibration.

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