*Acta Pharmaceutica Turcica*
*XLIII (1) (33-42) 2001*

# ANALYTICAL METHODS FOR DEXAMETHASONE

**S. SAEED-UL-HASSAN[1], FREDERICK J. ROWELL[2], L.S. JACKSON[3]**

[1] Faculty of Pharmacy, University of the Punjab (Allama Iqbal Campus) Lahore-54000, Pakistan
[2] Centre For Pharmaceutical and Environmental Analysis, School Of Health Sciences, University of Sunderland, Sunderland SRI 3SD, U.K.
[3] Horseracing Forensic Laboratory Limited, P.O.Box 15, Snailwell Road, New Market, Suffolk CB8 7DT, U.K.

**Keywords:** *Analytical methods; Dexamethasone*

There are various methods for the analysis of dexamethasone, the details of the methods are as follows:

## 1. Separation Methods

***i) High Pressure Liquid Chromatography (HPLC)-*** A number of different HPLC methods for the determination of dexamethasone and related steroids in biological fluids have been investigated by various workers. A sensitive and specific method for the quantitation of dexamethasone in plasma and urine has been described(1). The methods involve the extraction of dexamethasone from plasma or urine by dichlormethane followed by normal phase HPLC. The mobile phase was dichloromethane containing 5% 1-butanol and 0.3% water at a flow rate of 65 ml/h. The drugs were detected at 240 nm and the assay showed good linearity between 0-300 ng.

A sensitive, specific and reproducible high performance liquid chromatographic assay for corticosteroids in biological fluids has been described (2). This method also involved the extraction of samples with methylene chloride. Extracts were then washed with sodium hydroxide and then water. The mobile phase used was methanol-methylene chloride (3:97), at a flow rate of 2 ml/min. The drugs were detected at 254 nm and 280 nm and the assay was linear over the 0-500 $ng.ml^{-1}$ of steroid-concentration range.

An HPLC method has been described for the determination of prednisolone in plasma for small sample volumes(3). A mobile phase consisting of dichloromethane-ethanol-water, glacial acetic acid (500:30:30) was used in conjunction with a 10 μm porous silica column at a flow rate of 2 ml/min using ultraviolet (UV) detection at 254 nm. This method also involved the extraction of drug prior to the HPLC analysis. A linear relationship was observed over the concentration range of 25-150 $ng.ml^{-1}$.

Another HPLC method has been published for the analysis of dexamethasone and related compounds(4). The separation of corticosteroids was performed in the normal phase mode using a diol stationary phase. The eluent (a mixture of n-hexane: isopropanol (80:20) was chosen in order to achieve good selectivity and short run times. Flow rate was fixed at 1.5 ml. $min^{-1}$. Mass spectrometry was used to elucidate the

nature of the compounds isolated from illicit drug formulations and to confirm the structure of synthesized derivatives from the parent dexamethasone. The separation of 9 compounds was performed in 12 min and the detection limit was 2 ng.

A simple reverse phase HPLC method for the determination of steroid hormones in pharmaceutical preparations has been described(5). A mobile phase consisting of methanol:water (70:30 v/v) was used at a flow rate of 1.0 $ml.min^{-1}$ and the detection was made at 254 nm. A calibration curve was constructed in the range of 6.25 -100 $\mu g.ml^{-1}$ (for dexamethasone acetate, ethynylestradiol and norgestrel and from 3.12 to 50 $\mu g.ml^{-1}$ for prednisone).

A simple method(6) has been described for the determination of betamethasone (BTM) and dexamethasone (DEX) by high performance liquid chromatography. This method uses UV detection with silica as the stationary phase and dichloromethane-ethanol (34:1, v/v) as the mobile phase. The linear range of the method for BTM and DEX in 1.0 ml of sample solution was 5-50 nmol. The detection limits of BTM and DEX with an injection volume of 25 µl were 80 and 60 pmol respectively. This method was applied to the analysis of BTM and DEX in commercial tablets.

***ii) Thin Layer Chromatography:*** Thin layer chromatography (TLC) on silica gel with subsequent use of two different mobile phases to attain the separation of impurities in pharmaceutically important corticosteroids was recommended by Clifford et al. who also discussed the advantages and disadvantages of partition chromatography(7). Duthie et al. proposed the use of silica gel impregnated with 0.1 M sodium hydrogen carbonate and a mixture of light petroleum, diethyl ether, butanol and formic acid (100:50:30:2, v/v) as the mobile phase(8).

Hoebus et al. (9) have described the identification of 27 corticosteriod hormones by TLC. They used 13 different combinations of mobile phases. Of these combinations mobile phase I, dichloromethane, diethyl ether, methanol and water (77: 15:8:1.2 v/v), mobile phase II, diethyl ether: toluene: I-butanol saturated with water (80:15:5 v/v), mobile Phase III, acetic acid: carbontetra-chloride: heptane (40:30:30 v/v) and mobile Phase IV, I-butanol:acetic acid:water (60:20:20, v/v) showed the best separation of the corticosteroid hormones.

## 2. Immunoassays

***i) Principles of Immunoassays:*** Immunoassay techniques (10) have over the past 25 years allowed biochemists to understand many physiological, pathological and pharmacological processes. The initial studies by Yalow and Berson (11, 12) involved the use of radioactive iodine ($I^{125}$) as a label for the peptide hormone insulin and radioactive atoms are still one of the most commonly employed labels in the immunassay. The basic principle of competitive immunoassay is shown in the equation as.

$$2Ab + Ag + Ag^* \quad Ab\,Ag + Ab\,Ag^*$$

The unlabelled antigen (Ag) and labelled antigen (Ag*) compete for a fixed and limited number of specific binding sites on the antibody (Ab) molecules. After an incubation period the free and the antibody-bound antigen are separated from each other and the amount of labelled antigen in one of the fractions is determined. At higher concentrations of unlabelled antigen, fewer labelled antigen molecules will be bound by the antibody. Therefore a

calibration graph can be produced and from this graph the concentration of antigen in biological samples can be determined. The growth of immunoassay as a bioanalytical technique is due to a number of advantageous properties (13), which are as follows:

i. It is applicable in principle to the assay of any compound, only a specific antibody and a labelled antigen are needed and these are either available commercially or can be generated in the house.
ii. It is capable of high sensitivity, Amounts as low as $10^{-18}$ mol have been reported as being measurable, the degree of sensitivity is dependent on the avidity of the antibody for the antigen and on the detection systems available to measure the label.
iii. A little or no pre-treatment of sample is necessary.
iv. Many manufacturers produce equipment for the (semi) automation of immunoassays.

***ii) Enzyme Immuno Assay (EIA):*** There are various methods in which enzymes and enzyme active substances such as cofactors, prosthetic groups, substrat and inhibitors can be employed in immunoassay techniques. The advantages and disadvantages of enzyme labels compared with radio labels can be considered in general terms and are as follows:

**a) Advantages of enzyme labels**

i. No radiation hazards occur during labelling or disposal of waste.
ii. Enzyme-labelled products can have a long-shelf life.
iii. Equipment for enzyme assay can be inexpensive and is generally available.
iv. Homogeneous assays can be completed in a few minutes and are readily automated.
v. Heterogeneous assays are ideal for visual qualitative tests.
vi. Multiple simultaneous assays are possible.

**b) Disadvantages of enzyme labels**

i. Sample matrix constituents may affect enzyme activity.
ii. Assay of enzyme activity can be more complex than measurement of some types of radioisotopes.
iii. At present, homogeneous EIAs have limited sensitivity. Enzyme immunoassays can be further subdivided into two major groups.

***iii) Homogeneous (non-separation) assays:*** In these methods(14) the enzyme activity is altered by the immunological reaction when the carrier of the enzyme participates in an antigen-antibody reaction (e.g. EMIT). Moreover in this method small molecules (usually drugs) are labelled with an enzyme. When specific antibody is reacted with the enzyme-hapten conjugate the enzymatic activity is altered, in fact it is usually reduced. The effect of the antibody on the enzyme activity will be reversed by free hapten in the test samples. Thus in this method antibody and hapten-enzyme conjugate were mixed with the test samples and the results compared with those obtained when the same reagents were mixed with hapten free samples and with known amounts of hapten. The more drug present in the test sample, the more enzymatic activity was retained and so the more coloured product was generated from the substrate.

***iv) Heterogenous (Separation) assays:*** In these assays the enzyme activity was unaltered by antigen antibody reaction. In fact the main objective was to preserve as much activity as possible in both the enzyme

and antibody in the conjugate. Thus in the heterogenous systems, reacted antibody or antigen must be separated from the unreacted components whereas in the homogeneous assays no such separation is needed. The best known of these assays is the enzyme-linked immunosorbent assay (ELISA)

An enzyme immunoasssay for dexamethasone and flumethasone residues in milk of intramuscularly dosed cows has been described by Reding, J. et al(14). Its limit of detection for dexamethasone was 0-23 ng $ml^{-1}$ in milk and 2.60 ng $ml^{-1}$ in urine. The heterogeneous immunoassays were further subdivided into competitive and non-competitive types(15).

***a) Competitive immunoassay:***

Competitive assays make it possible to obtain an estimate of the amount of a particular antibody or antigen, even when these can not be isolated from the medium in which they are found. They can also provide useful information about the presence of common and distinct antigenic determinants. Antigen can be measured in a competitive ELISA in two ways. In the first, the microtitre plate is coated with the same antigen or antigen mixture and enzyme labelled antibody specific for the test antigen is added together with the sample (Fig. 1a). In the modification of this method, the enzyme labelled and sample are incubated together before being added to the antigen coated plate, which is slightly more sensitive, if the corresponding antigen is present in the sample, the enzyme labelled antibody will be prevented from binding. An alternative system utilises an antibody-coated micortitre plate. Here enzyme labelled antigen competes with the unlabelled antigen in the system for the antibody on the plate (Fig. 1b).

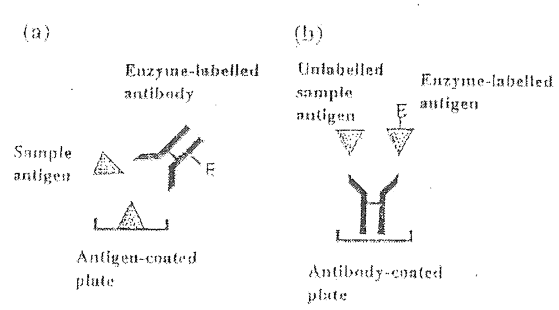


Fig. 1. Competitive ELISA for detection of antigen usiug (a) labelled antibody and (b) labelled antigen.

Competitive assays for measuring antibody have become more common. They are used to compare antibody specificity in studies of antigen structure with monoclonal antibodies. The microtitre plate can be coated with antigen and the test antibody competes with a fixed quantity of enzyme-labelled antibody for binding to the antigen on the plate (Fig 2a). Alternatively, the plate is coated with antibody and the sample and enzyme-labelled antigen added. Here the competition is between the antibody on the micortitre plate and the antibody in the sample (Fig 2b).

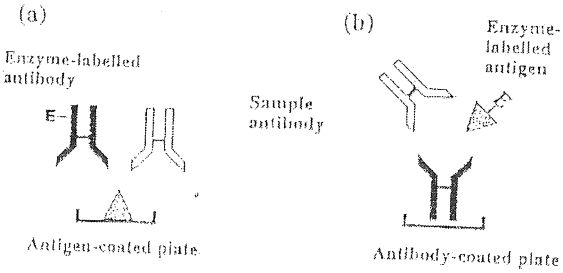


Fig. 2. Competitive ELISA for detection of antibodies through competition with (a) labelled antibody and (b) labelled antigen

***b) Non-Competitive Immunoassay:***

These are probably the simplest types of assays. They are also called indirect or sandwich ELISAs. Microtitre plates are coated with antigen. Sample is added and the bound antibody subsequently

detected by addition of an enzyme-labelled antibody specific for the bound antibody. This enzyme-labelled antibody is referred to as the detector antibody. The detector antibody is not always labelled directly and a second enzyme-labelled anti-globulin antibody, directed against the detector antibody is some times used (Fig. 3).

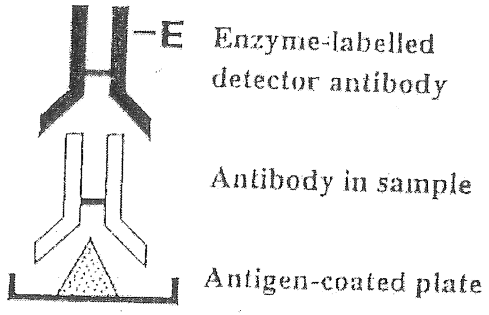


Fig. 3. Antigen –coated plate ELISA for antibody assay.

The two-site assay is also referred to as a sandwich assay. This ELISA uses a pair of antibodies, one bound to the plate and the other labelled. The first binds the antigen and the second enables identification of the antigen. They may be symmetrical if the same antibody reagent is used for capture and as detector, or are termed asymmetrical where these are different. It is essential that all of the antigen in the sample binds (Fig 4).

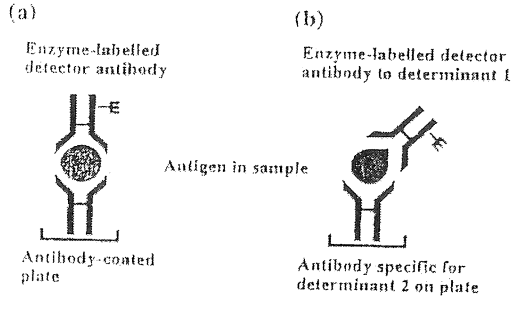


Fig. 4. The two site ELISA (a) Symmetrical (b) Asymmetrical, for antigen assay.

*v) **Radioimmuno assay (RIA):*** Radiolabelling procedures are relatively simple and many labelled compounds are commercially available, and because radiolabels are comparatively small they do not normally affect reaction kinetics (16). A number of different RIA methods for the determination of dexamethasone and related steroids in biological fluids have been investigated by various workers (17, 18).

Another RIA method (19) has been proposed for determining dexamethasone pharmacokinetics in clinically normal dogs during low and high-dose dexamethasone suppression testing. Comparison of pharmacokinetics for the low and high dose protocols revealed dose dependence as; area under the curve, mean residence time, clearance, and volume of distribution increased significantly when dexamethasone dosage increased.

However, radioactive labels also have drawbacks. Preparation of the labelled antigen involves real risks which are cumulative. The labelled antigen shows batch to batch variation and generally has a shelf-life limited to two months for $I^{125}$ although it may be shorter if the radioactive decay causes destruction to the molecular structure. Separation of reacted from unreacted labelled compounds is essential and hence a heterogeneous system is required. The separation process usually involves centrifugation after the addition of a reagent that will selectively precipitate one of the two phases. The supernatant is then removed by aspiration or decantation. Such a separation process is difficult to automate and has limited the development of automated radioimmunassay. Some separation methods have been developed for use in heterogeneous assays but none of these systems have been widely applied (20-23).

***vi) Enzyme Linked Immunosorbent Assay (ELISA):*** The use of enzyme linked immunosorbent assays (ELISAs) for dexamethasone and related steroids in equine urine have been described by various workers.

Rodriquez et al. (24) has described an ELISA method to detect several corticosteroids without hydrolysis of urine samples with a total assay time of 1.5 h for forty samples. Sensitivities obtained in this assay were 2.5, 3.1 and 12.5 ng $ml^{-1}$ for flumethasone, dexamethasone and betamethasone respectively.

Another ELISA method for dexamethasone in equine urine using a universal method of enzyme-labelling antibodies has also been described (25). The limit of detection obtained for dexamethasone was 2.1 ng $ml^{-1}$ and the method requires 2.5 h for analysis of forty duplicate samples. A new simple and sensitive competitive ELISA with a colorimetric end point has recently been developed for the measurement of dexamethasone in equine urine samples by S.S.Hassan (26). The method involves the use of pre-mixed reagent of untreated sheep antiserum and donkey antisheep alkaline phosphatase-labelled second antibody enables analysis of 40 duplicate urine samples within 75 mins. The assay has good precision (Sr. less then 5% for within and between assay runs) and is sensitive (LOD equivalent to 173 ng $ml^{-1}$ of diluted equine samples). The assay has been used to detect the drug over 20 hours in urine from a race horse following interamuscular injection of 20 mg of dexamethasone sodium phosphatase.

Another simple and sensitive competitive ELISA with a flourescent end point has been developed by S.S.Hassan (27) for the measurement of dexamethasone in equine urine samples. The use of premixed reagent of untreated sheep anti serum and alkaline phosphatase lebelled second antibody enables the analysis of 40 duplicate urine samples within 55 minutes. The assay has good precision (RSD less than 10% for within and between assay runs) and is reasonably sensitive (LOD equivalent to 174 ng $ml^{-1}$ of diluted equine sample). The assay has been used to detect the drug over 23 h in urine from a race horse following intramuscular injection of 20 mg of dexamethasone sodium phosphate.

Recently, another enzyme-linked immunosorbent assay and radioimmunoassay to determine serum and urine dexamethasone concentration in thoroughbreds after intravenous administration of the steroid has been described (28). These methods require 1.5 and 2.5 h of total assay time and detect dexamethasone in the range of 0.01 to 50 ng $ml^{-1}$.

***vii) Immunoassay on filters:*** During the past few years there has been an increased interest in the serodiagonosis of parasitic and microbial diseases using dot ELISA techniques (29), which are rapid and simple to perform and inexpensive. The dot ELISA procedure has a number of advantages over ELISA techniques using micortitre plate (30).

In dot-ELISA only a coloured product is formed and positive reaction can visually be assessed against the almost white background of filter disc which also acts as a control for non specific antibody binding, thus expensive ELISA photometers are not necessary (31). A dot-ELISA with a detection limit of 0.1 $\mu g\ ml^{-1}$ for detecting gentamicin in milk has been described by Ara et al. (32).

Another versatile dot-ELISA for detecting small peptides has also been described by Sithigorngul et al. (33). The conventional dot ELISA employs a sandwich format in which strips of

cellulose nitrate are impregnated with dots of capture antibody.

Recently Rowell et al. (34) reported a direct on-filter immunoassay for some ß-lactam antibiotics for rapid analysis of drug captured from the work place atmosphere. This has a limit of detection of 1.9 ng per filter.

A new simple on-filter immunoassay screen has been developed by S.S.Hassan et al. (35) for the detection and semi quantification of dexamethasone in equine urine samples. The assay consists of an indirect competitive ELISA in which dexamethasone in standards or samples competes with an immobilised dexamethasone-protein conjugate for binding to a complex of sheep anti dexamethasone antibodies complexed with alkaline phosphatase-labelled second antibody. The assay has a limit of detection of 390 $ng.ml^{-1}$ for a visual end point in which the colour intensity of spots developed in the presence of samples is compared with those of standard.

## 3) Other Methods

***i) Capillary Electrophoresis:*** A selective capillary zone electrophoresis (36) (CZE) micro assay was developed for the simultaneous determination of dexamethasone sodium phosphate and its major metabolite, dexamethasone in tears. The calibration was carried out in the biological matrix with indoprofen as an internal standard which allowed the separation of dexamethasone sodium phosphate and dexamethasone from the tear constituents. The limits of detection of the assay were 0.5 and 2.0 $\mu g\ ml^{-1}$ respectively.

A method for the analysis of corticosteroids in biofluids by capillary electrochromatography with gradient elution was described by Taylor et al. (37). Urine samples were first purified using solid phase extraction. The linearity of peak area following analysis was determined for hydrocortisone, dexamethasone and fluocortolone by regression analysis over a concentration range of 0.39-25 $\mu g\ ml^{-1}$. The limit of detection was 0.39 $\mu g.ml^{-1}$ for all three corticosteroids.

***ii) Immunoaffinity chromatography:*** Immunoaffinity chromatography (IAC) is a separation method based on specific and reversible interactions between an antigen (Ag) and a matrix-bound antibody (38) (Ab) and is capable of effecting high yield, single stage purification of many biomolecules including proteins of pharmaceutical interest, vaccines and virtually anything to which polyclonal or monoclonal antibodies (Mab) can be made (39-41).

A micellar electrokinetic capillary chromatography combined with immunoaffinity chromatogrpahy for identification and determination of dexamathasone and flumethasone in equine urine has been described by Xuelin, G.U. (42) which requires pretreatment of the sample. Use of a dexamethasone affinity column resulted in low background and detected levels as low as 1.1 $ng\ ml^{-1}$ and 2.7 $ng\ ml^{-1}$ for dexamethasone and flumethasone respectively in horse urine.

IAC has been combined with high performance liquid chromatography (HPLC) and the resulting high performance immunoaffinity chromatography (HPIAC) used to provide sensitive assays for components of complex mixtures (43-45) which are present at very low concentration, such as IgE in blood plasma. Thus immunospecific purification procedures have become established laboratory and commercial tools.

ELISA was compared with immuno-affinity chromatography combined with reversed phase high performance liquid chromatography (IAC-HPLC) for the detection of dexamethasone in equine urine by Neto et al. (46). The ELISA test showed a linearity in the range of 4-500 ng $ml^{-1}$ of urine. The IAC-HPLC confirmation method had a detection limit of 4 $ng.ml^{-1}$ for dexamethasone.

A comparison of immunoaffinity chromatography combined with gas chromatography-negative ion chemical ionization mass spectrometry and radioimmunassay for screening dexamethasone in equine urine was carried out by Stanley et al. (47). The limit of detection of dexamethasone by RIA immunoaffinity chromatography GC-MS were 10 pg $ml^{-1}$ and 132 pg $ml^{-1}$ respectively.

Another method for the determination of synthetic corticosteroid dexamethasone in equine urine was described by Creaser et al. (48) using coupled online immunoaffinity chromatographyhigh performance liquid chromatography with detection by UV or mass spectrometry. The limit of detection for dexamethasone with UV detection was 30 ng $ml^{-1}$ for a 10 ml urine sample. Atmospheric pressure chemical ionization mass spectrometry, with single ion monitoring, gave a limit of detection of 0.1 ng $ml^{-1}$ for dexamethasone.

A quantitative determination of several synthetic corticosteriods by gas chromatography-mass spectrometry after purification by immunoaffinity chromatography was described by Delahaut et al. (49). The estimated detection and quantification limits were 0.25 and 0.50 $ng.ml^{-1}$ respectively.

***iii) Other Hyphenated Methods:*** A method for the determination of dexamethasone in urine and faeces of treated cattle with negative chemical ionization-mass spectrometry was described by Courheyne et al. (50). The maximum levels found in urine and faces were 980 and 744 ng $ml^{-1}$ respectively.

Another confirmatory HPLC-MS/MS method for ten synthetic corticosteroids in bovine urine was described by Savu et al. (51) and the limit of detection achieved was 0.05-1.0 ng $ml^{-1}$ in urine.

***iv) Flow Injection Immunoassay:*** Flow injection immunoasssay (FI IA) is a relatively new approach that combines the advantages of flow injection analysis and immunoassay to perform semi or fully automated measurements of analyte concentrations (52).

A number of truly continuous FIIAs have been pubished(53,54) both in competitive and non competitive formats which do not require washing, incubation or regeneration steps. As a consequence of the continuous signal generation these assays can be coupled on-line to LC without requiring a change in the assay format.

A number of solid phase immunassays have been developed employing a range of binders(55), a binder being any molecule which exhibits molecular recognition for another molecule (ligant). Antibodies are the binders most commonly employed in ligand binder assay and both polyclonal and monoclonal antibodies have been used. The early assays employed Protein A or G as the solid phase absorbent for antibodies, however, a newer approach using thiophilic gels has gained popularity. Porath et al. (56) first described thiophilic gels which bind antibody molecules specifically and subsequently release them under the conditions. A draw back of Protein A and G columns is that elution of the antibodies requires

strong acid conditions whereas thiophilic gels require only a change in ionic strength from the binding and elution conditions and not a pH difference. This has resulted in a simple fluorescence flow injection immunoassay for theophylline (57).

Drug-enzyme conjugate of dexamethasone-subtilisin & dexamethasone-cellulase have been synthesized and their characterization with respect to their drug was carried. Protein incorporation ratio, immunoreactivity, enzyme activity and stability have been described by S.S.Hassan et al. (58).

**References**

1. Tseui, S.E., Ashley, J.J., Moore, R.G. McBride, W.G.: *J. Chromatogr.*145,213 (1978)
2. Rose, J.Q., Jusko, W.J.: *J. Chromatogr.* 162, 273 (1979)
3. Hartley, R., Brocklebank, J.T.: *J. Chromatogr.* 232,406 (1982)
4. Gaignage, Ph., Lognay, G., Marlier, M., Severin, M., Dreze. Ph.: *J. Chromatographia* 28, 623 (1989)
5. Santoro, M.R.M., Govato, E.B., Hackmann, E.R.M.: *Anal. Lett.* 26, 925 (1993)
6. Liu, K.R., Chen, S.H., Wu, S.M., Koy, H.S., Wu, H.L.J.: *J. Chromatogr. A* 676, 455 (1994)
7. Clifford, C., Wilkinson, J., Wragg, J.: *J. Pharm. Pharmacol.* 16 Suppl. 11TH (1964)
8. Duthie, A., Simmons, K., Urey, B.: *J. Chromatogr.* 44,193 (1969)
9. Hoebus, J., Dannels, E. R., Hoogmartens, J.: *J. Planer. Chromatogr.* 6, 271 (1993)
10. Blake, C., Gould, B.J.: *Analyst.* 109, 533 (1984)
11. Yalow, R.S., Berson, S.A.: *Nature.* 184,16 (1959)
12. Yalow, R.S., Berson, S.A.: *J.Clin. Invest.* 39,1157 (1960)
13. Veinryb, I., Schroff, J.R., Olson, D.R.: *Drug Metab. Rev.* 10, 271 (1979)
14. Reding, J., Sahin, A., Schlatter, J., Naegeli, H.: *J.Vet. Pharmacol. Therap.* 20, 198 (1997)
15. A Practical Guide To ELISA. Ist Ed.(P22) Kemeny, D.M. Pergamon Press Plc., Headginton Hill Hall, Oxford, England (1991)
16. Ekins, R.: Radioimmunassay And Related Procedures (In) Medicines 1977 Vol I International Atomic Energy, Vienna P241 (1978)
17. Meikle, W.A.: *Clin. Endocr.* 16,401 (1982)
18. Schalm, S., Skill, S., William, H., Go, V.L.W.: *Mayo Clin. Proc.* 51, 761 (1976)
19. Greco, D.S., Brown, S.A., Gauze, J.J., Weise, D.W., Buck, J.M.: *Am. J. Vet. Res.* 54,580 (1993)
20. Mattiasson, B., Ling, T.G.I.: *J. Immuno. Methods* 38,217 (1980)
21. Cais, M., Shimoni, M.: *Ibid.* 18, 317 (1981)
22. Cais, M., Shimoni, M.: *Ibid.* 18, 324 (1981)
23. Smith, K.O., Gehle, W.D.: *Methods Enzymol.* 70, 288 (1980)
24. Rodriquez, M.L., McConnell, I., Lamont, J., Cambell, J., Fitzgerald, S.P.: *Analyst* 119, 2631 (1994)
25. Robberts, C.J., Jackson, L.S.: *J. Immunol. Methods* 181,157 (1995)
26. Hassan, S.S.: *Pakistan J.Med. Res.* 36,75 (1997)
27. Hassan, S.S.: *Analytical Comm.* 33, 299 (1996)
28. Chen, C.L., Zhu, D., Gillis, K.D., Meleka-Boules, M.: *Am. J. Vet. Res.* 57, 182 (1996)
29. Pappas, M.G.: *Vet. Parasitology* 29, 105 (1988)
30. Pappas, M.G., Hajkowski, R., Hockmeyer, W.T.: *J. Immunol. Methods* 64, 205 (1983)
31. Burt, S.M., Carter, T.J.N., Kricka, L.J.: *J. Immunol. Methods* 31,231 (1979)
32. Ara, J., Gans, Z., Sweeney, R., Wolf, B.: *J.Clin. Lab. Anal.* 9,320 (1995)
33. Sithigorngul, P., Streton, A.O.W., Cowden, C.: *J. Immunol. Methods.* 141,23 (1991)
34. Rowell, F.J., Miao, Z.F., Reeve, R.N.,Cumming, R.H.: *Analyst* 122, 1505 (1997)
35. Hassan, S.S.: *Analytical Comm.* 35,249 (1998)

36. Baeyens, V., Varesio, E., Veuthey, J.L., Gurny, R.: *J. Chromatogr.* B 692, 222 (1997)
37. Taylor, M.R., Teale, P., Westwood, S.A.: *Anal. Chem.* 69,2554 (1997)
38. Bigio, M., Rossi, R., Borri, M.G., Casagli, D.M.C., Nucci, D., Baldari, G., Volpini and Borachi, D.: *J. Immunol. Methods* 123,1 (1989)
39. Jack, G., Wade, H.: *Trends In Biotech.* 5,91 (1987)
40. Janson, J.C.: *Ibid.* 2,31 (1984)
41. Muller, H., Tilburg, N.V., Derks, J., Breteler, E.K., Bertina, R.: *Blood* 58, 1000 (1981)
42. Xuelin, G., Boules, M., Ling Chen, C.J.: *Capill. Electrop.* 3,43 (1996)
43. Davis, G.C., Hein, M.B., Chapman, D.A.: *J. Chromatogr.* 366, 171 (1986)
44. Phillips, T., More, N., Queen, W., Thompson, A.: J. *Ibid.* 327, 205 (1985)
45. Yarmush, M.L., Weiss, A.M., Antonsen, K.P., Odde, D.J., Yarmush, D.M.: *Biotech. Adv.* 10, 413 (1992)
46. Neto, R.L.M.,Spinosa, H.S., Salvadori, M.C.J.: *Anal. Toxicol.* 21,393 (1996)
47. Stanley, S.M.R., Wilhelmi, B.S., Rodgers, J.P.: *J.Chromatogr.* 620,250 (1993)
48. Creaser, C.S., Feely, S.J., Houghton, E., Seymour, M., Teale, P.: *Ann. Comm.* 33,5 (1996)
49. Delahaut, Ph., Jacquemin, P., Colenonts, Y., Dubois, M., Graeve, J.D., Deluyker, H.: *J. Chromatogr.* B. 696, 203 (1997)
50. Courtheyne, D., Vercammen, J., Brabander, H.D., Vandenreyt, I., Batijoens, P., Vamoosthuyze, K., Peteghem, C.V.: *Analyst* 119, 2557 (1994)
51. Savu, S.R., Silvestro, L., Sorgel, F., Hagg, A.: *J. Mass Spectrometry* 31, 1351 (1996)
52. Palmer, D.A., Miller, J.N.: *Anal. Chim. Acta* 303, 223 (1995)
53. Kusterbeck, A.W., Wemhoff, G.A., Charles, P.T., Yeager, D.A., Bredehorst, R., Vogel, C.W., Ligler, J.: *J. Immunol. Methods* 135, 191 (1990)
54. Whelan, J.P., Kusterbeck, A.W., Wemhoff, G.A., Bredehorst, R., Ligler, F.S.: *Anal. Chem.* 65, 3561 (1993)
55. Miller, J.N. , Palmer, D.A., French, M.T.: *J. Pharm. & Biotech. Anal.* 9,115 (1991)
56. Porath, J., Maisano, F., Below, M..: *FEBS Lett.* 185,306 (1985)
57. Palmer, D.A., Evans, M., Miller, J.N.: *Anal. Proc.* 31,123 (1994)
58. Hassan, S.S.: Ph.D Thesis, University of Sunderland (England) P. 72 (1998)

Accepted: 30.06.2000