

High-Performance Liquid Chromatography for Hormone Assay

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Summary

High-performance liquid chromatography (HPLC) is a refinement of traditional column chromatographic techniques. The speed of analysis and the resolution are increased with new column-packing materials and eluant pumped through the column at high pressure. The potential for achieving measurements of hormones in small volumes of plasma or urine is limited, both in normal and pathological situations. Using HPLC with ultraviolet absorption, the detection limit is only nanogram amounts of hormones per milliliter of blood serum. The applications of the technique to specific hormones from recent and older literature will be used throughout this chapter to illustrate aspects of the technology.

Key Words: HPLC; UV absorption; reproductive steroids; adrenal steroids; thyroxine; insulin; angiotensins; melatonin; neuropeptides; endorphins; renin.

1. Introduction

High-performance liquid chromatography (HPLC) is often used for purification of samples prior to an indirect detection technique such as immunoassay (IA). Metabolic studies are also performed using HPLC to resolve the metabolic products. Some HPLC meth-

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ods come close to standards for reference technology. For peptide hormones the major uses of HPLC have been for purification, qualitative analysis, and structural determination. Clinical studies are sometimes dependent on indirect measurements when, for example, monitoring hormone treatment. Therefore, in insulin-treated diabetics, management of patients has been improved by monitoring glycated haemoglobins in blood samples, separated by HPLC with reverse phase (RP) and cation exchange. The quantitative analysis of hemoglobin A1C is used as a marker of insulin action and glucose control in those patients (*I*). With the introduction of new technologies for producing hormones, the testing of pharmaceutical preparations for purity is an area where HPLC has been particularly useful.

HPLC analysis of hormones in biological fluids has to compete in the laboratory with IA that is capable of measuring hormone concentrations rapidly in numerous small samples (10–50 μL) of body fluid equivalent to as little as 10 pg/mL. There are diurnal variations in the concentrations of hormones in blood as well as pulsatile secretory patterns. Several samples may need to be taken to interpret results with time. Measurement of a hormone concentration in plasma provides insight to the tissue exposure of the hormone. Plasma is therefore a more popular medium for analysis than urine. The latter still provides an important source of information because the steroid content of a 24-h urine collection is an integer of the daily production.

HPLC is an important chromatographic technique for hormones for several reasons.

1. High temperatures are not required.
2. Material can be recovered from the column eluates for further analytical procedures.
3. Resolution is superior to TLC and paper chromatography.
4. HPLC offers the potential and versatility for separation of different forms of hormones such as free and conjugated steroids, pro-hormones, hormones, and peptide fragments.
5. The range of detectors. UV absorption is the most useful of current detectors, other hormones can be detected with a refractive index or an electrochemical detector.

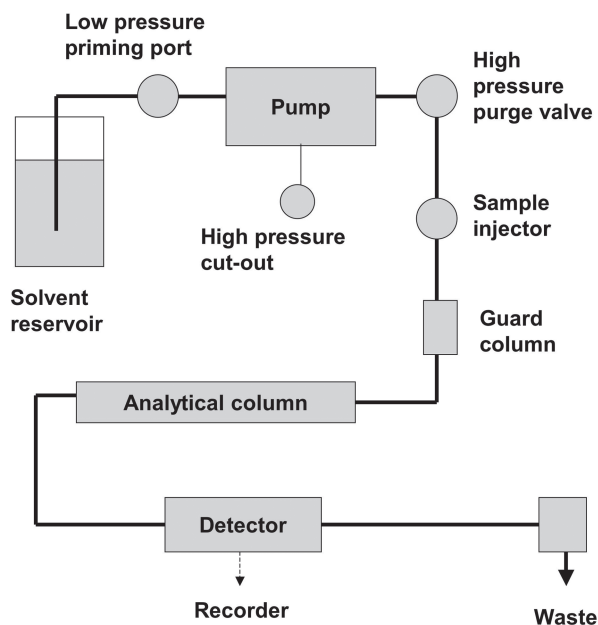


Fig. 1. Schematic of an HPLC system for hormone analysis.

2. Materials

Figure 1 is a block diagram of a typical HPLC system. The mobile phase is contained in the solvent and is delivered to the column through the action of a pump that controls eluant flow at up to 6000 psi. Gradient elution of the column may require more than one reservoir, pump, and eluting reagents. The flow of the separate pumps is regulated to generate a varying mixture of the eluting reagents with ability to have gradients of different shapes as part of the computer-controlled package. There are two main types of pump, the syringe and reciprocating pump. The most popular applications use constant flow of column eluant. The advantage of syringe pumps is that the flow is pulse free, this is particularly important with electrochemical detectors. A pressure display is important to monitor the outlet pressure. Changes in pressure indicate blockages or declining column conditions. The column is placed between the column injector and the detector. A system may also include provision for pre- or post-

column reactions (derivatization). The detector then couples to a recorder or back to the computer operating/data management.

2.1. Columns

Separation in HPLC is effected as components move in the liquid mobile phase through interactions with the packing of the column. Typically columns are 10–25 cm in length and less than 5 mm in diameter. Porous, silica-based packing materials, of 3–40 μM in particle size, of rigid material can withstand high pressure. Sinter disks (frits) are used to cap the ends of the column and act as filters. The packings can be coated with a stationary phase or be chemically bonded. The separation of hormones with HPLC can be effected by absorption, partition, ion-exchange, RP, and RP ion-pair chromatography.

High-performance silica and alumina columns give excellent separation of steroids, but are less popular these days. RP phases with C_{18} , C_8 , C_2 , and phenyl substitution have been used. The chromatography depends largely on partition so that separation will vary with the carbon chain length and the nature of the mobile phase. The size and consistency of the porous packing will also affect analytical performance. RP columns that have 60,000 or 80,000 theoretical plates per meter offer excellent resolution and sharp peaks permit detection to around 1 ng of many hormones (see **Table 1**) (2–14).

Cyano and amino phases have been used to effect separation of corticosteroids (15) and oestrogens (16). HPLC of polar oestrogens has been achieved on ion-exchange columns (17). Size exclusion analysis has been used for the separation of insulin and its degradation products (11). Micro bore columns of less than 2 mm id may permit increased sensitivity by narrowing the elution peak unless the volume of sample and the total mass of material in the extract cause a loss of peak shape and resolution. Antibodies to thyroxine (T4) were purified and subjected to proteolytic degradation. The resulting peptides were purified using microbore columns (18).

Naturally occurring mixtures of steroid hormones have a wide range of polarities, but steroids of similar polarity, that may be

Table 1
Reverse-Phase HPLC for Hormones

Hormone	Source	Reference
Gonadotrophin surge-attenuating factor	Ovarian follicular fluid	2
Corticotrophin-releasing hormone and POMC peptides	Human skin	3
POMC and ACTH peptides	Skin	4
α -melanocyte stimulating hormone	Phaeochromocytoma tissue	5
Recombinant IGF-1		6
Calcitonin	Incubation with enzymes and gut mucosa	7
Anabolic steroids	Urine	8
Proinsulins	Rat pancreas	9
Insulin and related peptides		10
Insulin	Pharmaceutical preparation	11
25-hydroxy-vitamin D	Serum	12
25-hydroxy-vitamin D	Serum	13
Arginine vasopression	Pharmaceutical preparation	14

derived from different metabolic pathways, tend to elute in clusters. Careful selection of the stationary phase from the range of commercially available products can enable a system to be devised with high selectivity (**19,20**). Silica packings, bonded to octadecyl or diol groups, are the most popular for general use. Supports differ in particle size, porosity, and levels of residual-accessible silanol groups. Synthetic polymers may be more inert than silica. The physical characteristics of packings have been studied with various solvent gradients. The resolution and elution order of steroids has been reported in a number of publications. There seems to be no easy means, however, to identify the most suitable packing for a particular separa-

tion. Selective differences cannot be firmly attributed to alkyl chain length or to shape of the packing.

Supports can have variable and often incomplete coverage of residual silanol groups, which affects separation, peak shape, and recovery. Some packings with uncapped silanol groups are chemically reactive with hormones owing to intramolecular hydrogen bonding. This leaves the phase acidic and may explain the instability of certain hormones. Aldosterone and 18-hydroxylated steroids are susceptible to a number of reactions on certain columns that can influence the quality of the HPLC result. Acid residues, such as residues found on uncapped HPLC supports, can lead to ring closure of such steroids with a bridge of C-18 to C-20 or C-21. In the presence of methanol this may lead to formation of methyl ethyl ketals. Other products, dimers, and isomers are possible leading to a number of peaks in the HPLC analysis of a single compound. These products can have retention times spread throughout a solvent gradient elution of a RP column. This may be disastrous in interpretation of a metabolic study unless products are characterised by other means. Such supports are not recommended for aldosterone and related steroids, e.g., 18-hydroxycorticosterone (21). The extent to which a packing is not covered can be determined by a methyl red absorption test (22).

Porous graphitic carbon is a new micro-crystalline packing material for RP chromatography that is chemically stable to strong alkali and acid. As it contains no unreacted silanol groups commonly found on silica based RP materials it is considered to be a pure RP adsorbent and may overcome some of the difficulties of silica-based columns described previously. Corticotrophin-releasing factor (CRF) and gonadotrophin-releasing hormone (GnRH) were among the hormones purified with columns of high carbon content (23).

Very nonpolar material will accumulate on an RP column and decrease separation but this can be reduced by the use of a guard column (30–70 mm in length) containing a larger (30- μ m particles) pellicular equivalent of the analytical column. In an assay for simultaneous assay of cortisol, prednisone, and prednisolone a C8 guard column was used before the C18 analytical column (24). Guard columns are cheap and can be dry-packed. The first few millimeters of packing from the analytical column can also be replaced at intervals.

2.2. Mobile Phases

Useful separations of hormones can be achieved using chromatography on silica with isocratic (fixed) mixtures of two (binary) solvents (25–27). RP columns eluted with polar binary solvent mixtures, usually methanol or acetonitrile with water, are now used widely for separations of hormones. The polarity of the solvent is an important factor in the separation of molecules using HPLC. The elution ability of a solvent is reversed with RP-HPLC, the more polar the phase, the longer the retention time. Other factors need to be considered. The lower the viscosity of a solvent the lower the pressure needed to operate the column. Solvents and any impurities they contain may affect the detection system (particularly the UV absorption). Solvents such as ethers form unstable peroxides when exposed to oxygen and may need stabilisers to combat this. Methyl-*t*-butyl ether is a useful substitute for diethyl ether. Tetrahydrofuran is a good solvent for higher molecular weight analytes. The eluting solvents should be degassed to avoid bubbles in the low pressure of the detector and filtered before use. Mobile phases need careful preparation. Aqueous components need to be adjusted for pH.

The separation of a range of related hormones is best achieved with gradient elution. Additional pH, ion-pair, and modifier effects can be incorporated. Retention times are reproducible between runs provided that the column is equilibrated to the starting solvent mixture. Methanol/water gradients effect the separation of the major adrenal steroids. Dioxane is a better choice for the separation of polar adrenal steroids and acetonitrile is preferred for resolving testicular steroids. Peak shape and resolution can be improved by maintaining the column at a fixed temperature above ambient, e.g., at 45–60°C when analyzing steroids, but this could lead to degradation of peptide hormones. At higher temperatures, the eluent viscosity is reduced (28). If working at ambient temperature it is advisable to have a room with well-controlled temperature to achieve reproducible retention times.

The use of three and four solvents in a mobile phase system has overcome the difficulties in choosing the appropriate column pack-

ing for a particular steroid separation. Systematic, statistical procedures for solvent optimisation have been developed (29–32). Derks and Drayer (33) reported the separation of very polar 6 α and 6 β hydroxylated metabolites of cortisol by isocratic elution from a silica column with water:chloroform:methanol. A computerized system for optimization of conditions for RP-HPLC with respect to temperature and gradient steepness has been tested for a number of compounds, including corticosteroids and androgens (34).

Phosphate can be incorporated into the mobile phase (35) and, with this system, the buffer anion and pH exert significant effects on the separation (36). Salts are used in the eluting solvent in the analysis of oestrogen conjugates, but this may in the long-term corrode the steel of the columns and tubing. Oestrogens can be effectively separated when silver nitrate is included in the mobile phase to give 2 g of silver nitrate with 60 mL methanol, 40 mL water at 0.55 mL/min (37). To prevent metallic silver building up on the column a water:methanol (50:50 v/v) mobile phase is used each evening to flush excess silver nitrate from the system. Even so, a small build-up can occur that requires a rinse with dilute nitric acid or replacement of the tubing when back-pressure rises.

Steroid conjugates can be separated using ion-pair chromatography. Andreolini et al. (38) show excellent separations of oestrogens on RP-18 packings by eluting with a gradient of acetonitrile/methanol and phosphate buffer containing cetyltrimethylammonium bromide. Cyclodextrins have been used for micellar chromatography of steroids with variable success (39–41). Trifluoroacetic acid has been used in eluting solvents for insulin (42), steroids (43), CRF and GnRH (23), and angiotensin II (44).

Gradient elution is usually necessary to elute a series of hormones after extraction from biological fluids. Gradient elution reduces analysis times and depending upon the gradient shape can optimize separation and improve peak symmetry. Nonlinear, stepped, and linear gradients of solvent proportions have been largely dictated by the available facilities for programming the pumps. Flow and temperature programming can also be used. Simultaneous variation in temperature and gradient steepness for RP-HPLC of steroids may

be useful for improved resolution of corticosteroids, but not substituted testosterone compounds (34).

2.3. Sample Injection

The sample injection system is usually a loop on a valve system controlled by opening and closing valves to direct eluant flow in several directions. The sample loop itself can be between 1 and 100 μL . A syringe with the sample, or an automated sample injection system, inserts the sample through a septum. Dead volume is kept to a minimum in the system by using narrow-bore stainless steel tubes with low dead-volume connections.

After extraction the hormones are usually dissolved in the mobile phase. The addition of a suitable macromolecular matrix, e.g., polyethylene glycol to the extracting solvent prior to evaporation can improve the recovery because the hormones may dissolve poorly in the mobile phase alone. Injectors that encompass rubber septa should be avoided. At the high instrument sensitivities, often used for the analysis of hormones spurious and irreproducible peaks, may occur in the chromatogram. These may reflect the action of injected solvents on the septum. Injection valves are, therefore, preferred.

2.4. Detection

The simplest detectors for hormones are single wavelength spectrophotometric detectors. Variable wavelength detectors are now common and very sensitive. Detection cells should have small dead volumes. Diode array detectors provide a repeated UV spectrum of the column eluate and, thus, for a component eluting in a single peak from the HPLC there is precise identification.

Peptide hormones are easily detected by UV absorption of peptide bonds at 210 nm or tryptophan residues at 280 nm. Static/dynamic light scattering has been used to detect insulin (11). Steroid hormones with the α , β unsaturated ketone in the A-ring absorbs UV light with maximum around 240 nm and extinction coefficients 12,000–20,000. Isolated carbonyl groups absorb with a

maximum at 280 nm (275–285 nm) and molar extinction coefficients of 17–155. The natural oestrogens have peak absorption at 280 nm because of the aromatic A-ring and can be detected themselves with sensitivity limits of 100–10 pg/mL. Although steroids can absorb UV below 200 nm, in practice it is difficult to attain a clear signal from noise without a reduction in sensitivity particularly when solvent gradients are used to elute the steroids. With some gradient elution systems it is necessary to correct for baseline variation by comparison of the response of the eluate from the analytical column with the flow of solvent alone through a reference cell. Interference in UV detection of a synthetic progestogen (Medrogestone) by endogenous steroids in HPLC analysis was achieved by reaction with oxalyl chloride and UV detection at 242 nm (45).

Nanogram quantities of steroid hormones can be detected by the use of fluorescence (46), refractive index (47), and electrochemical detectors (48,49). Melatonin has been detected by fluorescence (50) and electrochemical detection (51,52). Chemiluminescence has been used for proinsulin and C-peptide (53).

In some cases, it has been necessary to react the hormones in the eluate from the column with reagents to form UV-absorbing derivatives. Post-column derivatization methods are restricted to very fast reactions limiting the scope of application (46). Using HPLC with post-column derivatization melatonin was shown to be unstable in commercial formulations when exposed to light (54).

Most of the urinary steroid metabolites are inert in HPLC detectors. Reactive groups have been utilized in order to make derivatives for spectrophotometric detection. HPLC has, thus, been used to separate individual oxo-steroids after conversion to phenylhydrazone derivatives. 3 α -Hydroxysteroids are detected using a dehydrogenase reactor (55). Melatonin in pineal gland extracts were determined by HPLC with pre-column derivatization (56).

Several laboratories have demonstrated the variety and complexity of intermediates and products formed when radioactive steroids are incubated with steroid metabolizing tissues. Flow-through radioactivity detectors have been useful for examining the products of reac-

tions with labeled substrates (57,58). T metabolism in human hair follicles (59), the action of Formestane (a steroidal aromatase inhibitor) in breast cancer patients (60), and dehydroepiandrosterone sulphate (DHAS) transport across the blood–brain barrier (61) are recent examples. A number of metabolites of dexamethasone formed in vitro in human liver were found using this approach and later identified by LC coupled with mass spectrometry (MS) (62). The short resolution time of the sample components in the radioactive counting chamber limits sensitivity. Current detection limits for tritium are 10,000 dpm with flow cells incorporating scintillant (around 1% efficiency) to 1000 dpm (50% efficiency) when the column effluent is mixed with liquid scintillant before passing through a flow cell.

The coupling of HPLC with MS is more widely used for hormone assay than gas chromatography (GC)-MS. The coupling of LC with MS necessitates the removal of a solvent mobile phase that can flow at rates up to 2 mL/min. Interfaces that rely on heat for the production of a fine spray of solvent (Thermospray) are now relatively common. The spray is directly heated as the sample passes through a fine capillary leading from the HPLC to the MS. The fine droplets continue to vaporize as they pass into the source. A portion of this vapor and the ions produced in the ion source pass into the mass spectrometer through a sampling cone and the remainder is pumped away by the mechanical vacuum pumps. Direct ion evaporation involves evaporation into the gas phase of ions present in solution. This process is improved if there is a volatile buffer like ammonium acetate in the LC mobile phase. A secondary type of ionization uses a filament to initiate chemical ionization. Thermospray can be used in negative or positive ion modes.

In electrospray, an electric field is used to cause ions to evaporate from solution. Compressed air is used to force the sample from the HPLC through a fine capillary to a jet into the MS. This system operates at ambient temperatures and ionization is gentler than with thermospray. The combination of micro-bore column HPLC with thermospray or electrospray MS seem to be the most promising systems. Spray interfaces are used mainly with quadrupole MS. A disadvantage of mild ionization is in the lack of fragmentation that

reduces specificity of the technique as a detection system. Increased structural information can be obtained if molecular ions (or fragment ions) are induced to fragment further by collision induced dissociation in a collision cell placed between two MS systems (tandem MS). LC-MS is now used for a range of hormones, including steroids (63–69), neuropeptides (70), β -endorphins (71), enkephalins (72), melatonin (73), angiotensin I and II (74), arginine vasopressin (75), plasma renin activity (76), and thyroxine (77).

2.5. Identification

A homogeneous peak at an elution time that coincides with that of the reference compound under similar conditions are indications of the identity of material in a chromatogram. 3 Keto-4-ene steroids can be distinguished from other possible compounds eluted from the column by monitoring the UV absorption at further wavelengths using a photodiode array detector (78). Photodiode array has also been used for vitamin D metabolites (79). A mass spectrum is a fingerprint of any compound.

A separate analysis with a different column (preferably of opposite polarity) or a different gradient elution system adds confidence to the analytical specificity. Should the identification in each system coincide with the same standards it is highly probable that each chromatogram reflects the same steroid content. These criteria have not been rigidly applied in the published work relating to hormones. The combination of LC column retention time with a mass spectrum or UV absorption spectra comparable to those properties of reference compounds are taken as high standards in identification.

3. Quantitative Methods

The chromatographic peak height or area is measured manually or with the aid of an integrator and, ideally, the response of the analyte is compared to the response of an appropriate internal standard. The ratios of response for the analyte to the signal from the internal standard are plotted for the concentration range of interest. The concentration of an unknown amount of hormone in the sample is

determined from a calibration curve. There are a vast number of synthetic steroids and hormones available that can be used as internal standards. Because a number of steroid-based drugs are widely used in hospital patients, there is potential for interference in steroid assays. The use of two very different internal standards (e.g., 19-nortestosterone and 6 α -methyl prednisolone) (**80**) prevents erroneous results in the case of medication by either one of the steroids selected provided that they behave in a similar way to the analyte during the analytical procedure.

When internal standards are not used, the extraction and injection must be carefully controlled before peak response can be reliably derived from a calibration curve on injected standards. A deferred standard technique can be adopted in which a known amount of the analyte is injected in pure form some time after, but during the chromatographic run of the unknown sample.

One major drawback to HPLC lies with the inability of UV detectors to provide suitable sensitivity and selectivity for analysis of many of the hormones in plasma or tissue extracts. Coupling with IA provides the requisite sensitivity to many hormone detection systems. The eluates from an HPLC analysis can be collected, dried down, and reconstituted in buffer for IA. This has been used for steroids (**81–83**), CGRP (**84**), thyroxine (**85**), angiotensins (**86**), and gastric inhibitory peptide (**87**).

3.1. Preparation of Samples

In general the analysis of hormones in biological materials requires the following:

1. Treatment of the specimen—extraction, hydrolysis of conjugates.
2. Pre-treatment of samples to produce derivatives that will enhance separation or increase sensitivity of detection.
3. HPLC analysis and detection of the individual components including post-column derivative formation to enhance detection.

3.2. Extraction

Internal standards of tritium-labeled hormone can be added to a sample or extract in order to check recovery. The sample should be

left for several hours, e.g., overnight at 4°C, in order to attain equilibrium of labeled with endogenous hormone. Steroids have been traditionally extracted from aqueous solutions and tissues by use of organic solvents, e.g., dichloromethane, ethyl acetate. A high ratio of solvent to aqueous phase is required to avoid emulsions. Serum is usually mixed for several minutes by vortexing or rotation. The layers are separated by centrifugation and the aqueous layer is usually removed by suction. Freezing the aqueous phase (in a bath of dry ice and methanol) provides a useful method of retaining water in the tube while decanting an organic layer. To avoid large “solvent front” effects in the chromatogram the extracts are washed with 0.1 *M* base or with hexane to remove saponifiable lipids or nonpolar materials. The organic phase is partly dried by addition of anhydrous sodium sulphate. A completely dry extract is produced by evaporation under nitrogen or by rotary evaporation under vacuum.

Solid-phase extraction using small cartridges containing silica modified by a functional group covalently bonded to the surface, e.g., octadecylsilyl groups (Sep Pak C18, Waters Associates; Bond Elut C₁₈, Analytichem) are ideal for extraction and recovery of hormones including metabolites and conjugates that are very water soluble (88). Some highly polar metabolites of cortisol (notably 1β and 6α hydroxylated metabolites of cortisol that are important excretory products of the hormone in urine of newborn infants) (33) and aldosterone (found particularly in renal tissue) (89) are highly polar and are poorly recovered from water with organic solvents. SPE has been incorporated into a quantitative assay for insulin in blood (42) and plasma renin activity (76).

The particle size of the solid phase and character of the solvent requires that the sample be forced through or sucked through the columns. The following basic steps are utilized:

1. Column preparation.
2. Sample application and clean-up.
3. Analyte elution.

Methanol is passed through the solid phase to wet the surface of the packing material. A flush of water or buffer is then used to dis-

place the methanol. The volumes of washes used in these steps are not critical—typically being 2–5 mL. The sample is then passed through the column and the compound(s) of interest are retained. One or more washes with water or solvents can be used to selectively remove salts and other undesirable compounds. The compound(s) of interest are then eluted with a relatively small amount of solvent (such as methanol, acetone, or ethyl acetate) (2–5 mL) so that solvent can be subsequently evaporated rapidly to leave a dry extract. Certain undesirable compounds that are absorbed on the column may be selectively removed by washing with a specific solvent or buffer prior to final elution. Compounds of interest are eluted with a solvent selected on the basis of polarity and or acidity. These columns have also markedly improved the purity of mixtures injected onto HPLC columns, essentially acting as pre-columns.

The techniques based on extraction of solutes with solid matrix cartridges have been incorporated into automated analysis. The initial extraction is still a manual or semi-automated process. However, an automated sample—pretreatment procedure (90) was reported for the analysis of a synthetic steroid, Triamcinolone, in urine. An automated technique based on dialysis and SPE trace enrichment (ASTED) (91) has been applied to the analysis of cortisol and corticosteroids in serum (92).

Lipidex 1000 is inert with low polarity and with the absence of irreversible absorption. The gel acts as a solvent with high capacity for lipids. Inorganic and polar organic materials are readily removed with water. This extraction procedure is superior to the use of silica, which binds steroids strongly and causes decomposition of some steroids (93).

A powerful clean-up of samples can be achieved with immunoaffinity chromatography before LC-MS analysis. Antibodies to the analyte are supported on column packing support, such as Sepharose, and enrich the analyte of interest. Sepharose cannot withstand the high pressures of an HPLC system and is therefore an independent enrichment of sample. Rigid polymers such as polyhydroxyethylmethacrylate are now available that can be incorporated in a column on-line with the HPLC (94).

3.3. Pre-Column Derivatization

Steroid hydrazones formed by reaction of ketosteroids with 2,4-dinitrophenyl-hydrazine have strong UV absorption (maximum 260 nm and extinction around 10,000) as well as visible absorbance (maximum 350 nm, extinction 10,000) giving detection limits for DHAS of 80 ng/mL (95).

Kawasaki et al. (96) described the measurement of 17-oxosteroid conjugates in urine and serum by HPLC of dansyl hydrazine derivatives coupled with a fluorescence detector. Acidic metabolites of cortisol in urine (corticoic acids, formed by oxidation of the primary C-21 hydroxyl group) have been detected as the pyrenylmethyl-21-oic esters (97). Identification was confirmed by UV, fluorescence spectroscopy, and supported during evaluation by photodiode array and MS. Oestrogen and corticosterone in plasma were derivatized with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole before RP-HPLC and fluorescence detection (98,99).

3.4. Applications of HPLC for Hormone Assays

Many applications have already been cited in references utilized previously to illustrate methodological points. Some additional recent references in these areas have included:

1. Hormone assays in biological fluids and tissues—such measurements may be needed in clinical studies (100–102), outcome of experimental studies in vitro (103–110), and detection of hormone abuse (e.g., doping control in sports) (112–115).
2. Checks on other analytical technologies, e.g., comparison with results obtained by radioimmunoassay to reveal the extent and nature of crossreaction in the IA (84,116–119).
3. Purity and stability checks on pharmaceutical products and reference laboratory materials (120–124).
4. As one of a series of analytical maneuvers to achieve purification of a hormone.

It should now be clear that HPLC has an important analytical role in hormone assays as a stand-alone system for hormone analysis and as a purification technique within a series of steps to achieve high-quality specific assays.

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