Automation of Radioimmunoassays for Some Sex Steroids with Use of Both Iodinated and Tritiated Ligands

G. L. Hammond, L. Vlinikka, and R. Vihko

We describe an automated technique for estradiol, progesterone, and testosterone, in which System Olli 3000 pipetting and incubation units are used. After extraction or chromatography, steroids are redissolved in ethanol or buffer, and duplicate aliquots are arranged for radioimmunoassay in 24-tube blocks. Addition of antibodies, tracers (\(^{125}\)I or \(^{3}\)H), dextran-coated charcoal for separating free and bound ligands, and removal of a portion of the supernate for counting are all performed by the pipetting instrument. Incubations are at 37 °C in the incubation unit, or at 4 °C. After counting, steroid concentrations are computed from punch tape records by a Nova 840 computer. The management of assays in 24-tube units, and accurate simultaneous pipetting has reduced experimental error, and because there is no carryover, many different assays can be performed concurrently or in rapid sequence. Various scintillation media are compared.

Additional Keyphrases: analytical systems • System Olli 3000 • choice of scintillants • \(^{125}\)I or \(^{3}\)H-labeling • steroids • progesterone, testosterone, estradiol • data processing

In recent years an exponential increase in the research and clinical application of radioimmunoassays has created a demand for manipulative aids, to increase the precision and general efficiency of the technique. There now are several systems for sample preparation and the automation of subsequent assays. However, because of inherent problems of carryover, priming, cleaning, and the necessity for chemically inert components most of these systems tend to be rather inflexible and beyond the financial scope of most laboratories. The instrument of choice ought therefore to be extremely versatile and provide the operator with a wide variety of alternatives, in order to justify and optimize the initial capital outlay. To achieve this, we have adapted the pipetting and incubation modules of an existing System Olli 3000 automated clinical chemical analyzer for our radioimmunoassay needs.

The management of assays in unit blocks has become a familiar principle in many approaches to automation, but, unlike most, the system to be described makes use of a multipipetting unit capable of dispensing or aliquoting up to 24 volumes (50–2000 \(\mu\)l) simultaneously. This arrangement has several particular advantages: reagents may be added and samples and supernates apportioned more rapidly than by conventional "one-step" systems (24 100-\(\mu\)l transfers may be made within 20 s); suspensions such as dextran-coated charcoal (DCC)\(^1\) may be added to separate bound and free radioactive ligands (96 600-\(\mu\)l additions and mixings/100 s), thus shortening incubation time and increasing precision. Furthermore, the use of disposable or washable pre-arranged tips facilitates the availability of the machine for different assays, which may be run concurrently or in rapid sequence, because there is no longer a need to clean and prime components, or any possibility of carryover between samples or from one assay to another.

In this paper we present an evaluation of the adaptation of a System Olli 3000 automated clinical chemical analyzer for the automation of steroid radioimmunoassays by use of \(^{125}\)I- and \(^{3}\)H-labeled ligands. We also describe suitable scintillation media ("cocktails") that are available for "high sample load," low-volume scintillation counting.

Materials and Methods

Reagents

Apart from the reagents used for the radioimmunoassay of estradiol, details of antisera, the origins of tritiated steroids and cold steroid standards, as well as details of solvents used for the pre-radioimmunoassay

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\(^1\) Nonstandard abbreviations used: estradiol 6-CMO, 1,3,5(10)-estatrien-3,17\(\beta\)-dion-6-1one 6-carboxymethylxozime; BSA, bovine serum albumin; progesterone 11-HS, 11\(\alpha\)-hydroxy-4-pregn-3,20-dione 11-hemisuccinate; testosterone 3-CMO, 17\(\beta\)-hydroxy-4-androsten-3-one 3-carboxymethylxozime; DCC, dextran-coated charcoal; PBS, phosphate-buffered saline (9 g of NaCl per liter, 0.1 mol of phosphate per liter), containing 10 g of gelatin per liter.
extraction and chromatography of several other steroids have been published previously (1, 2). The antibody used in the estimation of estradiol was raised in a rabbit against estradiol 6-CMO/BSA. The equilibrium association constant measured by using [3H]estradiol at 4 °C from a Scatchard type plot was 1.1 × 10¹⁰ litres/mol, and, as can be seen from Table 1, the antibody was extremely specific. Nonradioactive estradiol was obtained from Steraloids, Inc., Wilton, N. H. 03066, and purified before use, on Sephadex LH-20 (3), as was the [2,3,6,7-3H]estradiol (90–110 kCi/mol) obtained from the Radiochemical Centre, Amersham, U.K. Estradiol 6-CMO, progesterone 11-hemisuccinate, and testosterone 3-CMO were purchased from Steraloids, Inc., and were iodinated with ¹²⁵I (Radiochemical Centre) and purified by thin-layer chromatography as described by Nars and Hunter (4). “Ready-solv VI” and “Ready-solv HP” were obtained from Beckman Instruments Inc., Fullerton, Calif. 92634; “Ria-Luma” and “Ria-Luma–PBS” were prepared and supplied by Lumac Systems AG, Aeschengraben 6 CH-4051, Basel, Switzerland. “Insta-Gel” was purchased from Packard-Becker B.V., Groningen, The Netherlands. Small 6-ml scintillation vials, which could be inserted into conventional glass 20-ml scintillation pots, were obtained from Sterilin Ltd., Teddington, Middlesex, U.K.

**Table 1. Relative Affinity of the Antiserum to Estradiol 6-Carboxymethyloxime/Bovine Serum Albumin for Some Steroid Ligands**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>1.000</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.009</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.003</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Androsterone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5β-Androstan-3β,17β-diol</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5β-Pregnane-3β,20α-diol</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt;0.00001</td>
</tr>
</tbody>
</table>

The protocol used in automating most of the radioimmunoassay procedures was essentially the same as that outlined for the manual approach, except that the dispensing of reagents and the removal of an aliquot of the supernatant fluid for counting were performed in units of up to 24 by the Olli dispenser 216; the optimal number of tubes per assay being 96. However, rapid routine assays for testosterone, estradiol, and progesterone for clinical purposes were developed with use of iodine-labeled ligands, the design of which was as follows: serum samples were pipetted either manually or by the dispensing unit into 100 × 12 mm glass extraction tubes, to which a 10-fold excess of solvent was added (for testosterone and estradiol, diethyl ether; for progesterone, petroleum ether, BDH Ltd., Poole, U. K., bp range 64–69 °C). Extraction tubes were tightly stoppered with plastic corks and vigorously shaken in a horizontal shaker for 10 min. Aqueous phases were frozen in a solid CO₂/ethanol mixture and the organic phase was decanted either directly into assay tubes (in the case of estradiol and progesterone) or into 10-ml graduated tubes (testosterone). Extracts of estradiol and progesterone were evaporated at 37 °C concurrently with assay tubes containing the respective standards in 100 μl of ethanol and a similar amount of extraction solvent; 100 μl of ethanol was also added to sample extracts. For this evaporation we used the Olli incubator 354, modified to include an air-tight manifold through which nitrogen could be directed by means of jets positioned over each tube; under the pressure of the inflowing nitrogen, solvent vapor was forced out of the unit via an exhaust pipe attached to the front of the unit. Extracts of testosterone were evaporated under a nitrogen stream in a water bath at 37 °C.
After the evaporation, 1 ml of PBS was added to the residue, the samples were sonicated for 5 min, and 100 μl was taken for the radioimmunoassay. Standards in 100 μl of ethanol were evaporated in the modified incubation unit as described for estradiol and progesterone, and 100 μl of PBS was added. Assay tubes were arranged on prelabeled cuvette code plates, by which units of 24 tubes could be transferred between blocks at different stages of the procedure. To samples and standards, 200 μl of antibody, at a dilution binding 30–40% of the radioactive steroid in the absence of cold steroid, was added by the dispenser 216. After 10 min at room temperature, 200 μl of the radioactive steroid (about 20 000 cpm) in PBS was added in a similar fashion. The incubation blocks containing the assay tubes were then inserted into the incubation unit and warmed at 37 °C for 50 min, after which the assay tubes were transferred to cold blocks and incubated at 4 °C for a further 10 min, before DCC was added. The DCC suspension was premixed at 4 °C on a magnetic stirring block, which could be easily removed from refrigerator to dispensing unit and replaced after use. In this way, the DCC was added at room temperature as a pre-chilled, even suspension, and assay tubes were retained at 4 °C in chilled blocks. After 500 μl of DCC was rapidly added and mixed, the assay tubes were reincubated at 4 °C for 15 min. They were then transferred to the Janetzki S70 centrifuge and centrifuged at about 2000 × g for 5 min at room temperature and 700 μl (or 70%) of the supernatant fluid was removed with the dispensing unit and redispensed into small plastic tubes, which were corked manually and the radioactivities counted for 1 min in an LKB-Wallac 1280 Ultrogamma Counter.

Assays in which 3H tracers were used could likewise be incubated at 37 °C for 50 min. The separation of bound and free radioactivities is performed similarly, but after the redispensing of 700 μl of the supernate into 6-ml counting vials, 2 ml of scintillation fluid was added, and the vials were capped and shaken before placing them into conventional glass 20-ml counting pots in an LKB-Wallac 81000 Liquid Scintillation Counter. Actual counts were recorded on punch tape and transferred by fast paper-tape reader to the Central Data Processing Unit (System Olli 4000) of the University Central Hospital. Results were calculated by a Nova 840 computer from laboratory terminals. The program was of a conversational type, written in extended BASIC computer language, essentially as described by Rodbard and Lewald (5).

Results

Antibody specificity. The specificity of the antiserum raised against estradiol CMO/BSA is shown in Table 1. The extremely low cross reactivity of the antiserum with other steroids of physiological importance enabled it to be used in a nonchromatographic radioimmunoassay of estradiol for clinical purposes. Similarly, the high specificity reported earlier for an antibody raised against 11α-hydroxyprogesterone 11-hemisuccinate/BSA (1) also permitted this antibody to be used in the routine clinical analysis of progesterone without chromatography. In fact, a comparison of values obtained with and without chromatography by a two-variable linear regression analysis resulted in correlation coefficients of 0.965 for estradiol (n = 20) and 0.996 for progesterone (n = 10), the relationship in each case being summarized by the equations y = 0.95x − 10.5 (pg/ml) and y = 1.06x − 0.13 (ng/ml), respectively (x = without chromatography, y = with chromatography).

Temperature and time. We evaluated the effect of increased temperature and shortened time of assay equilibration on the results for testosterone, progesterone, and estradiol (Figure 1) and saw no essential difference between the binding percentages of radioactive steroid in the absence of cold standards during equilibration incubations of 50 min at 37 °C, or 18 h at 4 °C. Testosterone and progesterone assays both tended to be more sensitive when incubations were conducted for 18 h at 4 °C, whereas the opposite was observed in standard curves obtained for estradiol under similar conditions.

Precision and accuracy. The precision of the automated methods for assay of testosterone, progesterone, and estradiol with iodinated tracers was evaluated by the repeated analysis (n = 10) of a serum pool to which known amounts of authentic steroids had been added, as well as a dilution of the pool with an equal volume of glass-distilled water. As can be seen in Figure 2, the
coefficients of variation were generally well below 10% in the physiologically significant ranges.

The accuracy of the method may be assessed from the same data, and we found a good correlation between added and recovered steroids in all three assays (Figure 2).

Ligands. The influence of iodinated as opposed to tritiated ligands on basic assay characteristics is summarized in Table 2. In all instances the titer of the working antibody increased, especially in the case of testosterone and progesterone. The sensitivity in terms of the dose/response curve for a range of nonradioactive standards increased in all assays except for estradiol. In terms of the blank values, the sensitivity of the methods increased in all cases. Both the smaller amounts of solvents used in the $^{125}$I assays and the increased sensitivity of the dose/response curves probably contribute to this increased sensitivity.

Antibody specificity. As reported earlier (6, 7), the specificity of antibodies may improve when $^{125}$I ligands are used instead of their $^3$H counterparts. In the present investigation, this was notable in the case of testosterone and progesterone, and additional details of the change in the specificities observed are given in Table 3. The increased specificity of the testosterone antibody is particularly advantageous if use of this antibody is considered in a nonchromatographic radioimmunoassay. In this respect, a 30% cross reactivity of the antibody with 5α-dihydrotestosterone represents an almost negligible contribution to the results obtained for samples from men (n = 9), and only about a 10% increment in the values obtained for normal women (n = 10). This is illustrated in Figure 3, in which a comparison is made between results obtained by the rapid method and those obtained by the conventional manual method after chromatography (2).

![Figure 3](image-url)
Effect of automation on precision. The impact of the application of automation alone on the precision of radioimmunoassays is difficult to assess due to the individual skills of various technicians. However, in Table 4 we compare the intra-assay (n = 10) coefficients of variation of the same low-, medium-, and high-value serum extracts when testosterone was estimated by use of: (a) manual pipetting of reagents and decantation of supernates for counting, in a protocol similar to that published previously (1); (b) a direct application of automation to the latter method; and (c) an incubation time of only 50 min at 37 °C and automated estimation. These assays were all performed by the same highly skilled technician at the same time. Similarly, intra-assay coefficients of variation (n = 10) can be compared between testosterone assays performed on the same occasion using 125I-labeled testosterone in a rapid method (incubation time of 50 min at 37 °C) and the manual 3H method, and such a comparison serves to indicate the impact on assay precision of the combination of automation and the 125I-labeled ligand. In the assays where a tritium-labeled ligand was used, we saw very little difference between the precision of various methods at a high concentration of testosterone (B/B0 = 30%), while at low (B/B0 = 71%) and medium (B/B0 = 56%) concentrations the coefficients of variations obtained in the manual assay were considerably higher than those in both automated approaches. However, this discrepancy was eliminated if one spurious count from a duplicate pair in the low serum pool samples, and one spuriously low count and one spuriously high count from two subsequent duplicate pairs in the medium serum pool samples were omitted. This suggested that errors had occurred in pipetting either samples or reagents during the manual preparation of assays. The influence of both automation and the use of 125I-labeled ligands did not increase the precision of the method at similar concentrations or regions of binding on the standard curve as compared with the automated methods with [3H]testosterone as tracer. In fact, at a similar binding range the CV for the 125I assay was slightly greater than that calculated for the 3H assay, but this may in part be due to the differences in the counting times used. 3H assays were counted to a minimum of 10 000 cpm or 10 min while 125I assays were counted for only 1 min (4000–7400 cpm range).

Scintillation fluids. Table 5 compares various scintillation “cocktails” used in this investigation, and
several salient points emerge as to which is most suitable for use in automated procedures that rely on the measurement of radioactivity in relatively large volumes of aqueous solutions. In terms of relative counts per minute (cpm), no difference in the apparent counting efficiency was apparent when 700 µl of PBS solution containing [3H]testosterone was counted in either 10 ml of "Insta-Gel" or only 2 ml of "Ready-Solv HP". Under similar conditions the respective counts obtained when we used only 2 ml of "Ria-Luma-PBS" or "Ria-Luma" represented 95% and 88% of those obtained on using 10 ml of Insta-Gel. Despite the slightly lower apparent counting efficiency achieved by "Ria-Luma-PBS" and "Ria-Luma," several characteristics inherent in their composition favor their use as high-capacity scintillators. Their solvent composition permits the formation of a single clear phase at sample loads of 38% between 15 and 30 °C, while "Ready-Solv HP" tended to form a heavy white precipitate at temperatures above 18 °C at PBS sample loads exceeding 26%. Their flash point was 47 °C, while "Ready-Solv HP" is highly flammable at room temperature. Considerably more "Ready-Solv HP" penetrated the polypropylene counting vials than was true for either "Ria-Luma-PBS" or "Ria-Luma" at 18 °C, and samples counted in "Ready-Solv HP" tended to lose counts markedly when stored at 18 °C for more than 48 h.

Two important features in the selection of a suitable cocktail for high-capacity liquid scintillation counting are the time required for sample equilibration and the subsequent stability of samples during storage before counting. In this respect, "Insta-Gel" required a pre-equilibration of at least 20 min before stable counts could be achieved, while all three high-capacity scintillators could be validly counted immediately. At temperatures between 15 and 18 °C all scintillators tested gave relatively stable counts over a period of 48 h. However, in the case of "Ready-Solv HP" precipitation and solvent losses occurring during storage at 18 °C may be responsible for losses in counts recorded after prolonged storage. In terms of cost per sample, all three high-capacity scintillators are markedly less expensive than "Insta-Gel", while both "Ria-Luma" and "Ria-Luma-PBS" are by far the most economical of the high-capacity scintillators.

Discussion

Recently a discrete clinical chemical analyzer (System Olli 3000) has been described in detail in connection with the multi-point analysis of enzymatic (8) and nonenzymatic (9) constituents of body fluids. When applied to radioimmunoassay, the dispensing and incubation modules exhibited several features that have enabled us to automate our assay procedure.

The arrangement of assay tubes in prelabeled code plates of 24 units facilitated their rapid transfer throughout the system, without the need for individual identification. We could also add reagents and portion the final reaction mixtures extremely rapidly in units of 24 with the dispensing module. This was particularly advantageous in minimizing the time-related stripping effect, characteristic of the DCC separation method (10). Despite this familiar drawback, the latter technique is widely used in protein binding assays in general, and although previous attempts have been made to automate this stage (11), none is as fast as that reported here, and furthermore those relying on pump mechanisms often suffer from blockages caused by the suspension settling out within the system. Apart from DCC, other separation techniques based on precipitation are widely used, including ammonium sulfate and polyethylene glycol as the separation medium, and a similar approach might also be engaged in the automation of assays which rely on their use. The use of prearranged units of 24 plastic pipette tips, which could be rapidly changed between each maneuver, eliminated the problem of carryover and enabled the machine to be used by several operators performing different assays, without an intermediate washing or priming of any part of the unit.

In the present work, samples and standards were not routinely pipetted by machine, but there is no technical reason why this should not be possible, and it may be especially useful in radioimmunoassays that can be performed directly on serum, or diluted serum samples (12).

In the case of steroid radioimmunoassays the reaction between antibody and steroid is thought to be rather temperature independent (13), but it is considered essential to perform assays under conditions of complete equilibrium to ensure that specificity is maximum during the reaction (14). We used the Olli incubator 354, which maintains a temperature of 37.0 ± 0.1 °C (range), to decrease the time required for the incubated mixture to reach a state of dynamic equilibrium. Although it is generally considered that increasing the incubation temperature results in a decreased affinity of antibody for antigen and a loss in sensitivity, there certainly are exceptions to this, as evidenced by our observation that the assay for estradiol was found to be slightly more sensitive, when the incubations were performed at 37 °C.

After incubations at 37 °C, an additional 5- to 10-min incubation must be conducted at 4 °C when DCC is used to separate bound and free ligands. This is due to the disequilibrium this method imposes on the binding system, which results in a rapid dissociation of the steroid–antibody complex at the higher temperature.

Although an objective comparison of automated and manual assays is difficult, a trial conducted on the same day by the same technician served to identify the advantages of automation. The precision of various assays was evaluated in terms of reliability, and the only obvious advantage of the application of automation was the elimination of highly spurious results, which inevitably occur when many samples and reagents are pipetted manually. The slightly higher coefficient of variation observed when using 125I-labeled testosterone in an automated assay was at least partly due to the much shorter counting time (1 min), because the coef-
sients of variation of 10 similar samples measured by using 

\( ^{125}\text{I} \)-labeled ligands in the assessment of testosterone assay accuracy and precision were very similar to those recorded using \(^3\text{H}\) ligands when a 3-min counting time was used (Figure 2). As reported previously (15), we used about 20,000 cpm of \( ^{125}\text{I} \)-labeled ligand per assay tube; however, even at a zero binding of about 60\%, this was obviously too little to ensure the precise counting of samples within 1 min, and to achieve a 10,000 cpm count rate, either about twice this amount of tracer should be used or the counting time should be increased.

In general, a major objective behind automation, besides decreasing experimental error, is increased assay speed. In this respect, the Olli 3000 dispensing unit has proved invaluable in enabling the rapid dispensing of reagents and aliquoting of final incubated mixtures. When used in conjunction with the incubation/evaporation module, the effective time required for assays of up to 96 tubes before counting could be decreased to less than 3 h. This is especially useful in combination with an \( ^{125}\text{I} \)-labeled tracer for the determination of estradiol, because these results are often required on the day of sampling.

Apart from the obvious advantages behind a decrease in counting time and costs, owing to direct gamma counting, the use of iodinated steroids has been observed by many workers to influence the quality of radioimmunassays in terms of antibody binding, sensitivity, and specificity. However, the literature is in disagreement as to the cause and extent of these influences (16). The exceptionally high specific activity that can be achieved when steroid derivatives are iodinated undoubtedly plays a significant role in increasing assay sensitivity, as demonstrated by Lindberg and Edqvist for estradiol (17), who also observed that parallel to increasing sensitivity, antibody titer greatly increased at higher tracer specific activities. Using a similar antibody and \( ^{125}\text{I} \)-labeled ligand, we have been unable to achieve such large increases in antibody titer or assay sensitivity. This may in part be due to our use of a tracer with relatively lower specific activity, but these phenomena were observed when both testosterone and progesterone were iodinated in an identical fashion and so it would seem that the affinity characteristics of the antibodies in question also play a significant role in determining the extent of the changes that take place (7). The extremely high increase in antibody titer that occurred when \( ^{125}\text{I} \)-labeled testosterone and progesterone were used instead of tritiated tracers was accompanied in both instances by increased sensitivity and specificity. The increase in specificity in the case of progesterone resembles that reported recently (6). It is important to note that changes in assay characteristics were never identical, and after each new iodinated tracer was prepared we found it necessary to reassess both antibody working dilution and assay sensitivity.

Previously, the major disadvantages attributed to the use of \(^3\text{H}\)-labeled tracers have been waste-disposal problems and the high cost of scintillation cocktails. The development of nonvolatile scintillators that have an extremely high capacity for buffer solutions may well alter this situation. Using only 2 ml of scintillant, we have been able to count 700 µl of our assay supernate without any apparent loss in counting efficiency, and by making use of the extremely high capacity of “Ria-Luma-PBS” it would be possible to again reduce scintillant volume by a factor of two, by using 3-ml counting vials. In this way, the technique has been found to be more suitable for automation, and at the same time generates far less bulk refuse. Furthermore, use of nonvolatile scintillants of high stability at temperatures up to 30 °C may well result in a significant decrease in liquid scintillation counter costs, because the expensive refrigeration unit included in most instruments would be unnecessary.

The choice between \( ^{125}\text{I} \)- and \(^3\text{H}\)-labeled ligands in assay procedures largely depends on the characteristics of individual antisera, although the number of assays and the time in which they must be performed also may influence the choice. Recent trends in radioactivity counter technology will obviously play a significant role in this respect, and a decrease in scintillator volume size and improvements in scintillator thermostability may have an important impact on the future development of cheaper and more economical liquid scintillation counting instruments.

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References

6. Scarabrick, J. J., and Cameron, E. H. D., Radioimmunoassay of progesterone: Comparison of \([1,2,6,7\text{-}^{3}\text{H}]\)-progesterone and progesterone-\([2\text{H}]\)-iodohistamine radioligands. J. Steroid Biochem. 6, 51 (1975).


