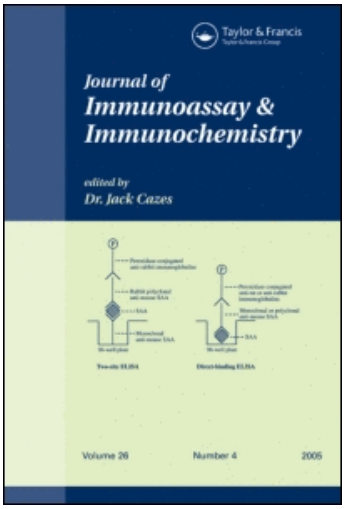

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Amphetamine Radioimmunoassay: Chemical Tuning of Specificity

Pa Mason[a]; B. Law[a]; AC Moffat[a]
[a] Home Office Central Research Establishment Aldermaston,

To cite this Article Mason, Pa , Law, B. and Moffat, AC(1983) 'Amphetamine Radioimmunoassay: Chemical Tuning of Specificity', Journal of Immunoassay and Immunochemistry, 4: 1, 83 — 98
To link to this Article: DOI: 10.1080/15321818308057001
URL: http://dx.doi.org/10.1080/15321818308057001

JOURNAL OF IMMUNOASSAY, 4(1), 83-98 (1983)

AMPHETAMINE RADIOIMMUNOASSAY: CHEMICAL TUNING OF SPECIFICITY

PA Mason, B Law and AC Moffat

Home Office Central Research Establishment

Aldermaston, Reading, Berks RG7 4PN.

ABSTRACT

Four [^{125}I]iodine labelled amphetamine derivatives were synthesised. Three of these proved useful in the development of radioimmunoassays for amphetamine. The assays, all using the same antiserum, had widely differing specificities for compounds closely related to amphetamine. Thus, chemical modification of the radiotracer was used to tune radioimmunoassay specificity without recourse to the production of different antisera.

(KEY WORDS: AMPHETAMINE RADIOIMMUNOASSAY IODINE-125)

INTRODUCTION

The specificity of an immunoassasy is often assumed to be solely dependent on a particular antibody, and ultimately on the chemical features of the immunogen used in its preparation. Attempts to develop immunoassays with high specificity towards a particular molecule have involved the preparation of immunogens in which the hapten has been conjugated to a large molecular weight protein in such a way as

to expose characteristic features of the hapten to the immune system. Similarly assays of broad specificity have been developed by conjugation of the hapten to the protein so that the features presented to the immune system have only group-specific characteristics. This latter approach is perfectly practicable when [^{3}H] labelled compounds are used as radiotracers.

When [^{125}I]iodine labelled compounds are used as radiotracers, they will often not bind to the antibody. This effect becomes more apparent when the hapten is a small molecule; the iodine atom is approximately the same size as a benzene ring. The only way to avoid this problem is to use a radiotracer where the iodine moiety is attached to the hapten via the original point of attachment to the protein so that it is 'hidden' from the immune system.

During the course of a series of experiments to produce iodine labelled drug derivatives for use in radioimmunoassay it became apparent that the specificity of an assay could be 'tuned' by simply altering the point or mode of attachment of the iodine to the hapten. It was predicted that a weakly bound radiotracer would be displaced from the antibody by compounds of similar structure to the hapten, thus producing a group-specific assay; a strongly bound radiotracer would only be displaced to any great extent by compounds very closely related to the original antigen.

The following is an account of the way in which 'chemical tuning' was used to develop assays of varying specificities for amphetamine-like compounds.

MATERIALS

Sodium [^{125}I]iodide (614MBq/μg) was obtained from Amersham International, Amersham.

Sodium toluene-4-sulphonchloramide (chloramine T) and all other chemicals and solvents, unless specified, were obtained from BDH Chemicals Ltd., Poole.

Sodium cyanoborohydride, 4-(4-hydroxphenyl)-2-butanone and 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester were obtained from Aldrich Chemical Co. Ltd., Gillingham.

Valeraldehyde was obtained from Koch Light Labs., Inc., Colnbrook.

Dioxane was purified by passage through a column of aluminium oxide (basic, Brockmann grade 1) to remove peroxides.

Analytical and preparative thin-layer chromatography (TLC) plates containing $F60_{254}$ UV indicator were obtained from Merck and pre-run in the appropriate system before use.

The following TLC systems were used:

(T1) Methanol:ammonia (0.88 S.G.) - 100:1.5

(T2) Cyclohexane:toluene:diethylamine - 75:15:10.

Bovine gamma-globulin (BGG, Cohn Fraction II) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., Poole.

Antiserum was obtained from an Amphetamine DAU Emit kit (reagent A) as supplied by Syva (UK) Ltd., Maidenhead.

High-performance liquid chromatography (HPLC) was carried out using a Waters 6000A HPLC pump (Waters Assoc., Northwich) fitted with a Rheodyne 7120 injection valve

(Phase Separations Ltd., Clwyd). Stainless steel columns (16cm x 5mm i.d. from Shandon Southern, Runcorn) were packed with Hypersil 5-ODS (Phase Separations Ltd). Column eluate was monitored with a Cecil Instruments 212 ultraviolet monitor (Cecil Instruments Ltd., Cambridge) and collected using a 2112 Redirac Fraction Collector (LKB Instruments Ltd., South Croydon).

The following HPLC systems were used:

(H1) Methanol:water - 50:50

(H2) Orthophosphoric acid:diethylamine:methanol: water - 5.6:3.7:125:365 - adjusted to pH 3.5 with sodium hydroxide solution (1M).

Gamma-counting was carried out in a NE 1600 counter (Nuclear Enterprises Ltd., Beenham) which had an efficiency of approximately 61% for [^{129}I]iodine. The counter was interfaced to a Commodore CBM Model 3032 data reduction system (Anaspec, Newbury).

Mass spectra were obtained using a VG-Micromass 16F mass spectrometer (VG Micromass, Altrincham) fitted with a single stage jet separator. The following conditions were used: emission, 100μA; electron energy, 70eV; source temperature, 200^{o}C. Data were collected using a VG 2250 Data System with the mass spectrometer scanning at 3 s per decade.

METHODS

The structures and synthetic routes of the compounds described in the following section are shown in Figure 1.

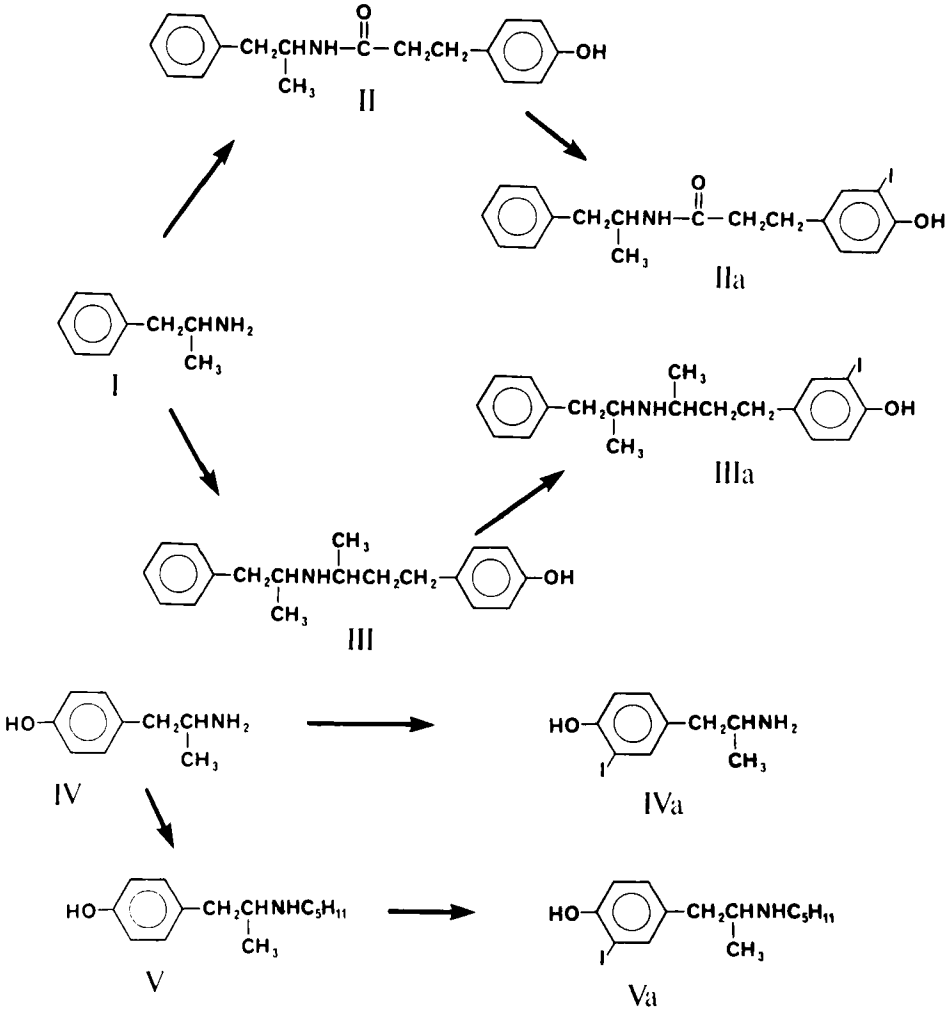

FIGURE 1. Structures of Amphetamine and Iodine Labelled Derivatives.

Synthesis of N-(1-methyl,2-phenyl)ethyl-3-(4-hydroxyphenyl) propionamide (II)

The title compound was prepared using a modification of the method of Bolton and Hunter (1) as outlined below.

A solution of 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester in dioxane (0.5ml, 100mg/ml) was added to 25mg of amphetamine base (I). The resultant mixture was stirred and left overnight in the dark at room temperature. The dioxane was then removed on a rotary evaporator and the residue redissolved in methanol:water (50:50, 2ml). Small aliquots (2µl) of this were then injected on to a HPLC column and chromatographed using eluent H1. Monitoring of the eluate at 255nm resulted in the detection of a major peak at retention volume (Rv) 10.8ml (Rv amphetamine = 1.9ml). Larger amounts of the reaction mixture were then processed by this method and fractions collected at Rvs 10-12ml. The identity of the title compound was confirmed by mass-spectroscopy (MS), molecular ion at m/z 283. The overall yield was approximately 32%.

Synthesis of N-(4-hydroxyphenyl)isobutyl amphetamine (III)

Amphetamine sulphate (200mg) and 4-(4-hydroxyphenyl)2-butanone (70mg) were dissolved in methanol (2ml). Sodium cyanoborohydride (60mg) was then added and the mixture stirred constantly. The pH was checked at five minute intervals and adjusted to 5.0 with methanolic potassium hydroxide (0.1M). When the pH remained steady (after approximately 1 hour) a further 60mg of sodium cyanoborohydride was added and the mixture left stirring overnight at room temperature after a final pH check.

The reaction tube was then placed in a fume cupboard and the pH was adjusted to 2.5 with concentrated hydrochloric acid (care - hydrogen cyanide is liberated at this

stage). The methanol was then removed on a rotary evaporator. The residue was redissolved in chloroform:isopropanol (3:1, 10ml) and washed with borate buffer (pH 8.6, 2ml). The organic phase was removed, dried under nitrogen, redissolved in methanol (0.5ml) and streaked on to a preparative TLC plate which was developed in system T2. Visualisation under ultraviolet light (254nm) demonstrated the presence of three major bands; Rf 0.6(A), Rf 0.4(B) and RF 0.27(C). Band A had an identical Rf to amphetamine. A thin band of the TLC plate (0.5cm) was then sprayed with Dragendorff reagent. Only band B gave a positive reaction (bright orange) with this reagent. The remainder of this band was then removed and eluted with methanol. The resulting solution was concentrated under nitrogen and rechromatographed in the same TLC system. Only one band was then visible upon inspection under ultraviolet light. This band was eluted and the identity of N-(4-hydroxyphenyl) isobutyl amphetamine was confirmed by mass spectral studies; molecular ion at m/z 283. Band C was similarly examined and was identified as (4-hydroxyphenyl) isobutanol. The yield of III was approximately 18%.

Synthesis of N-pentyl-4-hydroxyamphetamine (V)

To a solution of 4-hydroxyamphetamine hydrobromide (IV) in methanol (3ml, 150mg/ml) was added valeraldehyde (35µl). Sodium cyanoborohydride (50mg) was then added, the tube capped and stirred constantly on a magnetic stirrer at room temperature. At five minute intervals the pH was checked and adjusted to 5.0 with methanolic potassium hydroxide solution (0.1M). When the pH remained steady (after

approximately 1 hour) a further 40mg of sodium cyanoborohydride was added and the mixture left stirring for 16 hours after a final pH check. The reaction tube was then placed in a fume cupboard and the pH was adjusted to 2.5 with concentrated hydrochloric acid (care - hydrogen cyanide is liberated at this stage). After 10 minutes the methanol was removed on a rotary evaporator. The residue was redissolved in chloroform:isopropanol (3:1, 10ml) and borate buffer (2ml, 0.25M, pH 8.6) was added. The mixture was shaken, centrifuged and the organic layer removed and evaporated to dryness under nitrogen. The residue was then redissolved in methanol (0.5ml) and streaked on to a preparative TLC plate which was developed in system T1. Examination of the plate under an UV lamp (254nm) revealed the presence of a major band (Rf 0.53) running ahead of 4-hydroxyamphetamine (Rf 0.24). The band at Rf 0.53 was then removed and eluted with methanol. The identity of the title compound was confirmed by mass spectroscopy. Overall yield was approximately 37%.

Preparation of 3-iodo-4-hydroxyamphetamine (IVa)

4-Hydroxyamphetamine (IV) was iodinated by the chloramine-T method (2) as outlined below using both [^{127}I]iodine (i) and [^{125}I]iodine (ii).

(i) A solution of 4-hydroxyamphetamine hydrobromide in 50% aqueous methanol (2.5ml, 100mg/ml) was mixed with sodium [^{127}I]iodide solution (2ml, 40mg/ml). Chloramine-T (140mg/ml) in phosphate buffer (2.5ml, 0.1M, pH 7.4) was then added and mixed immediately. After 60 s the reaction was stopped by addition of sodium metabisulphite solution

(2.5ml, 150mg/ml). The pH was adjusted to 8.6 with sodium hydroxide solution (40%) and the mixture extracted twice with five volumes of chloroform:isopropanol (3:1). The organic phases were removed, pooled, washed with distilled water (10ml), dried with anhydrous sodium sulphate and evaporated to dryness under nitrogen. The residue was redissolved in methanol (3ml), small aliquots (2µl) of which were analysed by HPLC using solvent system H2. Monitoring of the column eluate at 255nm resulted in the detection of four peaks at Rvs 2.8, 8.4, 10.9 and 25.8ml. The first of these had identical Rv to 4-hydroxyamphetamine. The remaining three peaks were identified by mass spectroscopy: (Rv 8.4) 3-iodo-4-hydroxyamphetamine, (Rv 10.9) 4-toluenesulphonic acid and (Rv 25.8) 3,5-diiodo-4-hydroxyamphetamine. The overall yields were: 3-iodo-4-hydroxy amphetamine 18%; 3,5-diiodo-4-hydroxyamphetamine 25%.

(ii) A solution of 4-hydroxyamphetamine hydrobromide (10µl, 100µg/ml) in phosphate buffer (0.25M, pH 7.4) was added to sodium [^{125}I]iodide (10µl) in a glass centrifuge tube. Freshly prepared chloramine-T solution (10µl, 80µg/ml), in the same buffer, was then added. The tube was capped, the contents mixed thoroughly by vortexing and the reaction allowed to proceed for 40 s before being stopped by the addition of sodium metabisulphite solution (20µl, 80µg/ml). The solution was made alkaline by addition of borate buffer (200µl, pH 8.6, 0.25M). The mixture was shaken vigorously with chloroform:isopropanol (3:1) and passed through a phase separation filter paper which had been pre-soaked with the

solvent mixture. The tube was washed with further solvent (2 x 1ml) which was also passed through the filter. The organic filtrate was then dried with nitrogen at room temperature and redissolved in ethanol (6ml). Assessment of activity in a gamma-counter indicated that approximately 43% of the available [^{125}I]iodine had been incorporated into the product. Small aliquots (1µl) were then analysed using the HPLC system described above. Eluate fractions (1ml) were collected and their radioactive contents measured in a gamma-counter. There were two main peaks of radio-activity corresponding to the mono-and di-iodo derivatives. These were present in the ratio 95:5. A larger quantity of the reaction product was then processed by HPLC and the appropriate fractions collected, pooled and saved for future use.

Preparation of [^{125}I]iodine labelled derivatives IIa, IIIa and Va

Compounds II, III, and V all have 4-hydroxyphenyl groups which can be iodinated by the chloramine-T reaction. Accordingly, all three were iodinated by the procedure outlined for 4-hydroxyamphetamine above. In all cases the ratio of mono- to di-iodinated derivative was approximately 95:5 as determined by HPLC.

The purified mono [^{125}I]iodinated compounds (IIa, IIIa, IVa and Va) were then incubated with dilutions of the Emit amphetamine antiserum in an attempt to assess the affinity of the antiserum for each radiolabel. From the results of this study it was possible to optimise binding conditions

and develop RIAs using three of the four radiolabels. Only compound IIa proved unsuccessful in this respect.

Radioimmunoassays

Three RIAs were developed, all of which used the same antiserum with a different [^{125}I]iodine labelled amphetamine derivative. The three assays were based on the same overall protocol; 50µl of sample or standard were pipetted in duplicate into polypropylene micro-centrifuge tubes, followed by 100µl of the [^{125}I] label diluted in assay buffer and 100µl of Emit antiserum diluted in assay buffer.

For assay 1, antiserum was diluted 1:20 in phosphate buffer (pH 7.4) containing 0.5% BGG and 0.2% BSA. The label, IVa, was diluted in the same buffer to give approximately 550Bq in 100µl. The tubes were incubated for 30 mins at room temperature before addition of 250µl of saturated ammonium sulphate solution. The tubes were then incubated for a further 15 mins before being centrifuged (3 mins, 9000g). The supernatants were aspirated and the precipitates counted in a gamma-counter.

For assay 2, antiserum was diluted 1:40 in phosphate buffer (pH 7.4) containing 0.5% BGG and 0.2% BSA. The label, Va, was diluted in the same buffer to give approximately 550Bq in 100µl. Incubation, separation and counting were identical to assay 1.

For assay 3, antiserum was diluted 1:100 in phosphate buffer (pH 7.4) containing 0.2% BGG only. The label, IIIa, was diluted in the same buffer to give approximately 550Bq per 100µl. The tubes were incubated for 30 mins at room

TABLE 1

Relative Reactivity* of Amphetamine and Several Closely Related Compounds in Three RIAs Using the Same Antiserum with Three Different Radiolabels.

	Assay 1	Assay 2	Assay 3
AMPHETAMINE	1.0	1.0	1.0
METHYLAMPHETAMINE	2.5	1.67	0.69
FENFLURAMINE	0.15	0.40	0.03
BENZPHETAMINE	0.11	0.058	0.009
CHLORPHENTERMINE	0.045	0.080	0.004
PHENTERMINE	0.005	0.039	0.13
MEPHENTERMINE	0.004	0.005	0.01
EPHEDRINE	0.36	0.08	0.04
PHENMETRAZINE	0.002	0.022	0.01
PHENDIMETRAZINE	0	0	0
β-PHENYLETHYLAMINE	0.007	0.013	0.025
4-HYDROXYAMPHETAMINE	0.004	0.003	0.01
TRANYLCYPROMINE	0.16	0.11	0.06
PHENYLPROPANOLAMINE	0.04	0.03	0.02
PHENELZINE	0.04	0.005	0
DIETHYLPROPION	0	0	0
METHYLPHENIDATE	0	0	0

*Relative reactivity was measured at a level of 100ng/ml amphetamine.

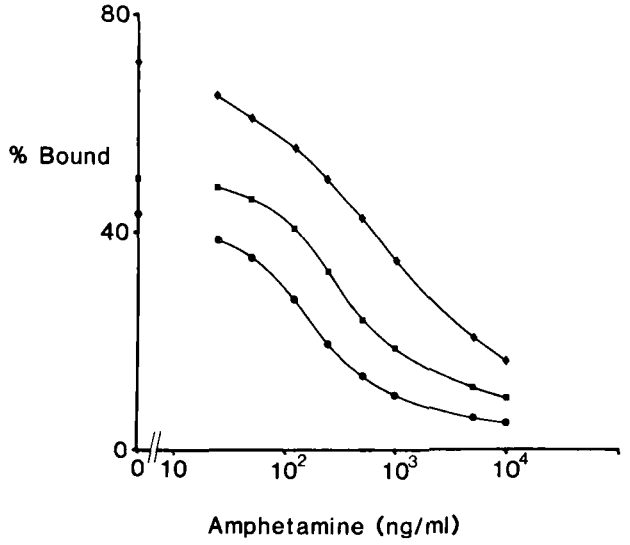

FIGURE 2. Calibration Curves for Amphetamine Obtained with Three different RIAS

● Assay 1, ■ Assay 2, ◆ Assay 3

temperature and 250µl of polyethylene glycol solution (27.5%) were added. After a further incubation of 10 mins the tubes were centrifuged (3 mins, 9,000g). The supernatants were then aspirated and the precipitates were counted in the gamma-counter.

The compounds listed in Table 1 were all tested for cross-reactivity in each of the three assays. This was done by comparing the calibration curve (see Figure 2) for each with that of amphetamine. The concentration of each compound which was required to give a depression of binding equivalent to that produced by 100ng/ml of amphetamine in each assay was calculated.

RESULTS AND DISCUSSION

The amphetamine antibody used in these experiments showed no significant binding with the radio-iodinated derivative IIa. The other three radiolabelled compounds, IIIa, IVa and Va, all showed appreciable levels of binding in the order IIIa > Va > IVa. The difference in levels of binding to the antibody of compounds IIa and IIIa was very great. These two compounds are very similar in structure (see Fig. 1) and have identical molecular weights; the only difference being that compound IIa has an amide bond linking the new side chain to amphetamine whereas compound IIIa has the alkyl side chain linked directly to the nitrogen of amphetamine. This feature is obviously a very important factor in determining the binding of the antibody to amphetamine-like compounds. Similar results have been reported by Budd (3) who found that the only factor which would increase binding of the Emit amphetamine antiserum to amphetamine-like compounds was the incorporation of N-alkyl side chains (the longer the chain, the more avidly bound). He did not, however, investigate the binding of amphetamine-like compounds with amide linked side chains.

The two other radiolabelled derivatives, IVa and Va, produced very similar results. Compound Va was bound more avidly by the antibody than compound IVa. The only structural difference between these two molecules (see Fig. 1) is that compound Va has an alkyl side chain on the nitrogen whereas compound IVa has no side chain. From this and the previous results it is apparent that the antibody does not

simply differentiate between an amide and an alkyl linkage on the nitrogen of amphetamine but has a distinct preference in its order of binding, alkyl > no substituent > amide. Although there is little information on the preparation of the amphetamine immunogen by Syva, these results indicate that the most likely mode of conjugation of amphetamine to a protein molecule was via an N-alkyl side chain.

The cross reactions of a number of amphetamine-like compounds in each of the 3 assay systems is shown in Table 1. Taken overall, Assay 3, using the most avidly bound radiolabel, appears to have the highest specificity for amphetamine whereas Assay 1, using the least avidly bound radiolabel, has the lowest. As predicted, weakly bound radiolabels are more easily displaced from the antibody than avidly bound labels. However, every compound did not conform to this general pattern. Some compounds showed their highest cross reaction with Assay 3 and one, fenfluramine, was most reactive in Assay 2.

In order to develop a highly specific RIA it is vital that the radiotracer be avidly bound and so the molecular structure of the radiotracer actually becomes more critical when a more specific antibody is generated. Obviously care must be taken when chemically tuning such systems that the RIA which is developed will detect the original drug molecule and not become totally specific for the modified hapten. This is likely to occur because antisera contain populations of antibodies directed not just against the hapten but also against the chemical bridge between hapten and protein (4).

In conclusion, it is possible to achieve wide variation in specificity of an RIA by chemical modifications of the radiotracer and hence the avidity with which it is bound by an antiserum. This point is extremely important when iodinated derivatives of low molecular weight compounds, such as drugs, are being prepared. Thus, a highly specific RIA for amphetamine can be made group specific to encompass any particular range of structurally related compounds by the choice of the appropriate iodinated label. This aspect of the specificity of radioimmunoassays has been largely overlooked and instead much effort has been expended on preparation of antibodies directed to specific and characteristic functions of a particular molecule.

REFERENCES

1. Bolton, A.E. and Hunter, W.M. The labelling of proteins to high specific activity by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 1973; 133:529-539.

2. Hunter, W.M. and Greenwood, F.C. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 1962; 194:495-496.

3. Budd, R.D. Amphetamine EMIT-structure versus reactivity Clin. Toxicol. 1981; 18:91-110.

4. Corrie, J.E.T., Hunter, W.M. and Macpherson, J.C. A strategy for radioimmunoassay of plasma progesterone with use of a homologous-site ^{125}I-labelled radioligand. Clin. Chem. 1981; 27:594-599.