EVALUATION OF AN IODINATION PROCEDURE FOR Arg^8-VASOPRESSIN

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SUMMARY

Arg^8-vasopressin (AVP) was labelled with iodine-125 using chloramine-T. The radiochemical yield of ^{125}I-AVP was 71% at the specific activity (S.A.) of 784 µCi/µg AVP (29 MBq/µg AVP) as estimated by self-displacement analysis. Immunoreactivity during storage was determined by the percent binding of ^{125}I-AVP with specific antiserum. Results indicate that chloramine-T allows high iodine incorporation with limited oxidation damage. Tracers produced were found stable for at least 5 weeks.

Key-words : Arg^8-vasopressin, ^{125}I-labelling, self-displacement analysis.

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INTRODUCTION

Arginine-8-vasopressin (Arg^8-vasopressin) (AVP) is the major regulator of water metabolism in man. This nonapeptide is present in human plasma at anticipated levels of 1 to 5 pg/mL (1). In order to measure these small concentrations it is necessary to work with a tracer of high specific activity. Although ^{125}I-labelling of AVP has been described (2), little data have been reported on the optimalisation of the iodination reaction. This study describes optimal reaction conditions for labelling AVP with ^{125}I as well as the properties of the reaction product.

MATERIALS

Synthetic Arg^8-vasopressin and normal rabbit serum (NRS) were supplied by Calbiochem (La Jolla, U.S.A.). The anti-AVP serum was raised in rabbits by coupling AVP with bovine thyroglobulin (3). The conjugated material (1 mg conjugate = 35 µg AVP) was injected intradermally at multiple sites on the back of the animals. Booster injections were given at intervals of 3 weeks (4). Goat-anti-rabbit serum (GAR) was produced at the Laboratory for Embryology and Comparative Histology, R.U.G.,Belgium. Bovine serum albumin (BSA) was purchased from Merck (Darmstadt, Germany). Sodium phosphate buffers (PB & PBS) were prepared using reagents obtained from Merck (Darmstadt, Germany). Disposable polypropylene tubes (12 x 75 mm) from Becton Dickinson (New York, U.S.A.) were used in iodination experiments. Carrier-free $Na^{125}I$ (IMS 30)

was supplied by The Radiochemical Centre (Amersham, U.K.). Carlo Erba (Milano, Italy) supplied chloramine-T. Sephadex G-25-Fine from Pharmacia (Uppsala, Sweden) was used as gel filtration medium.

METHODS

Iodination was carried out in polypropylene tubes using the chloramine-T technique as modified by Kagan and Glick (2). Purification of the iodination mixture was performed by gel permeation chromatography on a Sephadex G-25-F column (28 x 0.9 cm) with 0.25% (v/v) acetic acid containing 1.25 mg BSA/mL as eluate. Forty 1-ml fractions were collected from the column at an elution rate of 50 mL/hr. Of each fraction 10 µL was counted for 30 sec in a LKB-Wallac 1260 multigamma counter.

The radiochemical yield was calculated from the distribution of the radioactivity in the elution pattern.

The immunoreactivity of the tracer was assessed by its ability to bind to the specific AVP-antibody. Of each purified fraction the same quantity of radioactivity (approximately 10 000 cpm i.e. the concentration to be used in the assay) is incubated with an excess of antibody. After a 24-hr incubation time, free and antibody-bound fraction were separated using a soluble double antibody system. After centrifugation the radioacitivity in the pellet was counted. Immunoreactivity was expressed as a percentage ratio of the bound to the total (B/T) radioactivity of ^{125}I-AVP.

For estimation of specific activity by self-displacement, we used a radioimmunoassay procedure developed in our laboratory. AVP-standards, covering the range from 80 to 1.25 pg/mL, were prepared by doubling dilutions in PBS. In duplicate 400-µL aliquots of each standard solution were placed in a series of tubes. A separate series for the self-displacement experiment was set up containing 400 µL PBS. To both series 100 µL of AVP-antiserum (final dilution 1/800,000) was added. After a 24-hr incubation at 4°C, 100 µL (1 x 10^4 cpm) ^{125}I-AVP were added to the standard tubes, while for the self-displacement assay incremental tracer concentrations up to six times that used in the standard curve, were employed. Following a second incubation (48 hr ; 4°C), separation of free and antibody-bound antigen was achieved by adding 200 µL of pre-precipitated second antibody preparation (5). After a third incubation for 45 min at 4°C, 200 µL PBS was added to each tube. Mixtures were centrifuged (3500 rpm, 4°C, 20 min) and the radioactivity in each pellet was counted.

To monitor loss of immunoreactivity during storage, zero-standard tubes were analysed at different time intervals up to five weeks, and % B/T was calculated. After five weeks of storage an aliquot of the preparation was also rechromatographed on Sephadex G-25-F under the same conditions as described for the purification, to control for de-iodination of the tracer.

RESULTS

Labelling

Different sets of experimental conditions have been used to investigate the effects of actual chloramine-T concentrations on iodination yield. The initial conditions using 5 µg AVP, 71.4 µg chloramine-T and 0.5 mCi ^{125}I (18.5 MBq), gave a mean radiochemical yield of 74.2% (n=3) whereas the corresponding specific activity (S.A.) was only 67.2 µCi/µg AVP (2.5 MBq/µg). Incorporation of iodine into AVP was examined under conditions of varying the molar ratio of chloramine-T to AVP. In presence of excess ^{125}I (1 mCi = 37 MBq) the amount chloramine-T used was lowered from 71.4 to 50 and 35 µg separately, keeping the AVP quantity constant (5 µg). The radiochemical yield varied accordingly to 80.0, 89.5 and 71.8%, giving optimal incorporation at a chloramine-T/AVP ratio of 10/1 on a weight basis. In this case the S.A. was approximately 145 µCi/µg AVP (5.4 MBq/µg AVP). With respect to the optimal molar ratio previously determined, use of different amounts of AVP was also investigated (5, 2.5 and 1 µg), giving the highest S.A. by reacting only 1 µg AVP. Therefore we now use in routine iodinations : 1 µg AVP exposed to 10 µg chloramine-T and 1 mCi ^{125}I. The reaction is stopped by removal of excess iodine through addition of 15 mg BSA. Routinely, S.A.'s up to 783 µCi/µg AVP (29 MBq/µg) have been obtained. The radiochemical yield was never below 71% (n = 10).

Purification

^{125}I-AVP was purified by gel permeation chromatography. A typical chromatogram of iodination mixture on the Sephadex column is presented in Fig.1. ^{125}I absorbed by BSA (I) was eluted as the first peak, followed by a small peak of unreacted ^{125}I (II). Peak III corresponds to ^{125}I-AVP (mono-iodinated). The additional peak (IV) probably represents diiodo-vasopressin according to the findings of Husain et al.(6).

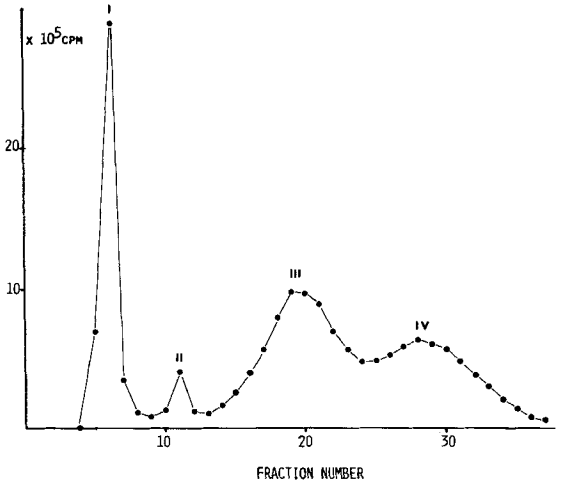

Fig. 1. Column elution pattern of an iodination mixture on Sephadex G-25-F.

Immunoreactivity

Immunoreactivity of the individual fractions eluted from the column was monitored by RIA (Fig.2). The first two peaks containing ^{125}I-BSA (I) and free ^{125}I (II) were not immunoreactive to AVP antiserum. In the ^{125}I-AVP peak (III), fractions of high radioactivity showed similar immunoreactivity. Since the same quan-

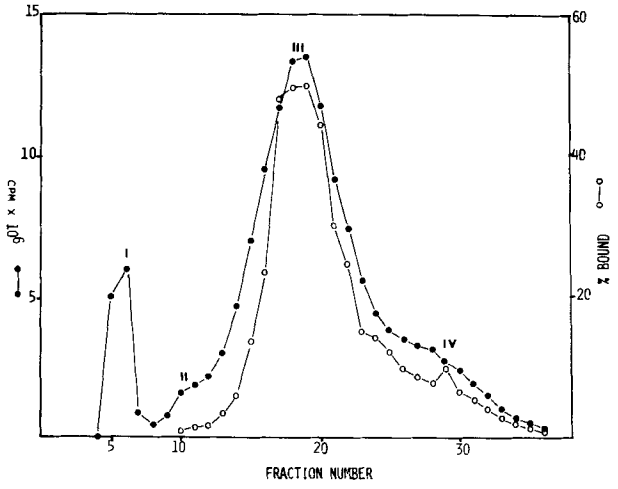

Fig. 2. The immunoreactivity of individual fractions eluted from a Sephadex G-25-F column, as determined by RIA.

tity of radioactivity of each fraction is used, fractions of the descending part of the AVP-peak, having the highest B/T values in the screening test, are selected for use in the RIA (2). Fresh ^{125}I-AVP preparation could be bound for more than 90% by excess antibody.

Specific activity (S.A.)

For the classical self-displacement procedure, ratios of bound to free radioactivity were plotted against mass of the AVP-standard. For each tracer increment, B/F ratios were calculated.

By superposition of these data upon the standard curve (Fig.3),

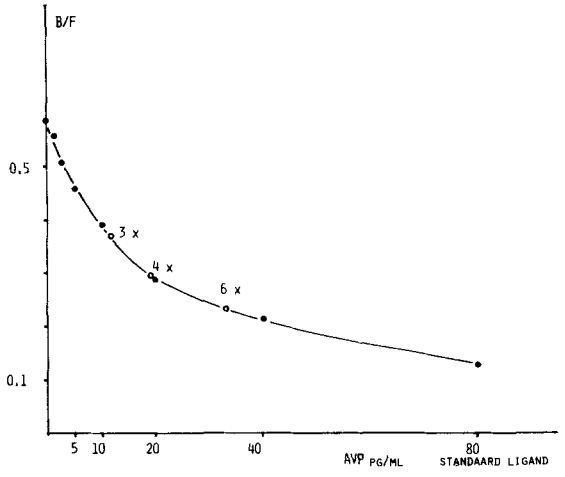

Fig.3. Standard curve showing superimposed self-displacement data. Mean mass of tracer per increment is 7.36 pg.

the amount of radioactive ligand displaced having the same B/F ratio. Each tracer increment causes a displacement of 7.36 pg indicating that the mean mass of tracer (δ), used in the standard curve, was 7.36 pg. Using the formula :

$$S.A. = \frac{T_c}{r.E.\delta}$$

where T_c is the total radioactivity (9398 cpm), r the conversion factor of dpm to pCi (2.22 dpm/pCi), E the counting efficiency of ^{125}I (0.8 cpm/dpm) and δ the mass of tracer (7.36 pg), the S.A. of ^{125}I-AVP was 719 Ci/µg AVP (26.6 MBq/µg AVP).

Similarly, by plotting interpolated doses against the corresponding cpm values (Fig.4) the intercept on the ordinate affords

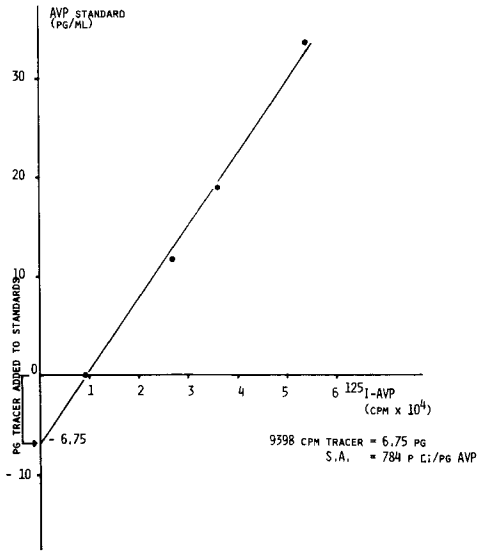

Fig. 4. Graphical plot of interpolated dose against cpm values added for the self-displacement analysis.

the mass of tracer (6.75 pg). The intercept on the abscissa (1×10^4 cpm) gives the radioactivity that was added to each standard. This graphical determination yields a S.A. of 784 µCi/µg AVP (29.0 MBq/µg AVP).

Stability

Up to 5 weeks no fall in percent bound in the zero-standard was found when the ^{125}I-AVP preparations were stored undiluted, at 4°C or -20°C. On rechromatography 83% of the radioactivity eluted in the protein peak indicating only a small loss of iodine after 5 weeks.

DISCUSSION

The chloramine-T method has been the method of choice for iodination of AVP. Results obtained indicate that by reducing the chloramine-T amounts, higher iodine incorporation can be obtained. By reacting only 1 µg AVP with 10 µg chloramine-T and 1 mCi ^{125}I (37 MBq), high specific activities are obtained with minimal iodination damage. In fact, preliminary results with an AVP-assay indicate that no significant damage to the immunologically active part, occurs.

To calculate S.A., there has been some controversy in literature whether to use the graphical plot (8) rather than the classical method (7). Morris (8) claimed that the classical analysis is unsatisfactory as it fails to allow for the fact that an unknown mass of labelled antigen is also present and competing for binding sites on the antibody in each antigen standard solution. A graphical plot was then suggested to circumvent this error. For this particular labelling, we found no discrepancies between the S.A.'s, calculated by both methods. According to Roulston's findings (9), this clearly indicates that there is no basis for the suggested error, and both calculation methods can equally well be used.

In conclusion, application of this improved and simple iodination technique together with the purification and storage prescriptions enables one that for routine uses few iodinations need to be performed. The high specific activity and immunoreactivity of the tracer are a prerequisite for development of a more sensitive and precise assay of AVP in human plasma.

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