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Vasopressin, oxytocin and synthetic analogues: the use of bioassays*

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Abstract: Bioassay procedures have been the prerequisite for detection, purification, elucidation of the structure and the synthesis of neurohypophysial hormones. After a review of the history of these bioassays and of the standard preparations including international standards, the current international standards and the bioassay methods prescribed by the pharmacopoeias are described. Some important methodological details are also mentioned.

Keywords: *Bioassay procedures; neurohypophysial hormones; oxytocin; arginine vasopressin; lysine vasopressin; deamino-oxytocin; felypressin; ornipressin; desmopressin; international standard preparations.*

1. Introduction

Progress in science is in most cases due to the introduction of new methods. In the case of the neurohypophysial hormones the progress from detection via purification, isolation, elucidation of the structure and, ultimately, confirmation of the structure by synthesis of the molecule and demonstration of identical effects, was based on relatively simple methods: the bioassays.

The bioassays for neurohypophysial hormones were derived from pharmacological methods used for the detection and qualitative description of these new principles. The only difference lay in the desire to quantify the pharmacological responses in order to be able to determine the amount of biologically active but unknown principle with which one was dealing.

2. History of the Bioassay Procedures for Neurohypophysial Hormones

The oxytocic activity of posterior pituitary lobe extracts was discovered by Sir Henry Dale in 1906 [1]. In the following 5 years, the aqueous extract or decoction of the posterior or infundibular lobe of the freshly excised bovine pituitary body acquired wide use and importance mainly in obstetrics but there was — at the time — no prospect of determining its efficacy by chemical methods. In 1912, Dale and Laidlaw [2] developed the first bioassay for uterotonic activity using isolated virgin guinea-pig uterus

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preparations in order to be able to determine the activity of extracts intended for therapeutic use. They wrote,

> "The method is essentially comparative and not absolute, so that an arbitrary standard must be adopted and renewed at intervals",

and gave the first description of the preparation of their sterilized standard extract and demonstrated its stability. This marked the very beginning of the history of the standard preparation, the mainstay of all bioassay procedures.

3. History of the Standard Preparations for Neurohypophysial Hormones (Table 1)

The first officially recognized standard for the uterotonic effect of posterior pituitary hormones was histamine (Roth, 1914) [3] adopted in USP IX (1916). However, it proved unsatisfactory, as did potassium chloride, which was suggested as a substitute for histamine (Spaeth, 1918 [4]). Later it was clearly demonstrated by Burn and Dale (1922 [5]) that the sensitivity of the isolated uterus to histamine or potassium chloride and to pituitary extracts was uncorrelated.

Table 1
History of the standard preparations

1916	USP IX	Histamine	Unsuitable
1918		Potassium chloride	Suggestion, unsuitable
1922	Burn and Dale	Acid extract, sterilized	Suitable, stable
1924	Smith and McClosky	Acetone-dried posterior pituitary lobe powder	Suitable, stable Carl Voegtlin
1925	USP X Reference standard		
1925	1st International Standard		Health Organization League of Nations Geneva Conference

0.5 mg Dried standard material = 1 IU (1 Voegtlin unit).

3.1 *History of the international standard preparations (Table 2)*

1st International Standard. The history of the international standard preparations began with the preparation of an acetone-dried posterior pituitary lobe powder by Smith and McClosky in 1924 [6] at the Hygienic Laboratory, Division of Pharmacology in Washington. At that time, Carl Voegtlin was head of the division, and on his recommendation the powder was accepted as the Posterior Pituitary Reference Standard for USP X and, in 1925, as the 1st International Standard by the Health Organization of the League of Nations at the Geneva Conference. This conference also defined a unit of activity for post-pituitary preparations as the activity of 0.5 mg of the dried standard material. However, this 1st International Standard consisted of an unknown number of individual preparations, since, according to the recommendation of the Geneva Conference of 1928, any laboratory could prepare a dry powder by Smith and McClosky's method and regard it as equipotent with the International Standard without

Table 2
History of the international standards for oxytocin, vasopressin and antidiuretic substances

1st	1925–1935	Acetone-dried posthypophysial powder, prepared by use of Smith and McClosky's method, 0.5 mg = 1 IU (Voegtlin-unit)
	1935	British standard for pituitary (posterior lobe), extract prepared and kept at the National Institute for Medical Research, London, since 1926
	1936	Shown to have the same biological activity as Smith and McClosky's preparation
2nd	1942–1957	International collaborative assay: approx. 15% more active, definition not altered. Precautions to protect from destruction during the war (Dale, 1942) [9]
3rd	1957–1979	International joint assay: 7% of 2nd. International Standard for oxytocic, vasopressor and antidiuretic substances, bovine for bioassay

having to prove it. It was 10 years before the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations decided to establish a single International Posterior Pituitary Standard and adopted the British Standard as the International Standard. This material had been prepared and kept at the National Institute for Medical Research in London and had been shown to have the same biological activity as the Smith and McClosky preparation (1936) [7].

2nd International Standard. Another batch of acetone-dried posterior pituitary powder superseded the 1st Standard in 1942 [8]. An international collaborative assay revealed that its activities were approximately 15% greater than that of the 1st International Standard, but the definition of the International Unit was not altered. Elaborate precautions were taken to protect the Standard from possible destruction during the Second World War (Dale, 1942) [9].

3rd International Standard. This again consisted of acetone-dried posterior pituitary powder and was established in 1957. An international collaborative assay [10] revealed that the 3rd Standard was 7% less potent than the 2nd Standard.

4th International Standard. This superseded the 3rd Standard in 1978. It consists of pure (98–99%) synthetic oxytocin ampouled together with human albumin and citric acid (for stabilization) and freeze-dried. An international collaborative assay against the 3rd International Standard showed its oxytocic activity to be 12.5 IU/ampoule [11]. At the same time international standards were established for the two vasopressins (Arg^8-vasopressin [11] and Lys^8-vasopressin [12]) containing highly purified synthetic peptides (Table 3). The international standard for desmopressin followed in 1980 [13].

The introduction of pure single substances was an important step forward for all laboratories performing bioassays on neurohypophysial hormones. The reason for this is that quantitative biological methods are only valid when used to compare identical substances, i.e. one can only compare oxytocin with oxytocin, and Lys^8-vasopressin with Lys^8-vasopressin. This imperative requirement could not be met by the use of an international standard containing both oxytocin and arginine vasopressin in approximately equal amounts, as extracted from the acetone-dried posterior pituitary powder.

Table 3
Current international standards for oxytocin and the different vasopressins

Name of international standard	Material specific activity	IU/Ampoule	Year of establishment
4th, Oxytocin, for bioassay	Synthetic oxytocin 584 IU mg^{-1}	12.5	1978 [11]
1st, Arginine vasopressin, for bioassay	Synthetic Arg^8-vasopressin 410 IU mg^{-1}	8.2	1978 [11]
1st, Lysine vasopressin	Synthetic Lys^8-vasopressin 329 IU mg^{-1}	7.7	1978 [12]
1st, Desmopressin	Synthetic desmopressin 1000 IU mg^{-1}	27	1980 [13]

With the establishment of the new international standards the accurate bioassay of oxytocin, Arg^8-vasopressin, Lys^8-vasopressin and desmopressin became possible.

It is remarkable that the International Unit, or Voegtlin Unit, survived in spite of the introduction of four highly purified synthetic peptides as international standard preparations, which could have led to the adoption of weight of peptide/ampoule as the standard. However, that would have forced clinicians to learn a new dosage system!

4. Synthetic Analogues of Neurohypophysial Hormones Having Acquired Clinical Recognition

In contrast to other successes of modern chemical synthesis, attempts to produce analogues and homologues of the naturally occurring posthypophysial hormones have been rather disappointing, only four out of at least 200 synthesized having acquired any sort of therapeutic recognition (Table 4). They have a few advantages over the natural compounds such as higher specific activity, a longer duration of action or higher specificity, e.g. in the case of desmopressin potent antidiuretic activity combined with only traces of vasopressor activity. This demonstrates that the natural compounds have developed to an almost optimal configuration during evolution.

5. General Requirements for Bioassay Procedures

The second element in a bioassay is the assay method itself.

A suitable bioassay method must fulfil the criteria shown in Table 5. One of these is high sensitivity, to enable it to detect traces of the target substance. This was very important for the detection and purification of the neurohypothysial hormones, but

Table 4
Synthetic analogues of vasopressin and oxytocin in therapeutic use

Deamino-oxytocin	= De-amino^1-oxytocin:	[14]
Felypressin	= Phe^2-Lys^8-vasopressin:	[15]
Ornipressin	= Orn^8-vasopressin:	[18] [19]
Desmopressin	= De-amino^1-D-Arg^8-vasopressin	[20]

Out of about 200.

Table 5
Criteria for suitable bioassay methods

High sensitivity
Linear dose–response relationship
Reproducible during single and repeated assay
Four-point assay design, doses given in randomized order

became less important after elucidation of their structure and the synthetic production of large amounts of the hormones.

A linear dose–response relationship over a reasonably wide dose range is a prerequisite for all sorts of quantitative measurements using pharmacological reactions. The reproducibility of the reaction within a single and between repeated assays is a basic requirement for enabling a sufficient number of administrations of unknown and standard to be given in randomized order so as to estimate the amount of unknown as precisely as possible. In most cases a "four-point assay" design is used, i.e. two different doses of the standard and two doses of the test sample are administered in randomized blocks of four administrations, the ratios between low and high doses being constant.

The four-point assay permits calculation of the amount of active principle contained in the test sample in terms (units) of the standard ± fiducial limits by use of standard statistical methods. It also reveals whether the dose–response curves of the standard and test samples are parallel (as they should be) or, more precisely, not significantly non-parallel. If they are not parallel, the assay is not valid. Other designs such as the 3 + 1 and the six-point assay are also in use.

6. The Current Bioassay Procedures for Neurohypophysial Hormones

Table 6 lists the currently used bioassay methods for neurohypophysial hormones, which are described in the pharmacopoeias. More detailed descriptions are also available [21].

Oxytocic activity is measured by determining the fall in chicken blood pressure, the contractions of the isolated rat uterus or the increase in milk ejection pressure in the lactating rat.

6.1 *The avian depressor assay*

Table 7 is an attempt to characterize the chicken blood pressure test according to a standard schema. This test uses a non-physiological response to oxytocin for its

Table 6
Most frequently used bioassay methods for neurohypophysial hormones

Oxytocic activity	Vasopressor activity
Chicken blood pressure (fall)	Rat blood pressure (increase)
Isolated rat uterus (contraction)	Rat diuresis (decrease)
Lactating rat (rabbit) mammary gland increasing milk-ejection pressure	

Table 7
Chicken blood pressure assay

Sensitivity:	20 mU Oxytocin c. 34 ng
Linear dose–response:	Yes [22]
Reproducibility:	Desensitization possible
Four-point assay design:	Yes
Detection of effect:	[23]
Introduction as bioassay:	[24] [25]
Official recognition:	USP XIV, 1 November 1950 BP 1 September 1953 Eur. Ph. Vol. III, 1975

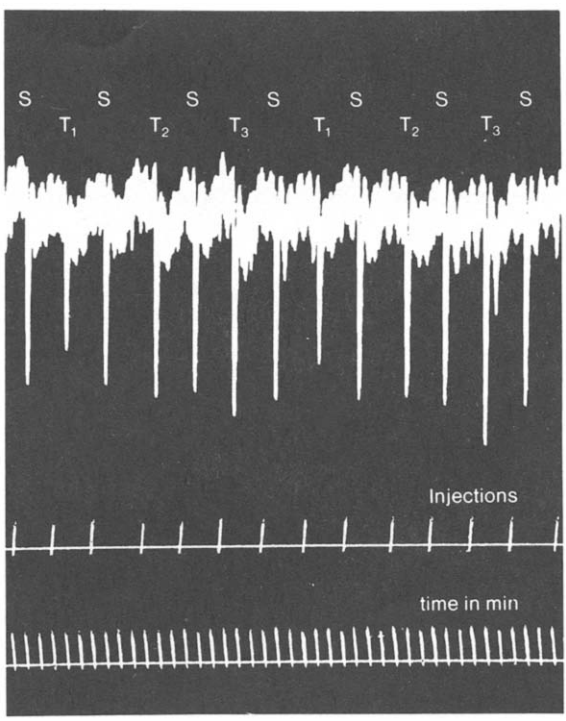

Figure 1
Oxytocin assay in the rooster. Representative section of an assay tracing. For explanation see text [21].

quantification. Figure 1 (Stürmer, 1968) [21] shows a representative section of an assay tracing. It demonstrates that a standard dose elicits the same effects over the recording time (the criterion of reproducibility is fulfilled). The administration of three different test doses (T_1 = 20% less than T_2, T_3 = 20% more than T_2) leads to quantitatively different responses. T_1 is less active than T_2, and T_3 is more active than T_2, which is nearly as active as the standard preparation.

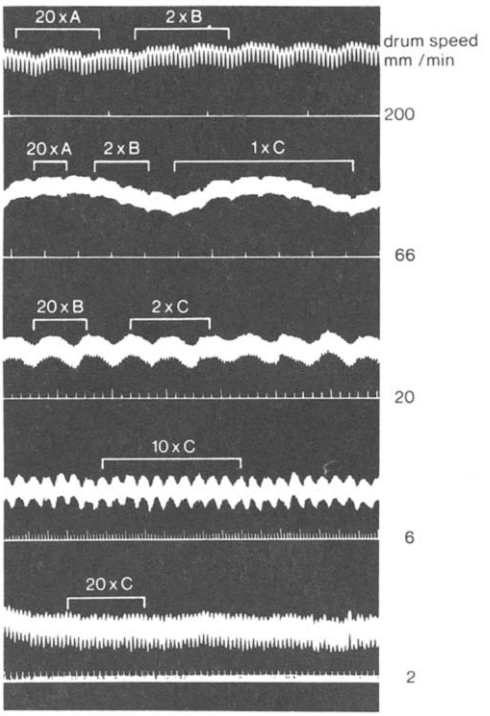

Figure 2
Blood pressure of a rooster recorded at different drum speeds. Time-base: short time markings, 10 s; long time markings, 60 s. For explanation see text [21].

In order to obtain good results, the investigator should know something about the regulation of blood pressure in order to time his injections correctly. Figure 2 [21] is a tracing of the blood pressure of a rooster recorded at different speeds. It shows three superimposed wave movements:

(1) the systolic–diastolic pulsation, corresponding to a heart rate of 100–200 beats/min (the A-wave);
(2) a slower series of waves with a frequency of 10–20/min which is synchronous with respiration (the B-wave);
(3) a slow wave with a frequency of about 1–1.5/min, probably reflecting central vasomotor tone (the C-wave).

At a low drum speed (2–3 mm min^{-1}) the C-wave is readily discernible. In my experience, it is important to ensure that the i.v. injections are always given when the C-wave is in the same phase, preferably when it has just begun to fall after reaching its peak. If measured at this point, the fall in blood pressure after a given dose of oxytocin is about 20% greater than if it is measured at the point where the C-wave begins to rise. That means that by use of an automatic timer for the injections, a random bias up to 20% is contracted.

The method gives good results (standard error <5%) if the number of injections of the test solution is not less than 10. Table 8 shows the distribution of the standard errors in 100 successive unselected assays conducted by five different technicians. It can be seen from the table that a standard error >5% was observed in only eight of 100 assays and that the mean relative standard error was only 2.9% ± 1.2 SD.

Table 8
Distribution of relative standard errors in 100 consecutive unselected oxytocin assays on chicken blood pressure performed by five different technicians

% Standard error range	0–1	1.1–2	2.1–3	3.1–4	4.1–5	5.1–6	6.1–7	7.1–8
Number of assays	1	28	34	23	6	6	1	1

Mean standard error $\bar{x}_{100} = 2.9 \pm 1.2\%$ (± SD).
Stürmer [21].

Table 9
Isolated rat uterus assay

Sensitivity:	10 μU oxytocin/10 ml c. 1.7 pg ml^{-1}
Linear dose–response:	Yes, small range
Reproducibility:	Depends on several factors
Four-point assay design:	Yes
Detection of effect:	[2]
Introduction as bioassay:	[26] [27]
Official recognition:	USP until October 1950 (guinea-pig) BP 1 September 1953 Eur. Ph. Vol. III, 1975

6.2 *The isolated rat uterus assay*

The isolated rat uterus assay (Table 9) is about 2000 times more sensitive than the chicken depressor method and appears fairly straightforward on the face of it. But in order to obtain good results a lot of pitfalls have to be avoided. The bath temperature, the ionic composition of the bath fluid, and isometric or isotonic registration can all influence the degree of uterine contractility. Moreover, a uterine contraction *in vivo* does not prove the presence of oxytocin. Bradykinin, for example, cannot be distinguished from oxytocin by this test (Fig. 3). But the rat uterus method is the best *in vitro* procedure available. For good results a total of 6–8 complete sets of four-points (24–32 reactions) is usually necessary.

6.3 *The milk-ejection assay*

The milk-ejection method (Table 10) works via a physiological effect of oxytocin: the increase in pressure in the large milk ducts converging in the nipple of a lactating animal due to contraction of the myoepithelial, or basket, cells of the mammary gland. The milk-ejection method is approx. 10–20 times more sensitive than the chicken blood pressure assay but approx. 10–20 times less sensitive than the isolated rat uterus preparation. The great advantage of the milk-ejection test is its relatively high specificity. Substances interfering with chicken blood pressure and isolated rat uterus response such as histamine, serotonin, acetylcholine, bradykinin, antiotensin I and II, and substance P are devoid of activity in this test. Today the standardization of highly purified synthetic posthypophysial hormones excludes the presence of other active principles, as

Figure 3
Isolated rat uterus; 10 ml bath. Contractions after increasing doses of bradykinin (A) and pure synthetic oxytocin (0). Doses of A: 1, 0.1 ng ml^{-1}; 2, 0.13 ng ml^{-1}; 3, 0.2 ng ml^{-1}; 4, 0.33 ng ml^{-1}. Doses of 0: 1, 0.044 ng ml^{-1}; 2, 0.066 ng ml^{-1}; 3, 0.088 ng ml^{-1}; 4, 0.11 ng ml^{-1}. Time: 1 min [37].

Table 10
Milk-ejection pressure measurement

Sensitivity:	10 μU oxytocin c. 0.2 ng
Linear dose–response:	Yes
Reproducibility:	Influence of anaesthesia
Four-point assay design:	Yes
Detection of effect:	[28]
Introduction as bioassay:	[29] [30]
Official recognition:	USP 0 BP 1 December 1978 Eur. Ph. 2nd revision

mentioned above. A disadvantage of the method is that a large rat colony must be kept if daily routine assays are to be carried out.

6.4 *The rat blood pressure assay*

The rat blood pressure assay (Table 11) for vasopressin, introduced in 1946, proved easy to set up, as well as reliable, extremely sensitive and capable of good discrimination. The method was further improved by Dekanski (1951, 1952), who pretreated the rats with α-adrenoceptor blocking agents in order to lower and stabilize their blood pressure. The rat blood pressure test superseded the dog and the spinal cat blood pressure tests used in the USA and the UK, respectively.

The most suitable anaesthetic for the rats in this test is urethane, although it has been dropped from the description of the method in the new edition of the European Pharmacopoeia and USP XXI and replaced by the wording "an anaesthetic favourable to the maintenance of a uniform blood pressure", owing to its cancerogenic potential. However, everybody familiar with the rat blood pressure assay knows that the only suitable anaesthetic is urethane. There is another important detail which merits mention,

Table 11
Rat blood pressure assay

Sensitivity:	2.5 mU Arg^8-vasopressin c. 6 ng 2.5 mU Lys^8-vasopressin c. 8 ng
Linear dose–response:	Yes
Reproducibility:	Good
Introduction as bioassay:	[31] [32, 33]
Official recognition:	USP XV, 15 December 1955 BP 1st September 1958 Eur. Ph. Vol. III, 1975

and that is that the body temperature of the anaesthetized rat falls to about 25°C during the assay without detriment to the sensitivity of the preparation. If body temperature is maintained at the normal level (37°C), the blood pressure falls and the animals die before the assay can be completed. This observation contradicts USP XXI, which tells us to "keep the animal warm during preparation and during the assay". The reason for this inter-continental difference is unclear.

6.5 *The rat antidiuretic assay*

The rat antidiuretic assay (Table 12) is not officially recognized by the leading pharmacopoeias at the present time, but it will appear in both the BP and the European Pharmacopoeia for testing the antidiuretic activity of desmopressin. Its antidiuretic effect is the main physiological effect of vasopressin, and was observed and studied some 60 years ago in various species including man [34].

There are fairly sophisticated methods using anaesthetized rats and very simple methods using unanaesthetized rats which provide, more or less, information and precision. Figure 4 shows a comparative assay in an anaesthetized rat. Use of a highly

Table 12
Rat antidiuretic assay

Sensitivity:	10 μU, c. 0.02 ng vasopressin
Linear dose–response:	Yes
Reproducibility:	Influence of anaesthesia
Four-point assay design:	Yes
Detection of effect:	Dogs [35]
Introduction as bioassay:	[36]
Official recognition:	USP 0 BP 0 Eur. Ph.* 0

* In preparation for desmopressin.

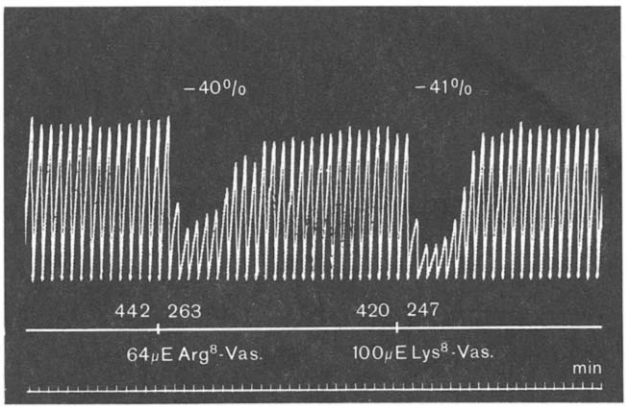

Figure 4
Differences in the antidiuretic action of Arg^8-vasopressin (3rd Standard) and synthetic Lys^8-vasopressin in the hydrated, ethanol-anaesthetized rat. The vertical lines indicate the diuresis in number of drops/min. The number of drops for a 10-min period before and after i.v. injection is denoted by the appropriate sign. The decrease in diuresis for each 10-min period is calculated in percentage. Time given in minutes. From our calculations both Arg^8-vasopressin and Lys^8-vasopressin appeared to exert an equal effect (i.e. −40 and −41%, respectively). The latter substance exerting a somewhat quicker onset and shorter duration than Arg^8-vasopressin. However, the assessment of activity depends upon the specific criteria chosen. Thus if one considers the results in terms of maximum decrease, then clearly Lys^8-vasopressin is the more active substance, but in terms of duration and effect, Arg^8-vasopressin exerts the more sustained antidiuretic action [38]. Reproduced from *Br. J. Pharmac. Chemother.* **15**, 544 (1960); by kind permission of Macmillan Press Ltd.

sensitive technique (1 drop = 5–7 μl of urine) reveals qualitative differences between the antidiuretic effects of Arg^8-vasopressin (3rd Standard) and Lys^8-vasopressin (synthetic). Although their overall antidiuretic activity is equal (−40 and −41% resp.), differences in onset of action, maximal effect and duration of action are apparent. Lys^8-vasopressin has a more rapid onset of action and a higher maximal effect than Arg^8-vasopressin, but a shorter duration of action.

These comparative assays are only possible if the antidiuretic substances are administered intravenously. If the duration of action of the antidiuretic effect is being studied, subcutaneous administration is employed, but absorption from the subcutaneous tissues varies as a function of the vasoconstrictor activity of the compounds.

7. Conclusions

The difficulties in standardization which arose in the sixties have now been partially overcome by the introduction of the International Lysine Vasopressin Standard and the International Desmopressin Standard; felypressin and ornipressin are still waiting for appropriate standard preparations.

Seventy-six years after the introduction of the first bioassay by Dale and Laidlaw, we still need them. Further developments, especially in the field of HPLC, may one day supersede the old bioassays, but for the time being the two methods complement each other when properly used.

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