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Chromatographic characterisation of an estrogen-binding affinity column containing tetrapeptides selected by a combinatorial-binding approach

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Abstract

In our work we performed a combinatorial synthesis in aqueous medium to prepare peptide libraries from which we would select amino acid sequences with binding properties towards estrogens. We prepared an affinity solid-phase by using a tetrapeptide with good selectivity and affinity towards the estradiol ($K>10^4$ M^{-1}). Samples of estrogens in buffer, in tap water and in river water were applied to our column in which they were retained ($k' > 116$). These could only be eluted in a few millilitres of methanol mobile phase. In all cases there were quantitative recoveries. The pre-concentration studies were promising.

Keywords: Binding constants; Affinity sorbents; Water analysis; Peptides; Endocrine disrupters; Estrogens; Steroids

1. Introduction

In affinity chromatography, a common goal is to prepare an immobilised molecular derivative which retains the ability to bind specifically to a mobile substance [1]. When such specificity is achieved, powerful separation methods are available for preparative isolation of the desired species, for preconcentrations of trace compounds, for the purification of analytes from complex mixtures and also as a method of analysis [2–4]. In the case of small analytes, the most widely used affinity columns are prepared by exploiting the antibody–antigen interaction; the antibodies were immobilised on the solid-phase and their great affinity for a specific molecule permits them to retain the analyte. These kinds of affinity columns are rather expensive, so over the last few decades, scientific research has tried to substitute them with synthetic systems with recognition properties similar to the natural ones.

The molecular imprinting techniques are giving good results in preparing tailor-made reagents (for a discussion of non-covalent molecular imprinting, see Ref. [5]). Most of these systems work well in non-polar environments, but recently template oligopeptides were obtained which also work in aqueous medium [6] showing high binding constants and good selectivity. The disadvantage of the molecular imprinted polymers (MIPs) is the necessity of a great

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quantity of imprinting molecule with a high purity degree. This aspect, above all, limits the use of this technique for preparing MIPs imprinted with very toxic molecules, or with low purity or very expensive compounds. Moreover, in the case of the template oligopeptides we had mixtures of oligopeptides with unknown amino acid sequence and so it would be difficult to define and immobilise them without losing their recognition properties.

Another approach to obtain synthetic recognition systems could be combinatorial chemistry [7,8], which allows us to prepare great libraries with an enormous number of well-characterised new compounds with different binding properties. Nevertheless, the experimental work to synthesise combinatorial libraries usually needs large and expensive instruments.

In our work we exploited these approaches and we performed a combinatorial solid-phase synthesis in aqueous medium using the amino acids as monomers—from each library we selected the amino acid sequence with the best selectivity and recognition properties. These applied syntheses allowed us to prepare an affinity column for the estrogens by using antibody-like oligopeptides that were less expensive and did not need drastic elution conditions.

2. Experimental

2.1. Chemicals

All chemicals for buffers, amino acids, *N*-hydroxy-succinimide and *N*,*N*-dicyclohexylcarbodiimide were from Merck (Darmstadt, Germany) and had a purity of 99.9%. The amino acids had an enantiomeric purity of 99.9%. All solvents of HPLC analytical grade were from Merck. The steroids with a purity of 99.9% were purchased from Sigma (Milwaukee, WI), whereas the tritium-labelled steroids were from Amersham (Uppsala, Sweden) and were in a toluene–ethanol (9:1) solution at a concentration of 1 mCi cm^{-3}. The specific activities of the markers were 85 Ci $mmol^{-1}$ for testosterone and 75 Ci $mmol^{-1}$ for estradiol. The liquid scintillation cocktail was Eco-lite, purchased from ICN (Costa Mesa, CA). We used polypropylene picovials purchased from Chemil (Padova, Italy) and from ICN for the counting of labelled samples.

2.2. Instrumentation

Radioactivity was counted by a Wallac 1410 liquid scintillation counter (Turku, Finland). The low-pressure chromatographic apparatus (peristaltic pump P-1, fractions collector FRAC-100, monitor UV-M and chart recorder REC-482) was from Pharmacia (Uppsala, Sweden).

2.3. Liquid chromatography

The solid-phase was Amberlite IRC-50 from Sigma, with superficial carboxylic groups (10 mmol/g of dried resin). The pore diameter was 16-50 mesh and it was stored at room temperature. The packed column dimensions were 1.7×1.0 cm. The chromatograms were recorded at 280 nm.

We used three mobile phases. The first was a phosphate buffer (50 m*M* phosphate, 1 m*M* EDTA, pH 7) and the second was phosphate buffer–methanol (50:50, v/v) and the last one was 100% (v/v) methanol. We used different flow-rates ranging from 0.2 cm^3 min^{-1} to 1.0 cm^3 min^{-1}. We applied 0.25 cm^3 of different steroid samples at a concentration of 2.5×10^{-5} *M*. The steroid solutions were prepared in phosphate buffer, tap water and river water. We integrated the chromatographic peak of the steroid and, from these data and the characteristics of the UV monitor, we obtained the concentration of the recovered steroid. Moreover, the recoveries were calculated by dividing the previous result by the initial concentration of the steroid and by multiplying it by 100. The capacity factor (k') was calculated as $(t - t_0)/t_0$, where t is the retention time of the eluted steroid, and t_0 the hold-up time. We determined it by applying 0.25 cm^3 of a phosphate buffer solution of sodium azide (2.5×10^{-5} *M*) to our column.

2.4. Solid phase syntheses

The working procedure, followed during the experimental phase, could be summed up on the flow chart reported in Fig. 1.

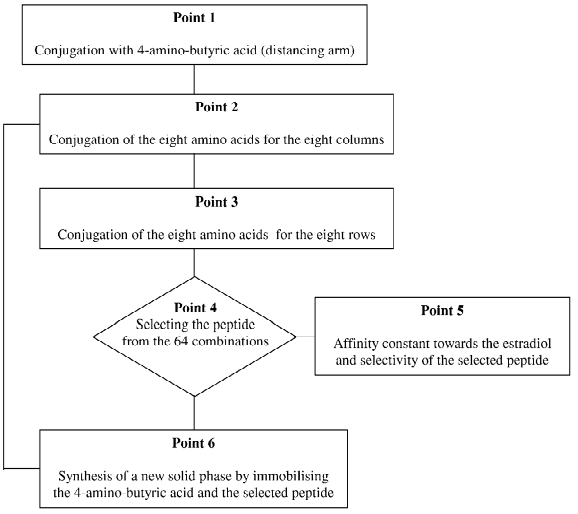

Fig. 1. Flow chart of the experimental work. The working sequence, which was followed to select a peptide with binding properties, was mainly made by first synthesis steps to prepare the amino acid library, the binding and selectivity properties of the 64 combinations were then put through a checking phase. The selected peptide was used as a starting solid-phase for a new working sequence.

2.4.1. Immobilisation of 4-aminobutyric acid as a distancing arm (point 1 in Fig. 1)

We activated the superficial carboxylic groups present on the solid-phase by following the *N*-hydroxy-succinimide-activated ester method [9]. One gram of the resin was suspended in 5 cm^3 of anhydrous *N*,*N*-dimethylformamide (DMF) and washed three times with the same volume of solvent. Following the literature, we added a solution of *N*-hydroxy-succinimide (10 mmol in 5 cm^3) in anhydrous DMF and then a solution of *N*,*N*-dicyclohexylcarbodiimide (10 mmol in 5 cm^3) in anhydrous DMF to the resin suspension. After mixing for 1 h at room temperature (RT), the beads were washed three times with anhydrous DMF and with carbonate buffer (0.15 *M* carbonate, pH 8.5) (again three times). Then we added 5 cm^3 of a mixture of 4-aminobutyric acid–ethanolamine (10:90, p/v) in carbonate buffer to the activated beads. The suspension was left to react for 2 h at RT under continuous mixing. The amino groups of these compounds reacted with the bead activated carboxylic groups. In this way we attached the 4-aminobutyric acid as a distancing arm (which had a final carboxylic group that could react again) to the beads; whereas the ethanolamine end capped the residual carboxylic groups on the bead surface, so they could not interfere with the consequent reactions. In the end the resin was washed three times with carbonate buffer and then stored at 4 °C until being used.

2.4.2. Preparation of the dipeptide library

The solid-phase (1 g), where the distancing arm had been attached, was washed three times with anhydrous DMF, suspended in anhydrous DMF (16 cm^3) and split into 64 tubes (0.25 cm^3/tube). We activated the carboxylic groups of the distancing arm using the *N*-hydroxy-succinimide-activated ester method, as previously described. After the washing step, we added an amino acid solution to each tube (point 2 in Fig. 1). The amino acids of the library were arginine (Arg), serine (Ser), proline (Pro), valine (Val), leucine (Leu), glutamine (Gln), glycine (Gly) and alanine (Ala). The amino acid concentration was 10 times that of the carboxylic groups of the distancing arm (1 mmol/g of dried resin) and was dissolved into the carbonate buffer. The α-amino group of the amino acid reacted with the activated carboxylic group of the distancing arm, so we could immobilise a first amino acid on the resin surface. After mixing for 2 h at RT, each tube was washed three times with carbonate buffer. We changed the dispensed amino acid after every eight tubes. In the end, we had 64 tubes that were divided into eight columns with eight tubes for each. Each column corresponded to a different amino acid, as shown in Table 1.

The 64 tubes were washed with anhydrous DMF and the α-carboxylic group of the first amino acid was activated by following the same procedure used for the carboxylic group of the distancing arm. After the washing step, we added the second amino acid solution to each tube. The second amino acid was bound by following the same synthesis procedure reported for the first one (point 3 in Fig. 1). We used the eight amino acids previously reported (Arg, Ser, Pro, Val, Leu, Gln, Gly, Ala) and we dispensed their solutions into eight different rows made up of eight tubes. The same amino acid was dispensed into each row (Table 1).

Table 1
Pattern of amino acid libraries

	Ala	Arg	Ser	Pro	Val	Leu	Gln	Gly
Ala	Ala–*Ala*	Arg–*Ala*	Ser–*Ala*	Pro–*Ala*	Val–*Ala*	Leu–*Ala*	Gln–*Ala*	Gly–*Ala*
Arg	Ala–*Arg*	Arg–*Arg*	Ser–*Arg*	Pro–*Arg*	Val–*Arg*	Leu–*Arg*	Gln–*Arg*	Gly–*Arg*
Ser	Ala–*Ser*	Arg–*Ser*	Ser–*Ser*	Pro–*Ser*	Val–*Ser*	Leu–*Ser*	Gln–*Ser*	Gly–*Ser*
Pro	Ala–*Pro*	Arg–*Pro*	Ser–*Pro*	Pro–*Pro*	Val–*Pro*	Leu–*Pro*	Gln–*Pro*	Gly–*Pro*
Val	Ala–*Val*	Arg–*Val*	Ser–*Val*	Pro–*Val*	Val–*Val*	Leu–*Val*	Gln–*Val*	Gly–*Val*
Leu	Ala–*Leu*	Arg–*Leu*	Ser–*Leu*	Pro–*Leu*	Val–*Leu*	Leu–*Leu*	Gln–*Leu*	Gly–*Leu*
Gln	Ala–*Gln*	Arg–*Gln*	Ser–*Gln*	Pro–*Gln*	Val–*Gln*	Leu–*Gln*	Gln–*Gln*	Gly–*Gln*
Gly	Ala–*Gly*	Arg–*Gly*	Ser–*Gly*	Pro–*Gly*	Val–*Gly*	Leu–*Gly*	Gln–*Gly*	Gly–*Gly*

The first eight amino acids, as with the other odd amino acids, were dispensed into tubes divided into eight columns by following the table, whereas the second eight amino acids, as with the other even ones, were dispensed following the rows reported in the table and where the amino acids are written in italics. The 64 cells represented the 64 amino acid sequences obtained by creating the libraries.

In the end we obtained 64 different dipeptides that constituted the first peptide library.

2.4.3. Preparation of the second library and other subsequent libraries

The dipeptide with the best binding properties and the best selectivity was chosen (points 4 and 5 in Fig. 1) as an initial solid-phase for the second library. We prepared 1 g of the resin with the selected dipeptide immobilised on its surface (point 6 in Fig. 1) by following the described procedures. We split this new solid-phase into 64 tubes and we bound a third amino acid to the activated α-carboxylic group of the second amino acid (point 2 in Fig. 1). The synthesis steps were the same as were reported in the previous paragraph for the first amino acid. In the end, a fourth amino acid was linked to the third one by the same synthesis procedures followed for the second amino acid (point 3 in Fig. 1).

From the second library we selected the immobilised tetrapeptide with the best recognition properties (points 4 and 5 in Fig. 1) and we used it as an initial solid-phase to create a third library (point 6 in Fig. 1) by binding a fifth (point 2 in Fig. 1) and sixth (point 3 in Fig. 1) amino acid. Also, the best esapeptide selected from the third library (points 4 and 5 in Fig. 1) was the base of a fourth library (point 6 in Fig. 1) and we immobilised a seventh (point 2 in Fig. 1) and eighth (point 3 in Fig. 1) amino acid by following the described procedures.

2.5. Checking of the binding properties towards the steroids (point 4 in Fig. 1)

For each library we suspended all the beads on which different amino acid sequences had been immobilised in 0.25 cm^3 of phosphate buffer 20 m*M* (20 m*M* phosphate, 0.13 *M* NaCl, 1 m*M* EDTA, 0.1% gelatine, pH 7.4), then we added 0.25 cm^3 of a tritium-labelled steroid solution (final dilution in tube 1:500) in phosphate buffer 20 m*M*. The beads were incubated overnight at room temperature under continuous stirring. They were spun down and 0.25 cm^3 of supernatant was transferred into picovials where the liquid scintillation cocktail (3 cm^3) was added. Non-specific binding of the labelled steroid was evaluated by replacing the beads functionalised with the peptides with beads completely blocked with ethanolamine. The total radioactivity of the labelled tracer was determined by dispensing an equal volume of labelled steroid (dilution 1:500) into two picovials where the liquid scintillation cocktail (3 cm^3) was added. We applied the described procedure first to the estradiol and then to the testosterone on the same beads. To remove the residual tritium-labelled estradiol from the beads of the library we washed them four times with a washing buffer (20 m*M* phosphate, 0.5% sodium dodecylsulphate, pH 7.4), and each time we incubated the beads for 30 min at room temperature under continuous stirring. We evaluated the concentration of tritium-labelled estradiol present in the washing fractions and we verified that this washing procedure was able to

remove all previous added estradiol (data not shown). Moreover, the beads were washed three times with carbonate buffer to eliminate the detergent and suspended in the phosphate buffer 20 m*M*. Now it was possible to check the binding of the testosterone to the dipeptides.

By counting the radioactivity we measured the free steroid that remained in solution after the reaction with the amino acid sequences. Also we measured the total radioactivity of the steroid tracer. We could calculate the bound steroid from these two experimental data and so we used the ratio (B/F) between the bound (B) and the free (F) steroid to express the affinity of each sequence towards the steroid.

2.6. Determination of the binding constants of the amino acid sequences (point 5 in Fig. 1)

For each selected amino acid sequence we determined the binding constants. The beads (1 g) on which the selected peptide had been immobilised were suspended in the phosphate buffer 20 m*M* (16 cm^3) and 0.25 cm^3 of the suspension was dispensed into tubes where 0.25 cm^3 of an estradiol solution in the same buffer was added. The added estradiol solutions had different concentrations ranging from 1×10^{-6} to 30×10^{-6} *M*. Moreover, we added a tritium-labelled estradiol (final dilution in tube 1:50) to the first dilution, so it was present in all following dilutions as they were prepared from this first one. The beads were incubated overnight at room temperature under continuous stirring. They were spun down and 0.25 cm^3 of supernatant was transferred into picovials where the liquid scintillation cocktail (3 cm^3) was added. Non-specific binding of the labelled steroid was evaluated by replacing the beads functionalised by the peptides with beads completely blocked by ethanolamine. The total radioactivity of the labelled tracer was determined by dispensing 0.25 cm^3 of labelled estradiol (1:50 as the first dilution) into two picovials in which the liquid scintillation cocktail (3 cm^3) was then added. The counting of the radioactivity allowed us to determine the free estradiol for each dilution and the total radioactivity for the first dilution. From these data we could calculate the total estradiol for each dilution. Furthermore, we calculated the bound estradiol by subtracting the free estradiol from the total one for each dilution. We plotted the ratio between bound estradiol and free estradiol versus the bound estradiol and we obtained the binding constants by fitting a linear regression through these data [10].

3. Results and discussion

The aim of our work was the synthesis of an amino acid sequence with recognition properties towards small-molecular-mass analytes such as the estrogens. Then we would use these synthetic recognition systems to prepare a solid-phase for a pre-concentration column, which exploited the peptide affinity to retain the analytes. We decided to choose the estrogens as these compounds have an important role in endocrine metabolism and in recent years were recognised as endocrine disrupters [11].

We prepared an amino acid library using as monomers eight amino acids: arginine, serine, proline, valine, leucine, glutamine, glycine and alanine. All of these amino acids are present in the estradiol binding site of the human steroid binding protein [12]. Only alanine does not belong to this sequence as we used it to substitute lysine and methionine, which have reactive groups in their lateral chains. In these preliminary studies we would avoid synthetic problems and so we simplified the composition of the library by eliminating these two amino acids. In this way we could easily obtain a known linear amino acid sequence.

As the solid-phase, we chose polystyrene cross-linked beads with carboxylic groups on the surface. Moreover these beads were suitable for the synthesis work because of their large size and so they were easy to handle and separate. Also, their characteristics made them a good solid-phase for carrying out liquid chromatography in order to obtain a new pre-concentration system.

Firstly, we immobilised a distancing arm, the 4-aminobutyric acid, to move the amino acid sequences away from the bead surface. In this way the oligopeptides should have better binding capability. Furthermore, the solution of the distancing arm was mixed with a solution of ethanolamine to obtain a more homogeneous distribution of carboxylic groups on the bead surface. Also, with this procedure we

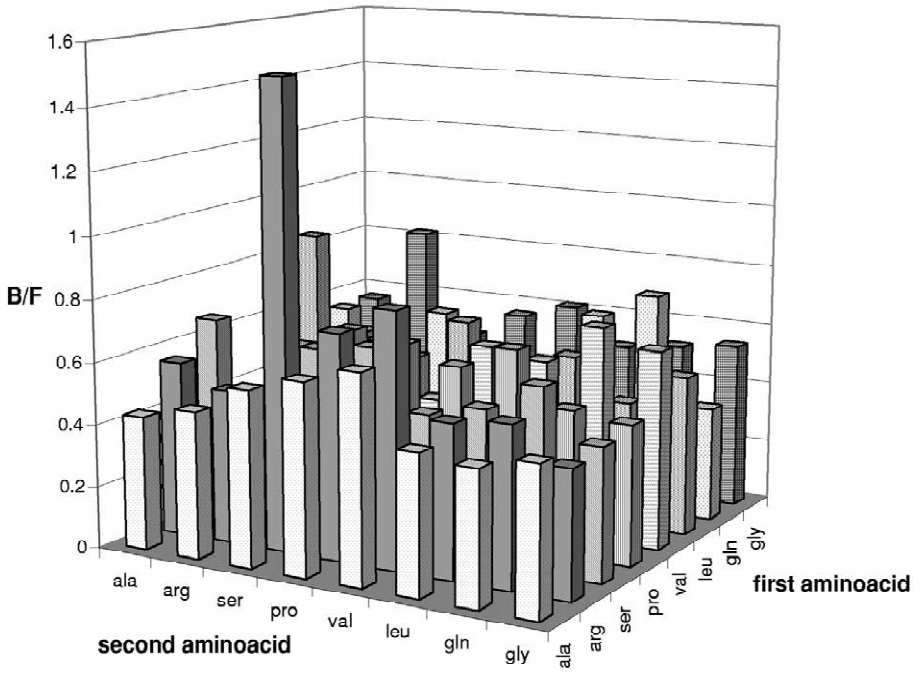

Fig. 2. Ratio (B/F) of bound (B) and free (F) estradiol of the 64 dipeptides composing the first library.

coated the residual carboxylic groups—end-capping them.

The beads with the immobilised 4-aminobutyric acid were the initial solid-phase to prepare the first amino acid library. Its pattern is shown in Table 1 and we immobilised two different amino acids as shown in each cell. Between the 64 dipeptides, the best binding properties towards the estradiol (Fig. 2) were shown by the sequence arginine–serine (B/F = 1.50) which also had the lower binding capacity towards the testosterone (B/F = 0.25). We determined the binding constant of this sequence and the ratio between the estradiol B/F and the testosterone B/F as reported in the first row of Table 2. The binding constant was rather high as we considered that the affinity of the human serum albumin [13] is 10^4–10^5 M^{-1} for the estradiol. Moreover we only had a linear sequence of three amino acids, including the distancing arm, without a complex protein structure.

Using this selected amino acid sequence as new starting solid-phase, we prepared a second library by following the described synthesis procedures and the pattern reported in Table 1. From this second library we were able to select a tetrapeptide with the same procedures followed for the dipeptide, and the sequence arginine–serine–serine–valine showed higher binding towards the estradiol (B/F = 3.35) and lower towards the testosterone (B/F = 0.33).

Moreover the binding constant increased (Table 2)

Table 2
Binding constants towards the estradiol and ratios ($R_{\text{estradiol/testosterone}}$) between the estradiol B/F and the testosterone B/F of the peptide sequences, which showed the best affinity towards the female steroid hormone

Peptide	10^4K (M^{-1})	$R_{\text{estradiol/testosterone}}$
Arg–Ser	3.1±0.5	6.0
Arg–Ser–Ser–Val	5.9±0.8	10.1
Arg–Ser–Ser–Val–Gly–Ser	8.1±0.9	5.6
Arg–Ser–Ser–Val–Gly–Ser–Gln–Ser	6.5±0.6	3.4

and the tetrapeptide was more selective towards the estradiol than the dipeptide.

Repeating the described synthesis and screening procedures we obtained an esapeptide and an octapeptide. The first had the sequence, arginine–serine–serine–valine–glycine–serine and it showed a B/F for the estradiol of 3.75 and 0.67 for the testosterone. The octapeptide was arginine–serine–serine–valine–glycine–serine–glutamine–serine and it showed a B/F of 3.54 and 1.04 towards the estradiol and the testosterone, respectively.

In Table 2 the binding constants are shown. We could observe that the value of the binding constants increased as far as the esapeptide, whereas the selectivity decreased after the tetrapeptide. The decreasing of selectivity was expected as we observed that, by increasing the number of amino acids bound to the solid-phase, the average binding of the amino acid sequences increased both towards the estradiol and towards the testosterone. This behaviour was reasonable as we always added a dipeptide on an amino acid sequence, which showed high binding properties, and so we started each time from a solid-phase with a higher affinity towards the steroids.

We chose arginine–serine–serine–valine to prepare a solid-phase for a pre-concentration column, as this sequence showed a good binding constant and the best selectivity.

We packed a small column with this solid-phase to carry out low-pressure chromatography. Then we applied different samples of steroids of the same concentration. Firstly, we tried to elute the steroids with a phosphate buffer but, as shown in Table 3, only testosterone and cortisol were eluted in a few millilitres of buffer with good recoveries. All the applied estrogens were retained within the column even when increasing the flow-rate to a maximum of 1 cm^3 min^{-1}. The capacity factors of the estrogens could be only estimated, as they remained in the column even after 70 column volumes. Furthermore we changed mobile phase by using a solution of methanol–phosphate buffer (50:50, v/v). As shown in Table 4, all the estrogens could be recovered in a few millilitres with good recoveries.

We verified the real binding properties of the tetrapeptide versus a blank column, which was packed only with polystyrene beads, completely end-capped with ethanolamine and it had the same dimensions as the tetrapeptide column. We applied the same steroid samples to this column as described before and all the steroids, even the estrogens, were eluted in a few millilitres of phosphate buffer with good recoveries (Table 5).

Table 3
Capacity factors of the steroids obtained by applying steroid solutions in phosphate buffer to the tetrapeptide column. The mobile phase was the phosphate buffer. The reported recoveries were the results of six independent experiments

Analyte	k'	Recovery (%)
Estradiol	>116.6	–
Ethynylestradiol	>116.6	–
Estrone	>116.6	–
Estriol	>116.6	–
Estradiol diacetate	>116.6	–
D-Norgestrel	>116.6	–
β-Sitosterol	>116.6	–
Testosterone	6.06	81–105
Cortisol	1.94	92–116

After these results, our tetrapeptide column seemed to work well, so we performed a loading test. We applied 100 cm^3 of phosphate buffer solution of estradiol at concentration of 1 ng cm^{-3} to the column. The flow-rate was 0.2 cm^3 min^{-1} during the loading. Moreover we washed the column with methanol 100% (v/v) (20 cm^3) at flow-rate of 0.5 cm^3 min^{-1}. We monitored the loading by fractionating each 5 cm^3 that came out of the column, and we recovered the entire methanol in one fraction. The estradiol present in the aqueous fractions was measured directly by enzyme-linked immunosorbent assay (ELISA) [14], whereas the solvent fractions

Table 4
Capacity factors of the estrogens obtained by applying steroid solutions in phosphate buffer to the tetrapeptide column. The mobile phase was phosphate buffer–methanol (50:50, v/v). The reported recoveries were the results of six independent experiments

Analyte	k'	Recovery (%)
Estradiol	4.29	96–108
Ethynylestradiol	3.62	85–97
Estrone	4.29	98–120
Estriol	4.17	91–113
Estradiol diacetate	3.11	92–113
D-Norgestrel	2.88	87–102
β-Sitosterol	3.70	93–106

Table 5
Capacity factors of the steroids obtained by applying steroid solutions in phosphate buffer on the blank column. The mobile phase was the phosphate buffer. The reported recoveries were the results of six independent experiments

Analyte	k'	Recovery (%)
Estradiol	4.15	88–104
Ethynylestradiol	3.87	87–102
Estrone	4.17	90–114
Estriol	4.11	93–111
Estradiol diacetate	3.22	98–111
D-Norgestrel	3.01	88–110
β-Sitosterol	4.12	95–114
Testosterone	3.98	91–107
Cortisol	2.08	94–115

were firstly evaporated, lyophilised and then reconstituted (1 cm^3) in phosphate buffer 20 m*M* before performing the immunoassay. The same test was performed with an estradiol solution in tap water at the same concentration. As reported in Fig. 3, in each case above 15 cm^3, the concentration of estradiol found in the following fractions increased until 1 ng cm^{-3} and then became constant. In fact the estradiol recovered in methanol was about 15–20% of the total loaded into the column.

Moreover, we applied 15 cm^3 of an estradiol sample in phosphate buffer, again at the described concentration and we fractionated the methanol every 0.5 cm^3. Then the fractions were evaporated, lyophilised and then reconstituted (1 cm^3) in phosphate buffer 20 m*M*. The estradiol concentrations were measured again by ELISA. We observed that now the loaded estradiol was recovered quantitatively by methanol and that it was present from 2.5 to 6.0 cm^3 as shown in Fig. 4.

We repeated this experiment with estradiol solutions at concentration of 10 pg cm^{-3} in phosphate buffer, tap water and river water. Moreover, after loading the sample, we introduced two washing steps by using firstly a deionized water solution at 10% (v/v) of methanol (10 cm^3) and then a deionized water solution at 20% (v/v) of methanol (10 cm^3). These steps were useful to eliminate possible interferences presented in the tap and river waters and also to decreasing the quantity of water in the methanol fractions thus accelerating the evaporation step. We checked that these washing solutions were not able to elute the estradiol and by so doing recover them. We measured the possible steroid present by ELISA. The results were that no estradiol was eluted with these washing solutions (data not shown). In the end we eluted the estradiol by methanol and the results were that in all cases the estradiol was recovered in a few millilitres and with good recoveries (phosphate buffer, 97–105%; tap water, 96–113%; river water, 94–120%). These

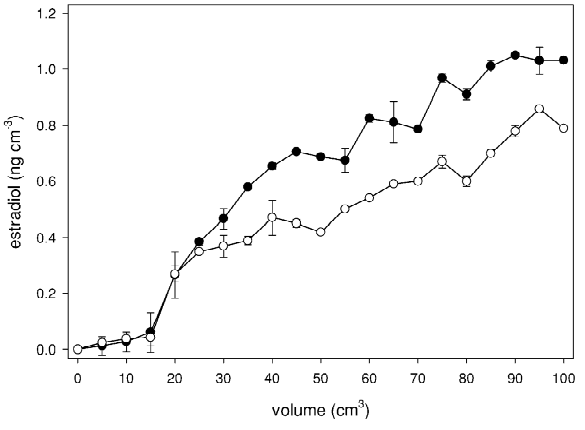

Fig. 3. Loading tests performed on the tetrapeptide column. Fractions in phosphate buffer (○) and tap water (●) were recovered from each volume of 5 cm^3, and the estradiol concentrations, measured by ELISA, were reported and compared to the mobile phase volume.

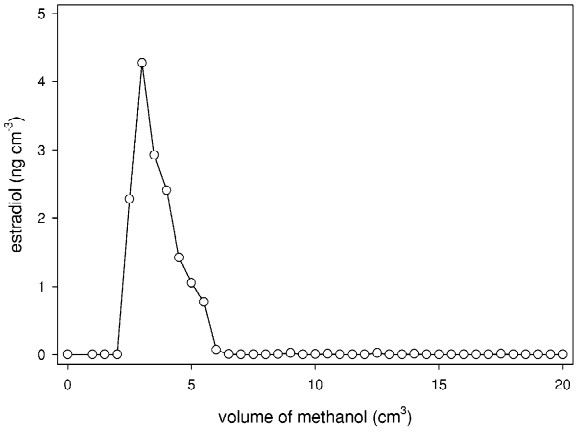

Fig. 4. Elution of the estradiol from the tetrapeptide column with 100% (v/v) methanol as mobile phase. Fractions were recovered from each volume of 0.5 cm^3, and the estradiol concentrations, measured by ELISA, were reported and compared to the volume of methanol.

recoveries were the results of six independent experiments.

4. Conclusions

This approach confirmed the feasibility of tailor-made reagents, which are water-friendly and work well in aqueous medium. Our procedures allowed us to check the binding capacity of the peptides during the creation of the libraries, and to select the sequence with greatest binding properties and best selectivity. Also, we avoided large screening work. The binding constants were high enough when taking into account the fact that we had small dimension recognition systems, and above all the tetrapeptide was able to retain the estrogens efficiently and selectively in the pre-concentration column.

The preliminary studies on the pre-concentration column were promising for the development of a new pre-concentration system as we obtained good recoveries. The column allowed us to concentrate the samples and so its application could be useful in increasing analytical performances.

Furthermore our approach to prepare synthetic recognition systems could be considered as a general method to obtain tailor-made reagents with antibody-like binding properties.

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