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Guidelines for analytical method development and validation of biotechnological synthesis of drugs Production of a hydroxyprogesterone as model

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Abstract

In connection with biotechnological synthesis of pharmaceutical drugs, validated methods for quantification of both product and substrate at different time intervals are essential for proper calculation of rate coefficients. In this field, there still exist no guidelines for analytical validation, unlike the situation in the bioanalytical field. Therefore, in this study the detailed guidelines by FDA for bioanalytical method validation were applied to a typical biotechnological process; the enzymatic synthesis of 9α-hydroxyprogesterone in *E. coli* using progesterone as substrate. The process liquid was extracted and analyzed using an HPLC–DAD system. The quality control (QC) samples of the product demonstrated excellent precision (C.V.<1.5%) and accuracy between 99.3 and 107%. The study showed that the recommendations and the validation terms for bioanalytical methods can be used also for biotechnological production, but with some important exceptions. The tolerances (C.V. values) of the validation terms should be much narrower; the internal standard (I.S.) must be present in the process liquid before the start of the process and must be much different in structure from the substrate (so as not to participate in the biotechnological process). In addition, the selectivity must be checked very frequently during the process due to the changes in the blank process liquid with time.

Keywords: Validation; Hydroxyprogesterones

1. Introduction

Validation of bioanalytical methods has been discussed frequently during the last 10 years in several meetings: in Arlington, VA, with a report published in 1992 [1], in London in 1999 and another one in Arlington in 2000 [2]. The main issue discussed was what type of analytical aspects should be investigated and reported in the validation protocol of a bioanalytical method in order to support bioavailability, bioequivalence and pharmacokinetic studies in man and animals. As a result of these discussions, the US Food and Drug Administration (FDA) has issued detailed recommendations for method validation of bioanalytical methods [3]. The International Conference on Harmonisation (ICH) has provided definitions of validation issues included in ''analytical procedures'' for the fields of

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bioanalytical methodology, pharmaceutical and biotechnological procedures [4–6]. However, this information is remarkably general and not at all written in practical terms or adapted to each respective field, such as the detailed FDA recommendations for bioanalytical methods (cf. Refs. [4–6] with Ref. [3]).

The suggested tolerances for the validation parameters in the FDA recommendations for bioanalytical methods [3] are rather wide, with C.V. <15%. There are no similar recommendations for pharmaceutical procedures but, the requirements are tighter, with C.V. <2% (Anders Karlsson, AstraZeneca R&D, Mölndal, personal communication). This is possible, since in the latter case the matrix is typically much simpler and the analyte concentration can be chosen freely, so that extremely low/high concentrations can be avoided.

There are no detailed recommendations for analytical procedures in the field of biotechnological production of drugs, in contrast to the recommendations made by the FDA for bioanalytical methods [3]. In fact, an extensive literature survey revealed only a few reports dealing with quantification issues in biotechnological processes. Indeed simple TLC methods have often been used and it is not always clear how the quantification has been done [7,8]. The term ''validation'' often appears for the measurement of cell growth [9] and cell distribution [10]. The absence of guidelines for analytical method validation in this field is surprising. It is important to validate the quantification of both substrate and product during the process, at definite time intervals, to ensure proper calculation of the kinetics of the process; i.e., the coefficients of substrate conversion, and production rates must be adequately calculated.

The aim of this study was to investigate whether the detailed guidelines and validation rules given by the FDA for bioanalytical methods can be used also in the field of biotechnological synthesis and, if not, to determine what modifications are required. The enzymatic synthesis of 9α-hydroxyprogesterone in *E. coli* with progesterone as starting material was used as a model. This process is a part of a large interdisciplinary project with the general goal to develop enzyme-catalyzed synthesis of chiral materials of recognized or potential commercial importance [11]. Before the quantification work was performed, an extensive product identification of the process was necessary due to the lack of commercially available product. That work is reported elsewhere [12].

It is our intention that the present article should serve as the basis for future discussions of recommendations and guidelines for analytical method development and validation applicable to the biotechnological field.

2. Experimental

2.1. Chemicals and solvent

Progesterone (PS) minimum 99% purity, 4-androsten-3,17-dione (AD), 11α-hydroxyprogesterone (11α-OH-PS), 11β-hydroxyprogesterone (11β-OH-PS), 20α-hydroxyprogesterone (20α-OH-PS) and 20β-hydroxyprogesterone (20β-OH-PS) were all of minimum 98% purity and obtained from Sigma (Stockholm, Sweden). *R*(+)-Bi(2-naphthol), minimum 99% purity was obtained from Sigma (Stockholm, Sweden). 9α-Hydroxyprogesterone (9α-OH-PS) was obtained by fermentation followed by preparative HPLC [12]. The organic solvents used were methylene chloride and acetonitrile of Lichrosolve-grade from Merck (Darmstadt, Germany) and methanol of HPLC Gradient Grade from J.T. Baker (Deventer, The Netherlands). The ethanol used was 99.5% pure and was obtained from Kemetyl (Haninge, Sweden). The water was obtained from the Milli-Q Academic Gradient A10 Biocel Synthesis system (Millipore, Sundbyberg, Sweden). All mobile phases were degassed in an ultrasonic bath before use.

2.2. Sample solutions

In the initial test of mobile phases the steroids were dissolved directly in the actual mobile phase. In the validation part, the components 9α-OH-PS and PS were prepared separately for the quality control (QC) samples and the calibration standard (CS). For the CS, 10 m*M* stock solutions of the steroids were prepared in ethanol; the stock solutions were mixed together and diluted with the fermentation process liquid to give concentration ranges for CS from 3 to 220 μ*M* for both PS and 9α-OH-PS, respectively.

The stock solutions were stored at 4 °C. All glassware used was first washed with ethanol and thereafter with water.

The culture media is described elsewhere [12]. Compounds added to the fermentations were: glucose (Merck, for biochemistry), riboflavin (Calbiochem, La Jolla, CA, USA), kanamycin (Sigma, St Louis, MO, USA), carbenicillin (Gibco-BRL), polypropylene glycol (PPG, BDH, Poole, UK) and isopropylthiogalactoside (IPTG). NaOH, H_3PO_4 and NH_4OH were of analytical quality (Merck).

2.3. Fermentations

The micro-organism used in the biotechnological process was *Escherichia coli* strain BL21 modified by insertion of a gene for the enzyme steroid 9α-hydroxylase from *Mycobacterium smegmatis*. The reaction was carried out in cylindrical glass fermentors with a working volume of 1.1 l. The culture was agitated continuously by a magnetic stirrer. The stirrer speed was coupled to the dissolved oxygen (DO) signal so that the DO at all times was kept above 30% saturation. The minimum stirrer speed was 200 rpm. The pH of the culture was kept constant at 7.2 by automatic addition of acid (3 *M* H_3PO_4) and base (3 *M* NaOH or 25% NH_4OH). The culture was aerated through a tube below the stirrer. The airflow was 0.5–1.0 l/min. The culture medium was either complex (LB) or minimal (M9). To decrease foaming, the chemical antifoam agent polypropylene glycol (PPG) was added before inoculation (0.2 ml/l). Before inoculation, antibiotics were also added to the fermentations (kanamycin, 50 μg/ml, and carbenicillin, 100 μg/ml). During the culture, expression of the enzyme that hydroxylates the steroid was induced by addition of isopropylthiogalactoside (IPTG, 0.1 m*M*, dissolved in 1 ml 70% ethanol). Prior to induction, riboflavin (5 μg/ml) was added. When the purpose of the fermentation was not to produce blank fermentate the substrate progesterone (200 μ*M*) was added at the time of induction and sometimes together with binaphtol (1 μ*M*) or AD (20 μ*M*) as internal standards, all dissolved in ethanol. The culture was run for 6–7 days after induction. Once a day, 8.5-ml samples were taken out with a syringe from a position near the bottom, close to the stirrer. Extra nutrition was supplied to the culture through a needle inserted in the headplate of the fermentor: glucose to 4 g/l in 20 ml was added once a day after the first day. We did not monitor the liquid volume in the reactor or the amounts of acid and base added for pH control.

2.4. HPLC instrumentation

All HPLC experiments were performed using a Hewlett-Packard HP 1100 chemstation with an automatic injector, equipped with a valve-switching unit with 10 port valves, a built-in diode-array UV detector and a work station PC (Agilent Technologies, Palo Alto, CA, USA). The DAD-detection made it possible to acquire spectra of all peaks in the chromatogram. The UV-pilot signal was recorded at 245 nm and 228 nm (bandwidth 4 nm, slit 2 nm), respectively. The UV spectra and 3D spectro-chromatograms were recorded from 190 to 400 nm. The screening experiments were carried out at ambient temperature. During the validation experiments the column was placed in a water jacket and its temperature was kept constant at 25.0 °C using a MN6 Lauda circulating water-bath (Lauda, Königshofen, Germany). The Agilent Technologies LC Chemstation software applications were used to assist in the quantification based on peak areas of standards and samples.

2.5. HPLC column

The column Kromasil KR100-3.5C18 (3.5 μm; 150×4.6 mm) with 100 Å pore size was obtained from Eka Chemicals (Bohus, Sweden).

Three different protections of the analytical column were investigated: a Kromasil KR100-5C18 guard column (10×4.0 mm), a graphite filter from ESA (ESA, Chelmsford, MA, USA) and a 0.5 μm frit A-431 from Upchurch Scientific (Oak Harbour, WA, USA). The 0.5-μm frit was later used in the validation.

2.6. Validation procedures

2.6.1. Solubility

The solubilities of PS and AD, the internal standard 11α-OH-PS and the product 9α-OH-PS in pure

water were investigated. An excess of each steroid, was added directly to three sample tubes, i.e., a total of 12 tubes for the four substances. Equilibrium was reached by ultrasonicating the sample tubes for 2 h followed by an adjustment to ambient temperature. After equilibration, the samples were centrifuged for 15 min at 1000 *g* and filtered through a 0.45-μm filter (GHP Acrodisc) to remove undissolved particles. The saturated samples were then injected into the Kromasil KR100-3.5C18 column. The saturating concentration (i.e., the solubility) of each sample was determined by use of a seven-point calibration curve obtained from standard concentrations prepared in the mobile phase and injected directly into the chromatograph. The measurements were performed in triplicate.

2.6.2. Extraction of the process liquid

A dose of 1.0 ml of the process liquid was added to 20.4 μl of the internal standard 11α-OH-PS and 1.5 ml methylene chloride. The mixture was (i) vortexed for 10 s, (ii) ultrasonicated for 30 min and (iii) placed in a shaking-machine for 120 min followed by (iv) centrifugation at 1000 *g* for 10 min. A 1.0-ml aliquot of the organic phase was transferred to a new test tube and evaporated to dryness under a stream of N_2 at 38 °C. The residue was re-dissolved in 670 μl mobile phase and transferred into an HPLC vial from which 50 μl was injected into the chromatographic system.

2.6.3. Preparation of samples

Two independent standard stock solutions of both analytes (9α-OH-PS and PS) were prepared. One of them (10.0 m*M*) was used to prepare the CS and the other (10.0 m*M*) was used to prepare the QC samples. Standard stock solutions were also prepared for the two internal standards, i.e., 11α-OH-PS (1.0 m*M*) and binaphthol (50 μ*M*). The spiked process liquid samples and the CS samples of PS and 9α-OH-PS were prepared by dilution of each stock solution to its final concentration using (i) blank fermentation process liquid, (ii) mobile phase or (iii) water. The samples were mixed thoroughly by vortexing for 30 s. The volume added from the stock solutions for dilution was always smaller than 8% of the total volume of the sample, so that the integrity of the sample was maintained. The stock solutions were kept in a refrigerator at 4.0 °C and the CS and QC samples were extracted as described for the process liquid given above.

2.6.4. Selectivity

The retention times of the analytes (i.e., the substrate, product and internal standard) were compared with retention times of similar components (hydroxyprogesterones). In addition, the selectivity of the method was assessed by comparing the chromatograms obtained after injection of blank process liquid without and with the addition of analytes. Each of the analytes was injected separately to ensure that no interfering impurities with the same retention times were present.

2.6.5. Calibration curve and linearity

The calibration curve consisted of eight calibration standards: 240, 200, 150, 100, 50, 25, 6.25, and 3 μ*M* of both 9α-OH-PS and PS. To the calibration standards were also added 20 and 1 μ*M* of the internal standards 11α-OH-PS and binaphthol, respectively. Two different approaches for including the internal standard (I.S.) were evaluated. In the first case, a 1.0-ml sample was taken out from the process liquid and to this sample was added 20.4 μl of a 1.0 m*M* solution of the internal standard 11α-OH-PS. In the second case, the internal standard binaphthol was added (1 μ*M*) directly into the fermentation process. The peak area ratio of the analyte to the internal standard was plotted versus theoretical concentrations. Calibration curves were obtained from least-squares regression analyses. No weighting was applied. The obtained calibration curves were used to calculate the concentration of product and substrate in the samples. The linearity of the method was checked by (i) evaluation of the regression coefficients and by (ii) plotting the response/sample concentration versus the logarithmic sample concentrations [13].

2.6.6. Precision and accuracy

The precision of an analytical method is the agreement within a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix [3]. The accuracy of an analytical method is the degree of

agreement between the true value of the analyte in the sample and the value determined. Both precision and accuracy can be calculated from the same analytical experiment.

Six samples of three different concentrations (18, 90 and 180 μ*M*) were analysed at three different occasions together with a calibration curve and the intra- and inter-day precision and accuracy were calculated. The accuracy was determined as the mean of the measure relative to the theoretical value and is reported in percentage (%). The precision is denoted by the intra- and inter-day coefficient of variation (C.V.%).

2.6.7. Limit of detection and quantification

The limits of detection (LOD) were experimentally estimated from the injection of continuously diluted standard solutions that were processed as the QC samples. The dilutions were made until the signal to noise ratio (*S*/*N*) for the analytes reached a value of three. The lower limit of quantification (LLOQ) was set as the lowest concentration in the standard curve (3 μ*M*) and the precision and accuracy were experimentally estimated at this concentration. The LOD and LLOQ were determined for both the substrate and the product.

2.6.8. Recovery

The absolute recovery was calculated by comparing the peak area ratios from the fermentation process liquid spiked with known amounts of 9α-OH-PS and PS (18, 90 and 180 μ*M*) versus peak area ratios of the same concentrations prepared in mobile phase and injected directly into the chromatographic system. Each determination was performed in six replicates.

The relative recovery was determined by comparing peak area ratios from fermentation process liquid spiked with known amounts of 9α-OH-PS and PS (18, 90 and 180 μ*M*) versus peak area ratios of the same concentrations prepared in water and extracted as described earlier. Each determination was performed in six replicates.

2.6.9. Stability

In the present study, the stability of the product (9α-OH-PS) as present in the fermentation process liquid was assured from spiked samples (18, 90 and 180 μ*M*) analyzed after 48 h at room temperature (bench-top storages) and after 4 months at −20 °C. The samples kept in the freezer were thawed and brought to room temperature and vortex-mixed before extraction and analysis. Each determination was performed in six replicates.

3. Results and discussions

3.1. Method development

3.1.1. Optimal stationary phase

In another study [12], an initial screening and investigation was made of several different promising HPLC columns, both chiral and non-chiral, in order to find the optimal column for the separation of the substrate from the product. This issue was somewhat complicated, since the product was not available as reference substance at the time [12]. Therefore, several similar hydroxylated progesterones were injected together with the substrate into various columns in order to see which one showed the best general performance for resolution of several hydroxyprogesterones from each other and from the substrate, progesterone. The injected steroid test-mixture contained the following six steroids: 11α-OH-PS, 11β-OH-PS, 20α-OH-PS, 20β-OH-PS, PS and AD. A good separation system for the steroids should be isocratic, have a high resolving power and a short total analysis time. The total analysis time depends on the retention of the most retained component, which should have a retention not longer than 20 min. On the other hand, the least retained compound of interest should, in a reversed-phase system, have a retention not less than 4 min in order to minimize interferences from polar matrix components. Kromasil KR100 with 3.5-μm particles had the lowest reduced plate height and gave the best resolution among columns tested and was therefore used in this validation study.

3.1.2. Optimal mobile phase

After finding the best column the mobile phase must be optimised. Fig. 1a,b shows chromatograms of the six steroids using two different organic solvents as modifiers of the mobile phase. In Fig. 1a

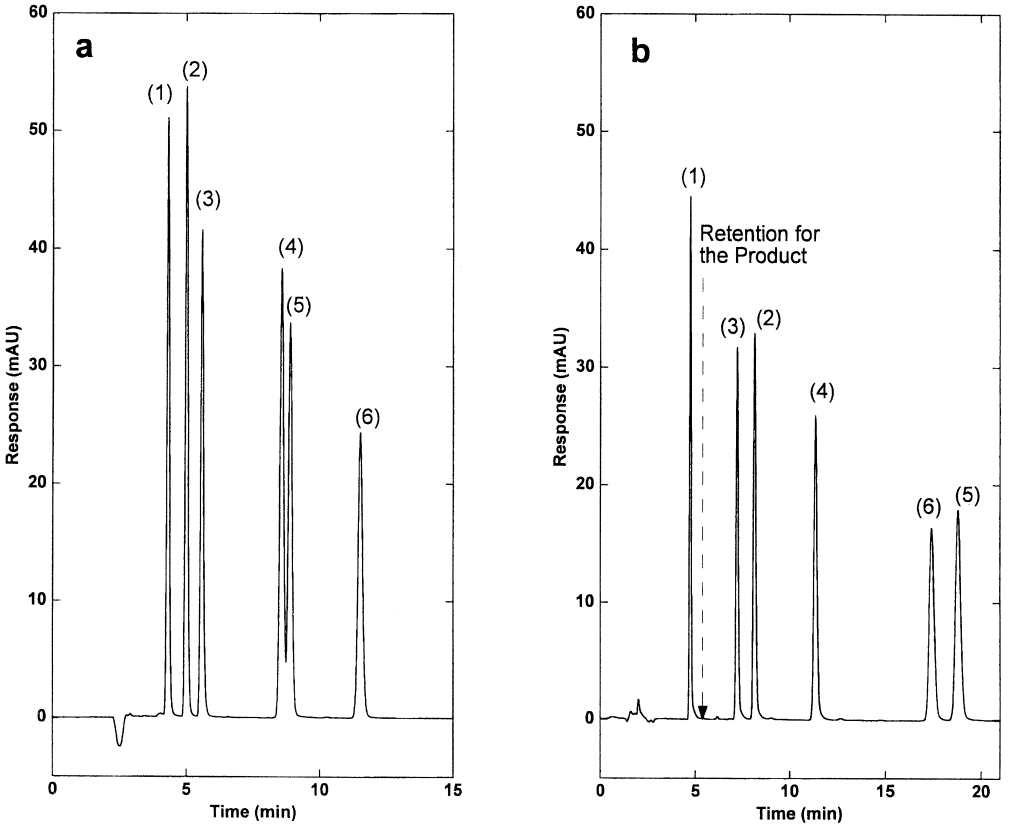

Fig. 1. Chromatograms obtained after the injection of 20 μl of a standard steroid mixture containing 10 m*M* of each analyte. Experimental conditions: HP 1100; column, Kromasil KR-100-C18 (particle size, 3.5 μm; *L*=150 mm; I.D.=4.6 mm); flow-rate, 0.70 ml/min; UV absorbance detection at 245 nm. The mobile phase was (a) methanol–water (80:20) and (b) acetonitrile–water (55:45). The eluted peaks are: (1) 11α-OH-PS; (2) AD; (3) 11β-OH-PS; (4) 20α-OH-PS; (5) PS; and (6) 20β-OH-PS.

the mobile phase consisted of a mixture of methanol and water, whereas in Fig. 1b it was a mixture of acetonitrile–water. The latter mobile phase gave a slightly lower reduced plate height and a much better resolution (cf. Fig. 1a,b). With acetonitrile as modifier in the mobile phase it was possible to separate all of the steroids. Switching modifier from methanol to acetonitrile changed the elution order of some steroids; 11β-OH-PS exchanged elution order with AD and 20β-OH-PS exchanged elution order with PS (cf. Fig. 1a,b). The retention time for 9α-OH-PS is indicated in chromatogram 1b by an arrow. Since acetonitrile–water as eluent showed the best performance this mobile phase was used in the validation study.

3.1.3. Column protection

It was necessary to protect the separation column with some type of guard column or filter while still maintaining the high resolution of the separation column. Unfortunately, when columns with high efficiency are used, the system is sensitive to the extra band broadening that always takes place when a guard column is inserted [14].

Three different approaches were evaluated: (1) a Kromasil KR100-5C18 guard column 10×4.0 mm, (2) a graphite filter from ESA and (3) a 0.5-μm frit from Upchurch. Of these, only the frit almost maintained performance, with only marginal decreases in resolution and symmetry as compared to the HPLC column without protection. Therefore, the

frit was used to protect the column in the validation study.

3.2. Evaluation of validation parameters

3.2.1. Solubility

The solubility in pure water was measured for each of the components and three measurements were done for each component. The mean values of the determinations were 519 μ*M* for 9α-OH-PS (C.V.=5.8%), 192 μ*M* for 11α-OH-PS (C.V.=1.3%), 33 μ*M* for PS (C.V.=8.2%) and 488 μ*M* for AD (C.V.=6.2%), respectively. The presence of an extra hydroxyl group in the hydroxyprogesterones thus resulted in a large increase in solubility compared to PS.

3.2.2. Selectivity

The selectivity is the ability of the bioanalytical method to measure unequivocally and differentiate among the analyte(s) in the presence of components which may be expected to be present in the matrix solution [2]. The great general selectivity of the present system was demonstrated already in Fig. 1b, where the best separation system was used to resolve six components similar to the product. The selectivity was further demonstrated by comparing chromatograms after injection of pure matrix solution and matrix solution to which analytes had been added. If no extra peaks originating from the matrix co-elute with the peaks to be quantificated the selectivity is good. It was observed for this biotechnological process that the blank process liquid did change composition with time. This is illustrated in Fig. 2,

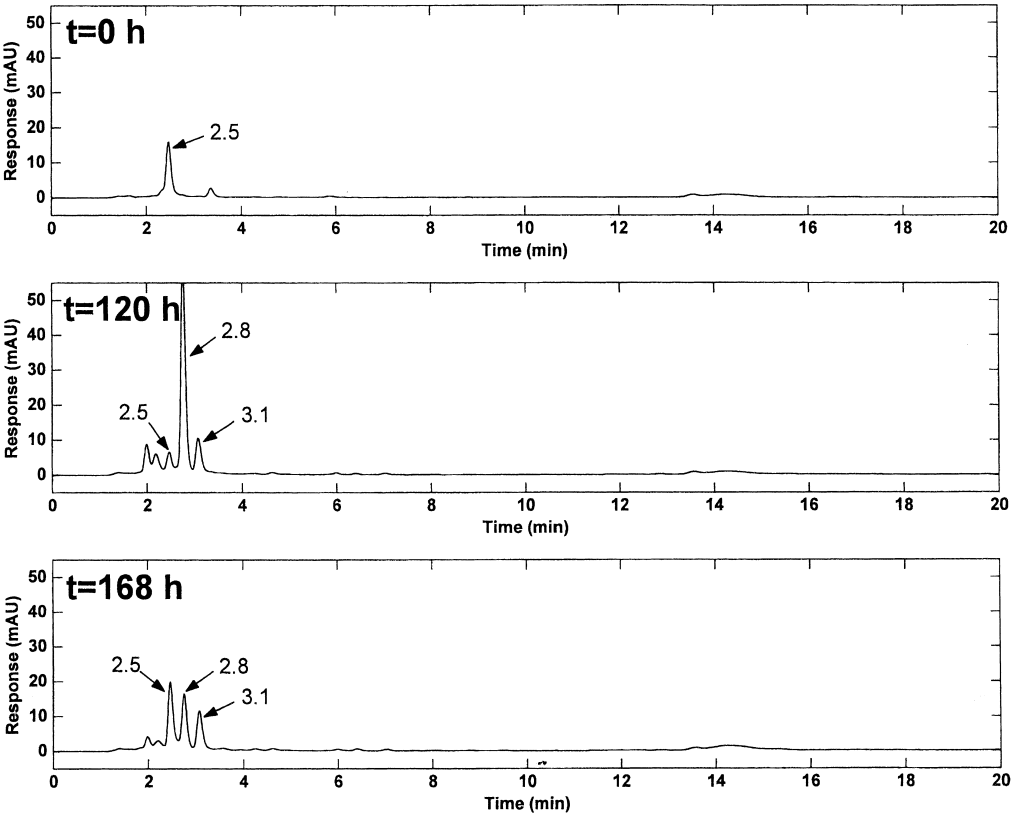

Fig. 2. Typical chromatograms of blank fermentation liquid at time zero, and 120 and 168 h. Experimental conditions as in Fig. 1b except that the eluent was monitored at 228 nm; a frit was used and the injection volume was 50 μl.

which shows the chromatogram obtained following injection of blank fermentation process liquids after different times of incubation. New peaks appear in the chromatogram and some old ones disappear, depending on the time elapsed after the start of the fermentation (cf. Fig. 2). This is probably a general phenomenon for biotechnological processes. One explanation for the ''dynamic baseline'' might be that the enzyme, of which the specificity for only the substrate is often not investigated, may also process other compounds and impurities. Another explanation is that various compounds become modified in the bacterial metabolic pathways to yield the energy and building blocks required for cell propagation. This phenomenon does not happen for analytical methods in the bioanalytical field.

The blank process liquid (at t=168 h) was also spiked in different ways. Fig. 3a shows blank process liquid spiked with two different internal standards, 11α-OH-PS (I.S. 1) and 1 μ*M* binaphtol (I.S. 2). Fig. 3b shows blank liquid spiked also with the product (9α-OH-PS) and the substrate (PS). No matrix substances from the process liquid interfered at the retention times of the analytes (cf. Figs. 3 and 2). If a mobile phase of methanol–water was used instead of acetonitrile–water the I.S. 11α-OH-PS interfered with the product.

3.2.3. Choice of internal standard and when to add it

For a proper quantitative determination and compensation for non-reproducibility in the extraction procedure a proper internal standard (I.S.) must be chosen. Initially, the hydroxylated steroid 11α-OH-

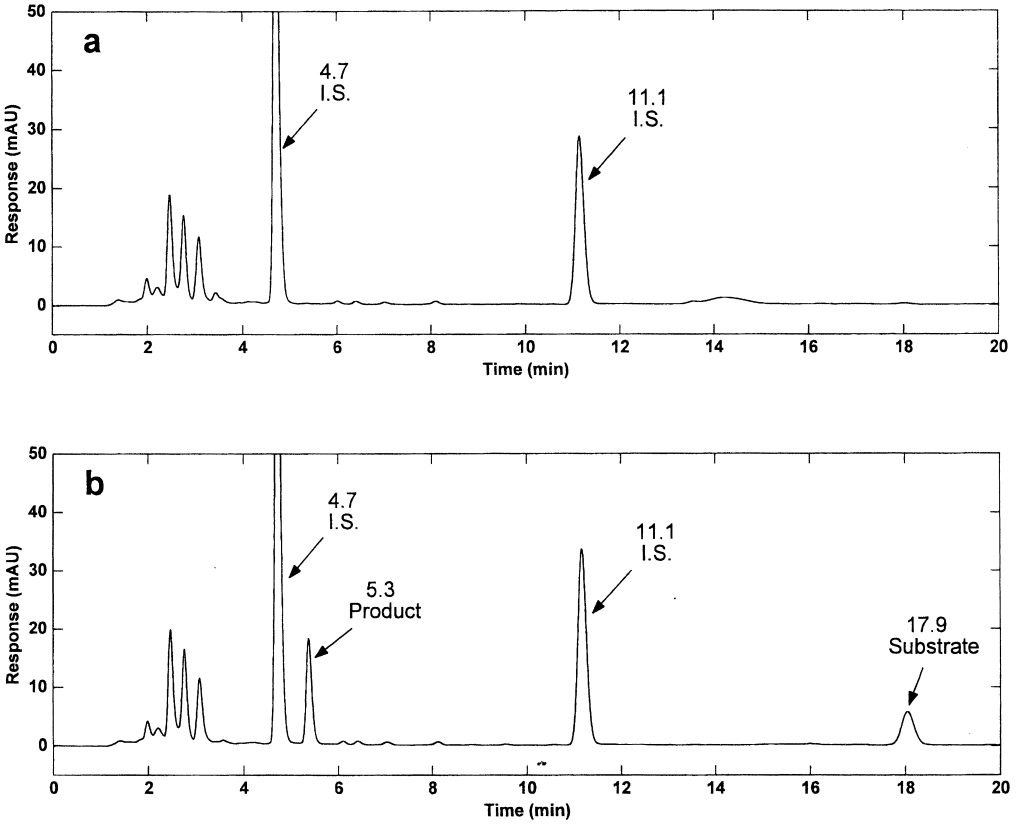

Fig. 3. Typical chromatograms resulting from injections of blank fermentation liquid after 168 h spiked with (a) 20 μ*M* 11α-OH-PS (I.S. 1) and 1 μ*M* binaphtol (I.S. 2), (b) 3 μ*M* 9α-OH-PS (product); 3 μ*M* PS (substrate); 20 μ*M* 11α-OH-PS and 1 μ*M* binaphtol. The other experimental conditions as in Fig. 2.

PS was chosen as I.S. because of its great structural similarity with the substrate and product. It is a general rule in bioanalytical methods development to choose an I.S. as similar in structure as possible to the analyte and the most important analyte in this case is the product. However, the I.S. 11α-OH-PS was added after the sample was taken out and not directly into the fermentation process. Therefore, this I.S. compensated only for inhomogeneities in the extraction procedure of the sample and for non-reproducibility in the working-up procedure. The I.S. did not compensate for inhomogeneities in the sampling, which was a problem with the heterogeneous process liquid, and because of the often great volume variations of the reactants during the fermentation process. The volume variations are due the following events in the fermentation reservoir; evaporation, automatic pH-adjustments, addition of extra nutrients and sample collection.

The reason why the internal standard 11α-OH-PS was not added directly into the fermentation process is its similarity in structure with the substrate, which implies the risk that also the I.S. should be consumed. In this case the I.S. might actually be consumed during the process, as it undergoes modification similar to the substrate. This is illustrated in Fig. 4, which shows the resulting chromatograms for samples taken at different times after start of the fermentation using two different types of I.S. One of the internal standards was 4-androsten-3,17-dione (AD), having a close structural similarity to substrate as well as product (and with 11α-OH-PS). The other I.S. was binaphthol, which, on the other hand, is structurally very different from both substrate and

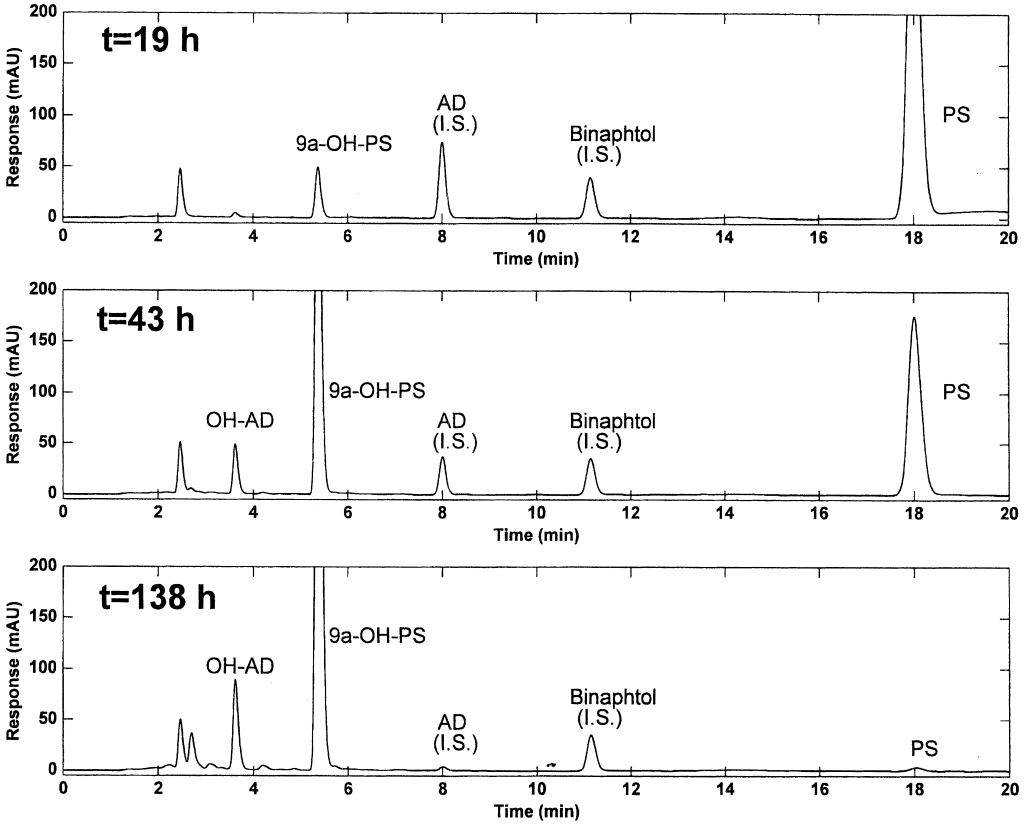

Fig. 4. Chromatograms resulting from injections of worked-up process liquid using two types of internal standards; the structural analogue AD and the structurally different and bulky binaphtol. The samples were taken after 19, 43 and 138 h. The fermentor contained from the beginning 20 μ*M* AD, 1 μ*M* binaphtol and 200 μ*M* PS. Experimental conditions as in Fig. 2.

product. Both AD and binaphthol had proper retention times with baseline separation under the condition used and showed good selectivities (cf. Figs. 4, 2 and 3). However the height of the AD peak decreased with time and a new peak appeared with a shorter retention time as compared to AD (cf. $t=19$ and 43 h in Fig. 4). By the end of the process the AD peak had almost disappeared (cf. $t=138$ h in Fig. 4). It can be concluded that the reason for this behaviour was that the enzyme also consumed AD and that the new peak that emerged contained hydroxylated-AD. On the other hand, the peak area of the bulky binaphthol was constant during the whole process (cf. Fig. 4 $t=19–138$ h).

Because of its inertness toward the enzymatic process, binaphtol turned out to be an excellent internal standard for this particular process. Due to its high UV absorbance a low concentration could be used (1 μ*M*) compared to that of the substrate, which was added at a concentration of 200 μ*M* at the start of the fermentation. Binaphthol as I.S. compensated very well for the large volume changes in the fermentor since it was added directly into the fermentor at the start of the process. This was demonstrated by running two fermentation processes; one where the fermentation vessel contained only PS and the other contained both PS and binaphthol.

3.2.4. Linearity

The linearity of an analytical method symbolizes its ability to elicit test results that are either directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [15]. What are the best criteria for linearity of the calibration curve? Traditionally, the linear correlation coefficient is a measure of the linearity, and according to Miller and Miller its value should be ≥0.99 [16]. But, according to Bildlingmeyer, a good linear correlation coefficient alone does not necessarily indicate a linear standard curve [13]. Instead, the linear coefficient should be accompanied by a graph in which the response/sample concentration is plotted versus the logarithmic sample concentrations and the deviation in the *y*-axis should not exceed 5% [13]. We therefore propose, for the best estimation of the degree of linearity in biotechnological production, the combined use of both the correlation coefficient and the graph discussed above.

The linearity of the peak area ratios versus the ''spiked'' concentrations was studied. Peak area ratio calibration curves were constructed for each component using a least-squares linear regression analysis from the injection of standard solutions of the mixture of the analytes at concentrations ranging from 3 to 220 μ*M*. The average slope of standard curves for 9α-OH-PS and PS were 0.0739 and 0.0797, with average intercepts of 0.0117 and 0.0013, respectively. The correlation coefficients (r^2) for the calibration curves were ≥0.9996. The logarithmic linearity plot showed that the deviation is <5% for both the product (max 2.9%) and the substrate (max 3.5%). According to both evaluation criteria the linearity of the system is thus excellent.

3.2.5. Precision and accuracy

The intra-day accuracy and precision calculated from six replicates of the product 9α-OH-PS extracted as described under experimental on the same day at three different concentrations are given in Table 1. Also the, inter-day precision and accuracy obtained for six replicates of 9α-OH-PS, which were analysed at three different days at three different concentrations, are given in Table 1. For 9α-OH-PS, at all three concentration levels, the intra-day and inter-day precisions were <1.5% and the accuracy varied between 99.3 and 106.9%.

If this were a bioanalytical application the values of the precision determined at each concentration

Table 1
Intra-day and inter-day accuracy and precision for quantification of 9α-OH-PS in fermentation medium at different concentrations

Spiked conc. (μ*M*)	Accuracy (%)	Precision (% C.V.)	*n*
Intra-day			
180	99.8	0.75	6
90	101.1	0.58	6
18	106.1	0.96	6
3*	106.9	0.90	6
Inter-day			
180	99.3	0.95	18
90	101.3	0.55	18
18	106.6	1.40	18

*LLOQ of the method.

level should not exceed 15% of the coefficient of variation (C.V.) except for the LLOQ where it should not exceed 20% C.V. The mean value for accuracy should be within 15% of the actual value at each concentration except at LLOQ, where it should not deviate more than 20% [3]. In pharmaceutical product analysis the same parameters should not exceed 2% (Anders Karlsson, AstraZeneca R&D, Mölndal, personal communication). In this context the deviation in accuracy and the found precision values are very low. As a result of the actual study we suggest tolerances between those applied in bioanalytical methods and those for product analysis. This is also motivated because the biotechnological process liquid is more complex than the matrix in pharmaceutical product analysis, but the concentrations determined are higher than in many bioanalytical assays. In view of this we suggest that the validation parameters should have C.V. values less than 10%. At LLOQ the acceptance criteria could, also for biotechnological processes, be a bit wider and precision within 15% and accuracy between 85 and 115% of the theoretical value should be acceptable.

3.2.6. Sensitivity

A clear distinction should be made between the limit of detection, LOD, and the lower limit of quantification, LLOQ. The LOD is defined as the concentration of analyte that result in a peak height three times the noise when injected into the chromatographic system. The LOD of the product 9α-OH-PS was 0.8 n*M* and for the substrate PS the value was 4.8 n*M* (both analytes dissolved in water). The LLOQ as determined from extraction from pure water was higher, 4.2 n*M* for 9α-OH-PS and 19.2 n*M* for PS, respectively. However, for this assay there is no need to determine such low concentrations and here the LLOQ represent the lowest points in the calibration curves, which is 3 μ*M*. The accuracy and precision observed at this point is given in Table 1.

3.2.7. Recovery

First we investigated if there were any significant differences between the recoveries during extraction if the analytes were extracted from the two different process liquids (i.e., complex medium, LB or minimal medium, M9). The complex medium has a dense yellow colour and is very heterogeneous. Fresh, 3- and 6-day LB and M9 media, were investigated. In addition, we considered it interesting to investigate whether ultrasonication improved the extraction recovery. This hypothesis has been proposed by Munoz and Rosés [17], since sonication of biological material destroys the cell membrane and hence should increase the contact between the sample matrix and the extraction solvent. We used the classical statistical method one-way analysis of variance (ANOVA) [16] to detect any significant differences ($P=0.05$). However, the ANOVA result showed that there were no significant differences between the two different media. The ANOVA showed further that ultrasonication did not improve the recovery significantly for either of the media.

The absolute and relative recoveries of the substrate and the product are presented in Table 2. Both compounds were extracted with similar recovery coefficients, probably due to the great similarities in their structures. The average absolute and relative recoveries of the product (9α-OH-PS) were 89.0 and 99.3%, respectively, at all concentrations investigated, whereas the corresponding values for the substrate (PS) were 91.8 and 101.1%. The absolute recovery in bioanalytical methods should preferably be above 90% and the relative recoveries above 95% [3].

Table 2
Absolute recovery and relative recovery for 9α-OH-PS and PS at different concentrations

Spiked conc. (μ*M*)	Absolute recovery		Relative recovery	
	9α-OH-PS (%)	PS (%)	9α-OH-PS (%)	PS (%)
180	91.1	95.2	96.8	103.0
90	90.7	91.0	97.6	100.9
18	85.1	89.2	103.4	99.4

Table 3
Stability studies of the product 9α-OH-PS in fermentation medium

Spiked conc. (μ*M*)	Mean values after 48 h at ambient temperature (% C.V.) $n=6$	Mean values after 4 months at −20 °C (% C.V.) $n=6$
180	179.8 (0.52)	176.8 (0.19)
90	91.6 (0.48)	90.8 (0.20)
18	19.5 (0.68)	19.0 (0.27)

3.2.8. Stability

The product (9α-OH-PS) was stable in the fermentation process liquid for 48 h at room temperature and during 4 months at −20 °C (Table 3). No proper determination of the stability of the substrate, PS, in the fermentation process was done. On the other hand, the stability of the product is the most important issue.

3.3. Calculation of rate coefficients

For complete quantitative monitoring of the process a time course study experiment was conducted concerning the transformation of PS to 9α-OH-PS in minimal (M9) culture medium. Samples were taken from the process liquid at 24-h intervals. Fig. 5a shows the corresponding chromatogram of a sample

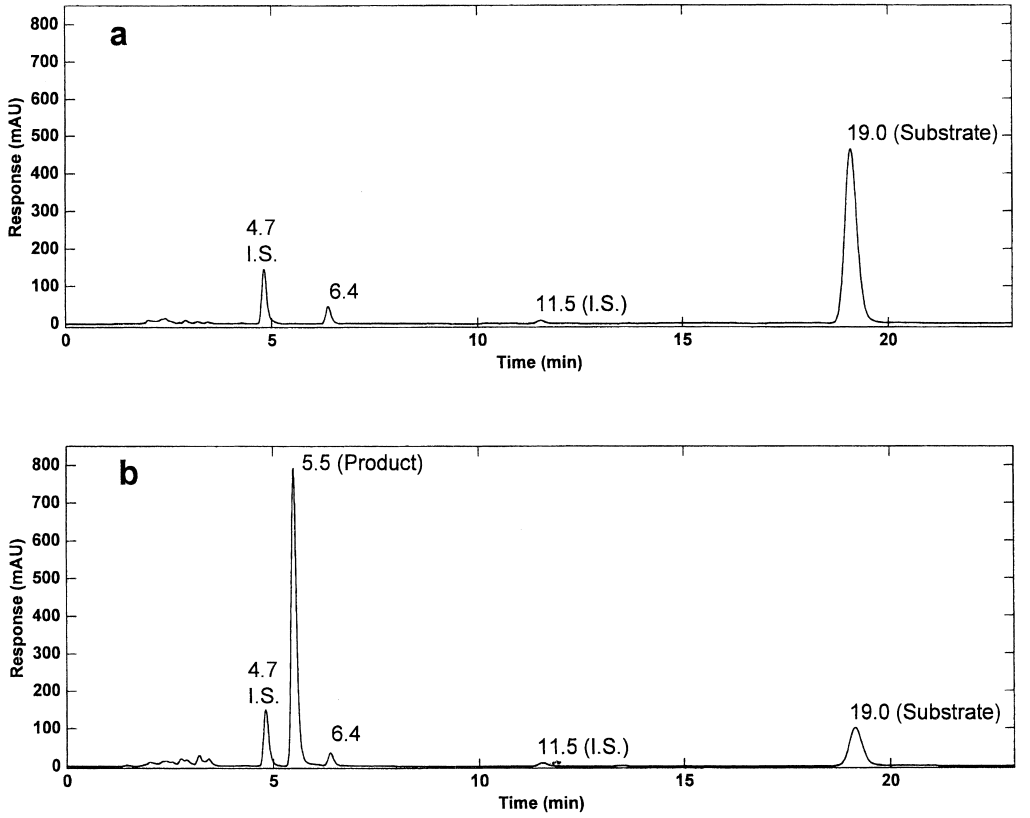

Fig. 5. Chromatograms obtained from injections of process samples taken (a) at the beginning of the fermentation process ($t=0$) and (b) at the end of the fermentation process ($t=172$ h). Chromatographic conditions as in Fig. 2, except that the eluent was monitored at 245 nm; 1 μ*M* binaphthol was added to the fermentor as internal standard and 20 μ*M* 11α-OH-PS were added before the extraction as internal standard.

taken at an initial stage of the fermentation process ($t=0$). The chromatogram obtained with a sample taken at the end of the fermentation process shows a new peak, the product (Fig. 5b). The time course (Fig. 6) shows that the product concentration increased and the substrate concentration decreased steadily until the fermentation process was terminated. Using linear regression on the midsections of the curves the substrate conversion rate was calculated to be 256 $\mu g\ h^{-1}\ l^{-1}$ and the product formation rate 255 $\mu g\ h^{-1}\ l^{-1}$. During a 172 h process in the 1.1l reactor, 43.6 mg 9α-OH-PS were formed from 53.8 mg l^{-1} PS.

The measurements by HPLC of the substrate and the product revealed that the kinetic constants of different fermentation batches for this synthesis differed much more than expected. For example, the production rate coefficient varied between 96 and 411 mg $h^{-1}\ l^{-1}$. This wide variation was indeed not recognised by the traditional TLC monitoring.

4. Conclusion

Validation of the analytical method used to follow the enzymatic synthesis of 9α-OH-PS in *E. coli* using progesterone as substrate is described. The detailed recommendations and rules formulated by the FDA [3] for validation of bioanalytical methods (i.e., quantification of pharmaceuticals in biological fluids) were applied and modified for proper quantification of this biotechnological process. The column Kromasil KR100-3.5C18 with the mobile phase acetonitrile–water (55:45) was used since this phase system showed particularly good performance for hydroxylated steroids.

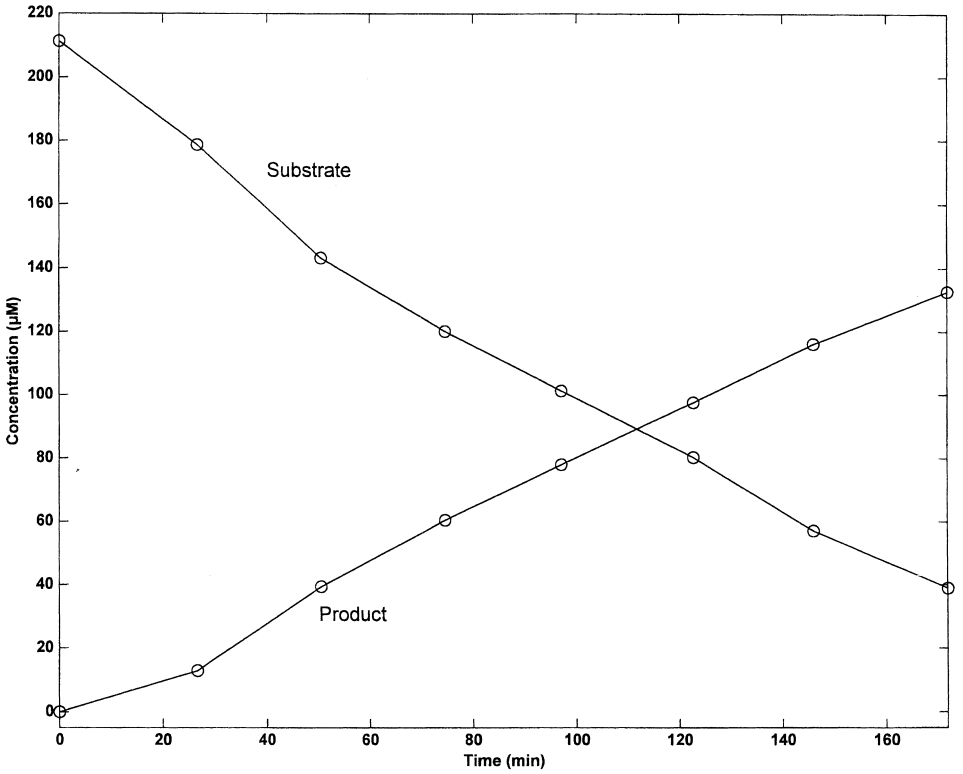

Fig. 6. Time course study of 9α-OH-PS and PS using the most proper internal standard, binaphthol added to the process liquid together with the substrate at the start of the fermentation.

The following validation terms were investigated: selectivity, linearity of the calibration curve, precision, accuracy, sensitivity, recovery and stability. The study showed that the recommendations and guidelines for method validation could be used preferably also for quantification of biotechnological production of compounds of pharmaceutical interest. However there are four important exceptions:

(1) The tolerances (C.V. values) of the validation terms can be much narrower; in this work 10% is suggested. This is between the accepted tolerances for bioanalytical methods and those for pharmaceutical product analysis.

(2) The internal standard (I.S.) must be introduced in the process liquid at the start of the process together with the substrate.

(3) The I.S. must be sufficiently different in structure from the substrate so that it will not participate in the enzymatic process.

(4) The selectivity must be checked frequently during the process due to changes of the blank process liquid with time.

From the quantitative data on the process obtained with the validated method the process substrate conversion rate was calculated to be 256 $\mu g\ h^{-1}\ l^{-1}$ and the product formation rate to be 255 $\mu g\ h^{-1}\ l^{-1}$.

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