Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 810 (2004) 251–257
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a method for the purity determination of (3β,20*R*)-4,4-dimethylcholesta-8,14,24-trien-3-ol(FF-MAS) in pharmaceutical products containing recombinant human albumin

Babak Jamali*, Steen G. Andersen, Helle Brødholt, Lene Wendel, Vibeke Bødstrup

Department of Analytical Development, CMC Development, Global Development, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Maaloev, Denmark

Received 28 January 2004; accepted 9 August 2004

Abstract

A simple and sensitive method has been developed and validated for purity determination of FF-MAS (also known as (3β,20*R*)-4,4-dimethylcholesta-8,14,24-trien-3-ol an endogenous substance usually present in the pre-ovulatory follicular fluid) at very low concentrations (200 ng per unit) in pharmaceutical formulations containing RECOMBUMIN® (recombinant human albumin) as the matrix. The paper focuses on development of the sample preparation for the product containing recombinant human albumin.

After removal of recombinant human albumin by precipitation using a mixture of water and ethanol, the FF-MAS was concentrated by evaporation using a vacuum centrifuge and the prepared sample was analyzed. The purity method was based on a reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet absorption detection at 250 nm.

The method was validated according to ICH guidelines. The method indicated a significant degree of specificity with good selectivity and no significant effect from the matrix. The limit of detection was found to be 0.3–0.8% (depending on the impurity) corresponding to 1.9–5.1 ng. The limit of quantification was found to be 0.8–2.5% (depending on the impurity) corresponding to 5.2–16 ng. The recovery was found to be between 90 and 101% for the FF-MAS, and 100–129% for the six known impurities. The tested range for FF-MAS was from 320 to 960 ng corresponding to 50–150% of the nominal concentration (640 ng, injection volume is 100 μl). The linearity of each compound (FF-MAS and the six impurities) was investigated. The squared correlation coefficient (r^2) was 0.999 for FF-MAS (50–150% level) and 0.977–0.998 for the six known impurities (at four levels: 0.20, 0.50, 1.00, 2.00%). The R.S.D. in the repeatability study was found to be 9.2% for the total amount of impurities, and 10.4% for single impurities. The R.S.D. in the intermediate precision study was found to be 10.9% for total impurities, and 12.0% for single impurities.

The validation results showed that the method was suitable for the purity analysis. The validated method was then ready for use for samples analysis of phase II clinical studies and the stability investigations of the pharmaceutical product.

Keywords: FF-MAS; 3β,20*R*-4,4-Dimethylcholesta-8,14,24-trien-3-ol; Recombinant human albumin; RECOMBUMIN; Sample preparation; Validation

1. Introduction

A very important part of method development is sample preparation, which is crucial for achieving a good and sensitive method. The matrix of the sample should be studied during method development, and the target of the sample preparation must be focused on developing a rather simple and practical work procedure. Good recovery for the compound(s) and good chromatography with no interaction from the matrix should also be achieved.

RECOMBUMIN®, a recombinant human albumin (rHA), is ultra-highly pure protein and is free from animal or human derived raw materials, and is structurally equivalent to

* Corresponding author. Tel.: +45 44434221; fax: +45 44434073.
E-mail address: bjam@novonordisk.com (B. Jamali).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.08.012

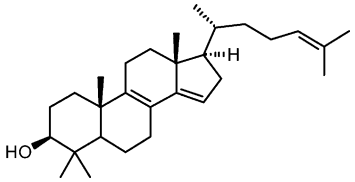

Fig. 1. Structure of (3β,20*R*)-4,4-dimethylcholesta-8,14,24-trien-3-ol also known as FF-MAS.

human serum albumin (HSA). RECOMBUMIN® is commercially available and has many application areas, which include as a matrix for formulation of a pharmaceutical product and cell culture medium for in vitro application [1,2].

The (3β,20*R*)-4,4-dimethylcholesta-8,14,24-trien-3-ol or follicular fluid MAS (also called FF-MAS) is a highly efficacious substance in the maturation of mammalian oocytes in culture, and has been developed as an in vitro fertilization improvement compound for treatment of infertility. The structure of (3β,20*R*)-4,4-dimethylcholesta-8,14,24-trien-3-ol (FF-MAS) is given in Fig. 1. FF-MAS at very low doses have been shown to improve markedly the quality of the mature oocyte, leading to significantly higher fertilization rates [3–6]. A pharmaceutical product has been developed containing a low dose of FF-MAS (200 ng per unit) for in vitro application. As the matrix for the formulation of the pharmaceutical product and as a part of the cell culture medium for in vitro application, RECOMBUMIN® was selected.

The aim of this paper is to describe the development of the sample preparation and validation of the HPLC purity method for the FF-MAS product containing large amounts of rHA. As part of the documentation, the method was validated according to official guidelines [7,8].

A unit (a vial) contains 200 ng of FF-MAS and approximately 2.5 mg of rHA giving a proportion between FF-MAS and rHA of approximately 1–12,500. The analytical studies during the development work of the purity method for the Active Pharmaceutical Ingredient have shown that at least 2500 ng of FF-MAS must be applied to the HPLC column for detection of impurities and/or degradation products at a 0.10% level. The injection volume for the purity method for the Active Pharmaceutical Ingredients is 10 μl. For application of 2500 ng of FF-MAS to the HPLC column for purity analysis of the FF-MAS product at least 13 units of 200 ng FF-MAS (2600 ng FF-MAS and 32.5 mg rHA) must be dissolved in 10 μl of a solution and then injected into the HPLC column. This was not possible in practice. Dissolving the samples in a larger amount of solvent, and injection of a large volume of a prepared sample was another possibility, which was tested. Unfortunately, the high amount of rHA in the samples resulted in problems with clog up and blocks the HPLC column. It was clear that a sample clean up combined with concentrating the FF-MAS in samples was necessary.

2. Experimental

2.1. Chemicals, materials and solutions

Deionized water (Milli Q water) was obtained from a Milli-Q system, acetonitrile (Rathburn RH1016), ethanol 99.9% (Merck art. 1.00983), FF-MAS standard, FF-MAS related substances as standards, API and Product (R&D Novo Nordisk A/S, Maaloev, Denmark).

2.1.1. Preparation of solutions

Extraction media was ethanol and water (as 90:10 (v/v)). The extraction media was manufactured separately by transferring 10 ml Milli Q water in a 100 ml measuring flask and filling up to the mark with ethanol 99.9%. Mobile phase for the HPLC system was Milli Q water as mobile phase A, and acetonitrile as mobile phase B.

2.2. Instrumentation

The HPLC systems used for the experiments were Waters 717 plus auto sampler, Waters 501 HPLC pump, Waters 2487 dual λ Absorbance UV detector and Millennium 32 Chromatography software (all from Waters A/S, Baldersbuen 46, 2640 Hedehusene, Denmark) and Dionex pump P580, Dionex ASI-100 Automated Sample Injector, Dionex Thermostatted Column Compartment TCC-100 and Dionex UV340S detector (all from Dionex Denmark A/S, Egegårdsvej 41, 2610 Rødovre, Denmark). The HPLC column was a Hypersil® BDS C-8, 250 mm × 4.6 mm, 5μPart No. 28205-20 from Phenomenex (http://www.phenomenex.com/). Branson 2510 Ultrasonic Cleaners bath (Branson Ultrasonic B.V., http://www.kellstromtool.com/branson.html), centrifuge (with rotating radius of 15 cm) and vacuum centrifuge GeneVac Ltd. (http://www.genevac.co.uk/), model HT-4, Automated Evaporator Cooler, Series II.

2.3. Quality controls and standard solutions

2.3.1. FF-MAS standard for calculation of recovery of sample preparation (20 μg/ml)

A solution of 20 μg FF-MAS/ml in acetonitrile was prepared by dissolving 25 mg FF-MAS standard in acetonitrile and filled up to the 25 ml mark with acetonitrile (1 mg/ml). This solution was then diluted 50 times by transferring 500 μl of the solution to a 25 ml measuring flask and adding acetonitrile to the mark.

2.3.2. FF-MAS LOD standard for control of the detection limit of the method

The FF-MAS standard was diluted 1–2000. FF-MAS (50 μl) standard was transferred to a 100 ml measuring flask and filled up with acetonitrile to the mark. The concentration of the solution corresponded to 0.20% level for a test solution of 200 ng/vial product.

2.3.3. Blind sample (extraction media prepared as a sample)

Extraction media (4.00 ml) was prepared as a sample. The blind sample was used for checking the background noise during the chromatographic runs.

2.3.4. Matrix sample

Matrix sample vials were prepared as 200 ng/vial samples. The matrix sample was used for checking the noise coming from the matrix (r-HA) during the chromatographic runs.

2.4. Preparation of sample

Fourteen units/vials (one vial/unit contains 200 ng FF-MAS) were used for one analysis. The content of the 14 vials was transferred to one 12 ml glass centrifuge tube. Extraction media (5.00 ml) was then added, and was shaken thoroughly. The mixture was then placed in ultrasonic bath for 10 min. To get a clear sample, the sample was centrifuged at 4000 rpm for approximately 10 min. Then, 4.00 ml supernatant (clear part of sample) from the top of the glass (approximately 1 ml white colored sediment will be left) was very carefully transferred to a new 12 ml centrifuge glass. The extraction media in the samples was then evaporated using a vacuum centrifuge. The sample in the glass was re-dissolved in acetonitrile (after full evaporation of extraction media) by adding 300 μl acetonitrile to the glass, while the glass was carefully rotated. The glass was then vibrated for approximately 5 s using a rotor-mixer and was put in ultrasonic bath for 10 min. The re-dissolved sample was centrifuged by 4000 rpm for approximately 10 min. Approximately 250 μl of the supernatant (clear fluid from the top) was then carefully transferred to a HPLC-vial with a liner vial for small sample volume. The sample was then analyzed.

2.5. Method conditions

The HPLC system was operated in gradient pump mode using a Hypersil® BDS C-8, 250 mm × 4.6 mm, 5 μm. The flow was 1.0 ml/min, the injection volume was 100 μl, the column temperature was 15 °C (±5 °C), the UV detection wavelength was 250 nm, and the runtime was 90 min. The pump gradient program was as follows: 0–60 min, 50% A and 50% B (isocratic); from 60 to 75 min, 0.1% A and 99.9% B (linear); from 75 to 77 min, 50% A and 50% B (linear); and from 77 to 90 min, 50% A and 50% B (isocratic).

3. Results and discussion

3.1. Development of the sample preparation

The target of the work was to develop a simple and practical work procedure for sample preparation for the HPLC purity analysis. Good chromatography with no interaction from the matrix and good recovery of all compounds was defiantly required. The last important issue was the number of vials used for the analysis as these are valuable and expensive.

Inspired from the sample preparation work in bio analytical area (with focus on extraction, evaporation and reconstitution), different cleaning and decontamination techniques were considered for removing the rHA [9–14]. For choosing decontamination technique and development of test preparation method, the following goals were brought into focus:

1. The method should be easy to work with (easy to run large number of sample).
2. The method should be performed in compliance with the working environment (no extraction by shaking using large amount of organic solvents, etc.).
3. The method should be specific, i.e. it must detain r-HA and not FF-MAS and related impurities.
4. The method should be sensitive (good and acceptable LOD and LOQ).
5. The method should be repeatable.
6. The method should have low cost (no expensive apparatuses or alike).

Based on the above mentioned goals, it was decided that the development work should be focused on a rather simple procedure.

To remove the rHA from samples, different methods were considered and tested. Soiled phase extraction seemed to be a good chose. But after test of a number of different soiled extraction columns it was concluded that the technique was not suitable for rHA as applications resulted in problems with clog up and blocks the soiled phase column. Precipitation using an organic solvent was found to be the easiest and most practical. Experiments with different organic solvents such as acentonitrile, methanol, isopropanol and ethanol showed that the precipitation of rHA by adding organic solvent was quite difficult as rHA is “clean” and e.g. do not contain different proteins, amino acids and salts as HA (human albumin). It was concluded that ethanol gives the best results and is the best organic solvent for the precipitation.

At the same time, the experiments showed that some water is needed in the media to get a good recovery. The procedure was that a mixture of ethanol and water (90:10) was added to the sample, the mixture was then centrifuged to separate the precipitated rHA, the ethanol and water were then evaporated from the sample, and the sample was re-dissolved in a smaller amount of acetonitrile. By this procedure, the FF-MAS and its related substances were concentrated. The evaporation of ethanol and water was performed using a vacuum centrifuge with a two steps program. The sample was re-dissolved in only acetonitrile to avoid transfer of possible residues of rHA (further micro precipitation), and because FF-MAS and the related compounds are very soluble in acetonitrile.

3.2. Theoretical calculation of applied FF-MAS to the HPLC column

The analytical studies during the development work of the purity method for the API had shown that impurities could be easily detected and quantified at 0.10% level (injection volume, 10 μl) with application of 2.5 μg of FF-MAS to the HPLC column. For this 14 vials/glasses were used for one analysis corresponding to 14 × 200 ng FF-MAS = in total, 2.8 μg FF-MAS. To make the practical performance of preparation effective, the vials were then pooled together. In practice, it was not possible to empty the vials totally. The recovery was not more than 87% (w/w). This was calculated to be only 87% of 2.8 μg FF-MAS from the 14 vials available, which corresponds to 2.4 μg FF-MAS. The 2.4 μg was then dissolved in 5 ml extraction media giving a concentration 0.48 μg FF-MAS/ml. After centrifuging, 4 ml supernatant was then selected corresponding to approximately 1.92 μg FF-MAS/4 ml extraction media. The extraction media in the sample was then evaporated and the dry substance was then re-dissolved in 300 μl acetonitrile. At this point, the sample concentration was then 6.4 μg FF-MAS/ml acetonitrile. The 100 μl samples were injected in the HPLC system corresponding to application of 640 ng FF-MAS on the column. This was four times less that the original concentration but this was deemed acceptable.

3.3. Control of recovery of FF-MAS in samples after sample preparation

To avoid unnecessary variation, the recovery of FF-MAS from the samples after the sample preparation was calculated as a quality check of the sample preparation. This was important to be sure that the correct and enough amounts were injected to the HPLC column. The recovery was calculated using the following FF-MAS standard:

$$\%\,\text{Recovery} = \frac{\text{FF-MAS peak area}_{\text{sample}}}{\text{FF-MAS peak area}_{\text{standard}}} \times 100$$

3.4. Validation

Validation was performed with respect to specificity and selectivity (the effect of the matrix on the chromatography), precision as repeatability and intermediate precision, accuracy and linearity (and range) of main peak and three known impurities, limit of detection (LOD), limit of quantification (LOQ), robustness and stability of the analytical solutions.

3.4.1. Specificity and selectivity (the effect of the matrix on the chromatography)

The method indicated a significant degree of specificity with good selectivity and no significant effect from the matrix. A chromatogram of a placebo and a sample can be seen in Figs. 2 and 3. As a quality control during sample runs, and as a part of the analytical procedure, it was decided to prepare and analyse a placebo during each run.

3.4.2. Precision as repeatability and intermediate precision

The precision of the method was investigated as repeatability and intermediate precision. The repeatability was estimated as within the lab variation, whereas the intermediate precision included the variation due to different groups in same lab conditions. The results obtained from different groups were totally independent (different mobile phase, HPLC systems, HPLC column, etc.). The design and results of the studies can be seen in Table 1. The R.S.D. in the repeatability study was found to be 9.2% for the total amount of impurities, and 10.4% for single impurities. The R.S.D. in the intermediate precision study was found to be 10.9% for total impurities and 12.0% for single impurities. The obtained repeatability and intermediate precision of the method was found to be satisfactory.

3.4.3. Accuracy, range and linearity

The accuracy of the method was demonstrated as % recovery using FF-MAS and three known impurities (ZK202914, ZK255305 and NNC 54-0148, Fig. 4) added to a matrix sample. The addition of compounds was done before the

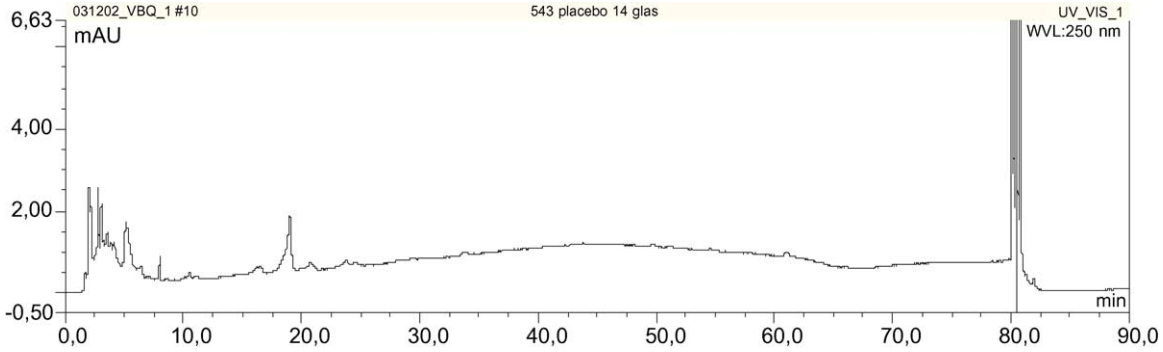

Fig. 2. Chromatogram of analysis of a placebo sample.

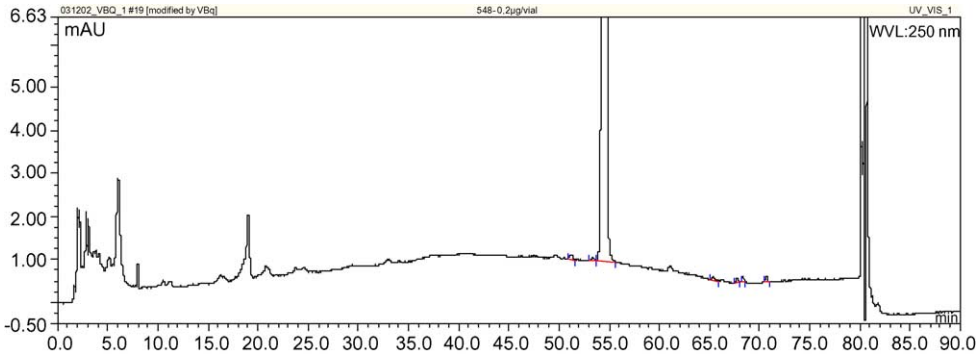

Fig. 3. Chromatogram of analysis of a sample.

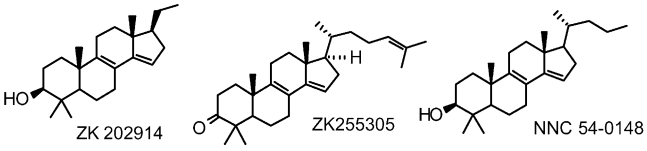

Fig. 4. Structure of three of FF-MAS related substances: ZK202914, ZK255305 and NNC 54-0148.

extraction media was added. The study was performed at four levels for impurities (0.20, 0.50, 1.00, and 2.00%), and one level (100%) for FF-MAS with two points at each level. The results for recovery of the three known impurities can be seen in Table 2. The recovery was found to be between 90 and 101% for the FF-MAS, and 100–129% for the three known impurities. The accuracy results as percent recovery were found to be good and satisfactory for the method. The tested range for FF-MAS corresponded to 50–150% of the nominal concentration (640 ng when injection volume is 100 μl). The range of method was well demonstrated to be from 320 to 960 ng FF-MAS. From the accuracy and range studies, the linearity of each compound (FF-MAS and the three known impurities) was investigated. The squared correlation coefficient (r^2) was 0.999 for FF-MAS (50–150% levels) and 0.977–0.998 for the six known impurities (at four levels: 0.20, 0.50, 1.00 and 2.00%). The linearity for the compounds was found to be good and acceptable.

Table 1
Design and results of the precision studies

No.	Group	Results, total imp. (%)	$\bar{x}$, total imp (%)	S.D., total imp.	Results, largest imp. %	$\bar{x}$, largest imp. (%)	S.D., largest imp.
1	Group 1, day 1, lab. A, HPLC 1	0.93	0.91	0.053	0.13	0.14	0.012
2		0.95			0.15		
3		0.85			0.13		
4	Group 2, day 2, lab. A, HPLC 2	0.92	0.84	0.093	0.16	0.160	0.010
5		0.74			0.17		
6		0.87			0.15		
7	Group 3, day 1, lab. B, HPLC 3	0.84	0.79	0.047	0.14	0.13	0.010
8		0.75			0.13		
9		0.77			0.12		
10	Group 4, day 2, lab. B, HPLC 4	0.85	0.75	0.095	0.17	0.14	0.023
11		0.75			0.13		
12		0.66			0.13		
$\bar{x}_{total}$			0.82	0.0894		0.14	0.0171

		Total impurities	Largest impurity
Repeat ability	$S.D._{pool}$	0.0753	0.0148
	R.S.D. (%)	9.15	10.40
	95% confidence interval	[0.65; 1.00]	[0.11; 0.18]
	95% confidence interval as percent of $\bar{x}_{total}$	[79.3; 122.0]	[78.6; 128.6]
Int. Precision	SD_{Total}	0.0893	0.0171
	R.S.D. (%)	10.86	12.02
	95% confidence interval	[0.63; 1.02]	[0.10; 0.18]
	95% confidence interval as percent of $\bar{x}_{total}$	[76.5; 124.4]	[71.4; 128.6]

Table 2
Results from recovery study of three known impurities (NNC 54-0148, ZK255305 and ZK202914)

Impurity	Determination no.	Level of spiking							
		0.20%		0.50%		1.0%		2.0%	
		0.0032 μg/ml		0.008 μg/ml		0.016 μg/ml		0.032 μg/ml	
		Result	$\bar{X}$	Result	$\bar{X}$	Result	$\bar{X}$	Result	$\bar{X}$
NNC 54-0148	1	132	126	136	129	113	120	–	123
	2	119		122		126		123	
ZK255305	1	123	121	141	124	124	115	–	100
	2	118		106		106		100	
ZK202914	1	119	117	126	121	114	118	–	119
	2	114		115		122		119	

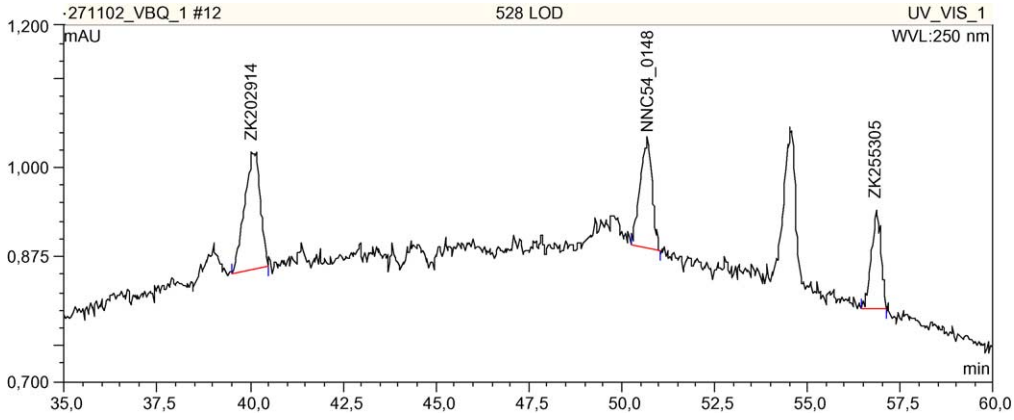

Fig. 5. Chromatogram of a LOD injection of three known impurities: ZK202914, ZK255305 and NNC 54-0148.

3.4.4. Limit of detection (LOD) and limit of quantification (LOQ)

For determination of LOD, solutions with low concentrations of the three known impurities were injected in order to find the concentration corresponding to a signal to noise ratio of 3 to 1 (as USP Pharmacopeia definition) [14]. These impurities were chosen randomly according to the variation in structure of FF-MAS impurities. The identified limit was confirmed by preparing two independent solutions of each impurity, and injecting these solutions on two different HPLC instruments. A chromatogram with impurities at the LOD level is given in Fig. 5.

The limit of detection was found to be 0.8% for ZK202914, 0.6% for ZK255305 and 0.3% for NNC 54-0148. All signals to noise ratios found were within the acceptance criteria.

Same procedure was followed for determination of LOQ but with solution with higher concentration of the three known impurities. The concentrations were corresponding to a signal to noise ratio of 10 to 1 (as USP Pharmacopeia definition) [14].

The limit of quantification was found to be 2.5% for ZK202914, 1.8% for ZK255305 and 0.8% for NNC 54-0148. All signal to noise ratios found were within the acceptance criteria. The LOD and LOQ of the method were found to be good and were accepted.

3.4.5. Robustness and stability of the analytical solutions

The robustness of the method was examined during the development of the method. Parameters such as different column, different instruments, and small variations in mobile phase were tested. No critical changes were observed.

The stability of the sample solution and standard solutions was investigated after three days at room temperature (to evaluate the time sample solutions can stay in the auto sampler may be critical).

The sample solutions and standard solution were found to be stable when stored at room temperature for 3 days but it was decided to choose only 2 days as durability due to the long runtime of the HPLC method. Generally, the method showed very good robustness.

4. Conclusion

A simple and sensitive method was developed and validated for purity determination of FF-MAS (also known as (3β,20*R*)-4,4-dimethylcholesta-8,14,24-trien-3-ol) at very low concentrations in pharmaceutical formulations containing recombinant human albumin (RECOMBUMIN®). A simple and practical sample preparation procedure was de-

veloped, and good chromatography with no interaction from the matrix and good recovery was achieved. The HPLC purity method was validated, and good and acceptable method performance was observed for all validation points.

In general, the results demonstrated that the HPLC purity method with the sample preparation that has been developed was suitable for the applied analysis. The validated method was used for analysis of samples from phase II clinical studies and product stability investigations.

Acknowledgment

The great help of Lars Klingberg Müller, and the support of Christian Grøndahl and Ole Norfang, is gratefully acknowledged.

References

[1] Aventis Behring, Recombumin®, January 2002, recombumin@aventis.com and http://www.deltabiotechnology.com/.
[2] J. Flensburg, M. Belew, J. Chromatogr. A 1009 (2003) 111.
[3] A.G. Byskov, C.Y. Andersen, L. Nordholm, H. Thøgersen, X. Guoliang, O. Wassamann, J.V. Andersen, E. Guddal, T. Roed, Nature 30 (1995) 374.
[4] C. Grøndahl, M. Lessl, I. Faerge, C. Helgele-Hartung, K. Wassermann, J.L. Ottesen, Biol. Reprod. 62 (2000) 775.
[5] I. Faerge, C. Grøndahl, J.L. Ottesen, P. Hyttel, Biol. Reprod. 64 (2001) 527.
[6] I. Faerge, B. Terry, J. Kalous, P. Wahl, M. Lessl, J.L. Ottesen, P. Hyttel, C. Grøndahl, Biol. Reprod. 65 (2001) 1751.
[7] ICH Harmonised Tripartite Guideline, Text on Validation of Analytical Procedures, International Conference on Harmonisation, 27 October 1994, http://www.ich.org/.
[8] ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodology, International Conference on Harmonisation, 06 November 1996, http://www.ich.org.
[9] R. Brodie, A. Peard, A. Roth, S. Persiani, F. Makovec, M. D'Amoate, J. Chromatogr. B 784 (2003) 91.
[10] J.F. Focant, E. De Pauw, J. Chromatogr. B 776 (2002) 199.
[11] S. Bajad, A.K. Singla, K.L. Bedi, J. Chromatogr. B 776 (2002) 245.
[12] C. Prakash, R.P. Koshakji, A.J.J. Wood, I.A. Blair, J. Pharm. Sci. 78 (9) (1989) 771.
[13] B.D. Kiss, K.B. Nemes, I. Klebovich, Chromatographia 57 (2003) 47.
[14] US Pharmacopeia, NF, United States Pharmacopeial Convention Inc., Rockville, USA, 2000.