

ATTACHMENT II: - ANALYTICAL STUDY REPORT

- The development and validation of analysis of HMR (hydroxymatairesinol) in formulation samples (Report as supplied by the sponsor)

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AN ANALYTICAL STUDY REPORT

BRST Project Title	The development and validation of analysis of HMR (hydroxymatairesinol) in formulation samples
BRST Study Code	A165
Sponsor Project Title	HM-3000
Sponsor Study Code	3000-IIID-001/A 3000-IIIF2-009/A 3000-IIID-002/A
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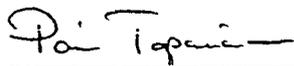
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8.3.2001

Date

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9.3.2001

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Appendix I: Quality Assurance Statement

LIST OF ABBREVIATIONS:

c.v.	coefficient of variation
conc.	concentration
DMSO	dimethyl sulfoxide
HMR	hydroxymatairesinol
HMR1 and HMR2	two diastereomers of HMR
HPLC	high-performance liquid chromatography
PEG300	polyethylene glycol, average molecular weight 300 g/mol
RP	reverse phase
s.d.	standard deviation
UV	ultra-violet

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SUMMARY

1 GENERAL

1.1 General Information

BRST Project Title:	The development and validation of analysis of HMR (hydroxymatairesinol) in formulation samples
BRST Study Code:	A165
Test Facility:	CRST Bioanalytics (BRST) Kiinamyllynkatu 10 FIN-20520 Turku Finland
Test Facility Management	Mika Scheinin
Principal Investigator:	Pasi Tapanainen
Sponsor:	Hormos Nutraceutical Oy Ltd Tykistökatu 6 A (BioCity) FIN-20520 Turku Finland
Sponsor Project Title:	HM-3000
Sponsor Study Codes:	3000-IIID-001/A 3000-IIIF2-009/A 3000-IIID-002/A
Start of Measurements:	06.11.2000
End of Measurements:	12.1.2001
Number of Samples Measured:	88
Date of Study Report:	08.03.2001

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1.2 Statement of Compliance

BRST Study Code: A165

Sponsor Study Code: 3000-IIID-001/A
3000-IIIF2-009/A
3000-IIID-002/A

Test Substance: HMR (hydroxymatairesinol)

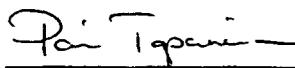
BRST Project Title: The development and validation of analysis of
HMR (hydroxymatairesinol) in formulation
samples

Principal Investigator: Pasi Tapanainen

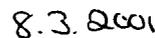
The study described in this report was carried out under my supervision and responsibility, and was based on the BRST method 166 version 01 and method 167 version 02. The laboratory procedures were performed according to CRST's Standard Operating Procedures.

The report is a complete and accurate account of the methods employed and the data obtained.

Principal Investigator:



Pasi Tapanainen
Research Scientist, CRST



Date

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2 MATERIALS AND METHODS

2.1 Instrumentation

Apparatus	Manufacturer	Art. No.
Vacuum concentrator	Heto	VR-MAXI
Cooling trap	Heto	CT60E
Vacuum pump	Vacuubrand	RD4
HPLC pump	Merck Hitachi	L-6200 Intelligent Pump
UV detector	Applied Biosystems	783A
Integrator	Merck Hitachi	D-2500 Chromato-Integrator
Analytical balance	Mettler Instrumente AG	Mettler Toledo AG204
Printer for the analytical balance	Mettler Instrumente AG	Mettler Toledo LC-P45
Water purification unit	Vartiainen	Orwa Filter, RO-60/30-DSP
Vortex shaker	Scientific Industries	Vortex Genie 2
Semipreparative column	Waters Corporation	Prep Nova-Pak® HR C18, 60 Å, 6 µm, 7.8 x 300 mm
Analytical column	Waters Corporation	Nova-Pak C ₁₈ 150 x 3.9 mm i.d. (4 µm)
Guard column	Waters Corporation	Nova-Pak inserts C ₁₈ 20 x 3.9 mm i.d. (4 µm)

2.2 Reagents

Reagent	Manufacturer	Art. No.	Quality
HMR mixture	Hormos Nutraceutical Oy Ltd.	HM-3000/rj13 and HM3000/rj14	purified by flash chromatography
HMR1	separated and purified at BRST		
HMR2	separated and purified at BRST		
Ethanol	Primalco		AaS
Dimethyl sulfoxide (DMSO)	Merck		SeccoSolv
PEG300 vehicle	Merck-Schuchardt		
Methanol	Merck		LiChrosolv
Acetic acid	Merck		100 %, p. a.
Aqua	self prepared		deionized

2.3 Glassware

Type	Used for	Manufacturer
Volumetric flask, Duran®, A	preparation of standard solutions	Hirschmann® EM techcolor

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2.4 Pipettes

Pipette type	Tip	Manufacturer
Finnpipette Colour 4027 (40-200 µl)	disposable	Labsystems
Finnpipette Colour 4027 (200-1000 µl)	disposable	Labsystems
Finnpipette Colour 4027 (1-5 ml)	disposable	Labsystems
Finnpipette Digital (5-40 µl)	disposable	Labsystems
Finnpipette Digital (40-200 µl)	disposable	Labsystems
Finnpipette Digital (200-1000 µl)	disposable	Labsystems
Finnpipette Digital (1-5 ml)	disposable	Labsystems

2.5 Other Consumables

Type	Used for	Manufacturer	Art No.
PP-tubes, 12 ml	preparation of solutions and evaporation of solvents	Greiner laborotechnik	
Injection needle, 100 µl	injection of samples	Hamilton Bonaduz AG	Microliter®*710
Membrane filters	filtering of samples	Schleicher & Schuell	Spartan 13/A, 0.2 µm
Sterile syringe, 1 ml	filtering of samples	Becton Dickinson S.A	Plastipak®
Falcon tubes, 15 and 50 ml	preparation of solutions and storage	Becton Dickinson Labware	
Microcentrifuge tubes, 1.5 and 2 ml	preparation of solutions and storage	Plastibrand®, Brand	
0.45 µm HV filter	filtration of mobile phase	Millipore Corporation	

2.6 HMR Solutions for Separation and Purification of HMR1 and HMR2

HMR mixture was dissolved in 30 % methanol/water (v/v) and filtered prior to separation and purification by HPLC. The amount of HMR in solution was about 350 mg/ml. Solutions were stored in refrigerator at +4 - +8 °C.

2.7 Reference Items

As reference compounds for preparing standards and control samples, HMR1 and HMR2, separated and purified at BRST, were used. The reference item mixtures were provided by Hormos Nutraceutical Oy Ltd (batches HM-3000/rj13 and HM-3000/rj14).

2.8 Control Zero Sample

In the analysis runs drug-free 50 % (v/v) DMSO/water-solution was used as a control zero sample.

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2.9 Stock Solutions

2.9.1 HMR1 Stock Solution in 30 % Methanol/Water

The stock solution (2.0 mg/ml) was prepared by dissolving HMR1 in 30 % methanol/water (v/v). For preparing 10 ml stock solution, 20.0 mg of HMR1 was weighed.

2.9.2 HMR2 Stock Solution in 30 % Methanol/Water

The stock solution (2.0 mg/ml) was prepared by dissolving HMR2 in 30 % methanol/water (v/v). For preparing 10 ml stock solution, 20.0 mg of HMR2 was weighed.

2.9.3 HMR1 Stock Solution in DMSO

The stock solution (2.0 mg/ml) was prepared by dissolving HMR1 in DMSO. For preparing 10 ml stock solution, 20.0 mg of HMR1 was weighed.

2.9.4 HMR2 Stock Solution in DMSO

The stock solution (2.0 mg/ml) was prepared by dissolving HMR2 in DMSO. For preparing 10 ml stock solution, 20.0 mg of HMR2 was weighed.

2.10 Preparation of the Reference Standards, Calibration Samples and Quality Assurance Samples

2.10.1 Preparation of the Reference Standards

The stock solutions in 30 % methanol/water were diluted with 30 % methanol/water (v/v) to get HMR1 and HMR2 concentrations of 2.0, 20.0, 100.0, 400.0 and 2000.0 µg/ml. They were further mixed and diluted to get reference standards containing HMR1/HMR2 0.5/0.5, 1.0/1.0, 10.0/10.0, 50.0/50.0, 200/200 and 500/500 µg/ml in 30 % methanol/water (v/v). These samples were used for determination of the linearity range of the assay.

For determination of the similarity of the quantitation of HMR diastereomers from 30 % methanol/water (v/v), DMSO and PEG300 formulation samples, HMR stock solutions containing 5 % PEG300 in 30 % methanol/water (v/v) were prepared. The stock solutions in 30 % methanol/water were diluted with 30 % methanol/water (v/v) to give HMR1 and HMR2 concentrations of 2.0, 20.0, 100.0, 400.0 and 2000.0 µg/ml. These were further mixed together and diluted with 30 % methanol/water (v/v) and PEG300 vehicle to get reference standards containing HMR1/HMR2 0.5/0.5, 5.0/5.0, 25.0/25.0, 100.0/100.0 and 500/500 µg/ml, and 5 % PEG300 in 30 % methanol/water (v/v).

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2.10.2 Preparation of the Calibration Samples

The stock solutions in DMSO were diluted with DMSO to give HMR1 and HMR2 concentrations of 2.0, 20.0, 100.0, 400.0 and 2000.0 µg/ml. These were mixed and diluted with water to get calibration samples containing HMR1/HMR2 0.5/0.5, 5.0/5.0, 25.0/25.0, 100.0/100.0 and 500/500 µg/ml in 50 % DMSO/water (v/v).

2.10.3 Preparation of the Quality Assurance Samples

To evaluate the inter and intra assay precision of the method, quality assurance samples at three different concentration levels were prepared. The quality assurance samples were made by mixing and diluting DMSO stock solutions of HMR1 and HMR2 with DMSO and water to get quality assurance samples HMR1/HMR2 of 1.0/1.0, 50.0/50.0 and 450/450 µg/ml in 50 % DMSO/water (v/v).

For ensuring that diastereomers HMR1 and HMR2 do not isomerize to each other during storage or analysis, samples containing 50 µg/ml of HMR1 or HMR2 in 50 % DMSO/water (v/v) and in solution containing 5% PEG300 in 30 % methanol/water were prepared. They were made by diluting DMSO and 30 % methanol/water (v/v) stock solution samples with water and DMSO, or 30 % methanol/water and PEG300 vehicle, respectively, to get samples containing 50 µg/ml of HMR1 or HMR2 in 50 % DMSO/water and in solution containing 5 % PEG300 in 30 % methanol/water (v/v).

2.10.4 Storing of the Reference Standards, Calibration Samples and Quality Assurance Samples

Reference standards, calibration samples and quality assurance samples were stored in microcentrifuge or Falcon tubes and kept in freezer. Under usage they were kept in refrigerator. For longer periods these standards and samples were stored at -70 °C.

2.11 Receiving and Storing of Samples

DMSO and PEG300 samples were provided by Hormos Nutraceutical Oy Ltd. They were stored at -70°C.

2.12 HMR Separation and Purification

Dissolved and filtered HMR mixture samples were injected repeatedly in 50-100 µl aliquots to the semipreparative HPLC every 10-12 minutes. Fractions containing HMR1 and HMR2 were collected in separate vials. Resulting solutions were evaporated to dryness, dissolved in 30 % methanol/water (v/v) and repurified by HPLC in a similar manner. After the second purification cycle HMR1 and HMR2 solutions were evaporated to dryness. Samples were stored at -20 °C or colder.

Separation and purification was achieved using an isocratic eluent system. The eluent used was 17 % ethanol/water (v/v) in the first separation and purification cycle and 18 % ethanol/water (v/v) in the second purification for HMR2 and 19 %

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ethanol/water (v/v) for HMR1. Flow rate was 3 ml/min. The wavelength of the UV-detector was set to 285 nm.

2.13 Analytical Procedure

2.13.1 Sample Preparation for DMSO Formulation Samples

Aliquots of 100 μ l of DMSO samples were diluted with 100 μ l of water. If extra dilution was needed, the required amount of 50 % DMSO/water (v/v) solution was added.

2.13.2 Sample Preparation for PEG300 Formulation Samples

Aliquots of 200 μ l of PEG300 samples were diluted with 3.8 - 49.8 ml (1:20 - 1:250) of 30 % methanol/water (v/v) depending on the HMR concentration of the sample.

2.13.3 HPLC Conditions

Analyses were performed with a Nova-Pak C₁₈ 150 x 3.9 mm i.d. column coupled with an integrated Nova-Pak C₁₈ guard column (Waters Co.). The mobile phase was a linear gradient from 30 % B to 47 % B in 12 min. (A = 0.1 % acetic acid in water and B = 80 % methanol / 20 % A, v/v). The flow-rate was 1.0 ml/min. A UV-detector was used for detection at a wavelength of 279 nm. The retention time values (t_R) were about 11 min for HMR1 and 10 min for HMR2.

2.14 Data Processing

The quantitation of the analytes was accomplished by HPLC-UV. The calculations of quantitation were based on peak areas of the analytes, using double logarithmic transformation.

The data from the HPLC-UV analyses was collected using a Merck Hitachi D-2500 Chromato-Integrator. Calculations were made by using GraphPad Prism 2.01 software (GraphPad Software, Inc.).

3 MODE OF ANALYSIS

According to the Analytical Study Plan, the study samples were analyzed in batches which consisted of one set of calibration samples, two sets of quality assurance samples, study samples, one sample of HMR1 and one of HMR2.

The time course of sample measurements during the analytical study is given in Table 1.

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Table 1. Sequence of measurements.

Document	Date of preparing	Date of analysing	Study samples Batch
HMR purification	21.9-11.10.2000	21.9-11.10.2000	
Method development	12-24.10.2000	12-24.10.2000	
Validation	25.10-2.11.2000	25.10-2.11.2000	
Study samples	6.11.2000	6.11.2000	Analysis1
Study samples	8.11.2000	8.11.2000	Analysis2
Verification of validation	25.10-2.11.2000	9.1.2001	
Study samples	9.1.2001	10.1.2001	Analysis3
Study samples	9.1.2001	11.1.2001	Analysis4
Study samples	9.1.2001	12.1.2001	Analysis5

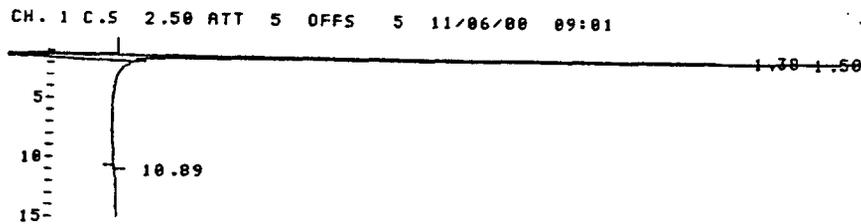
4 RESULTS

4.1 Validation Results

4.1.1 Selectivity

Under the specified chromatographic conditions good resolution between the analytes and DMSO- or PEG300-solution constituents was achieved. The retention time values (t_R) of HMR1 and HMR2 were about 11.0 and 10.0 min, respectively. Figures 1, 2 and 3 shows chromatogram examples of a drug-free DMSO-solution, a drug-free PEG300-solution, a spiked sample in DMSO, a spiked sample in PEG300, a study sample in DMSO and a study sample in PEG300.

a)



b)

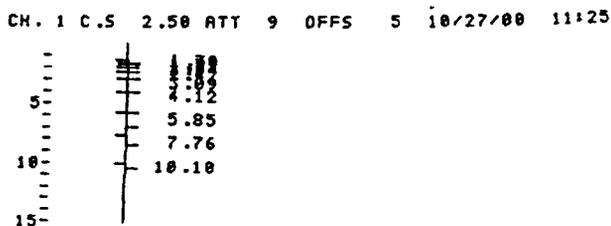


Figure 1. Chromatogram examples of a) a drug-free DMSO-solution, b) a drug-free PEG300-solution.

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4.1.2 Linearity

The linearity of the assay was ensured at the concentration range of 0.50 – 500 µg/ml. Calibration samples in 30 % methanol/water (v/v) were prepared twice and every sample was analysed in duplicate. Calibration samples in DMSO-solution were prepared twice and every sample was analysed in duplicate. Calibration samples in PEG300-solution were prepared once and every sample was analysed in duplicate.

Calibration samples contained 0.50, 1.0, 10.0, 50.0, 200 and 500 µg/ml of HMR1 and HMR2 in 30 % methanol/water (v/v). Calibration samples in DMSO- and PEG300-solutions contained 0.50, 5.0, 25.0, 100 and 500 µg/ml of HMR1 and HMR2. Figure 4. shows the standard curves of calibration samples in a) 30 % methanol/water (v/v), b) DMSO, c) PEG300 in a concentration range of 0.50 – 500 µg/ml. Characteristics of standard curves (slope, intercept and r^2) are presented in Table 2.

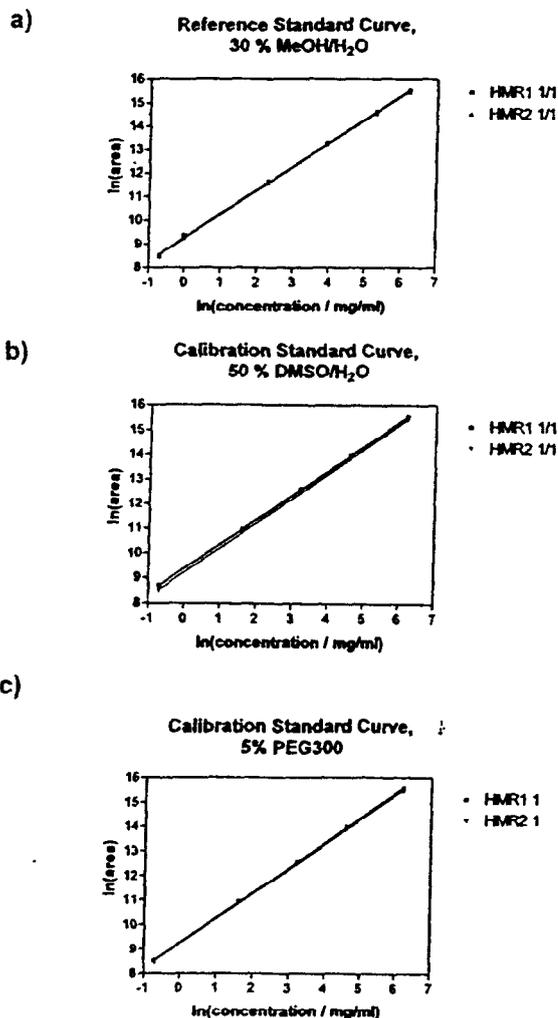


Figure 4. Standard curves of calibration samples in a) 30 % methanol/water (v/v), b) DMSO, c) PEG300 in a concentration range of 0.50 – 500 µg/ml.

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Table 2. Characteristics of standard curves (slope, intercept and r^2) in 30 % methanol/water (v/v), DMSO and PEG300 solutions in a concentration range of 0.50 – 500 µg/ml. Results are after double logarithmic transformation.

HMR1	calibration samples in 30 % methanol/water		
assay	slope	intercept	r^2
1/1	1.010	9.298	0.9996
1/2	1.004	9.342	0.9999
2/1	1.009	9.309	1.0000
2/2	1.004	9.339	1.0000
mean	1.007	9.322	1.000
s.d.	0.003	0.022	0.000

HMR1	calibration samples in DMSO-solution			calibration samples in PEG300-solution		
assay	slope	intercept	r^2	slope	intercept	r^2
1/1	0.998	9.373	1.0000	1.022	9.269	0.9998
1/2	1.002	9.361	1.0000	1.015	9.283	0.9998
2/1	1.002	9.364	1.0000			
2/2	1.003	9.364	1.0000			
mean	1.001	9.366	1.000	1.019	9.276	0.9998
s.d.	0.002	0.005	0.000	0.005	0.010	0.0000

HMR2	calibration samples in 30 % methanol/water		
assay	slope	intercept	r^2
1/1	1.009	9.225	0.9998
1/2	1.008	9.249	0.9998
2/1	0.991	9.311	1.0000
2/2	1.002	9.267	0.9998
mean	1.003	9.263	1.000
s.d.	0.008	0.036	0.000

HMR2	calibration samples in DMSO-solution			calibration samples in PEG300-solution		
assay	slope	intercept	r^2	slope	intercept	r^2
1/1	1.005	9.202	0.9999	1.014	9.202	0.9994
1/2	1.009	9.188	0.9998	1.007	9.245	0.9999
2/1	0.997	9.248	1.0000			
2/2	0.996	9.247	1.0000			
mean	1.002	9.221	1.000	1.011	9.224	0.9997
s.d.	0.006	0.031	0.000	0.005	0.030	0.0004

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4.1.3 Inter Assay Precision

The inter assay precision was evaluated from 10 sets of quality assurance samples, analyzed at 3 different concentration levels and during 5 days. The results are presented in Table 3.

Table 3. Inter assay precision of HMR1 and HMR2 in DMSO-samples.

HMR1								
conc. ug/ml	date	conc. found ng/ml	conc. ug/ml	date	conc. found ng/ml	conc. ug/ml	date	conc. found ng/ml
1.0	6.11.2000	0.991	50.0	6.11.2000	51.293	450.0	6.11.2000	437.716
		1.020			49.600			434.824
	8.11.2000	0.903		8.11.2000	49.122		8.11.2000	439.298
		0.979			50.714			441.215
	10.1.2001	(1.282)		10.1.2001	48.876		10.1.2001	438.610
		0.996			50.918			444.290
	11.1.2001	1.081		11.1.2001	49.997		11.1.2001	424.838
		1.097			51.815			436.670
	12.1.2001	1.000		12.1.2001	50.957		12.1.2001	450.690
		1.021			50.908			446.398
mean		1.01			50.42			439.45
s.d.		0.06			0.97			7.05
c.v. %		5.63			1.93			1.60
bias of mean		0.01			0.42			10.55
bias %		0.98			0.84			2.34
HMR2								
conc. ug/ml	date	conc. found ng/ml	conc. ug/ml	date	conc. found ng/ml	conc. ug/ml	date	conc. found ng/ml
1.0	6.11.2000	0.969	50.0	6.11.2000	51.574	450.0	6.11.2000	449.443
		0.984			50.261			445.563
	8.11.2000	0.899		8.11.2000	49.358		8.11.2000	447.327
		0.936			50.449			448.241
	10.1.2001	1.090		10.1.2001	48.423		10.1.2001	441.960
		0.927			49.636			447.208
	11.1.2001	0.967		11.1.2001	49.706		11.1.2001	429.091
		0.991			50.403			441.771
	12.1.2001	0.989		12.1.2001	49.861		12.1.2001	450.449
		0.986			50.127			446.890
mean		0.97			49.98			444.79
s.d.		0.05			0.82			6.20
c.v. %		5.25			1.64			1.39
bias of mean		0.03			0.02			5.21
bias %		2.62			0.04			1.16

() = Not included in calculations. Sample does not fulfil the defined criteria for quality assurance samples.

4.1.4 Intra Assay Precision

The intra assay precision was evaluated from quality assurance samples at three different concentration levels. The results are presented in Table 4.

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Table 4. Intra assay precision of HMR1 and HMR2 in DMSO-samples.

HMR1

conc. (ug/ml)	date	conc. found ug/ml	conc. (ug/ml)	date	conc. found ug/ml	conc. (ug/ml)	date	conc. found ug/ml
1.0	2.11.2000	1.11	50.0	2.11.2000	49.99	450.0	2.11.2000	437.66
		1.13			50.71			453.42
		1.01			48.83			437.52
		0.99			49.97			439.16
		0.98			50.20			432.22
		1.01			49.95			439.58
mean		1.04	mean		49.94	mean		439.92
s.d.		0.07	s.d.		0.61	s.d.		7.12
c.v. %		6.26	c.v. %		1.23	c.v. %		1.62
bias of mean		0.04	bias of mean		0.06	bias of mean		10.08
bias %		3.84	bias %		0.12	bias %		2.24

HMR2

conc. (ug/ml)	date	conc. found ug/ml	conc. (ug/ml)	date	conc. found ug/ml	conc. (ug/ml)	date	conc. found ug/ml
1.0	2.11.2000	1.04	50.0	2.11.2000	49.58	450.0	2.11.2000	444.90
		1.05			49.03			457.76
		0.97			48.71			440.89
		0.97			49.43			440.65
		0.94			49.38			436.10
		0.95			49.56			444.00
mean		0.99	mean		49.28	mean		444.05
s.d.		0.05	s.d.		0.34	s.d.		7.40
c.v. %		4.87	c.v. %		0.70	c.v. %		1.67
bias of mean		0.01	bias of mean		0.72	bias of mean		5.95
bias %		1.27	bias %		1.43	bias %		1.32

4.1.5 Limit of Quantitation

The quantitation limit of the assay was 0.50 µg/ml.

4.1.6 The Effect of Dilution of Samples

Two samples, Experiment2: 100.0 mg/ml in DMSO and 100 mg/ml (11.10.2000) in PEG300, were diluted three times and analyzed. Results are presented in Table 5.

4.1.7 The Verification of the validation

The validation was verified prior to analysis 3, 4 and 5. The batch consisted of one set of reference standards (5), one blank reference standard, one set of calibration samples (5), two sets of quality assurance samples (2 x 3), one control zero, one sample of HMR1 and one sample of HMR2. The verification of the validation fulfilled the criteria of acceptance defined originally in The Analytical Study Plan.

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Table 5. The effect of dilution of samples. a) Experiment2: 100.0 mg/ml in DMSO diluted three times and b) and 100 mg/ml (11.10.2000) in PEG300 diluted three times.

a)

HMR1			HMR2			HMR1 + HMR2		
date	diluting factor	conc. found ug/ml	date	diluting factor	conc. found ug/ml	date	diluting factor	conc. found ug/ml
6.11.2000	200	29.41	6.11.2000	200	63.79	6.11.2000	200	93.21
	1000	33.14		1000	70.87		1000	104.02
	4000	32.64		4000	69.19		4000	101.83
mean		31.73			67.95			99.69
s.d.		2.02			3.70			5.71
c.v. %		6.38			5.44			5.73

b)

HMR1			HMR2			HMR1 + HMR2		
date	diluting factor	conc. found ug/ml	date	diluting factor	conc. found ug/ml	date	diluting factor	conc. found ug/ml
8.11.2000	250	28.81	8.11.2000	250	62.61	8.11.2000	250	91.42
	1000	27.92		1000	60.38		1000	88.29
	2500	27.87		2500	59.27		2500	87.13
mean		28.20			60.75			88.95
s.d.		0.53			1.70			2.22
c.v. %		1.88			2.80			2.49

4.2 Study Samples

4.2.1 Study Sample Results

The results for all study samples are presented in Table 6. The criteria for the acceptance of an analysis run and the result of an individual study sample were originally defined in the Analytical Study Plan (in paragraphs 5.3. and 5.4.).

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Table 6. Concentrations of HMR1 and HMR2 in a) DMSO, b) PEG300 and c) DMSO formulation samples.

nq = not quantified (< 0.50 µg/ml)
nd = not detected

a) 3000-IIID-001/A (Covance study no. 1716/14)

DMSO Samples	HMR1 Conc. (mg/ml)	HMR2 Conc. (mg/ml)	HMR total Conc. (mg/ml)	Date of analysis
RangeFinderExp:0	nq	nq	nq	6.11.2000
RangeFinderExp:0.016	0.0052	0.0105	0.0158	6.11.2000
RangeFinderExp:0.080	0.0249	0.0526	0.0774	6.11.2000
RangeFinderExp:0.400	0.1238	0.2664	0.3902	6.11.2000
RangeFinderExp:2.0	0.6218	1.352	1.974	6.11.2000
RangeFinderExp:10.0	2.998	6.548	9.546	6.11.2000
RangeFinderExp:50.0	15.58	33.93	49.50	6.11.2000
Experiment1:0	nq	nq	nq	6.11.2000
Experiment1:0.016	0.0048	0.0100	0.0148	6.11.2000
Experiment1:0.080	0.0237	0.0500	0.0736	6.11.2000
Experiment1:0.400	0.1192	0.2558	0.3750	6.11.2000
Experiment1:2.0	0.5993	1.307	1.907	6.11.2000
Experiment1:10.0	3.043	6.640	9.683	6.11.2000
Experiment1:50.0	15.17	33.08	48.25	6.11.2000
Experiment2:0	nd	nd	nd	6.11.2000
Experiment2:1.5625	0.4811	1.047	1.528	6.11.2000
Experiment2:3.125	0.8994	1.940	2.840	6.11.2000
Experiment2:6.25	1.747	3.815	5.562	6.11.2000
Experiment2:12.5	3.660	7.942	11.60	6.11.2000
Experiment2:25.0	7.548	16.43	23.98	6.11.2000
Experiment2:50.0	14.53	31.37	45.90	6.11.2000
Experiment2:100.0	29.41	63.79	93.21	6.11.2000

b) 3000-IIIF2-009/A (RCC study no. 780636)

PEG300 Samples	HMR1 Conc. (mg/ml)	HMR2 Conc. (mg/ml)	HMR total Conc. (mg/ml)	Date of analysis
0mg/ml, 11.10.2000	nd	nd	nd	8.11.2000
0mg/ml, 12.10.2000	nd	nq	nq	8.11.2000
4mg/ml, 11.10.2000	1.084	2.325	3.409	8.11.2000
4mg/ml, 12.10.2000	26.55	57.18	83.73	8.11.2000
20mg/ml, 11.10.2000	5.588	12.07	17.66	8.11.2000
20mg/ml, 12.10.2000	5.516	11.89	17.41	8.11.2000
100mg/ml, 11.10.2000	28.81	62.61	91.42	8.11.2000
100mg/ml, 12.10.2000	1.051	2.243	3.294	8.11.2000

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c) 3000-IIID-002/A (Covance study no. 1716/12)

DMSO Samples: 1716/12 CHO	HMR1 Conc. (mg/ml)	HMR2 Conc. (mg/ml)	HMR total Conc. (mg/ml)	Date of analysis
Exp1: 0 mg/ml	nd	nd	nd	10.1.2001
Exp1: 8.431 mg/ml	2.733	5.657	8.390	10.1.2001
Exp1: 10.54 mg/ml	3.006	6.372	9.378	10.1.2001
Exp1: 13.17 mg/ml	3.689	7.672	11.36	10.1.2001
Exp1: 16.47 mg/ml	5.102	10.65	15.75	10.1.2001
Exp1: 20.58 mg/ml	5.301	11.17	16.48	10.1.2001
Exp1: 25.73 mg/ml	7.756	16.43	24.18	10.1.2001
Exp1: 32.16 mg/ml	9.630	19.35	28.98	10.1.2001
Exp1: 40.2 mg/ml	12.61	26.43	39.04	10.1.2001
Exp1: 50.25 mg/ml	15.70	33.17	48.86	10.1.2001
Exp1: 62.81 mg/ml	19.95	42.26	62.21	10.1.2001
Exp1: 78.52 mg/ml	23.09	49.06	72.14	10.1.2001
Exp1: 98.15 mg/ml	30.78	65.14	95.92	10.1.2001
Exp1: 122.7 mg/ml	41.20	86.57	127.8	10.1.2001
Exp1: 153.4 mg/ml	47.86	100.6	148.4	10.1.2001
Exp1: 191.7 mg/ml	63.75	134.4	198.2	10.1.2001
Exp1: 239.6 mg/ml	83.92	178.0	261.9	10.1.2001
Exp1: 299.5 mg/ml	113.4	241.4	354.8	10.1.2001
Exp1: 374.4 mg/ml	125.3	267.3	392.6	10.1.2001
Exp2: 0 mg/ml	nd	nd	nd	11.1.2001
Exp2: 47.67 mg/ml	14.95	31.18	46.12	11.1.2001
Exp2: 50.17 mg/ml	15.51	32.32	47.83	11.1.2001
Exp2: 52.82 mg/ml	15.87	33.06	48.93	11.1.2001
Exp2: 55.6 mg/ml	16.94	35.37	52.31	11.1.2001
Exp2: 58.52 mg/ml	18.26	38.18	56.44	11.1.2001
Exp2: 61.6 mg/ml	15.48	32.23	47.71	11.1.2001
Exp2: 64.84 mg/ml	20.09	42.09	62.18	11.1.2001
Exp2: 68.26 mg/ml	23.30	48.94	72.23	11.1.2001
Exp2: 71.85 mg/ml	22.86	47.98	70.84	11.1.2001
Exp2: 75.63 mg/ml	22.77	47.74	70.51	11.1.2001
Exp2: 79.61 mg/ml	25.60	53.39	78.99	11.1.2001
Exp2: 83.8 mg/ml	26.34	55.31	81.66	11.1.2001
Exp2: 88.21 mg/ml	26.36	55.38	81.73	11.1.2001
Exp2: 92.85 mg/ml	31.13	65.23	96.35	11.1.2001
Exp2: 97.74 mg/ml	31.89	66.80	98.70	11.1.2001
Exp2: 102.9 mg/ml	35.15	72.22	107.4	11.1.2001
Exp2: 108.3 mg/ml	38.06	78.31	116.4	11.1.2001
Exp2: 114.0 mg/ml	40.90	84.25	125.1	11.1.2001
Exp2: 120.0 mg/ml	38.82	80.29	119.1	11.1.2001

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DMSO Samples: 1716/12 CHO	HMR1 Conc. (mg/ml)	HMR2 Conc. (mg/ml)	HMR total Conc. (mg/ml)	Date of analysis
Exp3: 0 mg/ml	nd	nd	nd	12.1.2001
Exp3: 54.36 mg/ml	17.91	37.84	55.75	12.1.2001
Exp3: 57.22 mg/ml	18.98	40.05	59.03	12.1.2001
Exp3: 60.23 mg/ml	19.96	40.86	60.82	12.1.2001
Exp3: 63.4 mg/ml	9.770	20.51	30.28	12.1.2001
Exp3: 66.73 mg/ml	21.86	46.30	68.16	12.1.2001
Exp3: 70.25 mg/ml	22.61	47.86	70.47	12.1.2001
Exp3: 73.94 mg/ml	21.99	46.27	68.26	12.1.2001
Exp3: 77.84 mg/ml	24.57	52.13	76.70	12.1.2001
Exp3: 81.93 mg/ml	22.57	47.88	70.45	12.1.2001
Exp3: 86.24 mg/ml	28.81	61.13	89.94	12.1.2001
Exp3: 90.78 mg/ml	30.18	63.66	93.85	12.1.2001
Exp3: 95.56 mg/ml	15.36	32.43	47.79	12.1.2001
Exp3: 100.6 mg/ml	31.69	66.59	98.28	12.1.2001
Exp3: 105.9 mg/ml	26.29	55.74	82.03	12.1.2001
Exp3: 111.5 mg/ml	38.30	80.60	118.90	12.1.2001
Exp3: 117.3 mg/ml	38.15	80.19	118.34	12.1.2001
Exp3: 123.5 mg/ml	42.78	90.09	132.87	12.1.2001
Exp3: 130 mg/ml	46.14	97.04	143.18	12.1.2001

4.2.2 Reassays

No analyses had to be repeated according to the original criteria. The criteria for reanalysis of individual study samples were defined in the Analytical Study Plan (in paragraph 5.3.2).

4.3 Calibration Samples

No analysis run was rejected because of the calibration samples. The criteria for the calibration samples were defined in the Analytical Study Plan (in paragraph 5.3.A). The results and statistical evaluations of the calibration samples during the method validation are presented in Table 2 and Figure 4.

4.4 Quality Assurance Samples

No analysis run was rejected because of the quality assurance samples. The criteria for the quality assurance samples were defined in the Analytical Study Plan (in paragraph 5.3.B). The results and statistical evaluations of the quality assurance samples during the determination of the study samples and the method validation are presented in Tables 3 and 4.

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5 DEVIATIONS FROM STUDY PLAN

There were no deviations from the Analytical Study Plan.

6 DISCUSSION

The yields of separated and purified HMR1 and HMR2 were about 270 and 400 mg respectively. Purified HMR1 diastereomer contained maximum 0.5 % of HMR2, and HMR2 contained maximum 0.5 % of HMR1.

Results of validation and verification of validation fulfilled the criteria of acceptance defined in the Analytical Study Plan and the Analytical Validation Plan.

A total of 88 (22 + 8 + 58) formulation samples (DMSO and PEG300) were analyzed, after appropriate dilution, by HPLC-UV (CRST Method No. 167, version 02). The quality assurance samples fulfilled the criteria of acceptance defined in the Analytical Study Plan and the Analytical Validation Plan. The effect of dilution was found to be insignificant.

Obviously two PEG300 samples were incorrectly labelled by the sample provider. As seen in the results, amounts of HMR1 and HMR2 in sample 100 mg/ml (12.10.2000) were near the amounts found in sample 4.0 mg/ml (11.10.2000) and the amounts of HMR1 and HMR2 in sample 4.0 mg/ml (12.10.2000) were found to be near the amounts in sample 100 mg/ml (11.10.2000). In addition, the samples 100 mg/ml (11.10.2000) and 4.0 mg/ml (12.10.2000) were both slightly yellowish, which might be due to the high concentration of HMR, while all other samples were colorless. Most likely PEG300 sample 100 mg/ml (12.10.2000) should be 4.0 mg/ml (12.10.2000) and *vice versa*.

The method was found to be linear in the concentration range of 0.50 – 500 µg/ml. Average intra assay precision was 2.7 % and average inter assay precision 2.9 %. The limit of quantitation was 0.50 µg/ml.

7 ARCHIVES

The laboratory work book, chromatograms, derived data, records and all other study related data will be stored in CRST's archives for at least fifteen (15) years after the completion of the analysis. All final reports and study and validation plans will be stored in CRST's master schedule. The analytical methods (methods no. 166 version 01 and 167 version 02) will be stored in CRST's method schedule. After that period of fifteen years CRST shall contact Hormos Nutraceutical Oy Ltd to agree upon the further archiving of the material. All remaining study samples will be stored in a freezer at the test site at -20 °C or at lower temperature and retained there for six months after sending the analytical report. After this storage period the sponsor will decide whether the samples should be destroyed or sent to him for further storage.

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AN ANALYTICAL STUDY REPORT

APPENDIX I

Quality Assurance Statement

BRST Project Title	The development and validation of analysis of HMR (hydroxymatairesinol) in formulation samples
BRST Study Code	A165
Sponsor Project Title	HM-3000
Sponsor Study Code	3000-IIID-001/A 3000-IIIF2-009/A 3000-IIID-002/A
Date	08.03.2001

QUALITY ASSURANCE STATEMENT

CRST Bioanalytics, Analytical Study Number: A165

Analytical study title: The development and validation of analysis of HMR
(hydroxymatairesinol) in formulation samples

Sponsor Study Code: 3000-IIID-001/A
3000-IIIF2-009/A
3000-IIID-002/A

This analytical report has been inspected by the Quality Assurance Unit of University of Turku. The results described in this analytical report have been reviewed and the report was found to be an accurate reflection of methods employed and of the raw data.

Study-based inspections:

Phase inspected	Inspection	Report to Principal Investigator and Test Site Management
Analytical study plan	25. - 26.9.2000	2.10.2000
Separation and purification of diastereomers	5.10.2000	5.10.2000
Bioanalytical experiment	8.11.2000	24.11.2000
Results	7.12., 11. - 13.12.2000	13.12.2000
Analytical study report	28.2.2001	2.3.2001

Date 27.3.2001



Niina Nieminen
QA Inspector
Quality Assurance Unit, University of Turku

