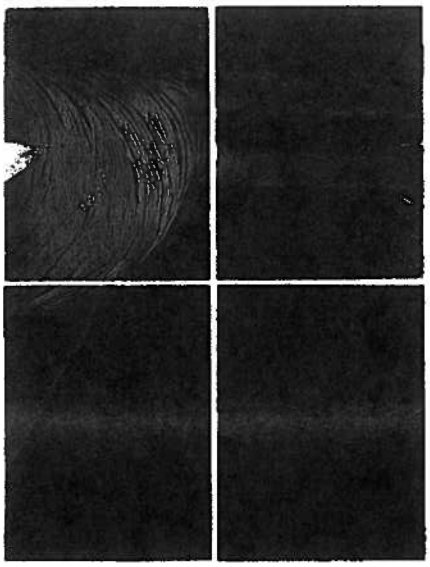

Cerep

STUDY NUMBER 8116H
FINAL REPORT

ADME: A-B Permeability
- Study of 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE -

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STUDY NUMBER 8116H

ADME: A-B Permeability

- Study of 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE -

Study Sponsor:

Attention:

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Study Period: From April 01, 2004 to April 06, 2004

Report Version: 1

Report Date: April 13, 2004

STUDY DIRECTOR

Maralee McVEAN, Ph.D.
Senior Scientist, Absorption and Pharmacokinetics

13 Apr 04
Date

CONTENTS

1. PURPOSE OF THE STUDY

The purpose of this study was to test compound 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE in the A-B permeability assay. This assay allows an estimation of the bioavailability across the intestinal barrier using the human intestinal epithelial cell line TC-7. The correlation between oral drug absorption in humans and the apparent drug permeability in TC-7 cells has been shown (Gres et al. 1998). This in vitro model of the intestinal barrier was also included in the report of the 46th ECVAM (European Centre for the Validation of Alternative Methods) workshop (Le Ferrec et al. 2001).

2. MATERIALS AND METHODS

2.1. ADME-Tox: Bioanalytical

2.1.1. General Procedures

Assay	Technique	Additional Information
HPLC-MS Screen	HPLC-MS, and HPLC-MS/MS	Full scan and product ion spectra; SRM conditions for quantitation, and ionization potential

2.1.2. Experimental Conditions

Assay	Test Compound	Analytical method
HPLC-MS Screen	200 µM (n = 1) acetonitrile/methanol/water (25/25/50, v/v/v)	HPLC-MS and HPLC-MS/MS

Abbreviations:
HPLC-MS/MS: HPLC coupled with tandem mass spectrometry (Instrumentation: Thermo Finnigan)
HPLC-MS: HPLC with mass spectrometry detection (Instrumentation: Thermo Finnigan)
HPLC: High performance liquid chromatography
SRM: Selected reaction monitoring

2.1.3. Analysis and Expression of Results

HPLC-MS Screen

Full scan HPLC-MS analysis was conducted on the test compound at 200 µM. Total ion current chromatograms and corresponding mass spectra were generated for each test compound in both positive and negative ionization modes. The precursor ions for MS/MS were selected from either the positive or the negative mass spectrum, as a function of the respective ion abundance. In addition, product ion HPLC-MS/MS analysis was performed in order to determine the appropriate selected fragmentation reaction for use in quantitative analysis. The final reaction monitoring parameters were chosen to maximize the possibility for quantitation of the test compound when present within a complex mixture of components. Finally, each test compound was assigned a rank number of ionization, which directly indicates its ease of quantitation.

2.2. ADME-Tox: *In Vitro* Absorption

2.2.1. General Procedures

Assay	Cell	Passage Number	Days in Culture	Reference Compound	Bibliography
A-B Permeability *(pH 6.5/7.4)*	TC7	15 passages in culture between passages 20 and 40	13 to 25	propranolol, ranitidine, vinblastine*	Gres et al. (1998)

Notes:
TC7 is a sub-clone of the Caco-2 cell line.
* Vinblastine is tested in the A-B permeability when the B-A permeability is also requested.

2.2.2. Experimental Conditions

Assay	Test Concentration	Biological Conditions	Analytical Method
A-B Permeability *(pH 6.5/7.4)*	≤ 50 µM in HBSS (n=2) 1 % DMSO	A-to-B flux at 37 °C with shaking 96-well transwell plate pH 6.5 in A and pH 7.4 in B Donor samples: time 0 and 60 min Receiver samples: time 60 min	HPLC-MS/MS

Note:
Multiscreen plate: from Millipore, 96-well plate.

Abbreviations:
A: Apical side
B: Basolateral side
DMSO: Dimethylsulfoxide
HBSS: Hank's balanced salt solution, from Invitrogen, catalog number 11201
HEPES: N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
HPLC-MS/MS: HPLC coupled with tandem mass spectrometry (Instrumentation: Thermo Finnigan)
HPLC: High performance liquid chromatography
MES: 2-(N-Morpholino)-ethanesulfonic acid, from Sigma, catalog number M-8652

A-B Permeability:

The working solution for each test compound was prepared at ≤ 50 µM in HBSS-MES (5 mM), at pH 6.5 from a 10 mM DMSO stock solution. ^{14}C-mannitol (approximately 4 µM) was also included in the working solution. The working solution was then added to the apical side with a final DMSO concentration of 1 %. HBSS-HEPES (5 mM), pH 7.4 was added to the basolateral side.
The following parameter was used for assessing the cell monolayer integrity:
^{14}C-D-Mannitol permeability < 2.5×10^{-6} cm/s

2.2.3. Analysis and Expression of Results

A-B Permeability

The apparent permeability coefficient (P_{app}) of the test compound in the apical to the basolateral direction was calculated as follows.

$$P_{app}(cm/s) = \frac{V_R \times C_{R60}}{\Delta t} \times \frac{1}{A \times (C_{D,mid} - C_{R,mid})}$$

where V_R is the volume of the receiver chamber. C_{R60} is the concentration of the test compound in the receiver chamber at time 60 minutes, Δt is the incubation time (60 minutes) and A is the surface area of the TC7 cell monolayer. $C_{D,mid}$ is the calculated mid-point concentration of the test compound in the donor side, which is the mean value of the donor concentration at time 0 minute and the donor concentration at time 60 minutes. $C_{R,mid}$ is the mid-point concentration of the test compound in the receiver side, which is one half of the receiver concentration at time 60 minutes. Concentrations of the test compound are expressed as peak areas of the test compound.

Recovery of the Test Compound from A-B Permeability Assay

The recovery of the test compound was calculated as follows:

$$\text{Recovery}\ (\%) = \frac{V_D \times C_{D60} + V_R \times C_{R60}}{V_D \times C_{WS}} \times 100$$

where V_D and V_R are the volumes of the donor and receiver chambers, respectively. C_{D60} is the concentration of the test compound in the donor sample at time 60 minutes. C_{R60} is the concentration of the test compound in the receiver sample at time 60 minutes. C_{WS} is the concentration of the test compound in the working solution. Concentrations of the test compound are expressed as peak areas of the test compound.

3. COMPOUNDS

3.1. Test Compounds

From:

CEREP I.D.	Compound I.D.	Reference Number	Batch Number	Submitted M.W.	Formula Weight	Stock Solution
8116-11	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE	23081	57	182.22	280.30	1.E-02 M DMSO

M.W.: Molecular Weight

The Certificate of Analysis is included in the Appendix.

3.2. Reference Compounds

In each experiment, the respective reference compounds were tested concurrently with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE in order to assess the assay suitability. The data were compared with historical values determined at Cerep. The assay was rendered valid if the suitability criteria were met, in accordance with the corresponding Standard Operating Procedure.

4. RESULTS

4.1. ADME-Tox: Bioanalytical

The individual data obtained with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE are reported in table 1 - 1.

The HPLC-MS total ion current chromatograms in positive and negative ionization modes, the full scan mass spectra, and the product ion spectra of the test compound obtained from HPLC-MS/MS screening are included in the Appendix.

Table 1 - 1

Individual Data

Assay Cerep Compound I.D.	Client Compound I.D.	Molecular Weight	FW	Selected ESI (+) Precursor Ion (m/z)	Product Ion (m/z)	Collision Offset (V)	Ionization Classification
HPLC-MS Screen							
8116-11	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE	182.22	280.30	183.2	138.0	-20	2.0

Notes:
Ionization Classification:
1 = Highly ionizable compound
2 = Intermediately ionizable compound
3 = Poorly ionizable compound

4.2. ADME-Tox: *In Vitro* Absorption

Cerep uses the following internal guidelines to classify the permeability of compounds:
Low permeability: Papp<2 x 10^{-6} cm/s, the absorption in human is likely to be permeability limited.
Medium permeability: Papp range: 2-20 x10^{-6} cm/s.
High permeability: Papp>=20 x10^{-6} cm/s, the absorption in humans is unlikely to be permeability limited.

Ranitidine, which has 50% absorption in humans, is used as the low permeability reference compound, as recommended by FDA (Guidance for Industry, 2000). Comparison of the compound tested to this reference compound may indicate a relative percent absorption of the compound.

The respective reference compounds (propranolol, ranitidine) validated the experimental conditions, since the suitability criteria (20-45 x 10^{-6} cm/s, 0.2-2 x 10^{-6} cm/s) were met.

The test compound 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE was classified to be of high permeability since the respective Papp was 73.3 x 10^{-6} cm/s.

Permeability:
The summary results obtained with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE are reported in table 2 - 1.
The individual data obtained with 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE are reported in table 2 - 2.
The individual data obtained with the reference compounds are reported in table 2 - 3.

Table 2 - 1

Summary Results

Assay Cerep Compound I.D.	Client Compound I.D.	Test Concentration (M)	Mean TC7 Permeability (10^{-6} cm/s)	Mean Recovery (%)
A-B Permeability *(pH 6.5/7.4)*				
8116-11	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE	5.0E-05	73.3	33

Table 2 - 2

Individual Data

Assay Cerep Compound I.D.	Client Compound I.D.	Test Concentration (M)	TC7 Permeability 1st (10^{-6} cm/s)	TC7 Permeability 2nd (10^{-6} cm/s)	Mean TC7 Permeability (10^{-6} cm/s)	Percent Recovery 1st (%)	Percent Recovery 2nd (%)	Mean Recovery (%)
A-B Permeability *(pH 6.5/7.4)*								
8116-11	2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE	5.0E-05	69.78	76.75	**73.3**	34	32	**33**

Table 2 - 3

Reference Compound Data

Assay Reference Compound	Test Concentration (M)	TC7 Permeability 1st (10^{-6} cm/s)	TC7 Permeability 2nd (10^{-6} cm/s)	Mean TC7 Permeability (10^{-6} cm/s)	Percent Recovery 1st (%)	Percent Recovery 2nd (%)	Mean Recovery (%)
A-B Permeability *(pH 6.5/7.4)*							
propranolol	5.0E-05	23.85	27.93	**25.9**	86	59	**72**
ranitidine	5.0E-05	0.20	0.20	**0.2**	93	89	**91**
vinblastine	5.0E-05	0.09	0.05	**0.1**	95	97	**96**

5. BIBLIOGRAPHY

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LE FERREC,E., CHESNE, C., ARTUSSON, P., BRAYDEN, D., FABRE, G. GIRES, P., GUILLOU, F., ROUSSET, M., RUBAS, W., SCARINO, M.L. (2001)

In vitro models of the intestinal barrier. The report and recommendations of ECVAM Workshop 46. European Centre for the Validation of Alternative methods. *Altern Lab Anim. 29:649-68.*

6. STORAGE AND RETENTION OF RECORDS

All documents generated during the performance of the study (raw data, various recordings such as QA audit reports, an original of the study report, study plan...) will be stored for a 10-year period in Cerep's archive rooms after achievement of the study. Only Cerep's authorized employees shall have access to the archives.

The original final report provided to the sponsor will be kept by the sponsor under its sole responsibility.

7. QUALITY ASSURANCE STATEMENT

The following audits were performed on this study:

	CALENDAR
Audit of Raw Data	April 6, 2004
Audit of the Final Report	April 13, 2004

Audit reports were established for each audit performed.
Audit report of the study report was transmitted to the Study Director for approval.

I certify that results presented in this report were generated using the materials and methods mentioned and that these results accurately reflect the Raw Data.

Kristin Herndon
Kristin Herndon

Quality Unit

APPENDIX

Certificate of Analysis and the HPLC-MS Total Ion Current Chromatograms, Full Scan Mass Spectra, and Product Ion Mass Spectra of the Test Compound

Version 1
April 13, 2004

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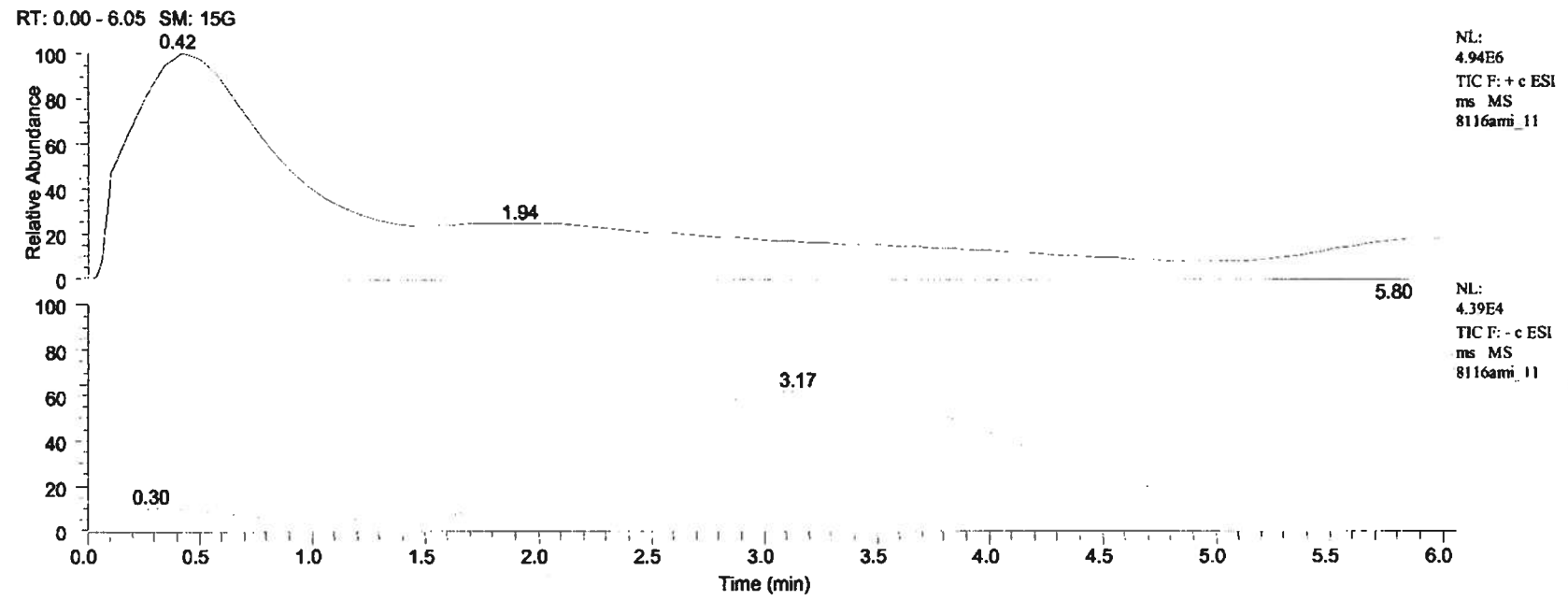

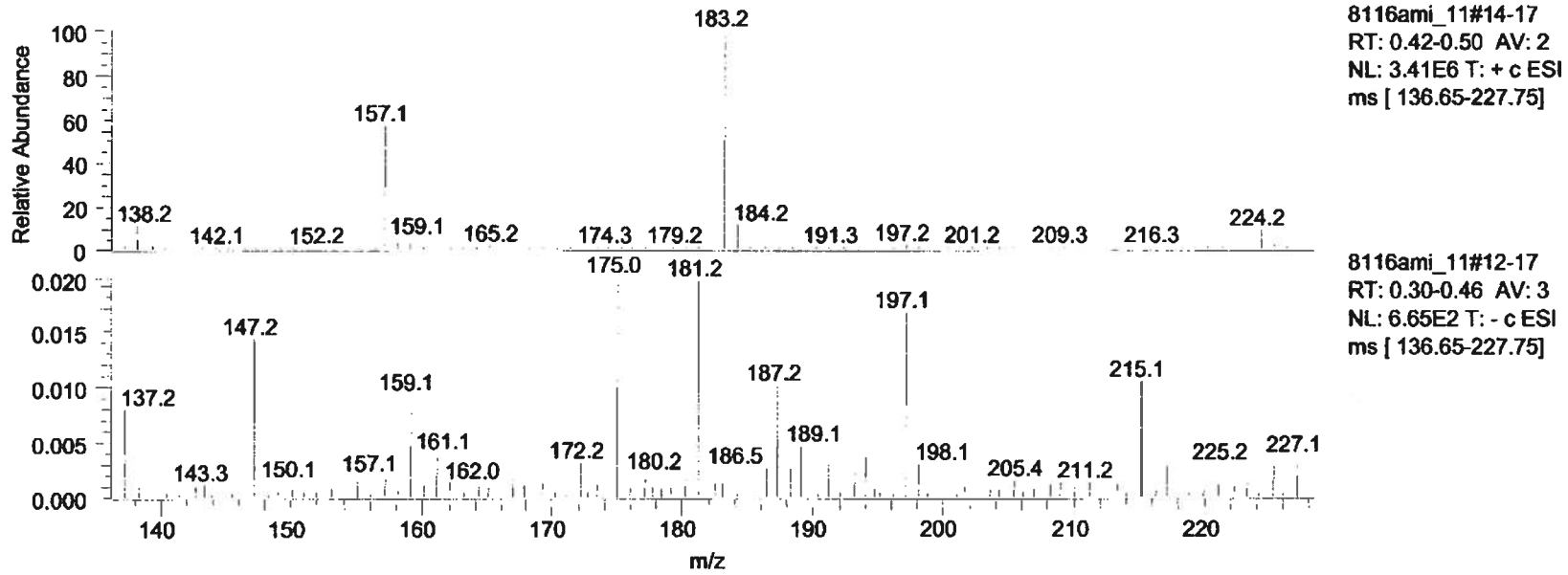

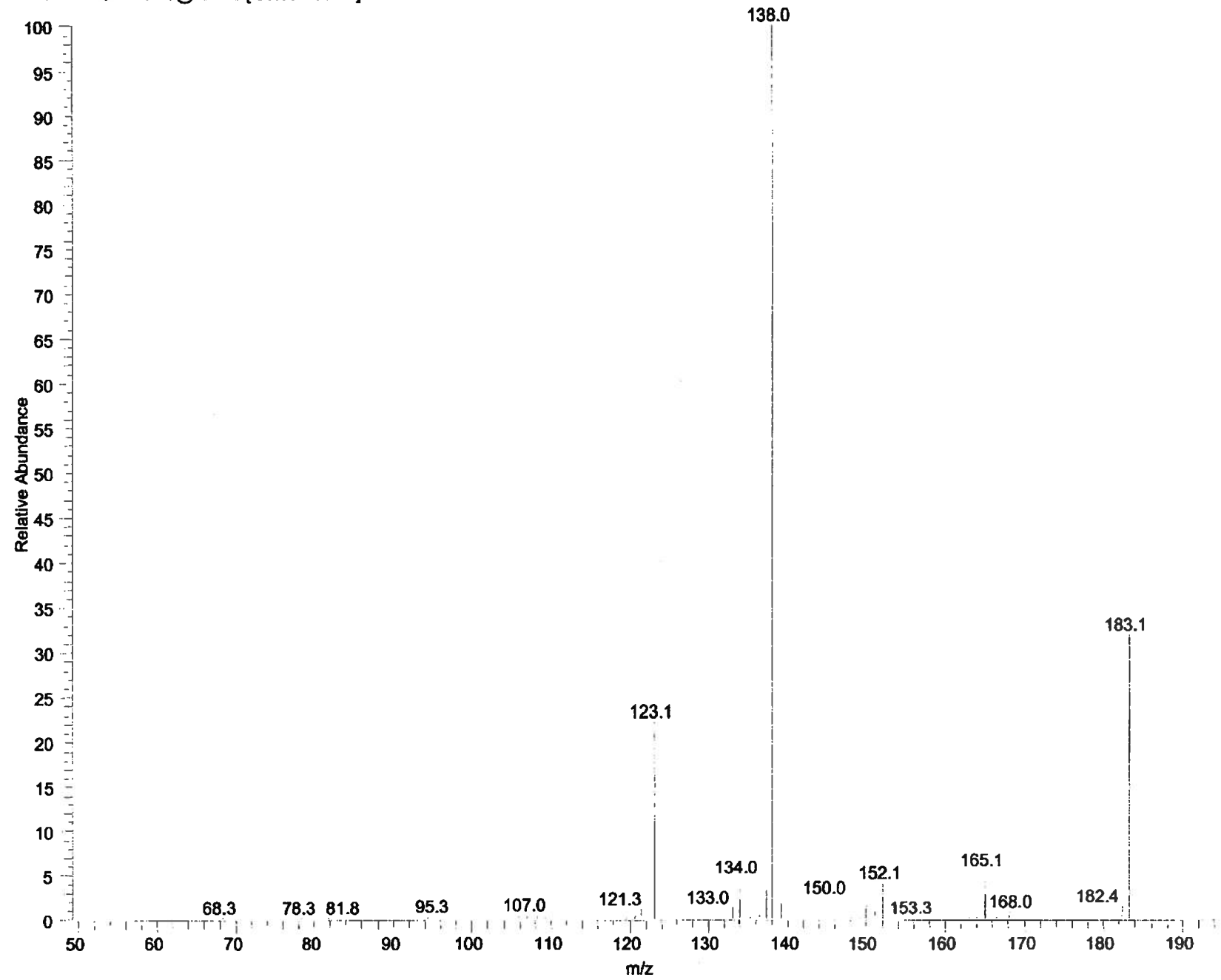
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T: + c ESI ms2 183.20@-20.00 [50.00-193.22]
Relative Abundance
m/z
138.0
183.1
123.1
134.0
165.1
152.1
150.0
68.3
78.3
81.8
95.3
107.0
121.3
133.0
153.3
168.0
182.4

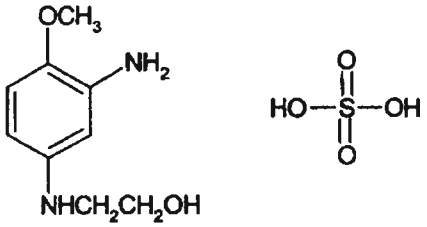
COSMITAL SA

Marly, 22.01.2004 LSY/BR/DOU/bs

CERTIFICATE OF ANALYSIS

Wella raw material no.: 23081 **Code**: A000157

Structure:



Molecular formula: $C_9H_{14}N_2O_2.H_2O_4S$ **Molecular weight**: 280.30
Molecular weight of free acid: 182.22

LEHMANN BLAU

Trade name: HC BLAU AC (ROBINSON). HC BLUE AC (CLARIANT)

Chemical name: 2-Amino-4-(2-hydroxyethyl)amino-anisole-sulfate

Name (INCI): 2-AMINO-4-HYDROXYETHYLAMINO-ANISOLE

CAS-No: 83763-48-8 **EINECS/ELINCS-No**: 280-734-8

Testing material

Sample name:
Batch: 57
Date of entry: 08.01.96

Sample no: R96000196
Study no.: A9803/086 and G2000/003
Expiry date: July, 2005

Results

Aspect: pale grey powder

Identity and Purity: The ^{1}H-NMR spectra confirmed the chemical identity of the test substance.

Purity: 99.6 area% (by HPLC)

Content: 99.1 weight% ($C_9H_{14}N_2O_2xH_2SO_4xH_2O$) } by
93.5 weight% ($C_9H_{14}N_2O_2xH_2SO_4$) } NMR

By-products: 0.062 weight% 4-Methoxy-1,3-phenylendiaminsulfate
82 ppm of iron

Solubility: 10g/l in water pH 2.8 (>5 weight% pH 8)
1 weight% in acetone/water 1:1 (pH 2.1)
9-10 weight% in DMSO
0.2 weight% in ethanol

Stability: The substance on storage in dryness and darkness to be stable → July 2005.

Stability in solution:
The stability over a total period of seven days was tested by HPLC. The test stock solutions (approx. 5 weight%) were stored at room temperature and in the absence of light.
Water solution: the results (t = 0h: 100.0%; 6h: 93.8%; 2d: 95.1%; 7d: 79.7%) confirm a low degradation (G2000/003)
DMSO solution: the results (t = 0h: 100.0%; 6h: 98.8%; 2d: 92.0%; 7d: 80.5%) confirm a low degradation (G2000/003)

Stability in solution:
The stability was tested over a period of seven days. The test solutions were stored at room temperature in the absence of light.
DMSO (ca. 10 weight%) : the results (99.8-100.1%) confirm a very good stability.
Acetone/water (ca. 0.5 weight%): the results (100.4-100.8%) confirm a very good stability.
Water (ca. 0.01 weight%): the results (100.2-99.2%) confirm a very good stability.

Remark: drying on $CaCl_2$ and then under high vacuum
Loss on water $\Rightarrow$ 8.8%

P: Dougoud