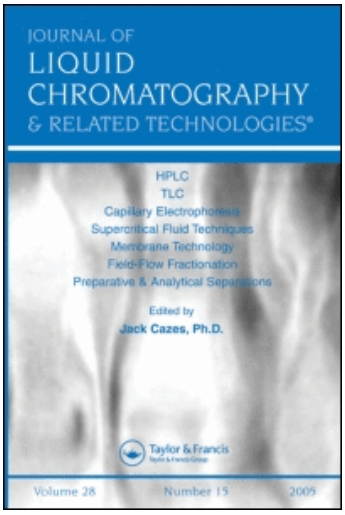

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Rapid and Sensitive Method for Determination of Cyclophosphamide in Patients Plasma Samples Utilizing Microextraction by Packed Sorbent Online with Liquid Chromatography-Tandem Mass Spectrometry (MEPS-LC-MS/MS)

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To cite this Article Said, Rana , Hassan, Zuzan , Hassan, Moustapha and Abdel-Rehim, Mohamed(2008) 'Rapid and Sensitive Method for Determination of Cyclophosphamide in Patients Plasma Samples Utilizing Microextraction by Packed Sorbent Online with Liquid Chromatography-Tandem Mass Spectrometry (MEPS-LC-MS/MS)', Journal of Liquid Chromatography & Related Technologies, 31: 5, 683 — 694

To link to this Article: DOI: 10.1080/10826070701853867

URL: http://dx.doi.org/10.1080/10826070701853867

Journal of Liquid Chromatography & Related Technologies®, 31: 683–694, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701853867

Rapid and Sensitive Method for Determination of Cyclophosphamide in Patients Plasma Samples Utilizing Microextraction by Packed Sorbent Online with Liquid Chromatography-Tandem Mass Spectrometry (MEPS-LC-MS/MS)

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Abstract: The aim of the present investigation was to develop a simple and fast method for the determination of cyclophosphamide in human plasma samples. Microextraction in packed syringe (MEPS) was used as an online rapid sample preparation method, followed by liquid chromatography with tandem mass spectrometry (LC-MS-MS) for the quantification of cyclophosphamide. The new method reduced the sample handling and the analysis time by several folds compared to liquid chromatography and UV detection. The limit of detection (LOD) was 0.005 μg/mL and the lower limit of quantification was 0.5 μg/mL. The accuracy of the quality control (QC) samples ranged from 95 to

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106%. The inter-day variation was within the range 5–9% while the intra day variation was between 1–5%. The calibration curve in plasma was constructed within the concentration range 0.5–150 μg/mL. The regression correlation coefficient (r) was ≥0.99 for all runs. The limit of detection improved by 100 time using MEPS-LC-MS/MS (0.005 μg/mL) compared to LLE-LC-UV (0.5 μg/mL). The present method was employed for the analysis of human plasma samples for more 170 patient samples. The concentrations obtained from LC-MS-MS were in good agreement with these obtained from LC-UV with the ratio of 1.02 ± 0.11. The present method is rapid, reliable, and robust and may be used for therapeutic drug monitoring of cyclophosphamide.

Keywords: Microextraction in Packed Syringe, Sample Preparation, Cyclophosphamide, Plasma, LC-UV, LC-MS/MS

INTRODUCTION

Cyclophosphamide is one of the most widely used anti cancer drugs.[1,2] It is used in the treatment of malignant diseases such as leukemia, lymphoma, and solid tumors. It is also used as an immunosuppresive drug in high doses prior to bone marrow transplantation, as well as in low doses for the treatment of autoimmune diseases. Cyclophosphamide is a prodrug that has to be activated. The drug is predominantly activated by liver cytochrome P450 enzymes.[3,4] Several fold variability in cyclophosphamide clearance have been reported in patients treated with the standard,[5] as well as with the high doses. The efficacy and toxicity of many anticancer drugs are related to parent drug and/or metabolite concentrations.[6] Therefore, a profound knowledge of the pharmacokinetic properties of the drug is essential. Furthermore, since anticancer agents often have narrow therapeutic windows, drug monitoring routines in clinical settings may be required for safe and efficacious therapeutic use.[7] Techniques such as gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance spectroscopy (NMR), GC–mass spectrometry (MS), liquid chromatography–mass spectrometry (LC–MS) have been used to measure cyclophosphamide in biological samples.[8–14]

The LC-MS method has recently become the method of choice for the quantitative bioanalysis of many anticancer agents. However, more development is to be expected in this area, such as the introduction of more sensitive and robust tandem mass spectrometres, high throughput analyses, and further optimization of the coupled LC systems.

Microextraction by packed sorbent (MEPS) is a new technique for miniaturised solid phase extraction that can be connected on line to GC or LC without any modification.[15–18]

The aim of the present study is to develop and validate a sensitive and rapid LC-MS-MS method utilizing online sample preparation, MEPS, to determine the cyclophosphamide in human plasma samples.

EXPERIMENTAL

Instrumentation

MEPS-LC-MS/MS

The MEPS syringe, (100 μL syringe, C2-sorbent) was obtained from SGE analytical (Melbourne, Australia). The high performance liquid chromatography (HPLC) instrument included two pumps, Shimadzu (Kyoto, Japan), and autosampler, CTC-Pal, (CTC Analytics AG, Zwingen, Switzerland). A Zorbax (50 × 2.1 mm, SB-C8, 3.5 μm) column obtained from Agilent (Calif., USA) was used as the analytical column. A guard column; Optiguard (C_8, 10 × 1 mm) was obtained from Optimize Technologies (Oregon City, USA). A Valco C4W valve, Valco Instruments (Houston, USA) was used as the gate valve between the liquid chromatograph and the mass spectrometer. The Milli-Q water used was obtained from Reagent Grade Milli-Q Plus water purification system (Millipore Corporation, Bedford, USA).

A gradient HPLC was used with mixer volume of 0.1 mL. Mobile Phase A was 0.1% formic acid in water and acetonitrile 90:10 (v/v) and mobile phase B contained 0.1% formic acid in water and acetonitrile 20:80 (v/v). The gradient started from 0% of phase B up to 80% from 1 to 4 min and then from 4 to 5 min isocratic at 80% of phase B and at 6.1 min phase B was set at 0% again. The flow rate was 150 μL/min and sample volume was 40 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass QII Z-spray (Waters Corporation, Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode. The scan mode was multiple reaction monitoring (MRM) using a precursor ion at (M + 1) m/z (m/z: 261 and 261) and after collisional dissociation the product ions 140 and 154 were used for quantification of cyclophosphamide and the internal standard.

The optimum mass spectrometric parameter settings were: capillary voltage at 3.1 kV, cone voltage at 27 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150°C and 300°C, respectively. Nitrogen was used, both as drying (400 L h^{-1}) and nebulizing gases (20 L h^{-1}), the vacuum was 2.10^{-5} in the mass analyzer and 2.10^{-3} in the collision cell. Argon was used as collision gas and collision energy was 22 eV. The gases were from ScanGas (Stockholm, Sweden). The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

LC-UV

The HPLC system consisted of a Shimadzu pump LC10AD (Kyoto, Japan) and CMA/240 autosampler with a 50 μL sample loop. The detector was a Milton Roy Spectro Monitor 3100 (PA, USA) and the UV detection wave length was 195 nm. The liquid chromatographic analysis was run in an

isocratic mode. The mobile phase was acetonitrile and 0.05 M phosphate buffer (20:80 v/v) and the flow rate was 0.4 mL/min. The separation was performed using a Phenomenex LC column, polar-RP 80A (4 μm, 3.0 × 150 mm).

Reagents and Materials

Cyclophosphamide (Figure 1) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Ifosfamide was used as the internal standard and obtained from ASTA Medica AG (Frankfurt, Germany). Acetonitrile, methanol, formic acid, and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Sample Preparation

Liquid-Liquid Extraction for LC-UV

Blood was withdrawn from a central venous catheter at different time points directly into heparinzed vaccutainer tubes. The samples were centrifuged at 3000 g for 5 min. Plasma was separated and stored at −20°C. Before use, the plasma was thawed at room temperature and centrifuged.

To prepare the calibration curve, stock solutions of cyclophosphamide (2.0 mg/mL) and iphosphamide (I.S. 1.0 mg/mL) were dissolved in distilled water. Serial dilutions were made, and 50 μL of the different concentrations were added to 1 mL plasma to reach a final concentration between 1.0–150 μg/mL (1.0, 5.0, 10.0, 25.0, 50.0, 100.0, and 150.0 μg/mL). The internal standard was added to each sample (20 μL). Quality control (QC) samples concentrations were prepared at the concentrations 20, 75, and 120 μg/mL. Plasma samples (0.25 mL) were extracted in 1.5 mL ethylacetate, shaking for 15 minutes and then centrifuged at 3000 g for 10 min. The

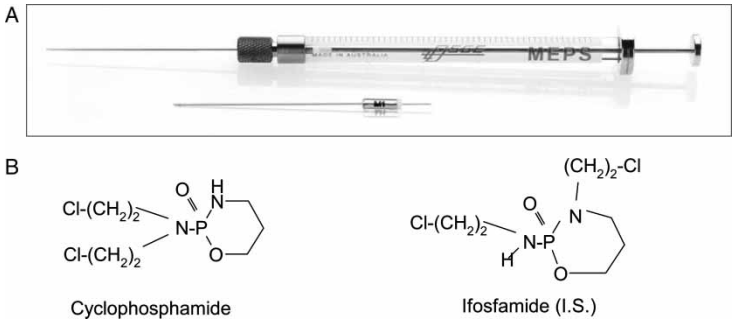

Figure 1. (A) Microextraction by packed sorbent syringe and (B) Chemical structures of cyclophosphamide and ifosfamide (I.S.).

organic layer was transferred into a new tube and evaporated to dryness. The residue was dissolved in 100 μL of mobile phase; 30 μL was injected into the HPLC system.

Microextraction by Packed Sorbent (MEPS) for LC-MS/MS

The calibration curve in human plasma was obtained in the concentration range 0.5–150 μg/mL (0.5, 0.75, 5.0, 7.5, 10.0, 25.0, 50.0, 100.0, and 150.0 μg/mL). Quality control (QC) sample concentrations were 20, 75, and 120 μg/mL. A plasma sample (50 μL) containing IS (40 μg/mL) was diluted with water (1:4) before MEPS extraction. The MEPS sorbent was manually conditioned with 50 μL methanol followed by 50 μL of water. After that, the syringe was connected to the autosampler and the spiked plasma sample (25 μL) was withdrawn into the syringe by the autosampler. The sorbent was then washed once with 100 μL of water/methanol 95:5 (v/v) to remove proteins and other interferences. The analytes were then desorbed by 30 μL methanol/water 95:5 (v/v) directly into the LC injector. Cleaning of the sorbent was carried out using 4 × 250 μL elution solution followed by 4 × 250 μL of the washing solution between every extraction. This step decreased memory effects, but also functioned as a conditioning step before the next extraction. The same packing bed was used for about 100-150 extractions before it was discarded.

Validation

Each calibration curve consisted of nine calibration points covering from 0.5 μg/mL to 150 μg/mL. Blank samples were run simultaneously. The plasma used for the calibration curve was collected and pooled from different objects. The peak area ratios for cyclophosphamide and internal standard were measured and a standard curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

where y is peak area ratio, x is the concentration, a is the curvature, b is the slope and c is the intercept. The calibration curves were quadric and the weight was $1/x$. The QC samples were prepared with the concentrations of 20, 75, and 120 μg/mL. The accuracy and precision were calculated for the QC samples at three different assays. To assess linearity, deviations of the mean calculated concentrations over three runs should be within ±15% from nominal concentrations for the non-zero calibration standards. At the lower limit of quantitation (LLQ) level a deviation of ±20% was permitted. The method was validated at optimized conditions.

Accuracy was defined as the degree of deviation of the determined value and the nominal value: [(measured value-nominal value)/nominal value] * 100. Precision (C.V. %) was defined as the percentage of standard deviation of the observed values divided by their mean values: [(standard deviation)/mean value] * 100.

RESULTS AND DISCUSSION

Calibrations

Calibration curves of plasma samples spiked with cyclophosphamide standards were obtained in the range 0.5–150 μg/mL, with 40 as internal standard. The coefficients of determination (r) were 0.999 (n = 6) using LC-MS-MS and 0.995 (n = 3) using LC-UV. Table 1 shows the back calculated values of standard calibration samples in human plasma.

Selectivity

The method selectivity was defined as non-interference with the endogenous substances in the regions of interest. LC-UV and LC-MS/MS analysis of the blank plasma samples showed no presence of an endogenous interference peak with the quantification of cyclophosphamide. More clean extract was obtained by MEPS-LC-MS/MS compared to LLE-LC-UV. Representative chromatograms of blank human plasma and spiked plasma are presented in (Figures 2 and 3).

Table 1. Back-calculated values of the calibration points of the plasma samples using LLE-LC-UV and MEPS-LC-MS/MS

		Mean conc. (n = 6)		Mean accuracy (%)		RSD (%)	
Compound	Concentration μg/mL	LLE-LC-UV	MEPS-LC-MS/MS	LLE-LC-UV	MEPS-LC-MS/MS	LLE-LC-UV	MEPS-LC-MS/MS
Cyclophosphamide	0.50	—	0.47	—	94	—	8
	0.74	—	0.69	—	93	—	7
	5.0	6.0	5.23	120	105	11	8
	7.5	7.8	8.00	104	107	7.8	6
	10	10.3	10.5	102	105	15	8
	25	24.8	25.7	99	103	2.5	7
	50	48.7	49.2	97	98	4.2	3
	100	104	98.5	104	99	1.2	5
	150	155	145	103	96	1.8	3

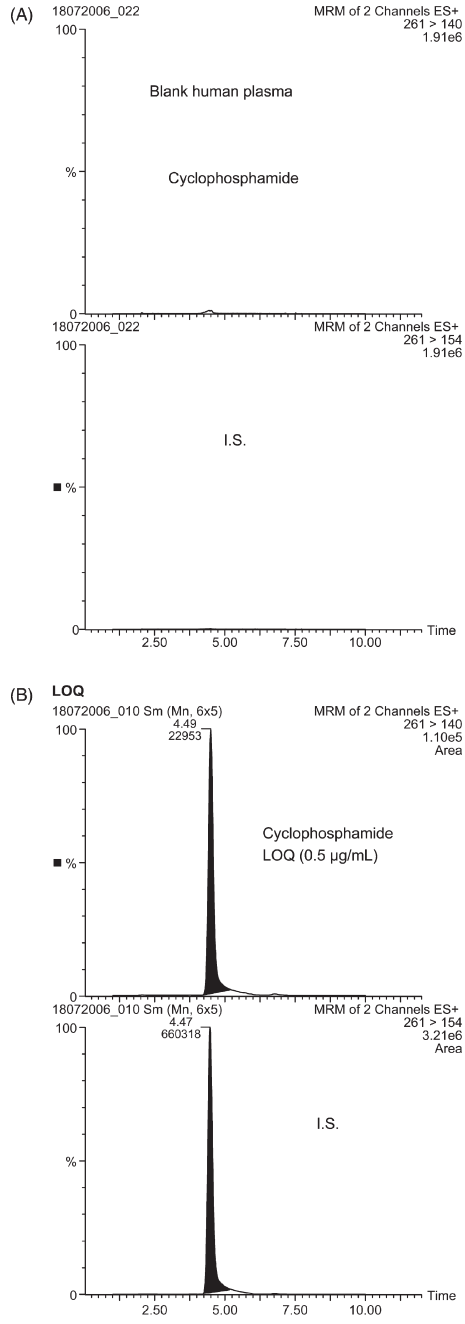

Figure 2. Representative chromatograms with mass spectrometric detection obtained from (A) human plasma spiked with cyclophosphamide 0.5 μg/mL (LLOQ) and I.S.; (B) blank plasma sample.

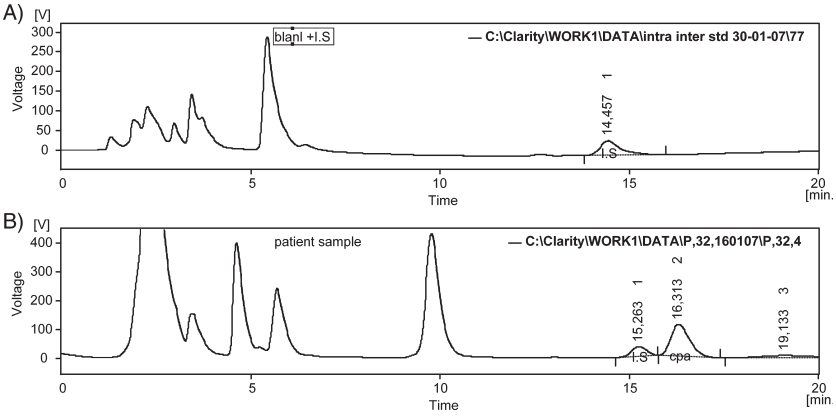

Figure 3. Representative chromatograms with UV detection obtained from: (A) Blank human plasma sample containing I.S.; (B) human patient plasma sample and I.S.

Accuracy and Precision

The QC samples were prepared in human plasma. The accuracy is determined as the percentage difference from the nominal concentration value of QC samples ($n = 12$) at three different concentration levels. The between-batch mean accuracy ranged from 91 to 106% using MEPS-LC-MS/MS and between 84 and 98% using LLE-LC-UV. The precision is determined by the percentage of the relative standard deviation (RSD) of the between-batch variations at three different concentration levels (QC samples). The data of between-batch variation of the precision were in the range 5.0–9.0% using MEPS-LC-MS/MS and 4.0–11% for the LLE-LC-UV method. The data of within-batch variation of the precision (RSD) were in the range 1.0–5.0% for MEPS-LC-MS/MS and 4.3–13% for LLE-LC-UV. The accuracy and precision results are summarized in Table 2.

Limit of Detection, Lower Limit of Quantification, and Carry-Over

LC-MS-MS

The limit of detection (LOD) was 0.005 μg/mL. The lower limit of quantification (LLOQ) in plasma was measured at 0.5 μg/mL. The precision (C.V.) for LOQ was 3% ($n = 12$). The carry over was investigated by injecting the elution solution after the highest standard concentration, which was lower than 0.1%. However, no carry over was observed after several washings (five times with methanol).

Table 2. Intra- and inter-day precision for cyclophosphamide in plasma using LLE-LC-UV and MEPS-LC-MS/MS

	Accuracy		Precision			
	Accuracy % (n = 18)		Intra-day (n = 6) (R.S.D. %)		Inter-day (n = 18) (R.S.D. %)	
Concentration μg/mL	LLE-LC-UV	MEPS-LC-MS/MS	LLE-LC-UV	MEPS-LC-MS/MS	LLE-LC-UV	MEPS-LC-MS/MS
120 (QCH)	85	95	4.3	5.0	4.0	7.0
75 (QCM)	98	91	11	1.0	9	9.0
20 (QCL)	84	106	13	5.0	11	5.0

LC-UV

The limit of detection (LOD) was 0.5 μg/mL. The lower limit of quantification (LLOQ) in plasma was measured at 5 μg/mL. The precision (C.V.) was 18% (n = 6).

Application of the Method

The method was applied for the analysis of plasma samples from clinical studies. Figure 4 shows the relation between 170 patient samples

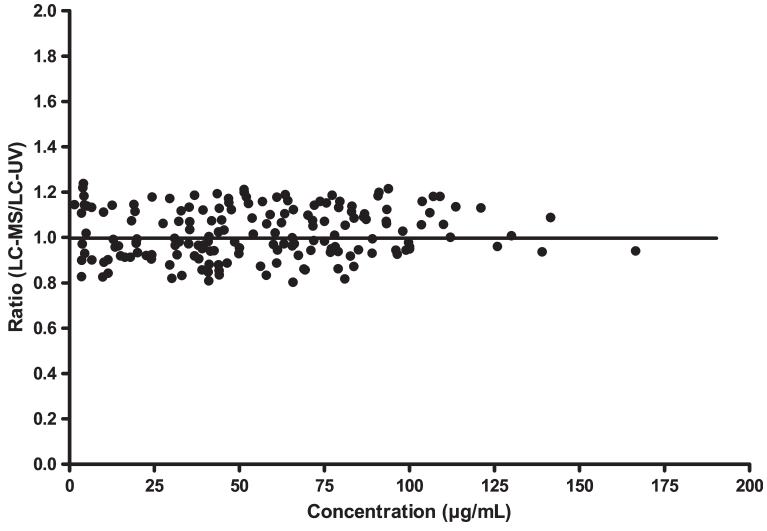

Figure 4. The ratio of patient plasma concentrations obtained by LC-MS/MS/LC-UV plotted against concentrations obtained using LC-MS/MS. The mean ratio 1.02 ± 0.11.

analyzed by LC-UV and LC-MS/MS. As can be seen, the ratio patient concentration analyzed using LC-MS/MS/LC-UV was 1.02 ± 0.11 ranging from 0.81–1.19.

Method Comparison

Gas chromatography or GC-MS belongs to the early methods that were used for cyclophosphamide analysis; however, it was connected with a number of problems.[14] Rapid development of LC-MS in combination with an increased need for therapeutic drug monitoring, made the LC-MS the method of choice in the determination of many anti cancer drugs including cyclophosphamide.

Many previous methods used for the determination of the neutral drugs, such as cyclophosphamide, ifosfamide, and carbamazepine was performed using LC-ESI-MS-MS in the ng/mL range in the positive ionization mode.[8] However, the sample preparation still is a problematic issue, since it is time and labour consuming. The need for online sample preparation combined with high throughput applications in bioanalysis has increased during the past decade. In this paper, we present a robust and online sample preparation technique that includes micro extraction in packed syringe (MEPS). The method is a miniaturized, fully automated, solid-phase extraction (SPE) technique that can be connected online to LC without any further work up procedure. On the other hand, it is important to mention that, further development of this assay is warranted to analyze both cyclophosphamide and its active metabolites that may contribute to the clinical effects of the drug. Other groups have adopted LC-MS techniques for the simultaneous determination of cyclophosphamide and its metabolites in plasma and tissue and have applied these assays in clinical and experimental trials,[7,8] however, these methods require both derivatization and long time

Table 3. Comparison between several cyclophosphamide measuring methods in plasma samples

Analysis method	Extraction method (sample volume)	Extraction time	Analysis time	LOD, ng/mL	Ref.
LC-MS/MS	MEPS (50 μL)	1.0 min	4.5 min	0.5	Present study
LC-MS/MS	LLE (250 μL)	35 min	2.0 min	3.1	[9]
LC-MS/MS	LLE (100 μL)	20 min	9.2 min	20.0	[11]
μLC-MS/MS	SPE (1000 μL)	20 min	11.5 min	0.04	[13]

work-up procedure, which probably is due to the heterogeneity and instability of the metabolites.

Until today, there was no method for on line sample preparation of cyclophosphamide and its metabolites. Such a method may play an important role to replace the old sample preparation methods and can be of a great advantage for therapeutic drug monitoring.

The present results were in complete agreement with those results obtained using the LC-UV method, which is mostly used by many centers for the routine analysis of cyclophosphamide. The present method provides an easy, robust, and rapid analysis for cyclophosphamide in human plasma, requiring only 50 μL plasma compared to other methods that require at least 100 μL of plasma. This is an important issue to consider when considering plasma sampling from paediatric patients. One important factor is that the present method does not require a work up procedure, which has to be considered in the clinical studies where a large numbers of samples are generated and the speed of analysis is important (Table 3).

ACKNOWLEDGMENT

This investigation was supported by grants from The Swedish Cancer Foundation and from The Swedish Children Cancer Society.

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Received June 10, 2007
Accepted September 6, 2007
Manuscript 6153