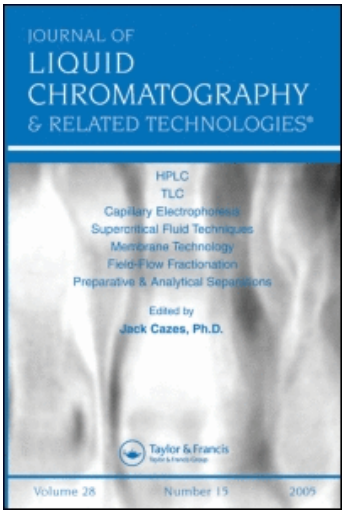

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Erythrocyte Porphyrins by Epi-Illumination Fluorescence Microscope with Capillary Electrophoresis

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To cite this Article Yao, Y. J. and Li, S. F. Y.(1996) 'Determination of Erythrocyte Porphyrins by Epi-Illumination Fluorescence Microscope with Capillary Electrophoresis', Journal of Liquid Chromatography & Related Technologies, 19: 1, 1 — 15
To link to this Article: DOI: 10.1080/10826079608006285
URL: http://dx.doi.org/10.1080/10826079608006285

J. LIQ. CHROM. & REL. TECHNOL., 19(1), 1–15 (1996)

DETERMINATION OF ERYTHROCYTE PORPHYRINS BY EPI-ILLUMINATION FLUORESCENCE MICROSCOPE WITH CAPILLARY ELECTROPHORESIS

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ABSTRACT

A micellar electrokinetic capillary chromatographic (MEKC) method is described for the simultaneous determination of zinc protoporphyrin (ZnPP) and protoporphyrin (PP) in whole blood. Reproducibility of migration times and peak areas of ZnPP and PP have been shown to improve significantly with the use of an internal standard.

Erythrocyte porphyrins were extracted from whole blood with N,N-dimethylformamide (DMF), followed by sample cleanup and preconcentration on C-18 cartridges. Analytical recoveries of the porphyrins were better than 88%. An epi-illumination fluorescence microscope was used for the detection of these porphyrins. Limits of detection were found to be 50 nM and 8 nM for ZnPP and PP, respectively.

INTRODUCTION

Naturally occurring porphyrins are intermediate metabolites of heme biosynthesis. Disturbances in the biosynthesis, caused by inborn or acquired defects of the corresponding enzymes, give rise to a family of disease called porphyria. Depending on the break of the metabolic pathway, different porphyrins are accumulated in body fluids and tissues. Certain diseases such as lead intoxication and iron deficiency anemia[1] result in protoporphyrin (PP) accumulating in the red blood cells as the Zn-protoporphyrin chelate (ZnPP), while in the case of erythropoietic protoporphyria, the unchelated PP accumulates[2]. Hence, erythrocyte porphyrin profile is an important diagnostic tool for the detection of such hematological disorders.

Separation and quantitative analysis of porphyrins extracted from human excreta and biological tissues have been conventionally performed by high performance liquid chromatography (HPLC) with fluorescence detection[3-5]. Different organic solvents have been used for the extraction of ZnPP and PP in whole blood[6-12]. Solvent systems which included mineral acid in the extracting solvent dissociate Zn from ZnPP[6-8], and separate extraction procedures were required for ZnPP and PP[8]. Other solvent systems such as acetone-DMSO mixture[9], aqueous acetone[10], and aqueous ethanol[12] have successfully been used for the simultaneous extraction of ZnPP and PP from whole blood. To date, there has been no usage of N,N-dimethylformamide (DMF) as the extracting solvent. In this paper, a method employing DMF as an extracting solvent for erythrocyte porphyrins and micellar electrokinetic capillary chromatography (MEKC) coupled with epi-illumination fluorescence microscope detection for their separation was described.

EXPERIMENTAL

Materials

Protoporphyrin IX (PP) and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) were obtained from Sigma (St. Louis, MO, USA). Mesoporphyrin IX (MP) was from Porphyrin Products (Logan, UT, USA), and zinc protoporphyrin IX (ZnPP) from Aldrich (Milwaukee, WI, USA). Sodium dodecyl sulphate (SDS) was purchased from Fluka (Buchs, Switzerland), and HPLC-grade DMF was from BDH (Poole, England). All other chemicals used

were of analytical-reagent grade. The electrophoresis buffer solutions were prepared with Milli-Q treated water (Millipore, Bedford, MA, USA) and filtered through a 0.45 μm membrane (Whatman, Arbor Technologies, Ann Arbor, MI, USA). Disposable solid phase extraction cartridges used were Sep-Pak Light C-18 cartridges and Sep-Pak Plus C-18 cartridges containing 120 mg and 330 mg, respectively, of 90 μm sorbent (Waters Associates, Milford, MA, USA). Disposable syringes were used to deliver liquids through the extraction cartridges.

Capillary Electrophoresis

CE was performed on 65 μm i.d. fused silica capillaries from Polymicro Technologies (Phoenix, AZ, USA). The detector system was similar to that described previously[13]. It consisted of a Nikon Labophot-2A episcopic fluorescence microscope (Nikon Corp., Tokyo, Japan) with a 100 W high pressure mercury lamp as the excitation source. Wavelength selection was accomplished through a filter block comprising of an excitation filter, a dichroic mirror, and a barrier filter. Excitation wavelength of 405 nm was selected with an interference filter (10 nm bandwidth, CVI Laser Corp., Albuquerque, NM, USA). The dichroic mirror reflects radiation below 560 nm and refracts radiation above 580 nm, and the barrier filter transmits radiation longer than 590 nm. A silicon photodetector (Model HC220-01, Hamamatsu Corp., Japan) was employed for the detection of fluorescence signal. Data acquisition was performed on a Hewlett Packard 3390A integrator (Palo Alto, CA, USA). Sample injection was performed hydrodynamically at a height difference of 10 cm for 15 s, corresponding to an injected sample volume of approximately 10 nL. Separations were accomplished at ambient temperature using a Spellman RHR30 high voltage power supply (Plainview, NY, USA).

New capillaries were rinsed with water for 30 min, followed by 1N NaOH for 10 min, and 0.1N NaOH for another 30 min. After a short wash with water, the capillary was filled with the electrophoresis buffer. When peak broadening or noisy baseline was observed, the capillary was rinsed successively with 1N HCl (2 min), water (2 min), 0.2N NaOH (5 min), water (2 min), and finally stabilized with electrophoresis buffer to restore to its original performance. At the end of the day, the above washing procedure was repeated, except that the NaOH solution was left in the capillary overnight.

Porphyrin Standards

Standard stock solutions of MP, PP and ZnPP were prepared by dissolving approximately 1 mg of each of the respective porphyrin in 1 mL of DMF. The concentrations of the porphyrin solutions were determined from their absorptivity[14]. As the porphyrins are photosensitive, all porphyrin solutions were protected from light whenever possible and were stored at -20°C.

Blood Samples

Whole blood from a healthy subject was treated with disodium ethylenediaminetetraacetate (EDTA) and stored in the dark at -20°C. Spiked blood samples were prepared by adding appropriate volumes (not more than 10 μL) of standard solutions containing ZnPP and PP to 500 μL of whole blood.

Extraction Procedures

Spiked blood (100 μL) was prediluted with 200 μL of 0.5N acetic acid and then extracted with 1500 μL of DMF containing MP as the internal standard. Extraction was achieved with vortex mixing for 1 min and sonicating in an ice-water bath for 5 min. The protein precipitate was packed by centrifugation at 2000*g* for 5 min. The yellowish supernatant was then transferred to a clean polypropylene vial.

Sample cleanup and preconcentration were carried out on a C-18 cartridge. The cartridge was first activated with 2 mL of methanol followed by conditioning with 1 mL of 0.5N acetic acid. The supernatant from the blood extract (1.5 mL) was diluted with 2 mL of 0.5N acetic acid to lower the solvent strength of the sample solution. This solution was loaded onto the cartridge at a flow rate of ca. 3 mL/min, and the cartridge was then washed with 1 mL of water and 1 mL of 20% aqueous DMF. Most of the water remaining in the sorbent was removed by forcing 10 mL of air through the cartridge using a disposable syringe. The retained porphyrins were then slowly eluted with about 0.3 mL (2 x 0.15 mL) of DMF.

RESULTS AND DISCUSSION

Electrophoretic Separations

The separation of the dicarboxylic porphyrins was performed using one set of the optimized conditions achieved employing the overlapping resolution mapping scheme, described in our previous paper[15]. Baseline resolution of the porphyrins was obtained with a 20 mmol/L CAPS buffer (pH 10.8) containing 20 mmol/L SDS and 8% (v/v) DMF, as shown in Fig. 1. From electropherograms of equimolar mixture of ZnPP and PP in DMF, the fluorescence of PP was found to be higher than that of ZnPP (data not shown). In fact, the ratio of the fluorescence peak areas of PP to ZnPP was found to be approximately 3. The difference in their fluorescence is mainly due to the difference in the excitation maximum of ZnPP and PP. In this study, fluorescence detection was selected with an excitation wavelength of 405 nm, which gives a maximum fluorescence for PP , whereas ZnPP emits maximally at an excitation wavelength of 417 nm.

Table 1 shows the migration time reproducibility for the porphyrins. When the capillary was flushed with electrophoresis buffer between runs, the run-to-run relative standard deviations (RSD) for the migration times of ZnPP and PP were more than 1% for 8 consecutive runs. A drift towards longer migration times and a gradual broadening of the porphyrin peaks were also observed. The situation did not improve with replenishment of the electrophoresis buffer at both electrodes. It was believed that the migration drift was, in large part, due to adsorption of the porphyrins onto the capillary wall, as noted by other workers[16,17]. To restore the performance, the capillary was washed with 1N HCl (2 min) and 0.2N NaOH (5 min), and rinsing with water in between. However, as a result of such acid and alkaline washes, the electroosmotic flow varied considerably, and the day-to-day RSD for the migration times were greater than 3%. Under such circumstances, reliability of peak identification based on migration time was poor. Besides variations in the electroosmotic flow, other factors such as temperature fluctuations due to Joule heating and inadequate temperature control would also contribute to migration irreproducibility. To improve analyte identification and migration reproducibilty in capillary electrophoresis, the uses of migration indexes[18] and normalization of the migration time of the analytes[18,19] have been reported. In this study, the approach of normalizing migration time by dividing the migration time of the porphyrins by the migration time of a reference standard

was utilized. MP was used as the reference standard, and the RSD values for the reproducibility of the normalized migration times are presented in Table 1. It is clearly evident that the migration time reproducibility was greatly improved with the use of normalized migration times. Day-to-day RSD values ranged from 0.2 - 0.64%, corresponding to a minimum of 4-fold improvement.

Table 1

Reproducibilities of the Migration Times and Peak Areas of ZnPP and PP, With and Without Normalization.

(I)	ZnPP	PP
	Migration Times in Min (% RSD)	
Run-to-run (n = 8)	7.81 (1.1)	9.15(1.1)
Day-to-day (n = 8)	7.82 (3.1)	9.26 (3.4)
(II)	**Normalized Migration Times (% RSD)[a]**	
Run-to-run (n = 8)	0.89 (0.26)	1.05 (0.12)
Day-to-day (n = 8)	0.89 (0.64)	1.05 (0.20)
(III)	**% RSD for Absolute Peak Areas**	
n = 5	12.6	12.5
(IV)	**% RSD for Normalized Peak Areas[a]**	
n = 5	4.3	2.1

a Migration times and peak areas were normalized by dividing by those of MP.

In addition to improvement in migration reproducibility, MP could also serve as an internal standard in quantitation. Run-to-run variations and quantitation errors due to diffusion during injection[20,21], accidental hydrodynamic flow (caused by unequal sample and buffer elevations), surging of solutions in the capillary due to changing of the electrolyte reservoirs, and the like could be minimized by the use of an internal standard[22]. MP was chosen as the internal standard as it is not naturally occurring in whole blood, and was well separated from the porphyrins of interest, such that it does not interfere with porphyrin quantitation. The structural resemblance between MP, PP and ZnPP (all contain 2 carboxylic acid groups) implies that their migration characteristics would possibly be similar. The large RSD values observed for the peak areas of ZnPP and PP (Table 1) could be partly attributable to the adsorption of the porphyrins. When the ratios of the peak areas of the analytes to that of the internal standard were taken as responses, RSD values dropped considerably, in agreement with literature RSD values for analyses with internal standard (e.g., comparison with Table 1 in ref. 22).

Extraction of Blood

Different solvents have been employed for the extraction of erythrocyte porphyrins. Solvents such as DMSO-acetone mixture[9], aqueous acetone[10] and aqueous ethanol[12] have been used. Attempts to adopt these extraction methods with analysis by MEKC were however, not successful. In the case of DMSO-acetone mixture, whole blood was first prediluted with aqueous acetic acid before extracting with DMSO-acetone mixture[9]. When the supernatant was injected into the MEKC system, broad peaks were observed, probably due to the pH gradient existing across the boundaries between the acidic sample zone and the alkaline electrophoresis buffer. It was also noted that broader peaks were obtained for porphyrins dissolved in aqueous acetone and aqueous ethanol, compared to porphyrins dissolved in DMF. It appeared that the choice of sample solvent to be injected has an effect on the peak efficiency. DMF is a strong solvent for the porphyrins, is water miscible, and is suitable for our MEKC analysis. To date, there has been no report on the use of DMF as the extracting solvent. The feasibility of extracting erythrocyte porphyrins with DMF was thus evaluated in this investigation.

Unspiked blood and blood samples spiked with ZnPP and PP were extracted with different volumes of DMF containing MP as the internal standard. After centrifugation, the yellowish extracts were directly injected into

the capillary column. Peak areas of ZnPP and PP relative to that of MP were calculated and compared with those of the standards, taking into account the amounts of ZnPP and PP extracted from unspiked blood. The analytical recoveries of the porphyrins obtained with different volumes of DMF are summarized in Table 2. As can be seen, larger volumes of DMF resulted in higher analytical recoveries of the porphyrins. Recoveries greater than 94% were obtained when extracted with 1.5 mL of DMF. Besides acting as an extraction solvent, DMF also precipitates proteinaceous materials from the blood. With smaller volumes of DMF, the blood proteins were probably not precipitated sufficiently and thus interfered with the porphyrins' recovery. Although filtration is not required, attempts were made to remove fine particles possibly present in the supernatant solutions, to prevent clotting of the capillary. Poor recoveries of the porphyrins were obtained when polytetrafluoroethylene (PTFE) filters were used. About 50% of ZnPP and PP were retained on the filter, while only about 38% was recovered for MP. Similar observations were also reported when porphyrin solutions were filtered through Nylon membranes[23,24]. Other filter which has lower affinity for porphyrins was recommended, if the filtration step was deemed necessary[24].

Table 2

Effect of Volume of DMF on the Analytical Recovery of Porphyrins Added to Normal Blood.

	% Recovery		
Volume of DMF (mL)	**ZnPP (220 pmol added)**		**PP (50 pmol added)**
0.3	62		78
0.7	74		84
1.5	94	98	

Despite the quantitative analytical recovery achievable with DMF extraction, there are drawbacks of direct injection of the supernatant solution. The capillary suffered from short column life as the performance of the capillary deteriorated beyond restoration after about 60 injections. This was not observed with injections of porphyrin standards. Although no other fluorescent compounds or fluorescence quenching compounds which interfered with the electropherograms were observed, it appeared that compounds capable of interacting with the capillary wall and shortening the life of the capillary were

simultaneously being extracted. The yellowish colour of the extract also suggested the presence of other UV-absorbing substances. In addition, the use of large volumes of DMF inevitably results in the dilution of the porphyrin concentration, hampering detection especially for samples containing low concentrations of porphyrins. A solution to both problems is to perform sample cleanup and preconcentration on a solid phase extraction (SPE) cartridge. Solid particulates could also be removed from the sample solutions after passing through the cartridges.

Extraction with Sample Cleanup

Sample preparation on SPE cartridges have been reported for porphyrins extracted from urine, faeces and tissues[24-26]. However, no such treatment on erythrocyte porphyrins has been performed. The conditions for quantitative extraction of erythrocyte porphyrins and subsequent sample cleanup on commercially available C-18 cartridges were investigated in this study. The performance of two different sorbent capacities, 330 mg (Sep-Pak Plus) and 120 mg (Sep-Pak Light) were compared. Supernatants of spiked blood extracted with 1.5 mL of DMF was first diluted with 2 mL of 0.5N acetic acid before loading into the C-18 cartridge. The purpose of the dilution step is to modify the polarity of the solvent so that porphyrins would be retained on the C-18 cartridge. Upon loading of the sample solution, a dark red band was formed near the inflow of the cartridge. No movement of the band was observed with subsequent washing with water and 20% aqueous DMF solution. When eluted with DMF, the red band diffused into broader bands, indicating differential elution. No signals were obtained from the first 120 mL and 350 mL of eluates from the Sep-Pak Light cartridge and Sep-Pak Plus cartridge, respectively. These volumes were needed to displace the residual water from the cartridges and to move the porphyrin band further down the cartridges. To elute the retained porphyrins, 600 mL of DMF was needed for the Sep-Pak Plus cartridge, while most of the porphyrins were detected in eluates of 300 mL for the Sep-Pak Light cartridge. The eluates were slightly yellowish in color, with most of the pigments still retained on the cartridges, demonstrating the effectiveness of the SPE cartridges in removing most of the matrix components. The Sep-Pak Light cartridge was judged to be superior as the analytes could be retained and collected with half the volume of eluant needed for the Sep-Pak Plus cartridge.

In most of the previous studies, blood samples were prediluted with an appropriate aqueous solution to achieve a more complete extraction[9,12]. Rossi et al.[9] reported a better extraction efficiency when the blood was prediluted with 0.5N acetic acid, instead of water. In our study, DMF extraction of unspiked blood, with and without predilution with acetic acid, followed by sample cleanup were compared. With predilution, extraction efficiencies of ZnPP and PP were both increased, with a considerable increase in the case of ZnPP. The increase is presumably attributable to the lysing of the erythrocytes, releasing the more strongly bound ZnPP[27]. The supernatants from DMF extraction with predilution with acetic acid, however, gave rise to broad peaks when injected directly into the MEKC system, probably due to the pH differences existing at the boundaries between the sample zone and the electrophoresis buffer. Hence with sample cleanup, exchange of the sample solution to one that is more appropriate for injection could be performed. The column life of the capillary was also prolonged as a consequence of sample cleanup. More than 120 injections could be carried out with reasonable performance of the capillary.

Analytical recoveries of ZnPP and PP from the Sep-Pak Light cartridge were first evaluated with standard porphyrin solutions in DMF, following the washing and elution steps described under Experimental. Near quantitative recoveries (> 95%) of the porphyrins were obtained, indicating minimum loss of analytes from the washing step and the effectiveness of DMF elution. Next, analytical recoveries of porphyrin standards spiked into normal blood, together with the concentrations of ZnPP and PP originally present in the blood sample were determined by the method of standard additions. A series of normal blood samples spiked with porphyrin standards were prepared for the calibration plot. These blood samples were then subjected to the extraction procedures and analysed by MEKC. The ratios of the peak areas of ZnPP or PP to that of MP were plotted against the amount of porphyrins added. Correlation between the relative peak areas and the amount of added porphyrins were generally good in the concentration range of 0.035 - 0.67 μmol/L for PP, and 0.16 - 2.9 μmol/L for ZnPP. Due to the higher fluorescence of PP than ZnPP, considerably lower concentration range was studied for the extraction recoveries of PP. Correlation coefficient values of at least 0.993 were obtained for both the ZnPP and PP lines. Extrapolation of the lines to the horizontal axis gave a measure of the porphyrins content in normal blood. The ZnPP value was found to be 0.27 μmol per liter of whole blood, and PP was found to be 0.03 μmol per liter of whole blood. These values fall into the range reported in literature[9]. To calculate the analytical recoveries of the porphyrins, relative peak areas of extracted ZnPP and PP were compared to those for the

standard solutions, corrected for the amount of porphyrins already present in the blood. The analytical recoveries are shown in Table 3. The recovery varied from 88 - 100%. The reproducibilities for the extraction recovery of ZnPP and PP were evaluated by repeating 5 analyses on blood samples from one normal human subject. RSD values were about 6% for both porphyrins.

Table 3

Analytical Recovery of ZnPP and PP Added to Normal Blood.

Compound	Added Amount (pmol)	% Recovery [a]
ZP	70	88
	240	99
	590	92
PP	15	96
	501	100
	120	98

[a] Spiked blood samples were prediluted with 0.5N acetic acid, extracted with DMF, cleaned up on Sep-Pak Light cartridges, and analysed by MEKC, as described under Experimental. The results represent the average of triplicate extractions. The concentrations of endogeous ZnPP and PP were subtracted prior to calculating recoveries.

Fig. 2 shows an electropherogram of a blood extract from a normal adult female human subject. The slight difference in the migration times of the porphyrins in Figs. 1 and 2 was probably due to matrix effects. Coproporphyrin I, which was extracted in some blood samples[8,11], was not detected in this study. Its presence , however, would not cause an interference problem, as it migrates between ZnPP and MP in our electrophoretic conditions. The limits of detection (LOD) determined from the actual injections of low concentrations of the porphyrins were found to be 50 nmol/L and 8 nmol/L for ZnPP and PP, respectively, at a signal-to-noise ratio of 3. With an injection volume of approximately 10 nL, the minimum detectable amounts for ZnPP and PP were about 500 amol and 80 amol, respectively. Further improvement in the LOD for ZnPP could be achieved with the proper choice of excitation wavelength. An excitation filter centered at 410 nm and wider bandwidth (e.g., 20 nm) should sufficiently cover the range of excitation

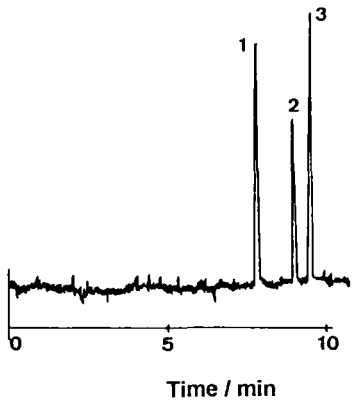

Figure 1. Optimized MEKC separation of ZnPP, PP and MP. Conditions: buffer, 20 mmol/L CAPS (pH 10.8), 20 mmol/L SDS, 8% DMF; capillary, 65 mm i.d. x 67.2 cm (45.2 cm to the detector); applied voltage, 21 kV. Peaks: 1 = ZnPP (0.2 mmol/L), 2 = MP (0.05 mmol/L), and 3 = PP (0.07 mmol/L).

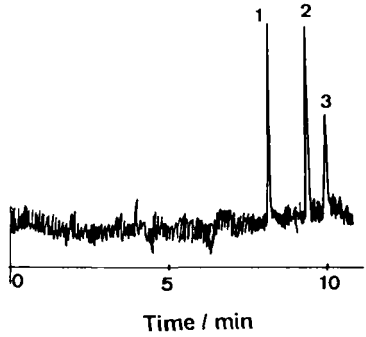

Figure 2. Electropherogram of a normal adult female blood extract. Electrophoretic conditions and peak identities same as in Fig. 1.

wavelengths required for maximum fluorescence of ZnPP and PP. Nevertheless, the sensitivity achieved with epi-illumination fluorescence microscopy detection was comparable to those reported using capillary electrophoresis / laser-induced fluorescence system[16], and approaching those reported for HPLC / fluorescence system[17].

CONCLUSIONS

This paper demonstrated that simultaneous extraction of ZnPP and PP from blood samples was possible with DMF. With sample cleanup on a C-18 SPE cartridge, quantitative analytical recoveries of ZnPP and PP of greater than 88% were achieved. Although the extracts were slightly yellowish in color, effective separation coupled with specific fluorescence detection permitted analysis of the porphyrins with very little interference by endogenous compounds. The porphyrins were identified by their normalized migration times, with respect to that of an internal standard, MP. The C-18 cartridge acted as a guard column to protect the capillary from undesirable adsorption of contaminants, thus aiding in prolonging the column life. It also improved the sensitivity of the method by preconcentrating the analytes. The LODs obtained for ZnPP and PP were 50 nmol/L and 8 nmol/L, respectively.

ACKNOWLEDGMENTS

The authors thank the National University of Singapore for financial assistance. Thanks also goes to Dr Philip Marriott of Royal Melbourne Institute of Technology (Australia) for advice and assistance in obtaining samples..

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Received April 19, 1995
Accepted May 9, 1995
Manuscript 3841