On-line Sample Preparation for High Throughput Reversed-phase LC/MS Analysis of Combinatorial Chemistry Libraries

Liang Tang*[1], William L. Fitch[1], Peter Smith[2], David Tumelty[1], Kathy Cao[1] and Steven W. Ferla[1]

Affymax Research Institute[1], 4001 Miranda Avenue, Palo Alto, CA 94304, LEAP Technologies, Inc.[2], PO Box 969, Carrboro, NC 27510, USA

Abstract: An on-line sample preparation method utilizing a time-programmed autosampler is described for high throughput liquid chromatography/mass spectrometry (LC/MS). This approach is particularly helpful for the LC/MS analysis of samples which require solvents incompatible with HPLC in the sample preparation process. The on-line sample preparation approach minimizes a bottleneck in throughput and improves sample recovery under some circumstances.

INTRODUCTION

Since liquid chromatography/mass spectrometry (LC/MS) combines the separation capability of liquid chromatography with the structural identification capability of mass spectrometry, it is extensively applied to the structural elucidation of combinatorial libraries, as well as other fields in pharmaceutical research. High throughput analysis is a requirement of contemporary LC/MS technology, and various automated sample preparation techniques have been developed to complement the speed requirements of high throughput analysis [1-4]. Effective use of robotic sample preparation techniques can maximize throughput in chemical analysis.

LC/MS has advantages over direct infusion of a sample into a mass spectrometer for analysis. The advantages include clean mass spectra and minimized interference from impurities in the sample [5]. However, chromatographic introduction can restrict the solvents which are utilized for sample preparation, as, for example, a strong organic solvent that can induce poor initial retention on a column during reversed phase LC/MS. In this research, an on-line sample preparation method is described which allows strong organic solvents to be used without compromising chromatographic quality.

*Address correspondence to this author at the Tularik, Inc., Department of Pharmacology and Preclinical Development. 2 Corporate Drive, South San Francisco, CA 94080, USA; TEL: 650-825-7071; FAX:650-825-7303; Email: ltang@tularik.com

A particular application of LC/MS at Affymax is high throughput single bead LC/MS for quality control of encoded combinatorial libraries [6,7]. In our previous method, samples were photocleaved in 100% methanol and solvent exchanged into methanol/water (10/90) prior to injection. This solvent exchange can be both a source of contamination and of poor recovery in trace analysis. An alternative approach to improving the chromatography would be to limit the injection volume. An injection volume of a few microliters of strong solvent would not lead to peak breakthrough. In practice, however, sufficient organic solvent must be used to ensure that the bead is immersed in the solvent during cleavage. Since trace analysis needs all the sensitivity available, injecting most of the sample is imperative. Difficulty would be encountered with only a few microliters of solvent. Furthermore, the identification of ligand in the encoded library is followed by a decoding experiment where the presence of residual ligand might interfere. Removal of 90% of the ligand from the bead for the LC/MS analysis is thus very important. The on-line sample preparation approach introduced in this article facilitates the automated mixing of the solvents for a sample. A comparison will be made between conventional sample preparation and on-line sample preparation. The application of on-line sample preparation in high throughput combinatorial library analysis will also be discussed.

1386-2073/01 $28.00+.00

EXPERIMENTAL SECTION

1. Instrumentation

LC/MS experiments were carried out using an Agilent (Palo Alto, CA) 1100 MSD (Model G1946A) quadrupole mass spectrometer, equipped with a vacuum degasser (Model G1322A), a binary pump (Model G1312A), autosampler (CTC PAL system from LEAP Technologies in Carrboro, NC), a thermostatted column compartment (Model G1316A) and a diode array detector (Model G1315A) equipped with a long-life deuterium lamp. The LC/MS instrument was controlled by ChemStation Rev. A07.01 from Agilent, and the autosampler was controlled by Cycle Composer V1.4.0. from LEAP Technologies. The autosampler and mass spectrometer were controlled by contact-closure.

2. On-line Sample Preparation

A single-cycle macro was written for the CTC PAL autosampler to execute the sample preparation steps and the injection. The macro was copied into the macro folder of Cycle Composer prior to its operation. The on-line sample preparation has the following steps:

Step 1: Wash injector syringe with solvent in bottle 1 (washing bottle), and rinse the syringe with the solvent in bottle 2 (solvent bottle).

Step 2: The syringe delivers a required volume of the solvent in bottle 2 to the first sample vial at the start of analysis, or next sample vial when a sample in the queue is being analyzed by LC/MS. The maximum volume of solvent delivered by a syringe depends on the syringe size. In this work, the syringe size is 100 μL.

Step 3: Mix solution in the sample vial one or more times.

Step 4: Wait for ready signal to inject the sample.

Step 5: Stop this sequence at the end of sample queue in a sample tray.

3. Reversed Phase HPLC Columns

A 30 mm x 3 mm (i.d.) Polaris2000 C_{18} column packed with 3 μm particles was obtained from MetaChem Technologies (Torrance, CA). A 50 mm x 4.6 mm (i.d.) Eclipse XDB-C_8 column packed with 3.5 μm particles was obtained from MacMod (Chadds Ford, PA).

4. Chemicals

Reagent grade reserpine and dioctyl phthalate were purchased from Aldrich Chemical Company (Milwaukee, WI). 4-Isopropylphenoxyacetic acid was reagent grade and was obtained from Lancaster Synthesis (Windham, NH). Fmoc-lysine was purchased from NovaBiochem (San Diego, CA). The benzimidazole was synthesized in-house. HPLC-grade water and acetonitrile were purchased from AlliedSignal, Burdick & Jackson (Muskegon, MI). Formic acid was HPLC grade from EM Science (Gibbstown, NJ). The water and acetonitrile HPLC mobile phases were prepared by adding formic acid to a concentration of 0.05% (v/v).

The polymer resins TentaGel HL-NH2, high loaded beads with mean particle size of 110 μm and capacity of 0.47 mmole/g, and macrobeads TentaGel MB Br with mean particle size 150 μm and capacity 0.48 mmole/g were obtained from Rapp Polymere (Tübingen, Germany). TentaGel resin coupled with photolabile linker and tags was prepared as described previously [7].

RESULTS AND DISCUSSION

1. On-line Sample Preparation

Typically, a sample for LC/MS analysis is prepared in a solvent which is appropriate for optimum HPLC performance. For gradient reversed phase HPLC, the injection solvent should be less hydrophobic than the initial composition of the gradient. However, solvents incompatible with optimal HPLC separation requirements are demanded under some circumstances. For example, the optimum solvents for cleavage of ligands which are linked to polymer resin with a photo-labile linker are strong organic solvents such as acetonitrile and dimethyl sulfoxide (DMSO) [8]. The direct injection of these solvents

onto a reversed phase HPLC column might give poor chromatography. If the analyte is hydrophilic, the injection solvent might elute the analyte in the void volume. Fmoc-lysine is a compound exhibiting such HPLC separation problems. Figure 1A illustrates the poor chromatogram obtained when 20 µL of 5 µM Fmoc-lysine in acetonitrile was loaded onto a 30 mm x 3 mm Polaris2000 C_{18} column. Part of the analyte is unretained. This problem can be easily eliminated by diluting the sample with water prior to injection onto the HPLC column. Figure 1B shows that normal separation was obtained if 20 µL of 5 µM Fmoc-lysine was injected in a solvent of 50:50 (v/v) acetonitrile/water.

The standard procedure to achieve the separation of Fmoc-lysine in acetonitrile as shown in Figure 1B involves opening the sample container, diluting the strong organic solvent with water, mixing the solvents, and re-closing the vial. This is a laborious process which is incompatible with a high throughput LC/MS analytical laboratory. Such a task could be more efficiently accomplished by using an on-line sample preparation procedure such as programming an autosampler to add a specific amount of pure water into a sample immediately prior to its analysis.

2. Effect of On-line Sample Preparation on Sample Recovery

A. Quantitative Measurement of Analyte Concentration by LC/MS

In order to evaluate the efficacy of on-line sample preparation and compare the experimental results of on-line sample preparation with those of

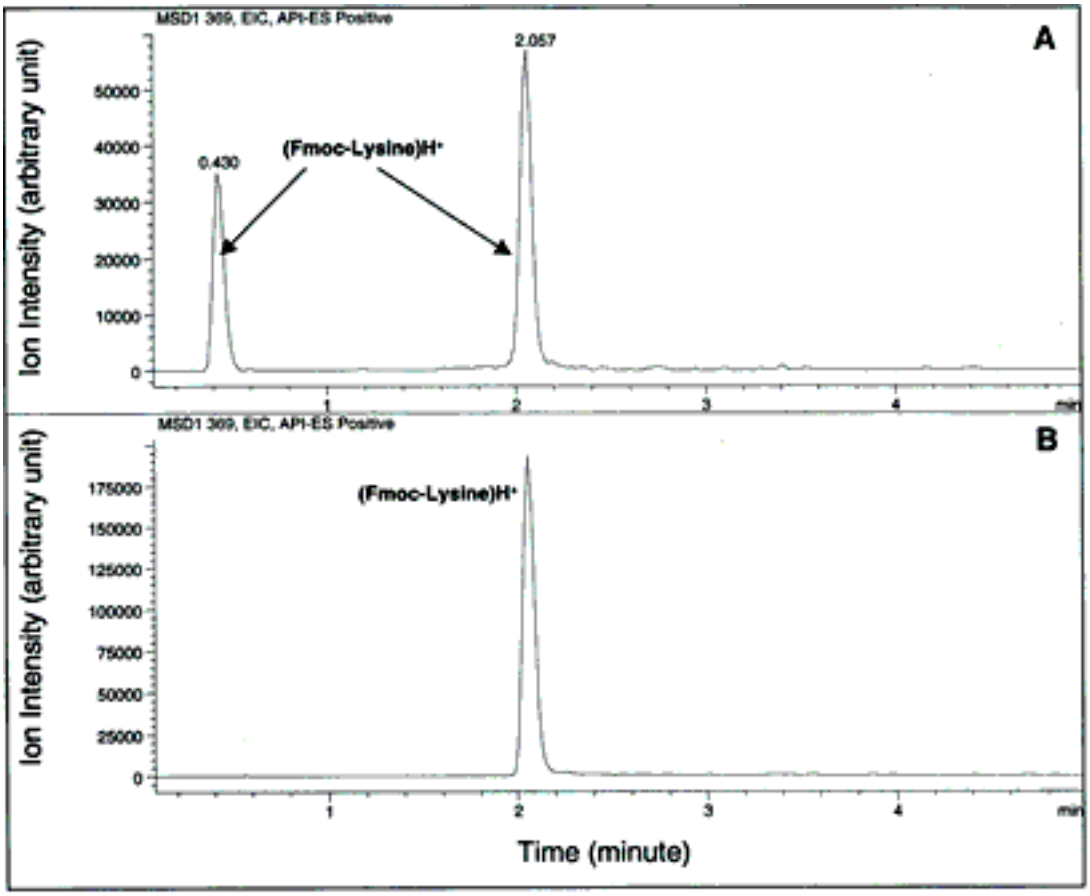


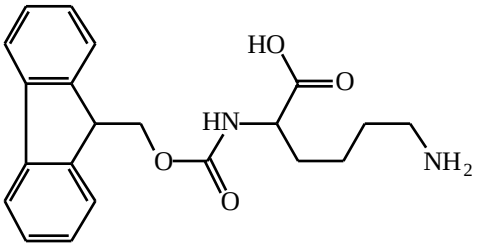
Fig. (1). Computer-reconstructed mass chromatograms of 37 ng Fmoc-lysine loaded on a 30 mm x 3 mm x 3 µm Polaris2000 C_{18} column. (A) Fmoc-lysine in 20 µL pure acetonitrile showed split-peaks in the chromatogram due to the large volume of organic solvent in the sample and the fast gradient. (B) Fmoc-lysine in 20 µL 50:50 (v/v) acetonitrile/water solution was effectively retained on the column and provided stronger ion signal under the same LC/MS conditions as in (A). Mobile phase, A = 0.05% formic acid in water (v/v) and B = 0.05% formic acid in acetonitrile (v/v). Gradient 5-95% B in 3 min. Flow rate 0.6 mL/min and column temperature 40^{o} C.

conventional sample preparation, the concentration of each analyte in solution was measured using LC/MS. Quantitative analysis was based on the peak area of the analyte analyzed by LC/MS in the selected ion monitoring mode. This evaluation focused on the low concentration range in which there might be concerns regarding the adsorption of analyte to the sample container or to the resin used during the combinatorial library synthesis, and the possible influence of sample preparation procedure on the detection limits. For quantitative analysis, an external standard calibration was used to avoid possible competition in adsorption due to the introduction of a reference standard to the samples. DMSO was selected to prepare external standard solutions because DMSO has versatile solubilization characteristics.

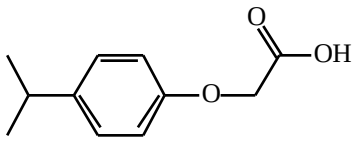
B. Chemical Properties and Sample Recovery

In the screening of a combinatorial library resulting from solid phase organic synthesis, it is assumed that ligand is best cleaved from a bead soaked in 100% organic solvent that can dissolve the ligand and release the ligand from the bead. Also, the use of solvents such as DMSO, acetonitrile and methanol is critical to achieve a good yield of the photocleavage product [8]. As described above, injection of samples dissolved solely in the strong organic solvents could be detrimental to chromatographic efficiency. Thus, on-line sample preparation might be necessary to obtain both high cleavage yield and a solution compatible with HPLC for robotic and high throughput analysis.

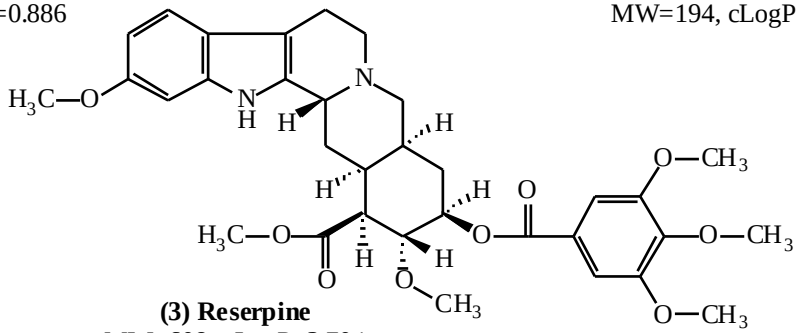
The compounds selected to study sample recovery yield by comparing conventional sample preparation and on-line sample preparation were Fmoc-lysine (1), 4-isopropylphenoxyacetic acid (2), reserpine (3), a benzimidazole derivative (4), and dioctyl phthalate (5). As shown in Figure 2, these compounds span the range of hydrophobicities from polar (cLogP<1) to extremely nonpolar (cLogP>8).



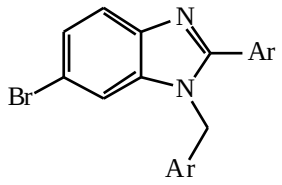
(1) Fmoc-Lysine
MW=368, cLogP=0.886



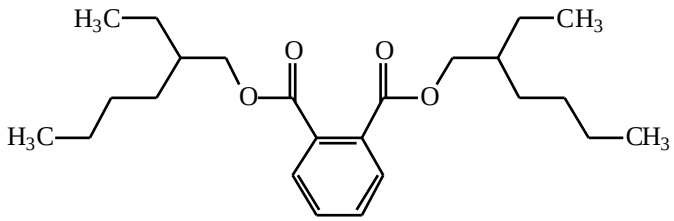
(2) 4-Isopropylphenoxyacetic acid
MW=194, cLogP=2.777



(3) Reserpine
MW=608, cLogP=3.704



(4)
MW=405, cLogP=7.560



(5) Dioctyl phthalate
MW=390, cLogP=8.706

Fig. (2). Structures, molecular weights, and cLogP values of the organic compounds used to study on-line sample preparation.

The single bead analysis procedure in use at Affymax leaves the bead in the sample container used for compound cleavage [6]. Subsequent decoding [7] of the same bead completes the analysis. To simulate the single bead analysis situation, 10 pmole of each model compound, which is approximately 10% of the ligand capacity on a resin bead used in the study, was used in each sample to investigate recovery yield. The experiment made use of two types of unmodified resin, TentaGel HL NH2 and TentaGel MB Br, which are extensively used in combinatorial library synthesis. A synthetic TentaGel resin with both photo-linker and dialkylamine tags [7] was also used in this test. In the investigation of recovery, experiments were carried out with a single bead in each sample container to observe if sample molecules would be adsorbed on the resin.

All model compounds were initially dissolved in DMSO. A 10 μL aliquot of a 1 μM DMSO solution of each compound was loaded into a glass conical vial, which either was empty or contained a single bead. Next, 20 μL pure water was added to each vial to yield a final concentration of 0.33 μM. For conventional sample preparation, water was manually added into each conical vial, which was then vortexed for 15 h to ensure solvent mixing and interaction of the sample with glass and porous resin bead [9] as well as to equal the time that would be used for normal sample storage prior to analysis. For on-line sample preparation, a 10 μL aliquot of the compound in DMSO solution was also vortexed for 15 h for the same reasons, and then 20 μL of water was added and mixed at least once by the CTC syringe injector before the sample was analyzed using LC/MS.

Fmoc-lysine was tested to see the effect of solvent mixing strokes during the on-line sample preparation. As depicted in Figure 3, one syringe-stroke of mixing was adequate to assure recovery. There was no evidence that the presence or absence of a resin bead affected sample recovery. Compounds 1-4 showed >70% recovery in all experiments. Only the most hydrophobic compound, dioctyl phthalate, showed a significant discrepancy between conventional and on-line sample preparation. The difference was probably the result of poor solubility of this compound in water. When the sample was prepared using the conventional approach, the material in the mixed

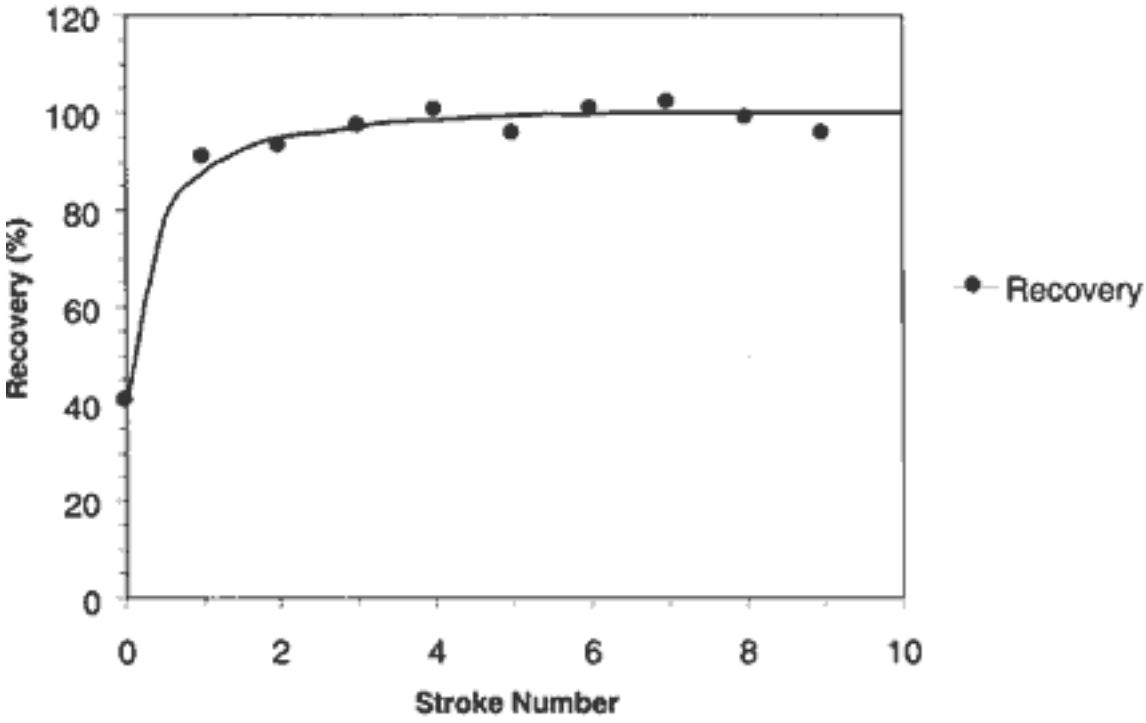


Fig. (3). Effect of sample amalgamation on Fmoc-lysine recovery using on-line sample preparation. Water (20 μL) was added to 1 μM Fmoc-lysine in acetonitrile (10 μL). A 100 μL syringe was stroked to full scale at a speed of 2 μL/sec per stroke. Fmoc-lysine was assumed to be completely dissolved in 1:3 (v/v) acetonitrile/water at concentrations of $\leq$ 1 μM. Fmoc-lysine in 1:3 (v/v) acetonitrile/water solution prepared by using conventional sample preparation with extensive vortexing was assumed to have 100% recovery in a glass vial.

Table 1. Effect of Sample Preparation on Dioctyl Phthalate Recovery

Approach (1)	Resin Bead	Amt.(pmole)	Ave. Amt.(pmole)	STD DEV	CV%	Recovery (%) (2)
	TentaGel HL NH_2	1.68	1.25	0.27	21.6	12.5
	Pre-mix	1.17				
		1.32				
		1.13				
		0.96				
	TentaGel MB Br	1.40	1.11	0.20	17.8	11.1
	Pre-mix	1.20				
		1.09				
Conventional		0.93				
Sample		0.93				
Preparation	TentaGel+Linker&Tag	1.25	1.15	0.07	6.3	11.5
	Pre-mix	1.21				
		1.13				
		1.12				
		1.06				
	No Bead	1.49	1.23	0.16	13.2	12.3
	Pre-mix	1.28				
		1.15				
		1.12				
		1.09				
	TentaGel HL NH2	5.66	5.89	1.07	18.2	58.9
	Online-Mix	4.62				
		6.45				
		5.31				
		7.40				
	TentaGel MB Br	6.05	6.29	0.22	3.5	62.9
	Online-Mix	6.26				
		6.58				
Online		6.11				
Sample		6.44				
Preparation	TentaGel+Linker&Tag	6.24	6.43	0.39	6.0	64.3
	Online-Mix	7.03				
		6.56				
		6.27				
		6.03				
	No Bead	5.83	6.34	0.56	8.8	63.4
	Online-Mix	5.73				
		7.09				
		6.51				
		6.52				

(1) 20 μL pure water was added into 10 μL dioctyl phthalate in DMSO solution with 1 μM as initial concentration.
(2) Dioctyl phthalate was assumed to be completely dissolved in DMSO at ≤ 1 μM. Dioctyl phthalate in DMSO solution with conventional sample preparation using vortexing was assumed to have 100% recovery in glass vials.

solvent had enough time to precipitate from solution phase before the solution was analyzed. However, on-line preparation optimized solvent mixing and minimized sample precipitation. In general, on-line sample preparation gave much higher recovery. As shown in Table 1, samples prepared on-line provided a recovery five times greater than that observed when using the conventional method.

CONCLUSIONS

On-line sample preparation can increase the efficiency of high throughput LC/MS analysis. In addition to reducing the probability of contamination that results from employing a manual preparation process, it can also increase sample recovery in the special case where solvent requirements for sample preparation and chromatography are incompatible. At a nominal concentration of 0.33 μM, there was no loss of compounds of moderate hydrophobicity (up to cLogP=7.5) due to adsorption onto TentaGel resins. At this concentration, only the most hydrophobic compounds (as illustrated by dioctyl phthalate of cLogP=8.7) are likely to precipitate during LC/MS sample preparation.

REFERENCES

[1] Watt, A. P., Morrison, D., Evans, D. C., *Drug Discovery Today*, **2000**, *5(1*), 17-24

[2] Harness, J. P., *Chim. Oggi*, **1999**, *17(1-2)*, 22-24

[3] Schmid, I., Sattler, I., Grabley, S., Thiericke, R., *J. Biomol. Screening*, **1999**, *4(1)*, 15-25

[4] Whitney, J. L., Kerns, E. H., Rourick, R. A., Hail, M. E., Volk, K. J., Fink, S. W., Lee, M. S., *Pharm. Technol.*, **1998**, *22(5)*, 76-82

[5] Tang, L., Kebarle, P., *Anal. Chem.*, **1993**, *65*, 3654-3668

[6] Lewis, K.C., Fitch, W.L., MacLean, D. *LCGC*, **1998**, *16*, 644

[7] Fitch, W. L., Baer, T. A., Chen, W. W., Holden, F., Holmes, C. P., MacLean, D., Shah, N., Sullivan, E., Tang, M. and Waybourn, P., *J. Comb. Chem*. **1999**, *1*, 188-194

[8] Holmes, C. P., *J. Org. Chem.*, **1997**, *62(8*), 2370-2380

[9] Rapp, W., In *Combinatorial Peptide and Nonpeptide Libraries*; Gunther Jung, Ed., VCH Verlagsgesellschaft, Germany, **1996**, Chapter 16, pp 425 – 463.