
HPLC IN ENZYMATIC ANALYSIS

SECOND EDITION

Edward F. Rossomando
University of Connecticut Health Center

with the collaboration of

Zdenek Deyl, Academy of Sciences of the Czech Republic

Jan Kehr, Karolinska Institute

David Lambeth, University of North Dakota

Ivan Mikšík, Academy of Sciences of the Czech Republic

Franco Tagliaro, University of Verona

Kathi J. Ulfelder, Beckman Instruments



WILEY-INTERSCIENCE

A JOHN WILEY & SONS, INC., PUBLICATION

New York • Chichester • Weinheim • Brisbane • Toronto • Singapore

CONTENTS

Preface	xix
Preface to the First Edition	xxi
Collaborators	xxiii
1. Application of HPLC to the Assay of Enzymatic Activities	1
<i>Overview</i>	1
1.1 Introduction	1
1.2 Anatomy of an Enzyme Assay	2
1.3 Classification of Enzymatic Assay Methods	3
1.3.1 Continuous Methods	3
1.3.2 Coupled Method	4
1.3.3 Discontinuous Method	5
1.3.4 HPLC as a Discontinuous Method	6
1.4 Criteria for the Selection of an Assay Method	10
1.4.1 Separation and Detection of Components	10
1.4.2 The Reaction Mixture	10
1.4.3 The Enzyme Sample	11
1.5 Summary and Conclusions	11
General References	12
2. Concepts and Principles of High Performance Liquid Chromatography	13
<i>Overview</i>	13
2.1 Introduction	13
2.2 The Introduction of HPLC	14
2.3 Basic Components and Operation	15
2.4 Coupling the Components: On the Perils of Ferrules	16
2.5 The Chromatogram	18
2.6 Interpretation of the Chromatogram	19
2.7 Selection of the Stationary Phase: Some Help from an Understanding of the Process of Separation	23
2.7.1 Gel Filtration Chromatography	24
2.7.2 Reverse-Phased Chromatography	25
2.7.3 Ion-Exchange Chromatography	30

2.8	Composition and Preparation of the Mobile Phase	35
2.9	Column Maintenance	36
2.10	Monitoring Column Performance	37
2.11	Summary and Conclusions	37
	General References	39
3.	Concepts and Principles of High Performance Capillary Electrophoresis	41
	<i>with Franco Tagliaro, Zdenek Deyl, Ivan Mikšič, and Kathi J. Ulfelder</i>	
	<i>Overview</i>	<i>41</i>
3.1	Introduction	41
3.2	HPCE: Definition, History, and Literature	41
3.3	Basic Components and Operations	42
3.4	The Process of Electrophoretic Separation	43
3.4.1	Electrophoretic Separation	43
3.4.2	Electroosmosis	44
3.5	Instrumentation in Detail	46
3.5.1	Injection	46
3.5.1.1	Hydrodynamic Injection by Pressure/ Vacuum Application	47
3.5.1.2	Electrokinetic Injection	47
3.5.2	The Capillary	48
3.5.3	Power Supply	49
3.5.4	Detection	50
3.5.4.1	Absorbance Detection	50
3.5.4.2	Fluorimetric, Electrochemical Detection, and other Detection Modes	51
3.5.4.3	Mass Spectrometric Detection	51
3.5.4.4	Indirect Detection	52
3.6	Separation Efficiency and Resolution	52
3.6.1	Theoretical Plate Number and Resolution	52
3.6.2	Practical Hints	54
3.7	Methods	55
3.7.1	Capillary Zone Electrophoresis (CZE)	55
3.7.2	Micellar Electrokinetic Chromatography (MEKC)	55
3.7.3	Capillary Isotachophoresis (CITP)	58
3.7.4	Capillary Gel Electrophoresis (CGE)	59
3.7.5	Capillary Isoelectric Focusing (CIEF)	60
3.7.6	Chiral Separations	60
3.8	Summary	61
	References	62

4. Strategy for Design of an HPLC System for Assay of Enzyme Activity	64
<i>Overview</i>	64
4.1 Setting Up the Assay	64
4.1.1 Analysis of the Primary Reaction	64
4.1.2 Analysis of Secondary Reactions	65
4.1.3 Selection of the Stationary Phase and Method of Elution	65
4.1.4 Modification of Reaction Conditions for the HPLC Assay Method	68
4.1.5 Understanding and Dealing with Secondary Reactions	68
4.1.6 Components of the Reaction Mixtures Can Cause Problems: Effects of Metals on Separation	71
4.1.7 Terminating the Reaction	73
4.1.8 Setting Up the Reaction Conditions	76
4.1.9 Detector Sensitivity	77
4.1.10 Summary and Conclusions	79
4.2 The Use of HPLC to Establish Optimal Conditions for the Enzymatic Reaction	81
4.2.1 Initial Decisions: Composition of the Reaction Mixture	81
4.2.2 Obtaining Initial Rate Data	82
4.2.3 Quantitative Analysis of the Reaction	83
4.2.4 Initial Rate Determination at Low Substrate Concentrations	85
4.2.5 The "Sensitivity Shift" Procedure	86
4.2.6 Substrate Analogs: Their Use in Limiting Secondary Reactions	86
4.2.7 Summary and Conclusions	87
References	90
General References	90
5. Strategy for the Preparation of Enzymatic Activities from Tissues, Body Fluids, and Single Cells	92
<i>Overview</i>	92
5.1 Introduction	93
5.1.1 The First Goal: Selection of the Biological Starting Point	93
5.1.2 The Second Goal: Determining the Extent of the Purification, or End Point	95

5.2	Preparation and Assay of Enzymatic Activities in Samples of Tissues, Organs, and Biological Fluids	97
5.2.1	Separation of Cellular from Extracellular Compartments	97
5.2.1.1	Samples Obtained Directly from an Organism	97
5.2.1.2	Samples Obtained from Tissue or Organ Culture	98
5.2.1.3	Samples Obtained from Biological Fluids	99
5.2.1.4	Samples Obtained from Cell Cultures	100
5.2.2	Assay of Activities in the Extracellular Compartment	100
5.2.3	Assay of Activities in the Cellular Compartment	100
5.3	Preparation and Assay of Activities in Intact Cells	103
5.4	Preparation and Assay of Activities in Subcellular Samples	103
5.5	Initial Purification and Assay of Activities in Cell-Free Lysates	105
5.6	HPLC for Purification of Enzymes: A Brief Background	106
5.7	Strategy for Use of HPLC in the Purification of Activities	107
5.8	Problems Related to the Assay of Activities Following Their Purification by HPLC	112
5.9	Summary and Conclusions	113
	General References	114
6.	Microdialysis: An In Vivo Method for the Analysis of Body Fluids	115
	<i>Overview</i>	<i>115</i>
6.1	Introduction	116
6.1.1	Principle of In Vivo Microdialysis	116
6.1.2	Extracellular Space	116
6.1.3	Microdialysis Probe	117
6.1.4	Dialysis Recovery	118
6.2	Technical Aspects of Microdialysis	119
6.2.1	Microdialysis Instrumentation	119
6.2.2	HPLC Analysis	121
6.2.3	Performing a Microdialysis Experiment on a Rat	122
6.2.4	Performing a Microdialysis Experiment on a Human	122
6.3	Applications of Microdialysis/HPLC in Enzymatic Analysis	123
6.3.1	Body Fluids Sampled by Microdialysis	123
6.3.1.1	Blood	123

6.3.1.2	Cerebrospinal Fluid (CSF)	124
6.3.1.3	Vitreous Humor	124
6.3.1.4	Synovial Fluid	124
6.3.1.5	Perilymph	124
6.3.1.6	Bile	124
6.3.2	Typical Analytes: Small Molecules	125
6.3.3	Estimating Enzymatic Activities	126
6.3.3.1	Studying Enzymatic Activities in Almost Intact Environments/Cellular Compartments	126
6.3.3.2	Measuring the Entire Time Course in a Single Experiment	126
6.3.3.3	Investigating the Effects of Cofactors and/or Drugs, in Small, Localized Tissue Structures	128
6.3.3.4	Testing Drugs That Do Not Penetrate the Blood-Brain Barrier	128
6.3.3.5	Estimating Enzymatic Activities Under Various Physiological/Pathological Stimuli	128
6.3.3.6	Microdialysis Sampling of Enzymes	129
6.4	Conclusions	129
	References	130
7.	Fundamentals of the Polymerase Chain Reaction and Separation of the Reaction Products	137
	<i>with Kathi J. Ulfelder</i>	
	<i>Overview</i>	<i>137</i>
7.1	Introduction: Polymerase Chain Reaction	137
7.2	Principles of Nucleic Acid Separation	139
7.2.1	Separation Mechanism	139
7.2.2	Classical Methods of DNA Analysis	141
7.3	CE Methods: Principles and Strategies for Nucleic Acids	141
7.3.1	CE Principles Related to Nucleic Acids	141
7.3.2	Buffer Systems	141
7.3.3	Intercalators	142
7.3.4	Typical Instrument Parameters	144
7.3.5	Detection	144
7.3.6	Data Analysis	146
7.3.7	Sample Preparation and Injection Considerations	146
7.3.8	Artifacts	150

7.4	PCR Applications	151
7.4.1	Forensic Analysis	151
7.4.2	Identification by Hybridization	151
7.4.3	Quantitative Analysis	153
7.4.4	Quantitative RNA-PCR	156
7.5	Conclusion: Future Applications	160
	References	160
8.	Applications of HPLC/HPCE in Forensics	164
	<i>with Franco Tagliaro, Zdenek Deyl, and Ivan Mikšik</i>	
	<i>Overview</i>	<i>164</i>
8.1	Introduction	164
8.2	Forensic Toxicology	165
8.3	HPCE Analysis of Illicit Drug Substances	165
8.4	Analysis of Gunshot Residues and Constituents of Explosives	172
8.5	Analysis of Pen Inks	175
8.6	Analysis of Proteins in Forensics	176
8.6.1	Separation of Proteins by HPCE	176
8.6.2	Separation of Protein Mixtures by Two-Dimensional Techniques	178
8.7	Analysis of Nonenzymatically Modified Proteins	179
8.7.1	Nonenzymatic Modifications to Keratins by Ethanol	179
8.7.2	Nonenzymatic Modifications to Collagen by Glucose	183
8.8	Enzymatic Activity Assay by Capillary Electrophoresis	185
8.9	Protein-drug Binding Assays	192
8.10	Nucleic Acids and Their Constituents	196
8.11	Conclusion	200
	References	200
9.	Survey of Enzymatic Activities Assayed by the HPLC Method	207
	<i>with David Lambeth</i>	
	<i>Overview</i>	<i>207</i>
9.1	Introduction	208
9.2	Catecholamine Metabolism	208
9.2.1	Tyrosine Hydroxylase	208
9.2.2	5-Hydroxytryptophan Decarboxylase	211
9.2.3	Dopa Decarboxylase (L-Aromatic Amino Acid Decarboxylase)	212
9.2.4	Dopamine β -Hydroxylase	215

9.2.5	Catechol <i>O</i> -Methyltransferase	219
9.2.6	Phenylethanolamine <i>N</i> -Methyltransferase	221
9.2.7	Monoamine Oxidases A and B	222
9.2.8	Arylsulfatase	224
9.2.9	Monoamine Oxidase and Phenol Sulfotransferase	225
9.2.10	<i>N</i> -Acetyltransferase	226
9.2.11	Acetyl-CoA/Arylamine <i>N</i> -Acetyltransferase	229
9.3	Proteinase	229
9.3.1	Vertebrate Collagenase	229
9.3.2	Dipeptidyl Carboxypeptidase (Angiotensin I Converting Enzyme, EC 3.4.15)	231
9.3.3	Luteinizing Hormone-Releasing Hormone Peptidase	235
9.3.4	Papain Esterase	235
9.3.5	Plasma Carboxypeptidase N (Kininase I, Bradykinin-Destroying Enzyme, EC 3.4.12.7)	237
9.3.6	Dipeptidase	238
9.3.7	Aminopeptidase	239
9.3.8	Enkephalinases A and B	239
9.3.9	Rhinovirus 3c Protease	241
9.3.10	Stromelysin	243
9.3.11	Dipeptidyl Peptidase IV/Amino Peptidase-P	244
9.3.12	Carboxypeptidase N	244
9.3.13	Renin	246
9.4	Amino Acid and Peptide Metabolism	247
9.4.1	Ornithine Aminotransferase	247
9.4.2	Glutamine Synthetase, Glutamate Synthetase, and Glutamate Dehydrogenase	249
9.4.3	Asparagine Synthetase	251
9.4.4	Tryptophanase	253
9.4.5	Dihydroxyacid Dehydratase	255
9.4.6	Glutaminy Cyclase	256
9.4.7	Leucine 2,3-Aminomutase	257
9.4.8	Diaminopimelate Epimerase and Decarboxylase	259
9.4.9	Lysine-Ketoglutarate Reductase	259
9.4.10	γ -L-Glutamylcyclotransferase	261
9.4.11	γ -Glutamylcysteine Synthetase and Glutathione Synthetase	261
9.4.12	Glutamic Acid Decarboxylase	262
9.4.13	Histamine <i>N</i> -Methyltransferase	262
9.4.14	Amino Acid Decarboxylase	263
9.4.15	Aromatic L-Amino Acid Decarboxylase	264

9.4.16	D-Amino Oxidase	264
9.4.17	Threonine/Serine Dehydratase	265
9.4.18	Tryptophan Dioxygenase	265
9.4.19	Tryptophan 2,3-Dioxygenase	267
9.4.20	Kynureninase	267
9.4.21	Kynurenine 3-Monooxygenase	268
9.4.22	<i>N</i> ⁵ -Methyltetrahydrofolate-homocysteine Methyltransferase	269
9.4.23	L-Alanine: Glyoxylate Aminotransferase	270
9.4.24	Tyrosinase	270
9.4.25	δ -(L- α -Amino adipyl)-L-Cysteinyl-D-Valine Synthetase	271
9.5	Polyamine Metabolism	272
9.5.1	Ornithine Decarboxylase	272
9.5.2	Spermidine Synthetase	273
9.5.3	Polyamine Oxidase	275
9.5.4	Diamine Oxidase	275
9.6	Heme Metabolism	276
9.6.1	δ -Aminolevulinic Acid Synthetase	276
9.6.2	5-Aminolevulinic Acid Dehydrase	278
9.6.3	Uroporphyrinogen Decarboxylase	278
9.6.4	Heme Oxygenase	279
9.6.5	Ferrocheletase	280
9.6.6	Protoporphyrinogen Oxidase	281
9.7	Carbohydrate Metabolism	283
9.7.1	β -Galactosidase	283
9.7.2	Lactose-Lysine β -Galactosidase	284
9.7.3	Arylsulfatase B (<i>N</i> -Acetylgalactosamine 4- Sulfatase)	285
9.7.4	Galactosyltransferase	287
9.7.5	Uridine Diphosphate Glucuronosyltransferase	287
9.7.6	α -Amylase (1,4- α -D-Glucaglucanohydrolase EC 3.2.2.1)	290
9.7.7	Lysosomal Activities	291
9.7.8	Sialidase	293
9.7.9	Cytidine Monophosphate-Sialic Acid Synthetase	294
9.7.10	Succinyl-CoA Synthetase	295
9.7.11	α -Ketoglutarate Dehydrogenase	299
9.7.12	Sucrose Phosphate Synthetase	300
9.7.13	6-Phosphogluconate Dehydratase	300
9.8	Steroid Metabolism	301
9.8.1	Δ^5 -3 β -Hydroxysteroid Dehydrogenase	301
9.8.2	11- β -Hydroxylase and 18-Hydroxylase	302

9.8.3	25-Hydroxyvitamin D ₃ -1 α -Hydroxylase	304
9.8.4	Cholesterol 7 α -Hydroxylase	304
9.8.5	3 β -Hydroxy- Δ^5 -C ₂₇ -steroid Oxidoreductase	306
9.8.6	Cytochrome P450 _{SCC}	306
9.8.7	Steroid 17 α -Hydroxylase/C ₁₇₋₂₀ Lyase (Cytochrome P-450 _{21SCC})	307
9.9	Purine Metabolism	309
9.9.1	Nicotinate Phosphoribosyltransferase	309
9.9.2	5'-Nucleotidase	310
9.9.3	Alkaline and Acid Phosphatase	312
9.9.4	Adenosine Deaminase	317
9.9.5	AMP Deaminase	317
9.9.6	Cyclic Nucleotide Phosphodiesterase	320
9.9.7	ATP Pyrophosphohydrolase	320
9.9.8	Hypoxanthine Guanine Phosphoribosyltransferase	322
9.9.9	Nucleoside Phosphorylase	323
9.9.10	Creatine Kinase	325
9.9.11	Adenosine Kinase	326
9.9.12	Adenylate Cyclase	327
9.9.13	cAMP Phosphodiesterase	330
9.9.14	Adenylate Kinase	333
9.9.15	Adenylosuccinate Synthetase	334
9.9.16	Dinucleoside Polyphosphate Pyrophosphohydrolase	336
9.9.17	NAD Glycohydrolase	337
9.9.18	Assay of Enzymes Involved in Cytokinin Metabolism	338
9.9.19	Xanthine Oxidase	339
9.9.20	Phosphoribosylpyrophosphate Synthetase	340
9.9.21	Guanase	342
9.9.22	Urate Oxidase	344
9.9.23	Glutamine: 5-Phosphoribosyl-1-pyrophosphate Amidotransferase	344
9.9.24	Thiopurine Methyltransferase	345
9.9.25	NAD Pyrophosphorylase	345
9.9.26	Nucleoside Diphosphate Kinase	346
9.9.27	ATPase	348
9.10	Oxygenations	348
9.10.1	Acetanilide 4-Hydroxylase	348
9.10.2	Ceruloplasmin	349
9.10.3	Aryl Hydrocarbon Hydroxylase (EC 1.14.14.2)	351
9.10.4	Hepatic Microsomal Testosterone Hydroxylase	352

9.11	Pterin Metabolism	353
9.11.1	Folic Acid Cleaving Enzyme	353
9.11.2	Dihydrofolate Reductase	353
9.11.3	Guanosine Triphosphate Cyclohydrolase I	357
9.12	Lipid Metabolism	360
9.12.1	Retinal Oxidase	360
9.12.2	Serum Cholinesterase	361
9.12.3	Carnitine Palmitoyltransferase I	362
9.12.4	Fatty Acid ω -Hydroxylase	363
9.12.5	Acyl-CoA: Alcohol Transacylase	363
9.12.6	Lipase	364
9.13	Modification of Proteins and Peptides	365
9.13.1	Tyrosine Protein Kinase	365
9.13.2	Adenosine Diphosphate-Ribosylarginine Hydrolase	366
9.13.3	Peptidylglycine α -Amidating Monooxygenase	367
9.13.4	Myosin Light Chain Kinase	369
9.13.5	Transglutaminase	369
9.13.6	Phosphotyrosyl Protein Phosphatase	370
9.13.7	Phosphotyrosine Phosphatases	371
9.13.8	Protein Phosphatase 2B (Calcineurin)	371
9.14	Vitamin Metabolism	372
9.14.1	Thiamine Triphosphatase	372
9.14.2	Lipoamidase	372
9.14.3	Pyridoxal Kinase, Pyridoxamine Oxidase, and Pyridoxal-5'-phosphate Phosphatase	373
9.14.4	Pyridoxine Kinase	373
9.14.5	Biotinidase	374
9.15	Xenobiotic Metabolism	374
9.15.1	ATP-Sulfurylase	374
9.15.2	Sulfotransferase	375
9.15.3	Glutathione S-Transferase	376
9.15.4	Adenosine 3'-Phosphate 5'-Sulfophosphate Sulfotransferase	380
9.15.5	Phenolsulfotransferase	380
9.15.6	Aryl Sulfotransferase	382
9.15.7	Cysteine Conjugate β -Lyase	383
9.15.8	UDP-Glucuronyl Transferase	384
9.15.9	UDP-Glucosyltransferase	385
9.15.10	Ethoxycoumarin O-Deethylase	385
9.15.11	Cytochrome P450 _{2E1}	386
9.15.12	Flavin-Containing Monooxygenase	387

9.16	Pyrimidine Metabolism	388
9.16.1	Dihydropyrimidine Dehydrogenase	388
9.16.2	Dihydroortic Acid Dehydrogenase	389
9.16.3	Cytidine Deaminase	389
9.16.4	β -Ureidopropionase	390
9.16.5	Dihydroorotase	391
9.16.6	Thymidylate Synthetase	391
9.17	Metabolism of Complex Saccharides and Glycoproteins	392
9.17.1	α -L-Fucosidase	392
9.17.2	α -N-Acetylgalactosaminyltransferase	392
9.17.3	GM ₁ Ganglioside β -Galactosidase	393
9.17.4	Aspartylglycosylaminase	394
9.17.5	β -Galactosidase and Glycosyltransferase	395
9.17.6	Glucose-1-phosphate Thymidyltransferase	396
9.17.7	CMP-N-Acetylneuraminic Acid: Glycoprotein Sialyltransferase	396
9.17.8	Thyroxine: UDP-glucuronosyltransferase	397
9.17.9	<i>trans-p</i> -Coumaroyl Esterase	397
9.18	Miscellaneous	399
9.18.1	Carboxylases	399
9.18.2	Carbonyl Reductase	400
9.18.3	6-Pyruvoyl Tetrahydropterin Synthetase	400
9.18.4	Pteroylpolyglutamate Hydrolase	401
9.18.5	Nitrogenase	402
9.18.6	Strictosidine Synthetase	403
9.18.7	Anhydrotetracycline Oxygenase and Tetracycline Dehydrogenase	403
9.19	Nucleic Acid Modification and Expression	405
9.19.1	DNA Topoisomerase	405
9.19.2	Chloramphenicol Acetyltransferase	405
9.20	Summary and Conclusions	407
	References	409
	General References	416
10.	Multienzyme Systems	418
	<i>Overview</i>	418
10.1	Introduction	418
10.2	Assay of Two Activities Forming Different Products from the Same Substrate	419
10.3	Assay of Two Activities Forming the Same Product from the Same Substrate	420
10.4	Formation of Two Separate Products from Two Separate Substrates by the Same Activity	426

10.5	Assay of a Multienzyme Complex by the Reconstitution Method	428
10.5.1	The Salvage Pathway: The Formation and Fate of IMP	428
10.5.2	The Degradation of IMP to Inosine	429
10.5.3	The Conversion of IMP to AMP	430
10.5.4	The Return of AMP to IMP	432
10.6	Assay of a Multienzyme Complex Using the HPLC Method	432
10.7	Summary and Conclusions	435
	References	435
	General References	435
	Subject Index	437