HANDBOOK of CHROMATOGRAPHY

Günter Zweig
Joseph Sherma
Editors-in-Chief

Ram N. Gupta

Drugs
Volume I

CRC Handbook of Chromatography Drugs

Volume I

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CRC HANDBOOK OF CHROMATOGRAPHY

Series Preface

This Handbook of Chromatography, Drugs by Ram N. Gupta, is one in a series of separate volumes devoted to a single class of chemical compounds or to compounds with a similar use pattern, like the prospective volumes on pesticides and terpenoids. When Volumes I and II of the Handbook of Chromatography were first published in 1972, the editors made an attempt to select the data so as to accomplish the coverage of most organic and inorganic compounds in a volume of about one thousand pages. However, during the ensuring ten years, the literature of chromatography, especially high-performance liquid chromatograph (HPLC), has grown to such an extent that, after an initial intent to update Volumes I and II, it was decided to publish separate volumes devoted to specific subjects. The present volume on the Chromatography of Drugs is an example of the expanded Handbook Series. In selecting Volume Editors, the Editors-in-Chief endeavored to select scientists with extensive knowledge and expertise in the chromatography of specific compounds. The Editor of this Volume, Dr. Ram N. Gupta is renowned in the field of chromatography of drugs, which is evident from the comprehensive and authoritative treatment of the subject found in this Volume. We have given each Volume Editor wide latitude in designing a format that would be most useful to the reader and do justice to the particular subject being covered. Subsequent volumes of this series will include the chromatography of pesticides, steroids, lipids and fatty acids, terpenoids, plant pigments, hydrocarbons, amino acids, inorganic compounds, polymers, and nucleic acids and associated compounds.

We invite readers to communicate with the Volume Editor for comments and corrections and to the Editors-in-Chief for suggestions for future volumes. The Editors-in-Chief want to thank Dr. Gupta for his outstanding effort and the cooperation of his associates.

Gunter Zweig, Ph.D.
Joseph Sherma, Ph.D.
Spring, 1981

PREFACE

In the last decade the most noted application of chromatography has been in the field of drug analysis. Demands for high sensitivity and selectivity in the analysis of drugs have been partly responsible for the development of sensitive and selective detectors and highly efficient columns for both gas (GC) and high-pressure liquid chromatography (HPLC).

As soon as a new drug is developed, the analytical laboratory of the pharmaceutical company proceeds to develop a sensitive analytical procedure to study the pharmacokinetics of the drug. In some cases, alternative analytical procedures are developed simultaneously in a number of centers where the drug is undergoing clinical trials. With rare exceptions, chromatographic procedures are used for the analysis of new drugs. In some instances, when the drug concentration per unit volume of the specimen is very low, immunoassays are also attempted.

In the last decade the role of the clinical chemistry laboratory has been augmented. In addition to providing analyses for different constituents both for diagnosing disease and for demonstrating symptomatic drug overdose, analyses are now being performed to monitor drug treatment. It is believed that the control of epilepsy has improved significantly with the measurement of therapeutic concentrations of antiepileptic drugs. This demand for therapeutic drug monitoring is being extended to more drug classes, e.g., antiarrhythmic drugs, antidepressants, neuroleptics, etc.

The American Society of Clinical Pathologists, the American Society of Clinical Chemists, and a number of European societies have made available voluntary quality control schemes whereby analysts can compare their performance in drug monitoring and detection with other analysts working in the field.

The spectrophotometric or colorimetric procedures used for routine quality control in the production of pharmaceuticals are being replaced by chromatographic procedures. These procedures have the capacity of detecting potentially harmful trace impurities in drugs. However, spectrophotometric or colorimetric procedures are still preferred in clinical laboratories for the rapid detection of some drugs in emergency situations, e.g., salicylates and acetaminophen.

In the last few years, there has been a phenomenal increase in publications describing the use of HPLC; however, GC is still popular and many improvements in instrumentation have been achieved. Thus, it is now quite convenient to use nitrogen-selective detectors in which the salt bead is heated electrically. With the introduction of flexible break-resistant capillary columns, GC has offered a new potential for ultratrace analysis in complex matrices. Another factor in favor of GC is that the mobile phase does not present a disposal problem.

In North America, quantitative thin-layer chromatography (TLC) is not as popular as it is in Europe. One advantage of TLC is the ability to separate a number of samples simultaneously, and the separated spots can be quantitated relatively rapidly by *in situ* densitometry. The spots can be made colored or fluorescent by spraying or dipping the plate in suitable reagents. Postcolumn reactions in HPLC to increase the detection sensitivity are still not very popular. TLC remains the method of choice all over the world for the qualitative detection of drugs of abuse. There have been advances in the manufacture of precoated TLC plates. High-performance TLC plates and chemically bonded reverse-phase TLC plates are now commercially available.

The purpose of this handbook is to provide a reference source of different chromatographic techniques available for the analysis of drugs.

I am grateful to Mrs. Elaine Moore for her skillful assistance in preparing the manuscript. Mrs. Diane Lewis helped to organize the filing system of reprints. Ms. Pamela Woodcock of CRC Press provided all the required editorial assistance.

Ram N. Gupta

THE EDITORS-IN-CHIEF

Gunter Zweig, Ph.D., received his undergraduate and graduate training at the University of Maryland, where he was awarded the Ph.D. in biochemistry in 1952. For two years after his graduation, Dr. Zweig was affiliated with the late R. J. Block, pioneer in paper chromatography of amino acids. Zweig, Block and Le Strange wrote one of the first books on paper chromatography which was published in 1952 by Academic Press and went into three editions, the last one authored by Gunter Zweig and Dr. Joe Sherma, the co-Editor-in-Chief of this Handbook. Paper Chromatography (1952) was also translated into Russian.

From 1953 till 1957, Dr. Zweig was research biochemist at the C. F. Kettering Foundation, Antioch College, Yellow Springs, Ohio, where he pursued research on the path of carbon and sulfur in plants using the then newly developed techniques of autoradiography and paper chromatography. From 1957 till 1965, Dr. Zweig served as lecturer and chemist, University of California, Davis and worked on analytical methods for pesticide residues, mainly by chromatographic techniques. In 1965, Dr. Zweig became Director of Life Sciences, Syracuse University Research Corporation (research on environmental pollution), and in 1973 he became Chief, Environmental Fate Branch, Environmental Protection Agency in Washington, D.C. In 1980, he was appointed Senior Science Advisor in the same agency. During his government career, Dr. Zweig continued his scientific writing and editing. Among his works are (many in collaboration with Dr. Sherma) the now 11-volume series on Analytical Methods for Pesticides and Plant Growth Regulators (Academic Press); the Pesticide Chemistry series for CRC Press; co-editor of Journal of Toxicology and Environmental Health; co-author of basic review on paper and thin-layer chromatography for Analytical Chemistry from 1968-1980; co-author of applied chromatography review on pesticide analysis for Analytical Chemistry, beginning in 1981. Among the scientific honors awarded to Dr. Zweig during his distinguished career are the Wiley Award in 1977, Rothschild Fellowship to the Weizmann Institute in 1963/64; the Bronze Medal by the EPA in 1980. Dr. Zweig has authored or co-authored over 75 scientific papers on diverse subjects in chromatography and biochemistry, besides being the holder of three U.S. patents. At the present time (1980/82), Dr. Zweig is Visiting Scholar in the School of Public Health, University of California, Berkeley, where he is doing research on farmworker safety as related to pesticide exposure.

Joseph Sherma, Ph.D., received a B.S. in Chemistry from Upsala College, East Orange, N.J., in 1955 and a Ph.D. in Analytical Chemistry from Rutgers University in 1958. His thesis research in ion exchange chromatography was under the direction of the late William Rieman III. Dr. Sherma joined the faculty of Lafayette College in September, 1958, and is presently full professor there in charge of two courses in analytical chemistry. At Lafayette he has continued research in chromatography and has additionally worked a total of 12 summers in the field with Harold Strain at the Argonne Nationa Laboratory, James Fritz at Iowa State University, Gunter Zweig at Syracuse University Research Corporation, Joseph Touchstone at the Hospital of the University of Pennsylvania, Brian Bidlingmeyer at Waters Associates, and Thomas Beesley at Whatman, Inc. Dr. Sherma and Dr. Zweig (who is now with U.S. EPA) co-authored Volumes I and II of the *CRC Handbook of Chromatography,* a book on paper chromatography, and 6 volumes of the series *Analytical Methods for Pesticides and Plant Growth Regulators.* Other books in the pesticide series and further volumes of the *CRC Handbook of Chromatography* are being edited with Dr. Zweig, and Dr. Sherma will co-author the Handbook on Pesticide Chromatography. A book on quantitative TLC (Wiley-Interscience) was edited jointly with Dr. Touchstone. Dr. Sherma has been co-author of seven biennial reviews of liquid chromatography (1968-1980) and the 1981 review of pesticide analysis for the journal *Analytical Chemistry.* Dr. Sherma has authored major invited chapters and review papers on chromatography and pesticides in *Chromatographic Reviews* (analysis of fungicides), *Advances in Chromatography* (analysis of non-pesticide pollutants), Heftmann's *Chromatography* (chromatography of pesticides), Race's *Laboratory Medicine* (chromatography in clinical analysis), *Food Analysis: Principles and Techniques* (TLC for food analysis), *Treatise on Analytical Chemistry* (paper and thin layer chromatography), and *CRC Critical Reviews in Analytical Chemistry* (pesticide residue analysis). A general book on thin layer chromatography co-authored by Dr. Sherma is now in press

at Marcel Dekker. Dr. Sherma spent six months in 1972 on sabbatical leave at the EPA Perrine Primate Laboratory, Perrine, Florida, with Dr. T. M. Shafik, and two additional summers (1975, 1976) at the USDA in Beltsville, Maryland, with Melvin Getz doing research on pesticide residue analysis methods development. He spent three months in 1979 on sabbatical leave with Dr. Touchstone developing clinical analytical methods. A total of more than 200 papers, books, book chapters, and oral presentations concerned with column, paper, and thin layer chromatography of metal ions, plant pigments, and other organic and biological compounds; the chromatographic analysis of pesticides; and the history of chromatography have been authored by Dr. Sherma, many in collaboration with various co-workers and students. His major research area at Lafayette is currently quantitative TLC (densitometry), applied mainly to clinical analysis and pesticide residue determinations. Dr. Sherma has written an analytical quality control manual for pesticide analysis under contract with the U.S. EPA and has revised this and the EPA Pesticide Analytical Methods Manual under a four-year contract (EPA) jointly with Dr. M. Beroza of the AOAC. Dr. Sherma has also written an instrumental analysis quality assurance manual and other analytical reports for the U.S. Consumer Product Safety Commission, and is currently preparing a manual on the analysis of food additives for the U.S. FDA, both of these projects also in collaboration with Dr. Beroza of the AOAC. Dr. Sherma taught the first, prototype short course on pesticide analysis, with Henry Enos of the EPA, for the Center for Professional Advancement. He is editor of the Kontes TLC quarterly newsletter and also teaches short courses on TLC for Kontes and the Center for Professional Advancement. He is a consultant for several industrial companies and federal agencies on chemical analysis and chromatography and regularly referees papers for analytical journals and research proposals for government agencies. Dr. Sherma has received two awards for superior teaching at Lafayette College and the 1979 Distinguished Alumnus Award from Upsala College for outstanding achievements as an educator, researcher, author, and editor. He is a member of the ACS, Sigma Xi, Phi Lambda Upsilon, SAS, and AIC.

THE EDITOR

Ram N. Gupta, Ph.D., is Assistant Clinical Chemist at the St. Joseph's Hospital in Hamilton and Associate Professor in the Department of Pathology at McMaster University, in Hamilton.

Dr. Gupta received his M.Sc. degree in 1962 and Ph.D. degree in 1963 in Organic Chemistry from McMaster University. He continued working in the Chemistry Department of McMaster University as a research associate until 1971 when he moved to the Department of Pathology at the same university.

Dr. Gupta has been elected as a fellow of the Chemical Institute of Canada. He is a member of the American Chemical Society, American Association of Clinical Chemists, Canadian Society of Clinical Chemists and the Association of Clinical Biochemists (U.K.). He is the author of more than 40 scientific publications.

His present research interests are the development of chromatographic procedures for the assay of drugs and other biochemicals in biological fluids.

THE CONSULTING EDITOR

Dr. Irving Sunshine is Chief Toxicologist at the Cuyahoga County (Cleveland), Ohio Coroner's Office; Professor of Toxicology in the Department of Pathology and Professor of Clinical Pharmacology in the Department of Medicine at the School of Medicine, Case Western Reserve University; Chief Toxicologist for the University Hospitals in Cleveland, Ohio; Director of the Cleveland Poison Information Center; and Editor-In-Chief for Biosciences for CRC Press, Inc. He is a Diplomate of both the American Board of Clinical Chemistry and The American Board of Forensic Toxicology and is on the Board of Directors of both these organizations.

Born in New York City, he obtained all his formal education in various Colleges of New York University, earning the B.Sc., M.A., and Ph.D. degrees. While earning his Ph.D., he taught chemistry in various colleges in the New York area and during the war, he worked during the "grave yard" shift on a pilot plant for the separation of uranium isotopes as a part of the "The Manhattan Project". His development in toxicology was encouraged by two memorable mentors, Dr. Alexander O. Gettler and Dr. Bernard Brodie.

To my teacher

Professor Ian D. Spenser

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PREPARATION OF SAMPLES

There are only a few cases where specimens can be analyzed by chromatography directly without prior manipulation of the specimen. In most cases, specimens are pretreated to concentrate the drug 10 to 100-fold and to remove constituents which are likely to either interfere with the analysis or affect the column or detector performance. A number of selected methods for the isolation of drugs have been described in Volume II of this Handbook series. Recent developments for sample preparation for chromatography will be described briefly. Techniques for sample preparation have been reviewed.[1,2]

A. Analysis Without Prior Concentration

Aqueous specimens in general are not suitable for analysis by gas chromatography (GC) employing columns packed with coated supports. However, uncoated polymer packings (e.g., Porapaks and Chromosorb century series) are commonly used for the analysis of volatiles in aqueous solutions. Filtered urine, diluted whole blood, or protein-free filtrates prepared using either trichloroacetic acid or sodium tungstate/sulfuric acid solutions can be directly injected.[3]

Aqueous solutions are quite suitable for analysis by reverse-phase high-performance liquid chromatography (HPLC). Thus, in a number of procedures for the determination of theophylline [4] and anticonvulsant drugs,[5] the plasma proteins are precipitated by the mobile phase, and the filtrate is injected directly onto the column. However, these procedures are somewhat nonspecific and co-administered drugs may interfere.[6]

Plasma has also been spotted directly on thin-layer plates. The proteins stay at the origin and satisfactory separation of the drug is achieved.[7] Plates with inert preadsorbent spotting areas are especially useful for directly spotting biological samples.

B. Liquid-Liquid Extraction

Liquid-liquid extraction based upon partition of an unionized compound between two immiscible solvents remains the most popular technique for the isolation of drugs from biological fluids. It is relatively economical and subject to batch operation. There are a number of parameters that can be varied.

Choice of Solvent

Solvents with widely different polarities are available. The greater the polarity of the extracting solvent, the greater the extraction of polar compounds and endogenous impurities that could interfere with the chromatographic analysis. During the development of a new procedure, a solvent of adequate polarity is selected empirically so that it will extract the desired compound with a recovery of a least 50 to 60%. In most cases, clean extracts are preferred to optimal recovery. Solvents that have appreciable miscibility with water (higher alcohols) have a tendency to carry polar impurities into the organic layer.

Many drugs are susceptible to degradation by prolonged heat in the presence of air and impurities. To avoid these degradative losses, high boiling solvents are evaporated either in a current of nitrogen or under vacuum. For convenience, low boiling solvents of comparable polarities can be selected that can be evaporated without the aid of either suction or a stream of nitrogen. For example, pentane can replace hexane or heptane, and dichloromethane can replace chloroform.

The choice of a solvent is also dependent on whether an upper or lower layer is required. The density of a required layer may sometimes be adjusted by using mixed solvents, e.g., chloroform may be made to float by mixing with ether or hexane.

All solvents are potentially hazardous. They are either inflammable or toxic. For laboratory safety, the number of solvents for routine use in the laboratory should be reduced. Unless absolutely essential, the use of ether should be avoided because of the danger of an explosion due to peroxide formation, and the use of benzene should be minimized because of its carcinogenic nature. Dichloromethane, pentane, ethylacetate, and toluene or a mixture of these water-immiscible solvents, are adequate to isolate a wide variety of drugs from different kinds of biological matrices.

To minimize the losses of drugs due to adsorption on glassware, the use of siliconized glassware has been recommended.[8] Glassware can also be treated with volatile compounds[9] or by the addition of compounds like trimethylamine to the specimen itself for the extraction of amines.[10] An excess of compounds (scavengers) with structures similar to the drug, but with significantly different retention times (or R_f values), has been added to the specimen to improve the extraction of drugs and to avoid losses due to adsorption.[11]

In a number of procedures, losses of the drug during evaporation of the solvent have been avoided by using a low organic to aqueous phase ratio and by analyzing the organic phase without further concentration.[12] A ratio as low as 1:80 of organic solvent [chloroform — isopropanol (4:1)] to urine has been used for the extraction of drugs of abuse at an alkaline pH.[13]

Choice of pH

The optimum pH for the extraction of a drug from a given specimen is determined empirically. Selection of pH depends not only on the pK (acidity or basicity) of the drug but also on protein binding. Both free and protein-bound drugs are extracted by solvents. However, drug-protein bonds are broken optimally at a pH which may be different from the optimal pH for the extraction of drug from the protein-free aqueous phase. Thus, propoxyphene (a basic drug) has been extracted from plasma at pH 4 with chloroform.[14] It has also been reported that dilution favors dissociation of protein bonds.[1] Changes in pH during extraction may cause degradation or transformation of the drug. Thus, norpropoxyphene is completely transformed to its amide at pH 13.[15]

Equipment

Culture tubes with Teflon-lined screw caps are preferred to classical separatory funnels as it is easier to handle batches of tubes than to handle batches of separatory funnels. Also, tubes can be centrifuged to break emulsions. Tubes of appropriate volume with a narrow diameter are selected. The tubes are filled only up to 80% of capacity so that enough space is available for proper mixing. The tubes are mixed at a slow speed either manually or mechanically with rotary mixers or tumble mixers.[16] Vortex mixing of plasma produces emulsions; layers are separated with difficulty, and the extracts produce extraneous peaks.

Different approaches to liquid-liquid extraction of a drug with the use of similar solvents or similar pH have been used. Some procedures are simple one-step techniques where the extraction is carried out for optimum yield of the drug, and the chromatographic system and the selective detection system assure specificity. Other procedures involve multistep extractions, separation, and transfer of layers to a different set of extraction tubes for an optimum selectivity, even if the final recovery of the drug is reduced. Examples of different extraction procedures for a few representative drugs are summarized in Table 1.

C. Ion-Pair Extraction

Improvement in the extraction efficiency of very polar compounds can be achieved by using suitably charged counter ions. For example, emepronium bromide has been

efficiently extracted from serum using perchlorate ion as the counter ion.[34] The recovery of drugs fom urine has also been improved by using bromocresol purple as the counter ion and chloroform as the extracting solvent.[35] In some cases, the ion-pair technique can improve the selectivity of extraction also.[36] These ion pairs are easily split in the subsequent chromatographic system or by further derivatization. Thus, when bromocresol purple adducts are analyzed by TLC, bromocresol purple ion stays at the origin and the drugs have their characteristic R_f values.[35]

In another form of ion-pair extraction, drugs are converted to stable derivatives. These derivatives have better extracting properties and better chromatographic characteristics. Thus, morphine has been extracted from plasma in the presence of pentafluoropentylbromide and tetrabutylammonium hydroxide. The pentafluorobenzyl derivative of morphine was obtained in quantitative yield on extraction with ethyl acetate.[37]

This technique has been used extensively for the preparation of alkyl derivatives of acidic drugs that have already been extracted from biological matrix into dichloromethane.[38,39]

D. Liquid-Solid Extraction

Despite its wide use, liquid-liquid extraction has some problems, e.g., formation of emulsions, use of relatively large volume of solvents as compared to specimen volume, poor recovery of polar compounds, and the need to dry the extracts with desiccants prior to evaporation. To overcome these problems, a number of solid supports have been introduced to adsorb the drug which is then eluted with a water-miscible or immiscible solvent to isolate the drug. Some of these supports are described here briefly:

XAD-Resins

These are nonionic hydrophobic polystyrene-divinylbenzene copolymer (Amberlite ®) resins developed by Rohm and Haas Co. Amberlite ® XAD-2 was introduced by Fujimoto and Wang[40] and has been mainly used for the isolation of drugs of abuse from urine.[41-44] This resin has also been used to a lesser extent for the extraction of drugs from blood.[45,46] Amberlite ® XAD-4 has been suggested for the isolation of drugs from blood.[47] For most of these applications, resin has been packed in suitable columns, and the extraction efficiency depends upon the rate of flow of urine and also of the eluting solvent. Prepacked, ready-to-use, disposable XAD-2 resin columns are available commercially (Brinkman Instruments Co.). For economy, improvised columns have been fabricated from plastic bags.[48] Recently, a batch procedure has also been described for the use of Amberlite ® XAD-2 resin.[49] In this procedure, resin is packed in 4 × 4 cm nylon bags. After adsorption of the drugs, the bags are eluted with different solvents of different pH values to collect acidic and basic drugs in separate fractions. The resin can be regenerated, and the bags can be used up to ten times.

Ion-Exchange Papers

The first alternative to liquid-liquid extraction was the suggestion by Dole et al.[50] to use Reeve Angel SA-2 cation exchange resin loaded paper to adsorb drugs of abuse from urine. The adsorption of drugs on resin papers can be carried out in clinics, and the papers can be mailed to reference laboratories for analysis. A number of reports have suggested that the use of ion-exchange papers leads to poor recoveries.[51-53] However, Kaistha reported use of this technique successfully for mass screening of drugs of abuse.[54] He and his colleagues have suggested a number of modifications to improve the efficiency of the ion-exchange paper technique.[55] The strong argument put forward by Kaistha in favor of this technique is that it is the most convenient and economical

Table 1
LIQUID/LIQUID EXTRACTION OF DRUGS

Drug (concentration range)	Plasma volume (mℓ)	pH	Solvent	Layer manipulation	Evaporation	Analytical technique	Recovery (%)	Ref.
Amitriptyline (50—500 ng/ mℓ)	2	9[a]	Hexane[b]	Upper layer collected	65°C/ air	GC/NPD	80	9
	3	i. 10.5	Hexane/ isoamyl alcohol (98/2)	Upper layer collected and extracted with 1 mℓ of 0.1 *M* HCl; Aq phase → step ii				
		ii. 10.5	Hexane/ isoamyl alcohol (98/2)	Upper layer collected	Ambient/ nitrogen	GC/NPD	50	17
	1—3	i. 2[c]	Pentane	Upper layer discarded; Aq phase → step ii				
		ii. 13	Pentane[b]	Upper layer collected	45—50°C (nitrogen)[d]	GC/NPD	60—70[e]	18
	1	i. 10	Hexane	Upper layer collected and extracted with 1 mℓ of 0.25 N H_2SO_4; Aq phase → step ii				
		ii. 13	Isopropyl ether[f]	Upper layer collected		HPLC (ion pair)	NA	19
	2	10	Hexane/isoamyl alcohol (98/2)	Upper layer collected	60°C/air	HPLC (normal phase)	65	20
Diphenylhydantoin (2—50 μg/mℓ)	0.2	i. 12	Ethyl ether	Upper layer discarded; Aq phase → step ii				
		ii. 6.8	Ethyl ether	Upper layer collected	Ambient/nitrogen	HPLC (normal phase)	86	21
	2	i. 2	Ethyl ether	Upper layer collected and extracted with 0.2 *M* phosphate buffer, pH 12; ether layer discarded and Aq. phase → step ii				
		ii. 2	Ethyl ether[b]	Upper layer collected	50°C/nitrogen	GC/FID	NA	22

	0.1	5	Toluene	Upper layer collected and extracted with 25 $\mu\ell$ of trimethylanilinium hydroxide; lower Aq layer used for injection		GC/FID	NA	23
	1	2	Chloroform	Upper layer discarded	NA/nitrogen	GC/FID	NA	24
Valproic acid (20—200 μg/mℓ)	0.2	2	Chloroform	Lower layer injected directly		GC/FID	NA	25
	0.05	2	Heptane	Upper layer collected and injected without prior concentration		GC/FID	NA	26
	0.5	2	Ethyl acetate	Upper layer collected	NA/nitrogen	HPLC (reverse phase)	95	27
	1	i. 2	Ethyl ether	Upper layer collected and extracted with 1 mℓ of 0.25 *N* NaOH; ether layer discarded and Aq layer → step ii				
		ii. 2	Chloroform	Lower layer injected directly		GC/FID	70—80	28
	0.25	i. 10	Pentane	Upper layer discarded				
		ii. 2	Pentane	Upper layer collected	50—53°C[g]	GC/FID; HPLC (reverse phase)	70	29
Carbamazepine (1—25 μg/mℓ)	1	7.4[h]	Ethyl ether	Upper layer collected	40°C/nitrogen	GC/NPD	80	30
	1	13	Benzene/ethyl acetate (4:1)	Upper layer collected	55°C/nitrogen	GC/FID	NA	31
	1	i. 7.2	Chloroform	Upper layer discarded. Lower layer evaporated; residue dissolved in 3 mℓ of methanol and 2 mℓ of 0.25 *N* HCl → ii				
		ii. 2	Hexane	Upper layer discarded; methanolic-Aq. layer → step iii				
		iii 13	Chloroform	Lower layer collected	NA/vacuum	GC/FID	95	32
	0.1	13	Dichloromethane	Organic layer spotted directly on a TLC plate[i]		TLC/Fl	95	33