- PCA: the precipitation of the drug substance from a solution in a primary solvent with the aid of a Compressed Anti-solvent, namely CO_2.
- SEDS: Supercritical solution Enhanced Dispersion of Solutions. A solution of the drug in a primary solvent is mixed in a nozzle with a stream of supercritical CO_2 that acts both as dispersant and anti-solvent. The particles form immediately and are collected on filter plates. This technique offers a number of very intriguing advantages such as very small particle size, very low residual solvent contents, or a low surface charge of the particles facilitating the formulation process.

It has been reported, that proteins or peptides crystallized via this technique fully retain their biological activity.

With these processes, crystal sizes down to and below 1 μm can be attained. So far, all these processes work in batch mode with small batch sizes, although attempts are being made to increase the batch size.

The particle formation, nucleation and growth, in all three techniques is rapid. Information on the nucleation and growth process are only emerging and more work needs to be done. Due to the nucleation and growth under very high supersaturations, the polymorphic form obtained might not be the stable one.

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See Colour Plates 111, 112, 113.

See also: **II/Crystallization:** Control of Crystallizers and Dynamic Behaviour; Polymorphism. **III/Supercritical Fluid Crystallization.**

Further Reading

Beckmann W, Budde U and Nickisch K (1998) Development of a seeding technique for the crystallization of the metastable. A modification of Abecarnil. *Organic Process Research and Development* 2: 298–304.

Blehaut J and Nicoud RM (1998) Recent aspects in simulated moving bed. *Analusis Mag* 26: M60–M70.

Boistelle R, Klein JP and Guyot-Hermann AM (1996) Eléments de cristallographie et de cristallogenèse à l'usage des industriels de la chimie et de la pharmacie. *S.T.P. Pharma Pratiques* 6: 111–140.

Brittain HG (ed.) (1995) *Physical Characterisation of Pharmaceutical Solids. New York*: Marcel Dekker.

Byrn A *et al.* (1995) Pharmaceutical solids: a strategic approach to regulatory considerations. *Pharmaceutical Research* 12: 945–954.

Grant DJW and Hiuch T (1990) *Solubility Behavior of Organic Compounds*. Chichester: John Wiley.

ICH-guidelines are found on the internet at www.eudra.org/emea.html. Guidelines Q3C discuss residual solvent content, Q3A the purity of drug substance and Q7A GMP considerations.

Jaques J, Collett A and Wilen SH (1994) *Enantiomers, Racemates, and Resolutions*. Malabar, Fla: Krieger.

Masters K (1991) *Spray Drying Handbook*, 5th edn. Burut House, Harlow, Essex: Longman.

Mersman A (ed.) (1995) *Crystallization Technology Handbook*, 1st edn. New York: Marcel Dekker.

Mullin JW (1993) *Crystallization*, 3rd edn. London: Butterworth-Heinemann.

Szejtli J (1982) *Cyclodextrins and their Inclusion Complexes*. Budapest Akademiai Kisdo.

Neutral and Acidic Drugs: Liquid Chromatography

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Introduction

Although the general topic of the high performance liquid chromatographic (HPLC) analysis of neutral and acid drugs encompasses a broad range of compounds that include antibacterial agents to vitamin supplements, many of the more common acid compounds are therapeutically active as analgesics, antipyretics, and antiinflammatories (e.g., aspirin, diclofenac, flurbiprofen, ibuprofen, indomethacin, ketoprofen, meclofenamic acid, mefenamic acid, naproxen, tolmetin and other compounds shown in **Table 1**). In many cases, this group of compounds are relatively small molecules and are structurally simple, containing only one or two aromatic rings and a single ionizable carboxyl group. Many of the common

Table 1 Recently published HPLC methodology for common acid drugs

Analyte	*Procedure*
General	The simultaneous analysis of twelve common nonsteroidal antiinflammatory drugs has been carried out by reversed-phase chromatography on a C18 column using acetonitrile–phosphate buffer as the eluent and detection at either 230 or 320 nm. The method has been used to evaluate the level of the analytes in human urine samples [5]. A fully automated on-line dialysis sample preparation procedure has been developed for assaying several nonsteroidal antiinflammatories in human plasma. In addition different strategies for improving analyte loss due to drug–protein binding are discussed [6]. A systematic investigation of the enantioselective separation of 28 α-alkylarylcarboxylic acids on an amylose tris(3,5-dimethylphenylcarbamate) chiral station phase has been carried out and the resulting retention data correlated to a series of molecular descriptors [7]. A [2.2.2]-bicyclooctane-based stationary has been developed for the chiral resolution of various profen enantiomers which functions via combination of hydrogen bonding, π–π face-to-face stacking, π–π face-to-edge interactions [8].
Aspirin	A high performance liquid chromatographic (HPLC) method has been developed for the simultaneous determination of aspirin and salicylic acid in transdermal perfusates. The compounds are separated on a C8 column 65 : 35 water–acetonitrile containing 0.2% phosphoric acid. For certain samples, a gradient was introduced by increasing the acetonitrile content of the mobile phase after the salicylic acid had eluted [9]. Aspirin and salicylic acid have been determined in skin and plasma samples by an isocratic reversed-phase method that uses a C18 column and 75 : 25 pH 2.5 phosphate buffer–acetonitrile as the eluent [10]. Gradient elution reversed-phase methods have been developed for measuring aspirin and warfarin in combination tablet formulations and in the presence of potentially related impurities that uses a C8 column operated at 40°C. In the first instance the starting conditions, which are maintained for 11 minutes, are 68 : 17 : 15 water adjusted to pH 2.6 with formic acid–methanol–acetonitrile. This is followed by a linear gradient over 15 minutes to 56 : 17 : 27 of the respective solvents and a hold time of an additional 38 minutes. When assaying for possible related substances, a second gradient step is used to 13 : 17 : 70 [11]. An isocratic reversed-phase assay has been reported for measuring aspirin, salicylic acid, and warfarin sodium. The separations are carried out on a C18 column using 23 : 5 : 5 : 67 acetonitrile–tetrahydrofuran–glacial acetic acid–water as the eluent with UV detection at 282 nm. The method has been used to study tablet dissolution [12]. An alternate isocratic reversed-phase method has been developed for measuring the analyte in model solution aerosols. It uses a C8 column with 44 : 5 : 5 methanol–THF–1 M phosphoric acid qs to 100 with water as the eluent and UV detection at 275 [13].
Diclofenac	An isocratic reversed-phase method has been reported for measuring the analyte and flurbiprofen in plasma. It uses a C18 column and 35 : 65 acetonitrile–0.1 M sodium acetate adjusted to pH 6.3 with glacial acetic acid as the eluent and detection at 278 nm [14]. The analyte has been detected fluorometrically as its photodecomposition product, carbazole-1 acetic acid, after on-line post column UV irradiation. Excitation and emission wavelengths of 286 nm and 360 nm are used respectively. The photoderivative is formed via loss of both chlorine substituents and ring closure [15]. Solid-phase extraction in combination with isocratic reversed-phase chromatography has been used to measure the analyte as well as indomethacin and phenylbutazone in human urine. The separations were performed on a C18 column using 42 : 58 acetonitrile–10 mM pH 4 acetate buffer as the eluent and detection at 210 nm [16]. The analyte has been assayed in the presence of oxybuprocaine in human aqueous humor by reversed-phase HPLC-EC using a C8 column coated with a hydrophilic polyoxyethylenepolymer. The detection limit for the analyte is 500 pg [17]. Pharmaceutical formulations containing the analyte, cyanocobalamin, and betamethasone have been assayed on a C18 column operated at 34°C employing 60 : 40 acetonitrile–water adjusted to pH 3.45 with acetic acid as the eluent and detection at 240 nm [18].
	See general methodology above [5].
Felbinac	See general methodology above [5].
Fenbufen	See general methodology above [5].
Fenoprofen	See general methodology above [6].

Table 1 *Continued*

Analyte	*Procedure*
Flurbiprofen	See general [5,6] and diclofenac [14] methodology above.
Ibuprofen	A reversed-phase method has been developed for studying the analyte and five metabolites that uses a 1.5 mm i.d. semi-microcolumn and a linear gradient over 70 minutes from 2 : 98 acetonitrile–phosphate buffer (pH 2.5 0.05 M) to 60 : 40 of the respective components. Both UV and EC detection were used to obtain information about glucuronation [19]. The analyte has been assayed in erythrocytes and plasma by an isocratic reversed-phase procedure following its liquid–liquid extraction with methylene chloride. The separation is carried out on a C18 column with 22 : 10 methanol–water acidified with perchloric acid to pH 3 as the eluent and UV detection at 222 nm [20]. Two cellulose-based chiral phases (Chiralcel OD and Chiralcel OJ) have been evaluated for their ability to resolve various aliphatic ibuprofen esters and the latter material in combination with nonaqueous eluents provided effective resolution of most of the esters [21]. The retention properties of a molecularly imprinted 4-vinylpyridine/ethylene glycol dimethacrylate polymer for (S)-ibuprofen have been evaluated using aqueous eluents. The novel packing, which is prepared via a multi-step swelling and thermal polymerization method, also has been used to partially resolve the enantiomers of ibuprofen metabolites, 2-hydroxy- and 2-carboxyibuprofen [22]. Both zonal elution and high-performance affinity techniques have been used to characterize the interaction of enantiomers of ibuprofen with immobilized human serum albumin [23]. The stereo specific analysis of two major metabolites of ibuprofen (hydroxyibuprofen and carboxyibuprofen) in urine has been carried out using a sequential achiral–chiral HPLC approach. The achiral separation was carried out under normal-phase conditions using a silica column and 98.2 : 1.8 hexane–ethanol with 0.05% trifluoroacetic acid (THF) as the eluent. The fraction containing the two metabolites is collected, evaporated to dryness, and redissolved in 92 : 8 hexane–ethanol with 0.05% THF (i.e. the chiral eluent). Subsequently, the chiral separation is performed on a Chiralpak AD CSP column [24]. A method has been reported for simultaneously determining the four major metabolites of the analyte in biological fluids. It uses a silica column and N-cetyl-N,N,N-trimethylammonium hydroxide dissolved in the eluent to dynamically modify it [25]. A column-switching system has been developed for measuring ibuprofen directly in plasma that employs three sequential separation steps: (1) the deproteinization and fractionation of 100 mL plasma samples with a polymer-coated mixed-function phase column, (2) concentration with an intermediate column, and (3) a final analytical separation. The reported dynamic range for the analyte is 0.1–250 mg mL^{-1} with intra-day and inter-day variation of less than 5.6% and 6.5% respectively and a detection limit of 25 ng mL^{-1} [26]. See general methodology above [5,6].
Indomethacin	The analyte has been assayed in plasma samples on a C18 column operated at 50°C in combination with 50 : 50 acetonitrile–6 mM phosphoric acid as the eluent and detection at 205 nm [27]. A study has been carried out to examine the loss of the analyte as well as ibuprofen and flufenamic acid in valve injectors for samples in non-eluting solvents [28]. Likewise, the assessment of injection volume limits when using on-column focusing with microbore LC has been evaluated [29]. See general methodology [5] as well as methodology for diclofenac [16] above.
Ketoprofen	An isocratic method has been developed for measuring the analyte in tissue samples following a two-step extraction procedure which involves an initial extraction into methylene chloride and back extraction following acidification with HCl into 95 : 5 isooctane–isopropanol. Subsequently, HPLC separation is carried out on a C18 column using 43 : 57 acetonitrile–water containing 0.1% glacial acetic acid and 0.03% triethylamine [30]. Zonal elution in combination with a novel mathematical approach has been used to characterize the mechanisms involved in the stereoselective binding of ketoprofen enantiomers to immobilized human serum albumin [31]. A stereospecific HPLC assay has been reported for the analyte in human plasma and urine that employes an amylose carbamate column and 80 : 20 hexane–isopropanol with 0.1% trifluoroacetic acid as the eluent [32]. See general methodology above [5,6].
Loxoprofen	See general methodology above [5].
Mefenamic acid	See general methodology above [5].

Table 1 *Continued*

Analyte	*Procedure*
Naproxen	Recently uniform-sized molecularly imprinted polymers for (S)-naproxen have been prepared using 4-vinylpyridine and ethylene glycol dimethacrylate as functional monomer and cross-linker respectively [33]. Subsequently, this material was modified further with a hydrophilic external layer via treating it with a 1 : 1 mixture of glycerol monomethacrylate and glycerol dimethacrylate. Likewise, molecularly imprinted polymers for the analyte have been prepared using 4-vinylpyridine and ethylenedimethacrylate [34]. In this latter study two different polymerization approaches were tested and materials prepared by the thermal route were found to have better chiral selectivity. See general methodology above [5,6].
Piroxicam	See general methodology above [5].
Sulindac	See general methodology above [5].

profens and compounds like ibufeac and xenbucin fit this general description as illustrated in **Figure 1**. However in some cases, with compounds such as indomethacin, caprofen, tolmetin, and zomepirac, additional functional groups are present which influence the chemical equilibria of the solutes and hence make generalization about separation conditions more difficult.

The most common of the non-steroidal analgesic/anti-inflammatories is aspirin, which is sold throughout the world as an over-the-counter (OTC) product and in combination with other non-acid analgesics as well as various other cough-cold agents. However in more recent years, compounds like ketoprofen and ibuprofen, which were first introduced as prescription products for the treatment of arthritis and related inflammatory disorders, are now available in OTC dosage forms and popular for their analgesic properties. When considered as a group, the number of pharmaceutically important acid analgesics/antiinflammatories is relatively small, but because of their widespread therapeutic usage, hundreds of assay procedures have been reported for these compounds, many of which are HPLC based.

In terms of the neutral drugs, perhaps the most common of these are hormones/steroids, certain vitamins (e.g. A, D and E), the pharmaceutical co-additives (e.g. alkyl parabens), and other miscellaneous compounds like the anticoagulant, warfarin, the coronary vasodilator, visnadine, and the antineoplastic agent, carubincin. Some of the more common examples of non-ionizable pharmaceutically active compounds are listed in **Tables 2** and **3** along with the accompanying analytical methodology that has appeared more recently. A number of these have been assayed by HPLC in combination with either mass spectrometry or an alternate detection technique to more commonly used UV monitoring. Also, unlike ionizable drugs that typically require buffers to obtain sufficient solute phase interaction, the neutral drugs are often separated using neat binary hydroorganic eluents in combination with either a C8 or C18 stationary phase. However, unlike the acid drugs, which contain at least one aromatic ring and are easily detectable using standard UV monitoring at 254 nm, a large number of them (i.e. the steroids) contain only a carbonyl group(s) (λ_{max} about 210 nm) and are less favourably detectable by UV monitoring.

Because of the very large number of HPLC procedures that have appeared for neutral and acid drugs, as well as for the related metabolites and degradation products of these, it is not possible to do an extensive review of them in the space limitations of the current chapter. Rather, a more general discussion of important principles and strategies in developing HPLC methods is presented, accompanied by representative analytical procedures for a number of the more important compounds that have appeared in the recent literature. However, for additional information the reader is referred to the comprehensive biennial reviews, *Pharmaceutical and Related Drugs*, that have appeared in *Analytical Chemistry*.

General Trends and Considerations

Over the last three decades HPLC-based methods have become increasingly popular and currently are the most widely used procedures for assaying pharmaceutical compounds. Their growing acceptance has resulted from the inherent versatility and reliability of the methodology, improvements in hardware, and ease of use and automation. Likewise, fundamental sample considerations such as thermal instability, low volatility, and matrix complexity have been important contributing factors.

The selection of the appropriate separation mode is dependent on the solubility and size of the analyte. Since most of the acid and neutral drugs are relatively small analytes that can be dissolved in either water or hydroorganic solvents, and are either neutral or their

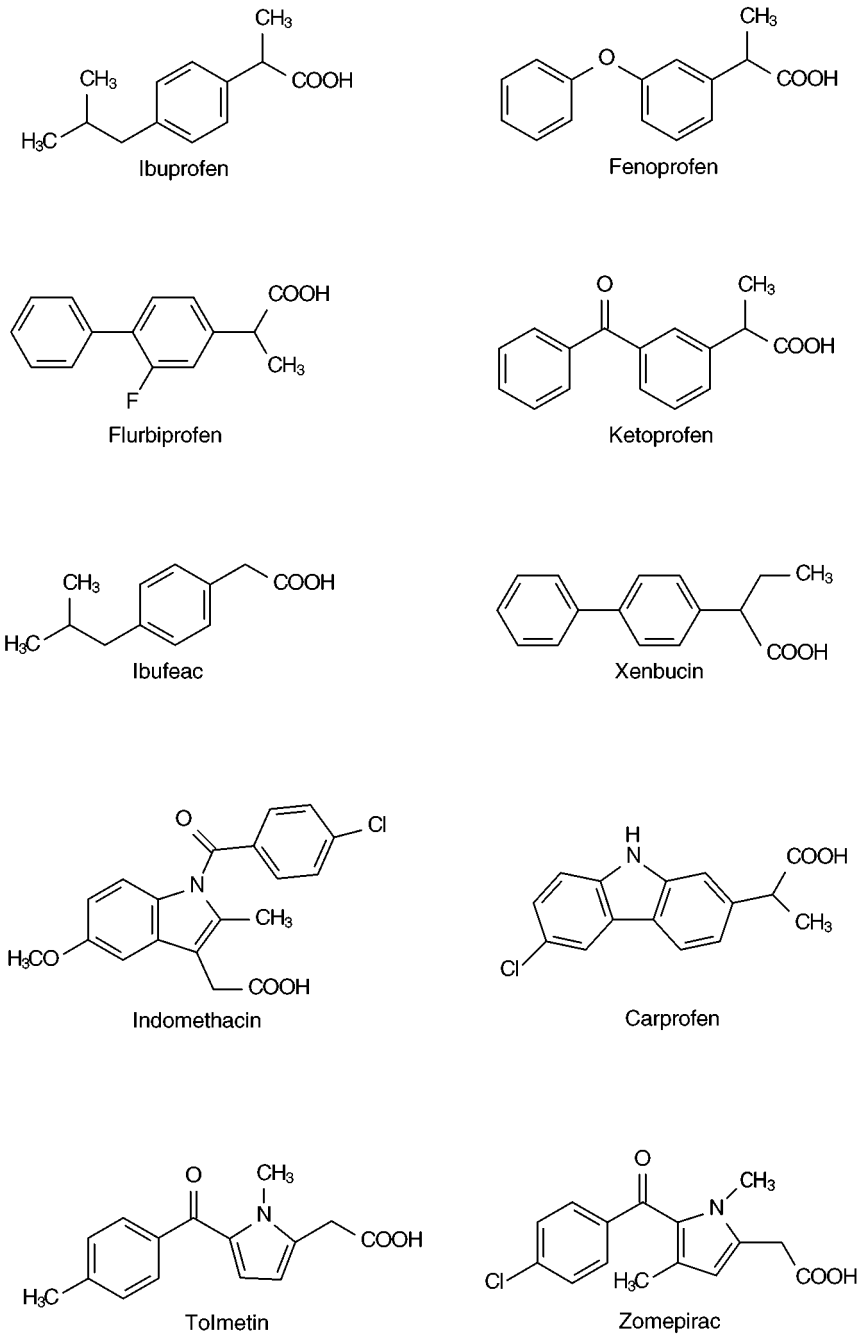

Figure 1 Examples of some common acid drugs.

chemical equilibria can be favourably controlled, reversed-phase (RP) procedures dominate the literature. However, in cases where structural differences are small (e.g. certain steroids, closely related impurities, or degradation products that result in only very minor changes to the analyte hydrophobicity) normal-phase conditions may provide better selectivity. In addition, if the intended application is to measure the level of a single active ingredient and to assure that it is within acceptable standards during manufacture of the formulated product, then simplicity, reliability, speed, and cost become important factors in selecting the overall approach. Typically in this situation, assays based on the use of isocratic eluents are more effective than those based on gradient elution conditions since they are easier to carry out by the less trained practitioner. Alternatively, if the analytical problem involves more complex samples or less

Table 2 Recently published HPLC methodology for steroids

Analyte	*Procedure*
General	Several reversed-phase HPLC methods have been developed for assaying a variety of corticoid alcohols and their corresponding esters and acetals derivatives. All are carried out with a C18 column in combination with several aqueous–acetonitrile eluents, either deoxycorticosterone or methylprednisolone as internal standards, and UV detection. In addition to the parent analytes studied (i.e. fluorocortisone and fluorocortisone acetate, triamcinolone and triamcinolone acetonide, dexamethasone and dexamethasone phosphate, 21-hydroxydeflazocort and deflazacort, betamethasone and betamethasone valerate), the methods have been used to examine the hydrolysis of the analytes in aqueous media at different pH and temperature conditions [35]. Several conjugated and unconjugated estrogens and related impurities have been assayed by reversed-phase chromatography in combination with postcolumn online photochemical derivatization via UV irradiation (254 nm) of the column effluent prior to fluorescence detection (excitation 280 nm; emission 410 or 312 nm). The photo induced modifications were useful for the identification of the various estrogens. The method has been used to evaluate raw materials and pharmaceutical formulations [36]. The influence of temperature on the high-performance chromatographic separation of steroids using eluents containing β-cyclodextrin has been studied [37]. The analysis of several binary drug mixtures have been carried out using either C18 or cyano bonded phases in combination with on-line post-column photochemical derivatization [38]. In another study, the simultaneous determination of glucocorticoids in plasma and urine using HPLC in combination with precolumn fluorimetric derivatization with 9-anthroyl nitrile has been considered [39]. The quantitative structure-chromatographic retention relationship of underivatized equine estrogens have been investigated [40]. The feasibility of using a combined liquid chromatographic–thermospray mass spectrometric–isotope dilution approach for measuring corticosteroids in human plasma has been evaluated. The selection of the eluent composition and its effect on the MS results are discussed [41]. The use of particle-loaded membranes to extract steroids prior to their HPLC analysis has been considered in terms of improvements in analyte stability and detection [42].
Beclomethasone	Metered-dose inhalers containing the analyte have been studied a simple isocratic method that uses a C8 column and 50 : 50 acetonitrile–water as the eluent [43]. HPLC methodology as well as other related assay procedures have been evaluated as a means of measuring the active ingredients and excipients found in commercial nasal sprays. The samples studied were highly viscous and contained a large number of particles in suspension. As such, special emphasis was paid to sample cleanup prior to analysis. Of the techniques examined, liquid chromatography was found best in terms of reproducibility and speed for assaying beclomethasone dipropionate, fluticasone dipropionate and benzalkonium chloride [44].
Betamethasone	Different hyphenated liquid chromatographic (LC) and mass spectrometric (MS) techniques have been evaluated for their use as fast direct analytical methods of measuring betamethasone in hydrolysed and non-hydrolysed urine. Following both the LC, thermospray, and mass spectrometric parameters separately several combined approaches were examined which included LC in combination MS–MS, coupled-column LC (LC–LC) combined with single quadrupole MS, and LC–LC–MS–MS. Neither of the three-step configurations (LC–MS–MS and LC–LC–MS) did not give satisfactory results. However, LC–LC–MS–MS analysis was found to meet the requirements of high sample throughput speed, selectivity, and sensitivity [45]. See general methodology above [35].
Cortisol	See general methodology above [41].
Cortisone	Electrochemically modulated HPLC, a technique developed by Deinhammer and co-workers, has been used to assay four common steroids including the analyte. The separations were carried out using a stainless-steel column containing both a tubular Nafion cation-exchange membrane and packing of porous graphitic carbon spheres, a mixture of 50 : 50 acetonitrile–water containing 0.1M-$HClO_4$/0.1M-$LiClO_4$ as the eluent, an open-circuit potential of $+$ 0.44 V vs. Ag/AgCl/saturated NaCl and detection at 258 nm. The unique aspect of this technique is the ability to adjust analyte retention by manipulating the applied column potential [46]. See general methodology above [41].
Deflazacort	See general methodology above [35].
Dexamethasone	On-line coupled immunoaffinity chromatography-reversed-phase high-performance liquid chromatography particle beam interfaced quadrupole ion trap mass spectrometry has been used to measure dexamethasone and flumethasone [47]. See general methodology above [35].

Table 2 *Continued*

Analyte	*Procedure*
Fluorocortisone	See general methodology above [35].
Fluticasone	The purity of batches of bulk drug substance has been evaluated via a combination of directly coupled HPLC–NMR spectroscopy and HPLC–MS. The separations are carried out on a C18 column using a multilevel gradient produced with D_2O and acetonitrile [48]. High-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry has been used to quantify fluticasone propionate in human plasma. The eluent was 50 : 50 water–ethanol and the mass spectrometer was set to monitor masses of 473.2 and 501.2 *m/z*, which correspond to the internal standard (i.e. the 22R epimer of budesonide) and the analyte respectively [49]. This same analyte also has been measured in plasma using an automated solid-phase extraction liquid chromatography tandem mass spectrometric approach [50]. See methodology for beclomethasone above [44].
Hydrocortisone	The analyte has been assayed in gel formulations by a reversed-phase isocratic procedure using a C18 column and 60 : 40 methanol–pH 7.5 0.02 M phosphate buffer as the eluent [51]. A normal-phase procedure has been developed for simultaneously measuring prednisolone and hydrocortisone in human serum. A silica column is used in combination with 266 : 120 : 26 : 0.8 n-hexane–dichloromethane–methanol–acetic acid as the eluent [52]. See methodology for cortisone above [46].
Lenonorgestrel	The orally active, progestational agent, levonorgestrel (I) in transdermal patches has been extracted with methanol and separated isocratically on a C18 column with 55 : 45 acetonitrile–water as the eluent and detection at 225 nm. The average recovery of 1 mg of the analyte from patches was 99% with an RSD of 0.4% [53].
Prednisolone	See general methodology [41] as well as methodology for cortisone [46] and hydrocortisone [52] above.
Prednisone	See general methodology [41] and methodology for cortisone [46] above.
Triamcinolone	An isocratic reversed-phase method has been reported for measuring triamcinolone acetonide in ointment preparations. The separations are carried out with a C18 column and either 35 : 65 acetonitrile–water or 55 : 44 : 1 methanol–water–acetic acid [54].
Triamcinolone	See general methodology above [46].

routine usage such as that encountered during drug discovery and product development, then specificity, versatility, and flexibility are the most important factors and gradient and multilevel elution, combination column, and novel detection approaches may not only be more convenient, but necessary. Additionally, gradient elution and alternate detection approaches may provide an important advantage in the detection and identification of trace impurities, especially if they vary significantly in their structures and polarity.

As noted above, an important trend in drug discovery and development has been the ever expanding usage of HPLC and the development of separation procedures that are based on reversed-phase conditions. An inspection of the recent pharmaceutical literature reveals that by far the most typical set of RP conditions used to measure neutral and acidic drugs employ either 150 or 250 mm conventional bore columns packed with either octadecyl or octyl 5 to 10 μm porous silica-based stationary phases in combination with either water–methanol or water–acetonitrile as eluents. Typically, octyl bonded phases are used for more hydrophobic analytes or if the analyte is easily resolved on an octadecyl phase, to reduce the amount of organic co-solvent in the hydroorganic eluent. An alternate approach to reducing the chain length of the bonded groups is to use shorter columns. For the neutral drugs, other retention modifying agents (i.e. those that are added to the eluent to suppress unfavourable equilibria) are not needed and for many of the common acid drugs, such as the non-steroidal analgesics/antiinflammatories (see **Table 1**), the most common eluent modifiers are simple buffers added to minimize ionization.

Chemical Equilibria and Methods Development

In the case of neutral drugs, pH control of the eluent is unnecessary. The reason for this is theoretically illustrated in **Figure 2** by the series of dashed lines labeled 1′, 2′, 3′ and 4′ (i.e. constant ln k' vs. eluent pH) for a homologous series of alkyl esters, such as

Table 3 Recently published HPLC methodology for miscellaneous acid and neutral pharmaceutical agents

Analyte	*Procedure*
Ascorbic acid	The stability of the analyte as well as ascorbyl palmitate and magnesium ascorbyl phosphate have been studied using an amino column and either 40 : 60 acetonitrile–pH 4 phosphate buffer or 70 : 30 methanol–pH 3.5 phosphate buffer as the eluents [55]. A review with 70 references has been published that considers the use of different detection methods as well as other HPLC conditions for assaying L-ascorbic acid, its dehydro oxidation product, D-isoascorbic acid, and its dehydro oxidation product [56].
Etoposide	A reversed-phase method, which uses a phenyl column, 23 : 77 acetonitrile–25 mM citric acid-50 mM sodium (pH 2.4) buffer as the eluent and electrochemical detection, has been developed for simultaneously assaying etoposide and its *O*-demethylated metabolite, etoposide catechol in human plasma. Prior to carrying out the chromatographic separation, the samples are extracted using chloroform and methanol. In addition, the long term stability of etoposide and etoposide catechol in human plasma containing ascorbic acid and stored at 27°C has been demonstrated and the procedure has been used to study the analytes pharmacokinetics in plasma following etoposide administration [57]. Several combined HPLC–fluorimetric detection procedures have been developed for measuring the analyte and related compounds in physiological media [58–61].
Parabens	Alkyl esters of p-hydroxybenzoic acid have been separated on a C18 column using 60 : 40 methanol–water as the eluent and detection at 254 nm [62].
Retinoic acid	A gradient elution method has been developed for measuring 9-*cis*-retinoic acid and its major metabolite, 4-oxo-9-*cis*-retinoic acid in human plasma. The analytes are first extracted with methyl-tert-butyl-ether and then separated on a C18 column by employing a multilevel gradient approach. The gradient components are pH 2.7 acetate buffer and methanol and detection is at 348 nm. The method is selective against endogenous compounds and potential metabolites (retinol, all *trans*-, 13-*cis*-, and 4-hydroxy-9-*cis*-retinoic acid) [63]. Normal-phase liquid chromatography/mass spectrometry has been used to study the photo degradation of retinoic acid. The chromatographic separations were performed on a silica column using 96.5 : 3.5 hexane–tetrahydrofuran containing 0.015% acetic acid as the eluent. Isomerization to form 5,6-epoxides occurred more readily in solution than in the solid form and 13-*cis* retinoic acid oxidized more readily than the all-trans isomer [64].
Vitamin A	UV, electrochemical, and particle beam mass spectrometry have been evaluated for use in assaying vitamins A and E in infant formula and related samples. Separations were carried on both a standard and micro bore C8 columns using hydroorganic eluents [65].
Vitamin E	See methodology for vitamin A above [65].

alkyl esters of p-hydroxybenzoic acid (i.e. methyl, ethyl, propyl, and butyl paraben). Shown in **Figure 3** is a representative chromatogram of the separation of this series of analytes carried out on a short column containing an octyldecyl reversed-phase packing using a simple binary hydroorganic eluent. Likewise, this separation demonstrates how retention is influenced by simple hydrophobic changes in the molecule and a plot of ln k' vs. carbon number is linear with a slope related to the incremental methylene selectivity. The addition of a polar substituent (e.g. a hydroxyl group) will result in a shift to shorter retention.

For many of the more common acid drugs, the most often used eluent modifiers are simple buffers that are added to control the protonation/deprotonation of the ionizable carboxyl group and hence to alter the reversed-phase retention properties of the analyte. A simple rule for reversed-phase separations is that by decreasing the extent of dissociation of the analyte one increases its interaction with the stationary phase, hence increasing its retention and the possibility of resolving like compounds. This also is illustrated in **Figure 2**, which shows how eluent pH influences the retention of a series of homologous acids with the general structure RCOOH, where R would be the alkyl chain in fatty acids or it would include all structural features in the molecule except the carboxyl group (e.g. as shown in Figure 1 where the basic structure of ibufeac is compared to the structure of ibuprofen which contains an additional methyl group). In the deprotonated form ($RCOO^-$), the acid drug elutes quickly from the column, whereas in the protonated form (RCOOH), it is retained to an increasing degree based on the hydrophobicity of the molecule. As in the case of the neutral compounds, the incremental addition of carbons to R results in a predictable incremental increase in

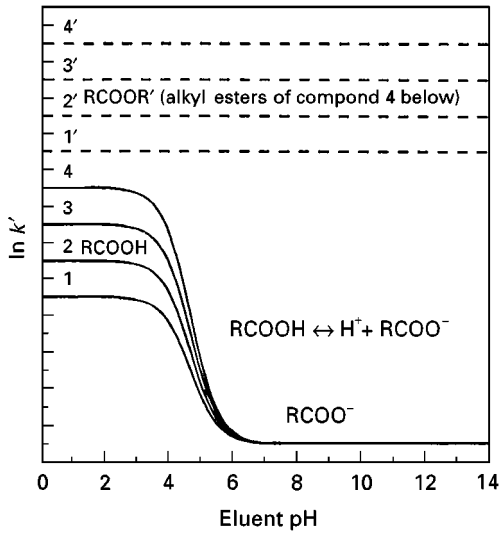

Figure 2 Influence of eluent pH on the retention properties of neutral (dashed lines) and simple monoprotic acid (solid lines) drugs. Curves 1–4 show the incremental change in ln k' as a function of incremental addition of carbon to simple ionizable solutes, and 1′–4′ show the continued incremental change when carbons are added to the alkyl ester portion of monoprotic compounds represented by solid line 4.

retention. This is illustrated by the family of retention curves in **Figure 2** (solid lines labeled 1–4). In the fully protonated form, as in the case of the neutral compounds, the relationship between retention and size (i.e. carbon number) of the aliphatic portion of the homologue is logarithmic.

Typically, the preferred set of conditions for the separation of simple acid drugs (i.e. monoprotic compounds) is in the plateau region of the curves shown in **Figure 2**. Experimentally, this is accomplished by adjusting the pH of the eluent to about 1 to 1.2 pH units below the pK_a of the carboxyl group which is approximately 4.7. When the eluent is buffered to about 3.5, the solute exists predominately in its uncharged form and its retention is governed by the hydrophobic interactions between the nonpolar portion of the molecule and the immobilized alkyl chains of the bonded phase. However, once the eluent pH is adjusted to this region, further adjustments to lower pH will have little effect on the quality of the separation, but will lead to increased chances of phase instability and decreases in the long term performance of the column. A working range for most silica-based bonded phases is 3.0 to 8.5 which is within a useful range for resolving most acidic drugs without the addition of more exotic eluent additives.

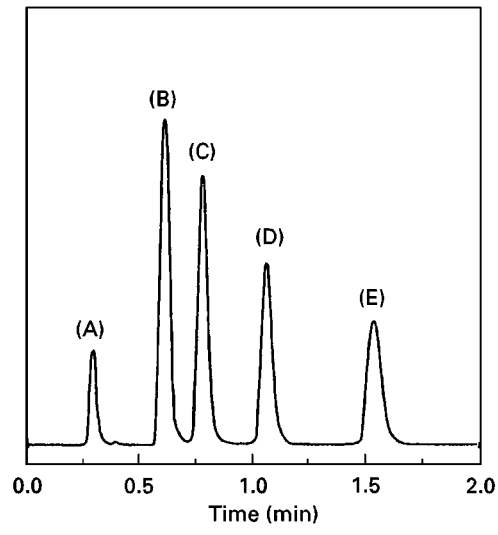

Figure 3 Reversed-phase separation of paraben homologues. Solutes: (A) unretained peak, (B) methyl paraben, (C) ethyl paraben, (D) propyl paraben, and (E) butyl paraben.

As the analyte becomes more complex in terms of the presence of multiple functionality and other structural features, it is often not possible to predict in advance many of the subtleties controlling retention. Considering some of the related non-steroidal analgesic/anti-inflammatories appearing in **Figure 1**, it is possible to predict that ibufeac will elute before ibuprofen, since the molecules are structurally very similar except ibuprofen contains an additional methyl carbon. However, it is much more difficult to predict how long indomethacin will be retained compared to tolmetin or the elution order of ketoprofen, fenoprofen, and flurbiprofen. Nevertheless for the latter set of compounds, since they are structurally similar, it is reasonable to suggest that an assay developed for ketoprofen might be a useful starting point for fenoprofen. Additionally, it is reasonable to suggest that the pH of the eluent for separating the first six compounds in **Figure 1**, which are all monoprotic acids should be in the 3.5 range, since the only important structural feature requiring eluent buffering, is the carboxyl group.

Representative Methodology

Summarized respectively in **Tables 1–3** are HPLC methods that have appeared recently for assaying some of the more common (1) non-steroidal anti-inflammatories/analgesics, (2) steroids, and (3) other miscellaneous neutral and acid drugs. These represent only a fraction of the numerous analytical procedures that have appeared and many of them are useful for measuring the analyte in biological materials as well as in formulated products. Shown respectively in **Figures 4–7** are representative separations of common non-steroidal anti-inflammatory drugs, aspirin and salicylic acid, several important steroids, and the fat-soluble vitamins A, D and E. In all cases the

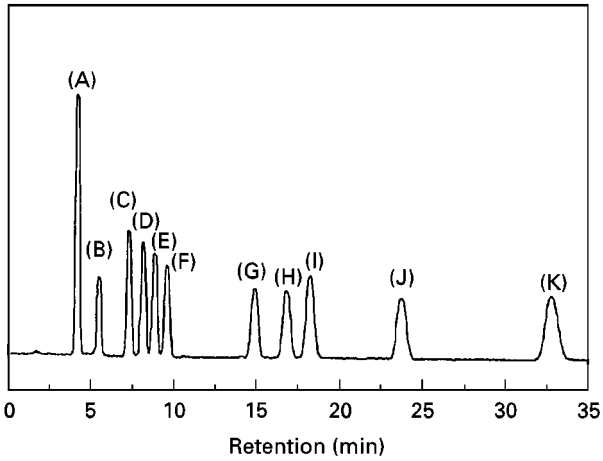

Figure 4 Reversed-phase separation of common nonsteroidal antiinflammatory drugs. Conditions: C_{18} column using 42 : 58 acetonitrile–phosphate buffer (pH 5.0) as the eluent. Solutes: (A) sulindac, (B) loxoprofen, (C) ketoprofen, (D) naproxen, (E) felbinac, (F) fenbufen, (G) flurbiprofen, (H) diclofenac, (I) indomethacin, (J) ibuprofen, and (K) mefenamic acid. (Chromatogram redrawn from Hirai, Matsumoto and Kishi, 1997.)

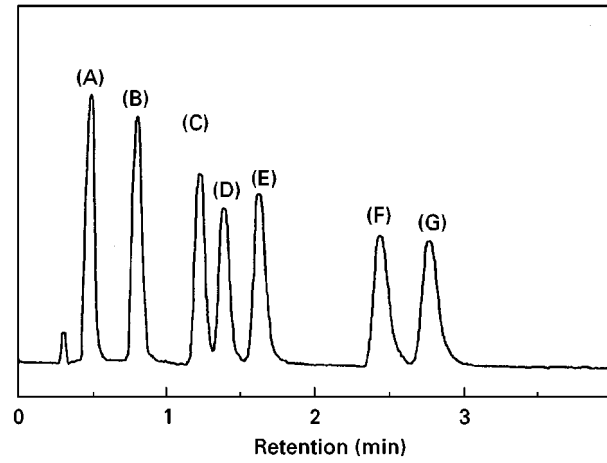

Figure 6 Reversed-phase separation of common steroids. Conditions: C_{18} column using 20 : 80 acetonitrile–phosphate buffer (pH 3.0). Solutes: (A) hydrocortisone, (B) corticosterone, (C) 11-α-hydroxyprogesterone, (D) unidentified compound, (E) 11-keto progesterone, (F) deoxycorticosternone, (G) 17-α-hydroxyprogesterone. (Chromatogram redrawn from *J. Chromatogr. Sci.* 1995, 33: 411.)

chromatograms were obtained under reversed-phase conditions using either an octyl or octadecyl column. In the first three examples standard bore columns were used in combination with UV detection and in the latter vitamin assay the separation was performed on a narrower 2.0 mm i.d. column using both UV and electrochemical detection.

In addition to the relatively common approaches in terms of separation mode and operating conditions discussed above, other important considerations have been addressed in the literature. These include analyte-cleanup prior to carrying out the HPLC separation, evaluation of optical purity, and the development of novel phases such as molecularly imprinted polymers as well as the use of alternate detection techniques like mass spectrometry and fluorescence to improve sensitivity and selectivity. In the latter instance, post-column photo-decomposition/derivatization has been used in methods developed for simple acid drugs such

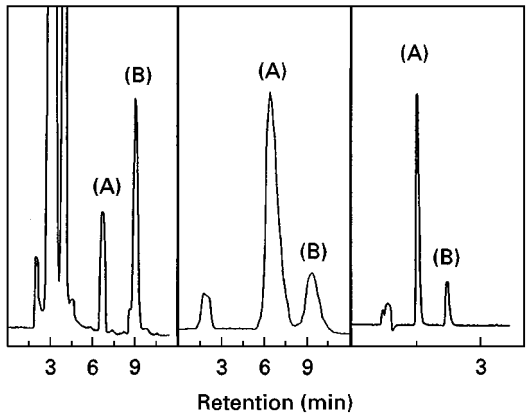

Figure 5 Reversed-phase separations of aspirin and salicylic acid. Conditions: left: C_8 column using 35 : 65 acetonitrile–water containing 0.2% orthophosphoric acid, centre: C_{18} column 25 : 75 acetonitrile–phosphate buffer (pH 2.5), right: C_{18} column 23 : 5 : 5 : 67 acetonitrile–tetrahydrofuran–glacial acetic acid–water. Solutes: (A) aspirin and (B) salicylic acid. (Chromatograms redrawn from McMahon, O'Connor, Fitzgerald, leRoy and Kelly, 1998, Pirola, Bareggi and DeBenedictus, 1998, McCormick, Gibson and Diana, 1997, respectively.)

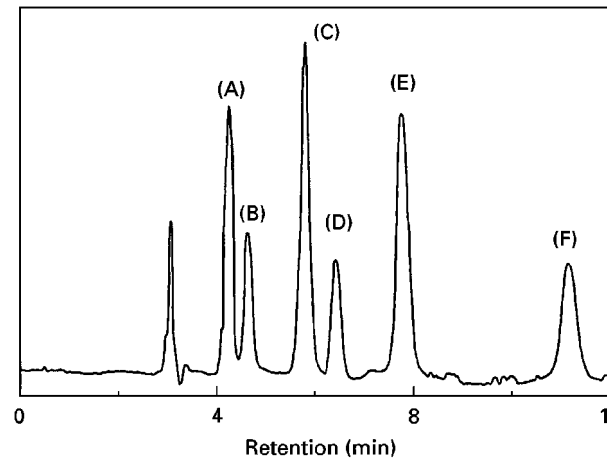

Figure 7 Reversed-phase separation of fat-soluble vitamins. Conditions: C_8 column using 92 : 8 methanol–water. Solutes: (A) vitamin A, (B) vitamin A acetate, (C) vitamin D_3, (D) vitamin E, (E) vitamin E acetate, and (F) vitamin A palmitate. (Chromatogram redrawn from Andreoli, Careri, Manini, Mori and Musci, 1997.)

as diclofenac, conjugated and unconjugated estrogens and related impurities, and binary drug mixtures containing either betamethasone, hydrocortisone, desonide, dexamethasone, or triamcinolone.

Further Reading

[1] Gilpin RK and Pachla LA (1999) *Analytical Chemistry* 71: 217R–233R.

[2] Gilpin RK, Jaroniec M and Lin S (1990) *Chromatographia* 30: 393.

[3] Jaroniec M, Lin S and Gilpin RK (1990) *Analytical Chemistry* 62: 2092.

[4] Horvath C and Mellander W (1997) *J. Chromatogr. Sci.* 15: 393.

[5] Hirai T, Matsumoto S and Kishi I (1997) *Journal of Chromatography B: Biomedical Sciences and Applications* 692: 375–388.

[6] Herraez-Hernandez R, van de Merbel NC and Brinkman UATh (1995) *Journal of Chromatography B: Biomedical Sciences and Applications* 666: 127–137.

[7] Booth TD and Wainer IW (1995) *Journal of Chromatography A* 737: 157–169.

[8] Pirkle WH and Yuelong L (1996) *Journal of Chromatography A* 736: 31–38.

[9] McMahon GP, O'Connor SJ, Fitzgerald DJ, leRoy S and Kelly MT (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 707: 322–327.

[10] Pirola R, Bareggi SR and DeBenedittis G (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 705: 309–315.

[11] Montgomery ER, Taylor S, Segretario J, Engler E and Sebastian D (1996) *Journal of Pharmaceutical and Biomedical Analysis* 15: 73–82.

[12] McCormick TJ, Gibson AB and Diana FJ (1997) *Journal of Pharmaceutical and Biomedical Analysis* 15: 1881–1891.

[13] Blondino FE and Byron PR (1995) *Journal of Pharmaceutical and Biomedical Analysis* 13: 111–119.

[14] Giagoudakis G and Markantonis SL (1998) *Journal of Pharmaceutical and Biomedical Analysis* 17: 897–901.

[15] Kuhlmann O and Krauss G (1997) *Journal of Pharmaceutical and Biomedical Analysis* 16: 553–559.

[16] Bakkali A, Corta E, Berrueta LA, Gallo B and Vincente F (1999) *Journal of Chromatography B: Biomedical Sciences and Applications* 729: 139–145.

[17] Kuhlmann O, Stoldt G, Struck H and Krauss G (1998) *Journal of Pharmaceutical and Biomedical Analysis* 17: 1351–1356.

[18] Gonzalez L, Yuln G and Volonte MG (1999) *Journal of Pharmaceutical and Biomedical Analysis* 20: 487–492.

[19] Kimura T, Shirota O and Ohtsu Y (1997) *Journal of Pharmaceutical and Biomedical Analysis* 15: 1521–1526.

[20] Sochor J, Klimes J, Sedlacek J and Zahradnicek M (1995) *Journal of Pharmaceutical and Biomedical Analysis* 13: 899–903.

[21] Ducret A, Trani M, Pepin P and Lortie R (1998) *Journal of Pharmaceutical and Biomedical Analysis* 16: 1225–1231.

[22] Haginaka J, Sanbe H and Takehira H (1999) *Journal of Chromatography A* 857: 117–125.

[23] Hage DS, Noctor TAG and Wainer IW (1995) *Journal of Chromatography A* 693: 23–32.

[24] Tan SC, Jackson SHD, Swift CG and Hutt AJ (1997) *Journal of Chromatography B: Biomedical Sciences and Applications* 701: 53–63.

[25] Kepp DR, Sidelmlann UG, Tjornelund J and Hansen SH (1997) *Journal of Chromatography B: Biomedical Sciences and Applications* 696: 235–241.

[26] Kang SH, Chang S, Do K, Chi S and Doo SC (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 712: 153–160.

[27] Sato J, Amizuka T, Niida Y, Umetsu M and Ito K (1997) *Journal of Chromatography B: Biomedical Sciences and Applications* 692: 241–244.

[28] Lough WJ, Mills MJ and Maltas J (1996) *Journal of Chromatography A* 726: 67–75.

[29] Mills MJ, Maltas J and Lough WJ (1997) *Journal of Chromatography A* 759: 1–11.

[30] Panus PC, Tober-Meyer B and Ferslew KE (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 705: 295–302.

[31] Zhivkova ZD and Russeva VN (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 714: 277–283.

[32] Carr RA, Caille G, Ngoc AH and Foster RT (1995) *Journal of Chromatography B: Biomedical Sciences and Applications* 668: 175–181.

[33] Haginaka J, Takehira H, Hosoya K and Tanaka N (1999) *Journal of Chromatography A* 849: 331–339.

[34] Haginaka J, Takehira H, Hosoya K and Tanaka N (1998) *Journal of Chromatography A* 816: 113–121.

[35] Gonzalo-Lumbreras R, Santos-Montes A, Garcia-Moreno E and Izquierdo-Hornillos R (1997) *Journal of Chromatographic Science* 35: 439–445.

[36] Gatti R, Gotti R, Gioia MG and Cavrini V (1998) *Journal of Pharmaceutical and Biomedical Analysis* 17: 337–347.

[37] Zarzycki PK, Wierzbowska M and Lamparczyk H (1996) *Journal of Pharmaceutical and Biomedical Analysis* 14: 1305–1311.

[38] Di Pietra AM, Andrisano V, Gotti R and Cavrini V (1996) *Journal of Pharmaceutical and Biomedical Analysis* 14: 1191–1199.

[39] Shibata N, Hayakawa T, Takada K, Hoshino N, Minouchi T and Yamaji A (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 706: 191–199.

[40] Novakovic J, Pacakova V, Sevcik J and Cserhati T (1996) *Journal of Chromatography B: Biomedical Sciences and Applications* 681: 115–123.

[41] Shibasaki H, Furuta T and Kasuya Y (1997) *Journal of Chromatography B: Biomedical Sciences and Applications* 692: 7–14.

[42] Lensmeyer GL, Onsager C, Carlson IH and Wiebe DA (1995) *Journal of Chromatography A* 691: 239–246.
[43] LeBelle M, Pike RK, Graham SJ, Ormsby ED and Bogard H (1996) *Journal of Pharmaceutical and Biomedical Analysis* 14: 793–800.
[44] Bernal JL, del Nozal MJ, Martin MT and Diez-Masa JC and Cifuentes (1998) *Journal of Chromatography A* 823: 423–431.
[45] Polettini A, Bouland GM and Montagna M (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 713: 339–352.
[46] Ting E-Y and Porter MD (1997) *Analytical Chemistry* 69: 675–678.
[47] Creaser CS, Feely SJ, Houghton E and Seymour M (1998) *Journal of Chromatography A* 794: 37–43.
[48] Mistry N, Ismail IM, Smith MS, Nicholson JK and Lindon JC (1997) *Journal of Pharmaceutical and Biomedical Analysis* 16: 697–705.
[49] Li YN, Tattam BN, Brown KF and Seale JP (1997) *Journal of Pharmaceutical and Biomedical Analysis* 16: 447–452.
[50] Callejas SL, Biddlecombe RA, Jones AE, Joyce KB, Pereira AI and Pleasance S (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 718: 243–250.
[51] Galmier MJ, Beyssac E, Petit J, Aiache JM and Lartigue C (1999) *Journal of Pharmaceutical and Biomedical Analysis* 20: 405–409.
[52] Doppenschmitt SA, Scheidel B, Harrison F and Surmann JP (1995) *Journal of Chromatography B: Biomedical Sciences and Applications* 674: 237–246.
[53] Prabhakar B and Deshpande S (1997) *Indian Drugs* 34: 699–701.
[54] Kedor-Hackmann ERM, Gianotto EAS and Santoro MI RM (1997) *Analytical Letters* 30: 1861–1871.
[55] Austria R, Semenzato A and Bettero A (1997) *Journal of Pharmaceutical and Biomedical Analysis* 15: 795–801.
[56] Barthelemy JP (1996) *Analusis* 24: 95–103.
[57] Cai X, Woo MH, Edick MJ and Relling MV (1999) *Journal of Chromatography B: Biomedical Sciences and Applications* 728: 241–250.
[58] Liliemark E, Pettersson B, Peterson C and Liliemark J (1995) *Journal of Chromatography B: Biomedical Sciences and Applications* 669: 311–317.
[59] Stremetzne S, Jaehde U and Schunack W (1997) *Journal of Chromatography B: Biomedical Sciences and Applications* 703: 209–215.
[60] Manouilov KK, McGuire TR, Gordon BG and Gwilt PR (1998) *Journal of Chromatography B: Biomedical Sciences and Applications* 707: 342–346.
[61] Robieux I, Aita P, Sorio R, Toffoli G and Boicchi M (1996) *Journal of Chromatography B: Biomedical Sciences and Applications* 686: 35–41.
[62] Thomassin M, Cavalli E, Guillaume Y and Guinchard C (1997) *Journal of Pharmaceutical and Biomedical Analysis* 15: 831–838.
[63] Dzerk AM, Carlson A, Loewen GR, Shirley MA and Lee JW (1998) *Journal of Pharmaceutical and Biomedical Analysis* 16: 1013–1019.
[64] Bempong DK, Honigberg IL and Meltzer NM (1995) *Journal of Pharmaceutical and Biomedical Analysis* 13: 285–291.
[65] Andreoli R, Careri M, Manini P, Mori G and Musci M (1997) *Chromatographia* 44: 605–612.
[66] Atlas of Chromatograms section (1995) *Journal of Chromatographic Science* 33: 411.

Supercritical Fluid Chromatography

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Introduction

The role of supercritical fluid chromatography (SFC) for the pharmaceutical scientist is based on its enhanced performance compared with high-performance liquid chromatography (HPLC). These advantages are speed, selectivity, and efficiency. Since diffusion is faster in a fluid than a normal liquid, SFC typically is five to ten times faster than HPLC. The mobile phase is less polar than the stationary phase in most SFC analyses so that the separation mechanism is similar to normal-phase HPLC. Thus, SFC can serve as a powerful complement to the vast majority of reversed-phase HPLC analyses. In terms of efficiency, SFC does not necessarily make the column more efficient. However, the tenfold lower viscosity of the mobile phase allows columns to be joined together. As many as eleven 20 cm × 4.6 mm 5-μm packed columns have been linked to yield over 250 000 theoretical plates. Packed capillary columns have been made even longer.

In order to demonstrate the utility of SFC for pharmaceutical analysis, the range of SFC in this field needs to be understood. A brief review of mobile phases, columns, and solutes (see the SFC instrumentation section) will suggest the current limits. The modes of application are summarized, indicating current and future usage. Finally, a thorough but by no means exhaustive listing of successful solute-specific applications serve as a reference for more detailed investigation.

Range of SFC

Mobile Phases

Carbon dioxide is by far the most popular choice as the primary mobile phase component. Since most