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Sample work-up by column techniques*

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Abstract: A review is given of the extraction and purification of biological samples by filtration through lipophilic neutral and ion-exchange dextran gels and their derivatives. Liquid-gel extraction, reversed-phase liquid chromatography and ligand-exchange chromatography are also discussed. The applications of Sephadex and Lipidex gels are reviewed, with special reference to extractions from biological fluids and solid samples and the extraction of metabolites. A number of selective isolation procedures are reviewed for estrogens, lignans, isoflavanes, isoflavones, catechol estrogens, ketonic compounds and ethynyl steroids.

Keywords: *Lipophilic dextran gels; liquid-gel extraction; ion exchange; reversed-phase chromatography; ligand-exchange chromatography.*

Introduction

Simple extraction and purification procedures are needed for high-resolution chromatographic and mass spectrometric analyses of compounds in biological materials. The need has increased with the introduction of capillary columns which are easily overloaded and deteriorate after injection of crude biological samples. This is true both in gas and liquid chromatography, although the latter can more readily be adapted for on-line pre-purification of the samples. Since work in this laboratory has involved analyses by gas chromatography–mass spectrometry (GC–MS) of metabolic profiles of steroids, bile acids and other lipid-soluble compounds, analyses of low levels of selected compounds and metabolic studies using stable isotope-labelling, the authors have investigated general methods for the isolation of group(s) of compounds to be analysed.

The work was started before the advent of high-performance liquid chromatography (HPLC); Sephadex was used as the matrix for attachment of non-polar and other substituents to provide gels for size exclusion and for normal- and reversed-phase chromatography in miscible solvent systems [1–11]. Although Teflon 'spaghetti column' and recycling systems yielded high efficiency separations [5, 6], the major advantages with the substituted Sephadex gels were their inertness, absence of adsorptive losses and high capacity. Ion exchangers with variable polarity could be prepared via a

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chlorohydroxypropyl derivative [10, 12] and were useful for group separations of conjugates of steroids and bile acids [13, 14] and phospholipids [12, 15, 16].

The extraction and purification methods presently used in the authors' laboratory are to a large extent based on filtration of the samples through small beds of lipophilic neutral and ion-exchanging derivatives of Sephadex. The aim is to retain either the compounds to be analysed or the interfering material. This paper reviews some of the methods available and their applications.

Materials and Methods

Lipophilic Sephadex derivatives

Table 1 gives schematic structures, names, abbreviations and references to the synthesis of the most commonly used gels. Three neutral lipophilic derivatives are commercially available, differing in polarity by the type and degree of substitution. In Sephadex LH-20 all the original hydroxyl groups of the cross-linked dextran matrix are hydroxypropylated. The hydroxyl groups of this derivative are further reacted with olefin oxides [9] or glycidyl ethers to give the less polar gels. The commercially available Lipidex gels carry alkoxy groups with an average chain length of 15 carbon atoms. Gels with other polarities and somewhat different properties have been synthesized by reactions with olefin oxides with other chain lengths [9], styrene oxide [9], cyclohexene oxide [24] and 23,24-oxido-5β-cholane [25]. The latter derivative was made in an attempt to obtain chiral properties in steroid separations but this goal was not achieved.

A variety of anion-exchanging derivatives have been prepared by reactions of chloro- or bromohydroxypropyl derivatives of Sephadex LH-20 with different amines [10, 12]. In addition to the materials listed in Table 1, piperidinohydroxypropyl Sephadex LH-20 has been synthesized and used extensively in bile acid separations [26, 27]. Other cation exchangers have been made [8, 13], but those listed in Table 1 have been found most useful in the authors' analytical work.

The chloro- and bromohydroxypropyl intermediates can be used to synthesise stationary phases with other properties. A mercapto derivative may be mentioned [10] that could find application as a selective sorbent in analyses of organic mercury compounds.

The methods of synthesis of the Sephadex derivatives are simple and reproducible. It is possible to prepare gels with a wide range of polarities. Addition of mixtures of water, alcohols, chlorinated hydrocarbons, aromatic and aliphatic hydrocarbons will create reversed- or normal-phase systems with different degrees of polarity, depending on the gel and the solvent mixture [8, 9]. With appropriate solvents or solvent proportions, size exclusion will predominate [6, 8, 9] and will depend on the degree of cross-linking and swelling of the Sephadex matrix.

The possibility to vary the type and content of hydroxyalkyl groups in the ion exchangers is of importance for the design of combined ion-exchange and partition chromatographic systems. The range of solvents and solvent mixtures that may be used with the ion exchangers is also increased, which is important in work with complex mixtures of polar and nonpolar compounds that are difficult to dissolve. Non-specific adsorption of polar compounds seems to be minimized by hydroxyalkylation of the matrix.

Gels of the type listed in Table 1 can be used repeatedly for long periods of time (months to years). Although they may be regenerated in the column after use, it is often

Table 1
Lipophilic neutral and ion-exchanging derivatives of Sephadex used in column clean-up procedures

Schematic structure*	Name or abbreviation	Content of substituents†	Chromatographic systems	Refs
Seph–O–CH_2CHCH_3 | OH	Sephadex® LH–20‡	52% HP	Polar normal-phase	[8, 17]
Seph–O–CH_2CHCH_3 | O–$CHCH_2O(CH_2)_nCH_3$ | CH_2OH	Lipidex® 1000§	10% HA	Polar reversed-phase	[9, 18]
	Lipidex® 5000§	50% HA	Reversed-phase or non-polar normal-phase	[9]
Seph–O–CH_2CHCH_3 | O–$CHCH_2SO_3^-$ H^+ | CH_2OH	SP–LH–20	1.5 meq/g	Cation exchange or ligand exchange	[19]
Seph–O–CH_2CHCH_3 | O–$CHCH_2SO_3^-$ H^+ | $CH_2O–CH(CH_2)_mCH_3$ | CH_2OH	SPHA–LH–20‖	1 meq/g 25% HA	Cation exchange or ligand exchange in less polar or reversed-phase solvents	[19]
Seph–O–CH_2CHCH_3 | O–$CHCH_2\overset{+}{N}H(C_2H_5)_2OH^-$ | CH_2OH	Lipidex®-DEAP§ DEAP–LH–20	1.5 meq/g	Anion exchange	[12, 14]
Seph–O–CH_2CHCH_3 | O–$CHCH_2\overset{+}{N}(C_2H_5)_3OH^-$ | CH_2OH	TEAP–LH–20	1 meq/g	Anion exchange	[20, 21]
Seph–O–CH_2CHCH_3 | O–$CHCH_2O(CH_2)_nCH_3$ | $CH_2O–CHCH_2\overset{+}{N}(C_2H_5)_3OH^-$ | CH_2OH	TEAPHA–LH–20 TEAP–Lipidex	0.7 meq/g and 40% HA	Anion exchange in less polar or reversed-phase solvents	[22, 23]

* Hydroxyalkylated (HA) gels were originally made with C_{11}–C_{14} olefin oxides (Nedox) [19] but commercial gels are glycidyl ethers.

† Hydroxypropyl (HP) and hydroxyalkyl (HA) groups as % of gel weight; ion-exchanging capacity as meq/g dry weight was determined by titration with sodium hydroxide or hydrochloric acid.

‡ Pharmacia Fine Chemicals, Uppsala, Sweden.

§ Packard Instruments Co., Downers Grove, IL, USA.

‖ m = 9 to 12.

more convenient to do this in batches. For analytical applications gel beds of 0.5–5 ml are easily prepared by gravity flow (or the application of slight pressure, using nitrogen) in siliconized glass columns equipped with a Teflon end-piece (with or without stopcock) covered with Teflon gauze to support the gel. The end-piece can be pulled out to empty the column after use. Washing is achieved with aqueous and non-aqueous alcohols and with mixtures of chloroform and methanol. Ion exchangers are converted to the appropriate forms by washing with 0.1–0.5 M solutions of acid, base or salts in water or in 70% methanol. When not in use, the gels are stored at +4° in methanol or aqueous methanol; cation exchangers in sodium form, anion exchangers in acetate form.

Applications

Extraction of biological fluids

Classical solvent extractions of biological fluids are likely to be replaced by solid extraction methods. Solvent extractions may seem to offer greater flexibility in choice of polarity, and nonpolar solvents have frequently been used to avoid extraction of polar interfering compounds. However, if the distribution of endogenous compounds is not known (e.g. among blood components), extraction methods which are too selective may give incomplete yields, depending on the nature and origin of the sample. This may not be detected by conventional recovery experiments. Thus, it is probably better to use a solid extraction method that extracts a broader spectrum of compounds and then permits purification by choice of a suitable elution scheme. Solid extractions are usually faster, easier to automate, do not give emulsions, and often result in concentration of the sample to a volume of organic solvent smaller than that of the original aqueous solution. Solvent volumes (with impurities) and solvent toxicity may also be easier to minimize.

The authors have used Amberlite XAD-2 [28, 29], Sep-Pak C_{18} [30] and Lipidex [31–33] for the extraction of steroids, bile acids and some types of xenobiotics from urine, bile, plasma and milk. Each of these sorbents has properties different from the others. In some cases the yield in the subsequent purification of extracted compounds is influenced by the method of extraction (probably due to the effects of co-extracted material); Amberlite XAD-2 gave better final yields of estradiol than Sep-Pak C_{18} in analyses of this steroid in cytosol fractions from rat uterus [34].

Amberlite XAD-2. This is a polystyrene resin introduced by Bradlow in 1968 for the quantitative extraction of steroids and steroid conjugates from urine [28], and has been widely employed for extraction of many types of amphipathic compounds. However, the relatively low capacity of the resin, the need for a low flow rate and the presence of sites with strong affinity for anions [35] were drawbacks and led to the search for other types of material. Although the problem of anionic sites may be overcome with a wash of aqueous triethylamine sulphate or sodium sulphate [35], Amberlite XAD-2 has now been largely replaced by octadecylsilane-bonded silica, which readily extracts compounds of a wide polarity range from aqueous solutions such as urine, enzymatic hydrolysis mixtures, etc. [30]. The alkyl-bonded silicas have a higher capacity, can be used at higher flow rates and will, under certain conditions, extract nonpolar compounds that are not extracted by Amberlite XAD resins.

When compounds to be extracted are bound to proteins, e.g. in plasma or milk, conditions must be used which minimize hydrophobic or ionic interactions between solutes and proteins. The choice of method depends on the nature of the interactions

involved. Bile acids are extracted from plasma after 10-fold dilution with 0.1 M sodium hydroxide [29, 36]. Unconjugated hormonal steroids in plasma, which may be strongly bound to carrier proteins, are extracted after dilution with an equal volume of physiological saline and the sorbent bed is kept at 64°C [22, 35, 37]. Conjugated, i.e. charged, steroids are only partially extracted from plasma and milk under these conditions but are quantitatively recovered at 64°C when the sample is diluted with an equal volume of 0.5 M triethylamine sulphate [35, 38]. Bile acids can also be extracted with this method. Neither of these conditions gives quantitative extraction of less polar lipids [35], e.g. cholesterol and its esters, triglycerides and phospholipids which are present in lipoproteins and chylomicrons. While this may be an advantage and result in a cleaner extract, nonpolar compounds, e.g. xenobiotics, dissolved in lipoproteins will also be lost in the extraction. The methods for disruption of lipoproteins mentioned below may be of value also in extractions with alkyl-bonded silica.

Lipidex 1000 *and* 5000. These can both be used to extract lipids and lipid-soluble compounds from aqueous media. Lipidex 1000 is preferred for steroids, whereas Lipidex 5000 gives better yields of nonpolar chlorinated pollutants in milk (Norén and Sjövall, to be published). The yields depend on the polarity of the compounds to be extracted and on the nature of the solution (protein binding, aggregation of nonpolar molecules in water, inclusion in lipoproteins). Steroid hormones of low and medium polarity (three oxygen substituents or less) in urine are readily extracted by passing the sample through a bed of Lipidex 1000 in water [31]. In contrast to the case with Amberlite XAD-2 and octadecyl-bonded silica, conjugated steroids are extracted poorly or not at all. If the Lipidex bed is washed with alkaline water, acidic steroids including estrogens are eluted [23]. Thus, extraction with Lipidex gels closely resembles extraction with a solvent of medium polarity and differs markedly from extractions with octadecyl-bonded silica and Amberlite XAD-2. This is also seen from the yield of solids from urine: the two latter sorbents extract 2–3% of the total solids, while Lipidex 1000 extracts less than 0.1% [31].

More polar neutral compounds can be extracted from urine by addition of 5% (v/v) pentylamine to the sample. This effect is obtained only with primary amines having 4–6 carbon atoms [31]. The mechanism is not known but may involve interactions between the alkyl groups of the amine and the gel. Potential reactions between the amine and extracted compounds present a drawback with this method.

Pentylamine inhibits protein binding, disrupts lipoproteins and promotes extraction of steroids and lipids from plasma [31] and of lipids and nonpolar pollutants from milk [33]. When milk is mixed with an equal volume of water–methanol–*n*-pentylamine (17:2:1, v/v) and shaken with Lipidex 1000 for 2 h, a clear aqueous supernatant and a gel phase containing the lipids are formed [33]. The mixture can be poured into a column for washing and elution of compounds to be analysed. However, aminolysis with formation of fatty acid pentylamides is a drawback. This is avoided and the same or better yields of lipids and nonpolar pollutants are obtained by dilution of the milk with formic acid–methanol instead of the pentylamine solution (Norén and Sjövall, to be published). The gel bed can be washed with increasing concentrations of methanol in water to remove unwanted polar compounds. These may then be extracted by passage of the solution through a Sep-Pak C_{18} cartridge after evaporation of the methanol.

Protein binding is a major factor affecting yields in extractions of plasma samples. It can be minimized by dilution and heating [37, 39], or by addition of pentylamine [31] or ion-pairing agents [32]. The method to be used depends on the analytes concerned.

However, because of protein binding, flow rates have to be lower in extractions of plasma (0.5 ml min^{-1} cm^{-2} column cross section) than of urine (2–5 ml min^{-1} cm^{-2}) [31, 32].

Lipidex gels are useful in ion-pair extractions [32]. While unconjugated bile acids can be quantitatively extracted by Lipidex 1000 from an acidified aqueous solution [40], this is not the case with more polar conjugated bile acids (Fig. 1). This selectivity is of value when only unconjugated acids are to be extracted, e.g. after alkaline or enzymatic hydrolysis of conjugated bile acid fractions. Extraction of all types of bile acids can be achieved by addition of 0.03 M decyltrimethylammonium bromide to the aqueous solution [32]. In the case of urine, the quaternary ammonium salt can be added as a solid; plasma and bile are diluted with a solution of appropriate strength to yield a final concentration of 0.03 M counter-ion and 0.25 M phosphate buffer, pH 7. Among a variety of water soluble tertiary and quaternary ammonium ions tested, only decyltrimethylammonium gave satisfactory results; the nonyl, dodecyl and hexadecyl homologues gave little or no extraction. Differences in interaction of the alkyl chains with those in Lipidex, and differences in critical micellar concentrations might explain this result. The concentration of decyltrimethylammonium ions is important; when the critical micellar concentration is exceeded the bile salt is eluted with the aqueous wash (Fig. 2). The amount eluted after extraction with the optimal concentration of counter-ion corresponds to the bile acid salt with decyltrimethylammonium. No counter-ion is taken up by Lipidex in the absence of the bile acid. This suggests that an ion-pair formed in the aqueous phase is sorbed by the gel phase.

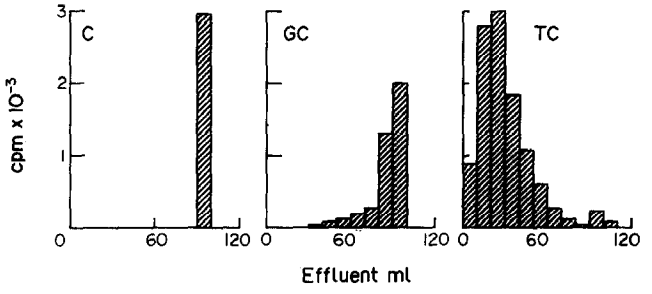

Figure 1
Extraction of bile acids with Lipidex 1000. ^{14}C-Labelled cholic (C), glycocholic (GC) and taurocholic (TC) acids were dissolved in 10 ml 0.3 M NaCl. Acetic acid was added (to 0.5 M) and the solutions were passed through a 4-ml bed of Lipidex 1000 followed by 50 ml 0.5 M acetic acid in 0.3 M NaCl. The bed was washed with 20 ml water and 20 ml methanol was used for elution of extracted bile acids (adapted from [32] and [40]).

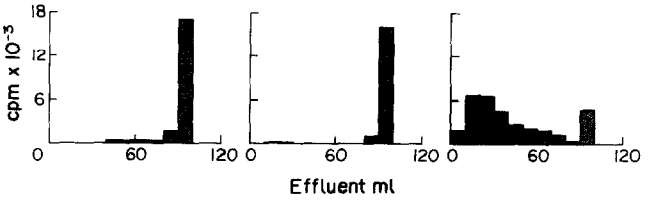

Figure 2
Effect of concentration of decyltrimethylammonium bromide on the extraction of ion-pairs of taurocholate with Lipidex 1000. The bile acid was dissolved in 10 ml 0.3 M NaCl containing counterion at 0.02 M (left panel), 0.03 M (centre) and 0.05 M (right), and passed through a 5-ml bed of gel followed by 60 ml of the same solvent. The bed was washed with 20 ml water, and 10 ml methanol used to elute the extracted bile acid (adapted from [32] and [40]).

The extraction of cholic, glycocholic and taurocholic acids added to plasma is illustrated in Fig. 3. Sulphates and glucuronides of bile acids are also efficiently extracted. It is probably important for the extraction that the polar glucuronides form ion-pairs with both the bile acid and glucuronic acid moieties.

Extraction with Lipidex is an alternative or complementary procedure to extraction with Sep-Pak C_{18} and similar sorbents. Elution of extracted material is achieved with solvents that are compatible with subsequent subfractionation on beds of lipophilic ion exchangers. When ion pairs are extracted, passage of the eluate through a suitable ion exchanger will remove the counter-ion, and the analytes can then be purified on an ion exchanger of opposite charge.

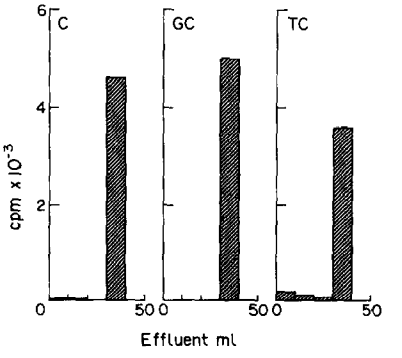

Figure 3
Ion-pair extraction of bile acids with Lipidex 1000. ^{14}C-labelled bile acids (C, GC, TC, cf. Fig. 1) were equilibrated with 5 ml plasma, which was then diluted with an equal volume of 0.06 M decyltrimethyl-ammonium bromide in 0.5 M phosphate buffer, pH 7, and passed through a 5-ml bed of Lipidex 1000. The bed was washed with 10 ml counter-ion solution (0.03 M in 0.25 M buffer) and 10 ml water and the bile acids eluted with 10 ml methanol. The leakage of TC is attributable to the flow rate being too high (adapted from [32] and [40]).

Extraction of tissue and faecal samples

Solid biological samples can be extracted with a combination of solvents and solids [41, 42]. Lipidex and octadecyl-bonded silica are the preferred sorbents for most sample types, although Amberlite XAD-2 has been used when bile acids were analysed in alkaline digests of tissues [43]. Enzymatic digestion of tissue samples to release the analytes (cf. [44]) is also compatible with solid extraction. However, the authors have not yet applied this method in steroid and bile acid analyses.

An example of a combined extraction procedure is the analysis of bile acids and sterols in faeces [42]. Lipid-soluble compounds are extracted by refluxing in aqueous ethanol, ethanol and chloroform–methanol. In order to remove inorganic and water-soluble contaminants, the residue after evaporation of the organic solvents is suspended in acidified water, which is then passed through a bed of Lipidex 1000. The less polar compounds are extracted by the gel and the aqueous effluent is passed through a Bond-Elut C_{18}-cartridge which extracts conjugated steroids and bile acids. Subsequent purification steps can then be the same as for the extraction of biological fluids.

When lipids are present in excess it may be a problem to obtain an appropriate slurry of the extract in water. Aggregated lipid-soluble compounds may then pass through the Lipidex bed without being extracted. This problem can be avoided by adding methanol to the water, on a similar basis to the method using methanol for the extraction of milk lipids, as discussed. It is likely that the alcohol decreases formation of lipid aggregates and micelles while increasing the miscibility of liquid and gel phases, thereby facilitating the transfer of lipid-soluble materials into the gel phase. In agreement with this interpretation, sterols in bile and aqueous suspensions of faecal extracts can be quantitatively sorbed on Sep-Pak C_{18} after addition of an equal volume of methanol (J. Sjövall and M. Axelson, to be published). In the absence of methanol extraction is poor. Although the more polar steroids and bile acids will appear in the eluate, they may be

recovered after removal of the methanol followed by passage of the solution for a second time through the sorbent. The extracted material may then be separated into crude fractions by stepwise elution with aqueous methanol and finally with chloroform–methanol.

Another method, used for extraction of steroids from tissues [41], is to evaporate a lipid extract obtained with hexane–2-propanol (3:2, v/v), together with a suitable amount of Lipidex 1000 in a rotary evaporator. The gel is then slurried in water and poured into a column for washing and elution. Part of the cholesterol, phospholipids and other glycerides will be washed out with water as discussed above, but most of the hormonal steroids are retained and can be eluted with 85% v/v methanol. This solvent also elutes extracted phospholipids and cholesterol. The former are retained by a Sep-Pak C_{18}-cartridge attached to the outlet of the Lipidex column, and cholesterol is removed by dilution of the effluent with water to give 70% methanol that is then passed through a small bed of Lipidex 5000. This forms a reversed-phase system in which cholesterol is retained while the hormonal steroids pass through. The effluent is evaporated to give recoveries of unconjugated steroids through the entire procedure of about 90% [41]. A residue of 0.5–1 mg is obtained after extraction of a rat testis weighing about 1 g and containing about 22 mg of lipids. The major contaminants are phospholipids which constitute 60–65% of the total lipids originally present. The reduction in weight is sufficient to permit further purification of the steroids on an analytical HPLC column equipped with a guard column.

Methods for solid extraction can be improved considerably, as demonstrated by several papers at this Symposium describing their incorporation into automated HPLC systems. In many cases, however, the concentrations of compounds to be analysed are very low (typically pg/ml or pg/g), so that relatively large samples have to be extracted and purified. Lipidex gels are simple to use and have a high capacity; they are therefore suitable for preliminary extraction and enrichment prior to purification and analysis by high-resolution methods.

Separation of groups of metabolites

The metabolism of lipid-soluble endogenous and exogenous compounds often involves conjugation with glucuronic acid, sulphuric acid, amino acids, etc. prior to excretion. The conjugates themselves may be further metabolized. Metabolic profiles of the different classes of conjugates may differ greatly, depending both on the specificity of the conjugating enzymes and on the nature of any subsequent metabolism. Intact conjugates are difficult or impossible to analyse by GC–MS, so that group separation of the classes prior to hydrolysis is therefore of importance. This is most readily achieved with ion exchangers. In addition to the group separation obtained, the behaviour of metabolites on ion exchangers provides valuable information about their nature. For example, derivatives of bile acids and sterols can give very similar mass spectra and their identification is greatly facilitated by separation of neutral and acidic steroids prior to analysis by GC–MS. The authors have usually used cation followed by anion exchangers in series, since the compounds to be analysed have been neutral or acidic. The column volumes employed are between 0.5 and 3 ml for analytical purposes, corresponding to an ion-exchanging capacity of 0.1–1 meq. The choice of solvent is governed by the solubility of the sample and the need for simultaneous separation by partition chromatography. It is often convenient to pass the eluate from the preceding solid extraction directly through the ion exchangers, after modification of the solvent mixture if required.

Cation exchangers. In the analysis of acidic compounds, the cation exchanger is used to remove basic contaminants. It is also employed to remove the counter-ions present in ion-pair extractions. Incomplete sorption of acids by a weak anion exchanger can also occur when the extract has not been passed through a cation exchanger, possibly through the formation of endogenous ion pairs.

When the biological extract is soluble in 70% v/v methanol, SP-Sephadex C-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) has been used in the H^+-form for the analysis of steroids [21, 23, 25], bile acids [42] and bile alcohols [45]. However, when large extracts of faeces have to be purified, solubility problems require the use of methanol–chloroform–water mixtures, together with a lipophilic ion exchanger (SP-LH-20 or SPHA-LH-20) which swells in the solvent used. This is particularly the case when positively charged metabolites, of xenobiotics, for example, are to be purified [46] (Egestad, Petersson, Åhlberg, Darnerud, Rafter, Bergman, Gustafsson, Wachtmeister and Sjövall, to be published).

The hydroxyalkylated SPHA-LH-20 can be used to combine cation exchange and reversed-phase partition [19]. A bed of this gel in H^+-form in 70% v/v aqueous methanol will retain both cationic compounds and nonpolar lipids, e.g. cholesterol, while more polar steroids elute with the void volume and can be passed onto the anion exchanging bed.

The lipophilic cation exchangers are useful for clean-up of extracts in analyses of cationic drugs. There are, however, few examples of such applications [47, 48] and further studies would be of interest. They are also useful for the separation of metabolites conjugated with cysteine and glutathione from those conjugated with, for example, *N*-acetylcysteine, glucuronic acid or sulphuric acid [46]. Those conjugates with a free amino group are sorbed and can be eluted by addition of ammonia to the solvent. Such preliminary fractionation may help to simplify the HPLC analysis of complex mixtures of conjugates.

The strong cation exchangers in H^+-form can catalyse decomposition of acid-labile compounds. They can also catalyse useful reactions, e.g. methylation of carboxylic acids when used in non-aqueous methanol. Their catalytic properties can be used to advantage and permit a combination of group separation and derivatization on the same column (J. Sjövall, M. Axelson, to be published).

Anion exchangers. Lipid-soluble compounds containing phenolic or carboxylic acid groups and compounds conjugated with different acids may be isolated according to acidity by use of lipophilic anion exchangers [13, 14, 21]. The weak anion exchanger DEAP-LH-20 (Lipidex-DEAP) originally synthesized [12–14], has been replaced in many applications by the stronger TEAP-LH-20 [20–23]. Several groups are using piperidinohydroxypropyl Sephadex LH-20 first described by Goto *et al.* [26].

As in the case of cation exchangers, the choice of solvent and polarity of the anion exchanger is governed by the solubility of the sample. Aqueous 70% v/v methanol or ethanol has usually been used for group separation of bile acid and steroid conjugates, but methanol–chloroform–water mixtures can be used with TEAP-LH-20 and TEAPHA-LH-20; this solvent mixture is needed when large extracts of, for example, faeces are to be purified (Egestad *et al.*, to be published; see above). It is an advantage if the acids can be eluted with a volatile displacer which is readily removed following chromatography. When this is not possible, buffers may be selected which are needed in

a subsequent step, e.g. enzymatic hydrolysis [13, 21]. The chemical stability of the solute in acids and alkali has also to be considered.

The separation of different groups of steroids on TEAP-LH-20 is shown in Fig. 4. Mono- and diphenolic acids which are not sorbed on the weak DEAP-LH-20 are readily sorbed on TEAP-LH-20 in the OH^--form and separated from neutral compounds [21, 22]. They may then be eluted with solvents (e.g. methanol, methanol–chloroform) saturated with CO_2 (Fig. 4). This yields a very clean phenolic fraction (see below) although larger volumes of CO_2-saturated solvent also elute the weaker carboxylic acids (bile acids, fatty acids) (J: Sjövall and M. Axelson, to be published). However, the latter compounds are more conveniently eluted with 0.1 M acetic acid in aqueous alcohol [14]. Higher concentrations of acetic acid (0.8 M) have been used for the elution of glucuronides of neutral steroids [49]. Glucuronides of both neutral and phenolic steroids (possessing a free phenolic group) are eluted with 0.4 M formic acid in aqueous alcohol [21] (Fig. 4). Alcoholic acetate buffer solutions of increasing ionic strength (0.1–0.5 M) and apparent pH (5.0–10.0) have been used for the group separation of more acidic conjugates such as glycine and taurine conjugates, monosulphates and disulphates [13, 14, 21]. The anion exchangers have also been used for group separation of cysteine, glutathione and *N*-acetylcysteine conjugates of xenobiotics [46].

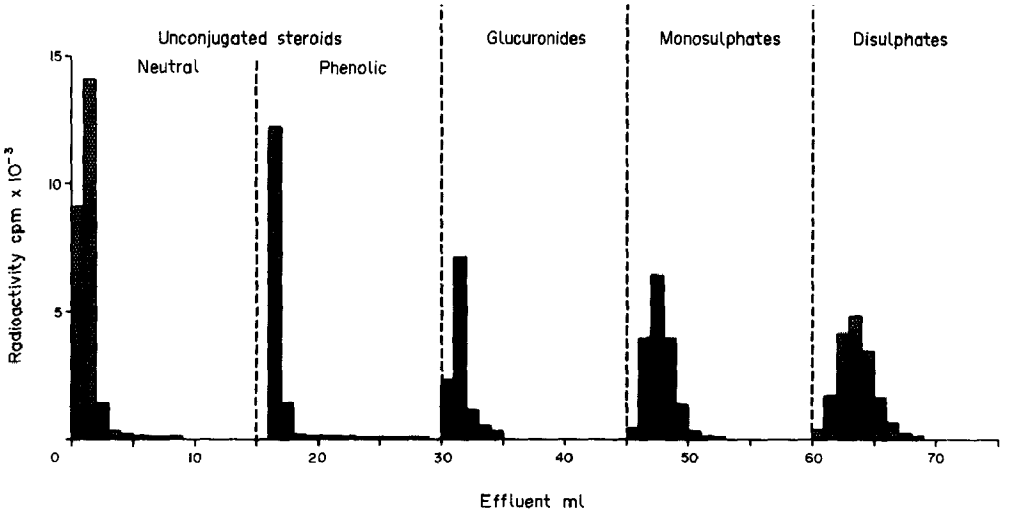

Figure 4
Group separation on TEAP-LH-20 (40 × 4 mm, OH^--form in 72% v/v aqueous methanol) of radioactively-labelled neutral steroids (cortisol, progesterone), phenolic steroids (estrone, estradiol, estriol), steroid glucuronides (testosterone and estriol glucuronides), steroid monosulphates (dehydroepiandrosterone sulphate) and steroid disulphates (5-androstene-3β,17β-diol disulphate) added to an extract of urine (from [21] with permission).

The systems described have been developed for the quantitative isolation of major groups of steroids, bile acids and conjugate classes. Clearly other types of acids or mixed conjugates will appear in the same or overlapping fractions. When required, the resolution of the chromatographic system may be increased by modification of the buffers and/or the use of mixed chromatographic systems. For example, the hydrophilic DEAE-Sephadex (Pharmacia) has been used in methanol to obtain a combination of ion exchange and adsorption of aromatic steroids, permitting group separation of aromatic

from non-aromatic steroid conjugates of the same class [50]. Reversed-phase systems may be obtained with TEAPHA-LH-20 [22].

Considerable purification is achieved by passing a sample through an ion exchanger before and after enzymatic or chemical hydrolysis. In the case of bile acids and steroids, acidic conjugates are converted into neutral compounds or weaker acids. Thus, they will appear in another fraction from the anion exchanger, and only compounds with the same properties will change in the same way. This purification is essential in the analysis of bile acids in urine, where much larger amounts of conjugated neutral steroids are present, and in the analysis of steroid conjugates in urine from patients with liver disease, where bile acids may be present in much higher concentrations.

Finally, the purification of biological samples by passage through ion exchangers can be essential for quantitative derivatization to form labile derivatives which may hydrolyse, e.g. in the presence of the remaining acids [51].

Neutral lipophilic gels. These gels are used for separation according to polarity, in order to obtain fractions that can be further purified or analysed by HPLC or analysed by GC–MS. Suitable normal-phase systems of low polarity are formed with Lipidex 5000 in hexane–chloroform (95:5 to 60:40, v/v) [9, 5]. Very nonpolar systems with hexane–toluene have been used to purify alkyl- and alkenyl-diacylglycerols from each other and triacylglycerols [20]. Systems of higher polarity are formed with Sephadex LH-20 in hexane–chloroform (7:3 to 5:5, v/v), to which a low percentage of ethanol or methanol may be added. Saturation with water may improve the reproducibility [5]. A wide variety of solvent mixtures has been described for various separation purposes; their use in steroid separations has been reviewed [52]. Depending on the degree of swelling of the gels, size exclusion effects may become important [6]. Normal-phase systems are convenient since they permit the use of volatile solvents which are easy to evaporate. A simple pre-separation step on a lipophilic gel may protect HPLC columns from overloading, memory effects and deterioration [34].

Reversed-phase systems are formed with Lipidex 5000 in methanol–water–chloroform (or ethylene chloride) (60:40:10 to 95:5:25, v/v) [9]. The addition of chloroform induces swelling of the gel and improves the efficiency. Systems of this type are useful when nonpolar compounds are to be removed from an extract [37, 51], and have also been used with TEAPHA-LH-20 [22]. Removal of nonpolar contaminants may also be achieved by filtration of the extract in 70% v/v methanol through Lipidex 5000, as discussed above.

Reversed-phase systems of higher polarity can be obtained with Lipidex 1000 in methanol–water–chloroform–*n*-butanol (50:50:5:5 or 40:60:3:7, v/v) [18]. Equilibration of this system requires the gel to be swollen in the organic solvent mixture first. When a gel bed has settled in the column, it is washed with the complete solvent and the system then remains stable. The polarity of the system makes it suitable for group separation of compounds such as polar bile acids, prostaglandins and their esters [18] and bile alcohols [45].

The neutral lipophilic gels have been used extensively in two specialized areas: for selective isolation of steroid mono- and disulphates, and for purification of products after derivatization reactions. The former application involves the use of Sephadex LH-20 in chloroform–methanol (1:1, v/v) containing 0.01 M NaCl or KCl [4, 53]. In contrast to the free acids and a number of other salts [4], the sodium and potassium salts of steroid sulphates are strongly retained by the gel phase and can be separated from each other

and from most other contaminating material in plasma. Retention increases with increasing chloroform content [4, 14]. This method may be useful in clean-up procedures for the sulphated metabolites of other endogenous compounds and drugs.

Sephadex LH-20 was first used by Engel and coworkers for the qualitative clean-up of mixtures after derivatization of steroids with *O*-methoxyamine hydrochloride and silylating reagents [52]. Essentially quantitative yields were achieved by use of Lipidex 5000 in hexane–pyridine–hexamethyldisilazane (98:1:1, v/v) [37] or (98:1:2, v/v), with the addition of dimethoxypropane [22]. The mixture in this solvent is filtered through a 70 × 4 mm bed of gel in 1–2 min, and the derivatized compounds are then collected for analysis. Analogous methods are generally useful when polar compounds are converted into highly nonpolar derivatives.

Selective isolation procedures

Separation methods with higher selectivity than that of the simple ion exchange techniques described above can be based on specific substituents in unconjugated endogenous or exogenous compounds. A few methods of this type have been developed and used in the analysis of biologically active compounds.

Estrogens, lignans, isoflavanes and isoflavones. These and other compounds containing one or two phenolic hydroxyl groups are sorbed by TEAP-LH-20 in OH^--form and can be eluted with CO_2 in the solvent as discussed above [21, 22, 54–56]. If present, weak carboxylic acids contaminate this fraction, but can be removed by passing the solution after evaporation of CO_2 through TEAP-LH-20 in bicarbonate form, since this retains only the carboxylic acids. If required, the latter may be eluted again with addition of CO_2 or acetic acid to the solvent, a method that can be used for the purification of bile acids and prostaglandins from biological materials (J. Sjövall and M. Axelson, to be published).

The phenolic compounds can be subfractionated into mono- and diphenolic species by passing the effluent from TEAP-LH-20 through DEAE-Sephadex (Pharmacia) in base form [56, 57]. Monophenolic compounds are only weakly retained and elute with the neutral solvent, while diphenolic compounds require the addition of CO_2 for elution. This method has been used for the selective isolation and analysis of lignans, isoflavanes and isoflavones from human and animal urine, where they were detected by use of the group isolation procedures discussed in the present review [56–59]. The same methods can be used for the analysis of their dietary precursors, which are glycosides [57, 58].

Catechol estrogens. Because of their sensitivity to oxygen and alkali, catechol estrogens are degraded and cannot be isolated with TEAP-LH-20 in base form. Although not degraded by the bicarbonate form, they are not retained on this system. In borate form, however, TEAP-LH-20 will retain and protect the catechol estrogens from oxidative decomposition [60]. Specific complex formation with borate has been utilized for a long time in chromatographic systems for compounds with a *cis*-1,2-diol structure [61]. Monophenolic compounds are only weakly retained and are eluted with neutral solvents. Catechols are then eluted by saturation of the solvent with CO_2, recoveries in this step being about 80% [60]. A detailed study of an analogous method using polar Sephadex ion exchangers has been published [62].

The isolation methods for phenolic compounds are simple and 10–20 samples can be

processed in a few hours. The purity of the fractions is high and recoveries (with the exception of catechols) are over 90%.

Ketonic compounds. Most biologically active neutral steroid hormones possess a 3-oxo group. Bile acids with this group have been implicated in colon carcinogenesis. A selective purification method has therefore been developed for their analysis. It is based on the observation that oximes of ketonic compounds are positively charged in methanol and are retained by a lipophilic strong cation exchanger in H^+-form [63]. The mobilities depend on the position and number of oxime groups and on the presence of neighbouring groups. Oximes of 7-, 12-, 17- and 20-oxosteroids are eluted much faster than those of 3-ketosteroids [19, 63, 64]; moreover, syn- and anti-isomers may be separated [65]. The biological sample is first passed through SP-LH-20 in H^+-form, and, if required, through TEAP-LH-20 in OH^--form. Oximes are prepared of the neutral steroid or bile acid fractions and then passed through a small bed of SP-LH-20 in H^+-form in methanol. Non-ketonic compounds appear in the solvent front, while oximes of 7-, 12-, 17- or 20-oxo compounds are eluted later with methanol and oximes of 3-oxo compounds are displaced with 5% v/v pyridine in the solvent. Oximes are readily derivatized for GC–MS analysis; the purity of the fractions from, for example, plasma or faeces is high and the recoveries of picogram amounts through this procedure are 80–90% [63].

The method may be applicable to the analysis of other compounds possessing oxo groups. For example, experiments with prostaglandins (PG) show that the oxime of 6-keto $PGF_{1\alpha}$ is retained much more than that of PGE_2 by SP-LH-20.

Ethynyl steroids. Synthetic steroids often contain specific substituents upon which selective purification procedures may be based. Ethynyl steroids, widely used as contraceptives, can be isolated by ligand exchange chromatography on SP-LH-20 in silver form. The principle is old and has been used with conventional ion exchangers for similar purposes [66]. Although the hydrophilic SP-Sephadex C-25 in silver form can be used in 70% v/v methanol [23], smaller column beds and solvent volumes are needed with SP-LH-20 [67]. In an analytical procedure the biological extract (e.g. eluate from Sep-Pak C_{18} or Lipidex 1000) is passed through SP-LH-20 in H^+-form and subfractionated on TEAP-LH-20. Following enzymatic hydrolysis and extraction with Lipidex 1000, the eluate from the latter column is passed through SP-LH-20 (10 × 8 mm in Ag^+-form on top of 30 × 8 mm in H^+-form obtained by washing a bed of 40 × 8 mm with an appropriate amount of silver nitrate). Ethynyl steroids are quantitatively sorbed and are then displaced with methanol saturated with acetylene [67]. This purification scheme is useful both for GC–MS analysis of low levels of ethynyl steroids in urine and plasma [23], and for the separation of radioactively-labelled metabolites into groups according to their mode of conjugation and retention of the ethynyl group, as illustrated in Fig. 5.

Summary and Conclusions

Some of the purification procedures discussed in this paper may appear tedious. They have been designed for research purposes and not for routine analyses. However, only those parts need be selected that are appropriate to a special application: moreover, column techniques can be automated. Because of their high capacity, the gel beds and solvent volumes can be kept small. Many of the procedures give only two fractions, one

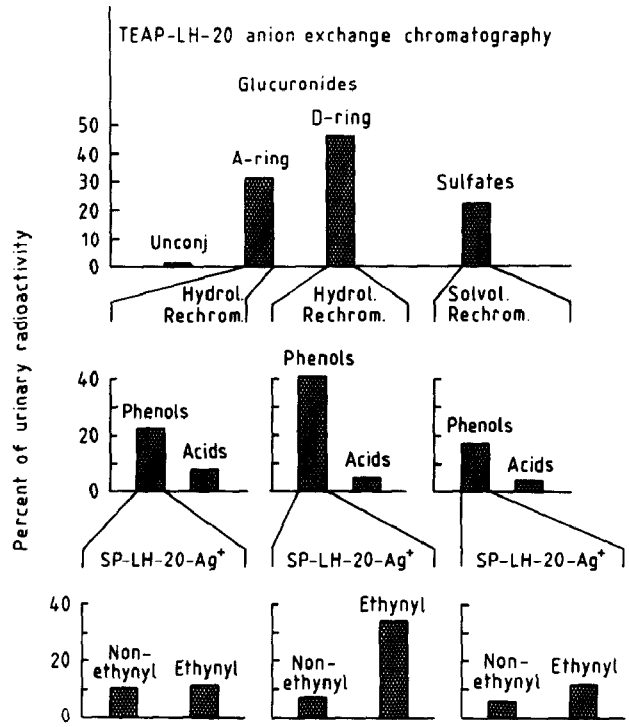

Figure 5
Sequential column work-up of a urine sample from a subject given 50 μg ^{3}H-labelled ethynylestradiol. Groups of conjugated steroids from TEAP-LH-20 were hydrolysed or solvolysed, liberated steroids were isolated in a phenolic fraction from a second TEAP-LH-20 column, and steroids retaining the ethynyl group were sorbed by SP-LH-20 in Ag^{+}-form. GC–MS analysis showed that 85% of the tritium in the ethynyl fractions was attributable to ethynylestradiol (adapted from [49]).

of which contains the compounds to be analysed: the number of evaporations and transfers are kept to a minimum. Recoveries of added compounds, also at the pg level, are typically 80–90% through an entire procedure, except when the compounds are sensitive to the acid or basic ion exchangers themselves. Similar sample work-up schemes may be designed with bonded-silica materials. Future studies will be expected to indicate the relative merits of lipophilic gels and bonded silica for the preliminary preparation of biological samples in bioanalytical studies on low levels of endogenous and exogenous compounds.

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