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MANUAL AND AUTOMATED ENRICHMENT PROCEDURES FOR BIOLOGICAL SAMPLES USING LIPOPHILIC GELS

JOHAN UUSIJÄRVI, BÖRJE EGESTAD and JAN SJÖVALL*

Department of Physiological Chemistry, Karolinska Institutet, Box 60 400, S-104 01 Stockholm (Sweden)

SUMMARY

Aspects of the use of lipophilic gels in manual sample preparation procedures are reviewed. Neutral gels with a controlled hydrophobicity are used for sorbent extraction of non-polar and medium polarity compounds from biological fluids. Acidic amphiphilic compounds can be extracted as ion-pairs with decyltrimethylammonium ions. Solvent or detergent extracts of tissues or faeces can be mixed with hydrophobic gels for transfer of analytes from a solvent to a gel phase, permitting subsequent sample preparation in gel bed systems. Hydrophobic gels, alkyl-bonded silica and polystyrene matrices can be used in series for extraction of compounds with a wide range of polarities. Group fractionations are performed on neutral and ion-exchanging lipophilic gels to yield fractions of neutral, basic and acidic metabolites within selected polarity ranges. Selective isolation of phenolic acids on a strong anion exchanger, of ethynylic steroids on a strong cation exchanger in silver form and of oximes of ketonic steroids on a strong cation exchanger in hydrogen form is possible.

A computerized system for automatic sample preparation is also described. It consists of an extraction bed, a cation-exchange column and an anion-exchange column. The pumps and switching valves are arranged so that the columns can operate in series or parallel for isolation of neutral, basic and acidic metabolites of amphiphilic compounds and for regeneration of the column beds. Fractions can be collected, or the effluent from the column beds can be diluted with water to permit sorption on a solid phase. The applicability of the automated method to the analysis of bile acids and metabolites of mono(2-ethylhexyl) phthalate is demonstrated.

INTRODUCTION

The analysis of endogenous and exogenous compounds in biological samples by high-resolution methods such as gas chromatography (GC), high-performance liquid chromatography (HPLC) and mass spectrometry (MS) usually has to be preceded by extraction and enrichment procedures. The biological mixtures are complex, the concentrations of the compounds of interest may be very low, and the requirements for purity are high in metabolic studies with isotopes and

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MS. The sample preparation procedures may be based on distribution in liquid–liquid or liquid–solid systems. Work in this laboratory has concentrated on the use of liquid–solid and liquid–gel extraction–fractionation procedures. Methods were developed for synthesis of lipophilic derivatives of Sephadex carrying specific functional groups for selective retention of analytes or contaminants [1–6]. The early development occurred before the introduction of solid column packings with chemically bonded phases [7], and Sephadex was chosen as matrix because of its known inertness, absence of adsorptive properties and high capacity. Methods for purification and group separation of steroids and bile acids in different conjugated forms were developed using solid-phase extraction and lipophilic ion exchangers [8,9]. In recent years, similar principles have been used in systems based on chemically bonded silica phases, either off-line or in on-line HPLC configurations [10]. Because we have found the systems based on lipophilic Sephadex derivatives to be versatile, inexpensive and simple, this paper reviews some of the applications of these gels to the extraction, group fractionation and selective isolation of lipid-soluble metabolites and their conjugates from biological materials. Various aspects have been discussed in previous reviews [11–17], and this paper focusses on more recent applications and those of some general interest. The design of an automated, computer-controlled system for sample work-up is also described.

EXPERIMENTAL

Chemicals, chromatography and mass spectrometry

Solvents were of analytical grade (Merck, Darmstadt, F.R.G.) and redistilled in an all-glass apparatus. Water was deionized and purified with a Milli-Q® cartridge (Millipore, Bedford, MA, U.S.A.). Acids and ammonia (Merck) were used as received. Sepralyte® (Analytichem, Harbor City, CA, U.S.A.) was washed with chloroform–methanol, methanol and water before use. SP-Sephadex C-25 (Pharmacia, Uppsala, Sweden) in sodium form was washed with aqueous ethanol and ethanol at 70°C. Prior to use it was converted into the hydrogen form [18]. Lipidex-DEAP (Packard, Downers Grove, IL, U.S.A.) in acetate form was washed with aqueous ethanol and ethanol at 70°C [19].

Cholic, glycocholic and taurocholic acids labelled with ^{14}C at C-24 were from Amersham (Amersham, U.K.). Mono(2-ethylhexyl) phthalate (MEHP) labelled with ^{14}C was synthesized from [^{14}C]phthalic anhydride [20,21]. This was diluted with unlabelled MEHP and an oral dose of 400 mg kg^{-1} was given to a guinea pig [20,21]. Urine was collected and a sample was processed by the automated sample preparation procedure. Radioactivity was counted in an LKB 1211 Minibeta liquid scintillation counter using OptoPhase HiSafe II as scintillating liquid (Pharmacia-LKB, Uppsala, Sweden). Fractions containing ^{14}C were analyzed by HPLC (LDC pumps and gradient mixer, Milton-Roy, Riviera Beach, FL, U.S.A.) using a semipreparative μBondapak C_{18} column (15 cm × 19 mm, Waters Assoc., Milford, MA, U.S.A.) and a linear solvent gradient of 30–60% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 30 min at a flow-

rate of 5 ml min^{-1}. UV absorption at 254 nm (LDC SpectroMonitor III) and radioactivity (Trace 7140, Packard) were monitored.

Fast-atom bombardment (FAB) MS was performed with a VG 7070E double-focussing mass spectrometer and a VG 11-250 data system (VG Analytical, Manchester, U.K.) An aliquot of the samples, corresponding to 25 μl of urine, dissolved in 10 μl of 50% aqueous methanol was applied under a slight stream of nitrogen to the FAB target already covered with the glycerol matrix. The sample was bombarded with 8-keV xenon atoms, and negative-ion spectra were recorded between *m/z* 800 and *m/z* 80 at a scan rate of 10 s per decade and a resolution of 1000.

Automated sample processor

The components of the automatic sample preparation system are listed in Table I and the configuration of the system is shown in Fig. 1.

The extraction columns (E) were filled with 100 mg of Sepralyte. The cation exchanger (CX) was SP-Sephadex C-25 in hydrogen form, bed size ca. 0.6 ml. The anion exchanger (AX) was Lipidex-DEAP in acetate form, bed size ca. 3 ml (capacity ca. 1 mmol of chloride ions).

The pumps, valves and fraction collector were controlled by an ND 100 computer (Norsk Data, Oslo, Norway) via a digital input/output device. Interfaces to the pumps, valves and fraction collector were constructed by Mr. N. Hammar in this laboratory. Several real-time programs were written in Fortran, permitting different processes to be executed simultaneously. Individual protected files can be created for the particular processes required. Written reports of the status of the processes can be obtained at any time.

RESULTS AND DISCUSSION

Off-line use of lipophilic Sephadex derivatives

Most studies in this laboratory have been carried out on steroids and bile acids. The metabolism of these compounds is analogous to that of many groups of lipophilic substances. Thus, methods of analysis of metabolic profiles or individual compounds of the latter groups can have similar designs, and the steroids are useful model compounds in analytical studies. In recent years we have applied analogous methods to the analysis of several xenobiotics and their metabolites.

Extraction with lipophilic gels. Solid-phase extraction gained widespread acceptance in steroid and bile acid analysis following the introduction of Amberlite XAD-2 as a sorbent for urine [22,23] and plasma [24,25]. Problems with "irreversible" binding [26], later shown to be due to the presence of ion-exchanging groups [27], prompted us to study other sorbents. Hydroxyalkylated Sephadex derivatives [5] were proposed as alternatives to styrene–divinylbenzene copolymers [28]. Lipidex 1000, containing 10% (w/w) of alkyl groups with an average chain length of fifteen carbon atoms (Packard), was found to extract efficiently steroids of low and medium polarity from aqueous solutions [29]. Unconjugated bile acids [30] and phenolic steroids with two oxygen substituents [31–33] are equally well extracted, provided they are in protonated form [30,31].

TABLE I

COMPONENTS OF THE AUTOMATED SAMPLE PREPARATION SYSTEM

Component	Abbreviation[a]
Pumps and valves	
HPLC pump, Model 302 (pumphead 5S), Gilson[b]	Pump 1, pump 3
HPLC pump, Model 396 (max. flow-rate 4.8 ml min^{-1}), LDC/Milton Roy[c]	Pump 2
HPLC pump, Model 396 (max. flow-rate 27 ml min^{-1}), LDC/Milton Roy	Pump 4
Cheminert® three-way slider valve with pneumatic actuator, LDC/Milton Roy	3W1, 3W2, 3W3
Six-position rotary valve with pneumatic actuator, Model 5012P, Rheodyne[d]	6P2, 6P3
Six-position rotary valve (Rheodyne) with electric actuator, Model SV-II, Eldex[e]	6P1
Sample injection valve, Model 5020, Rheodyne	IV
Fraction collector, Model 201, Gilson	FC
Columns	
PTFE tubing 5.0 mm O.D. and 3.0 mm I.D., length 80 mm with adjustable endpieces of stainless steel, 3.1 mm O.D. attached to 1.6 mm (1/16 in.) O.D. stainless-steel tubing to connect to the PTFE tubing. Each endpiece was covered with PTFE gauze	E
Omnifit® 6.5 mm I.D. chromatography column, length 100 mm, with one variable length endpiece[f]	CX
Cheminert® Model LC-9, 9 mm I.D. chromatography column, glass-tube shortened to 100 mm, LDC/Milton Roy	AX
Tubing and fittings	
PTFE tubing 3.2 mm (1/8 in.) O.D., 1.6 mm I.D.	
Conventional 1/4-28 TPI fitting system for flanged PTFE tubing	
Flange-free 1/4-28 TPI fitting system, Alltech[g]	
Kel-F® Tees and Crosses for 1.5 mm I.D. tubing and 1/4-28 TPI fittings	T and X

[a]As used in Fig. 1.
[b]Gilson, Villiers-Le-Bel, France.
[c]Laboratory Data Control/Milton Roy, Riviera Beach, FL, U.S.A.
[d]Rheodyne, Cotati, CA, U.S.A.
[e]Eldex Laboratories, Menlo Park, CA, U.S.A.
[f]Omnifit Ltd, Cambridge, U.K.
[g]Alltech Associates, Deerfield, IL, U.S.A.

In contrast, polar steroids with four or more oxygen substituents and conjugated steroids and bile acids are poorly or not at all retained by the gel. Addition of pentylamine to the aqueous solution enhances the extraction of polar neutral steroids [29], but later studies indicate that this method should not be used because of artifactual formation of pentylimines of oxosteroids [34].

Comparisons of Amberli XAD-2, octadecylsilica and Lipidex 1000 for extraction of metabolites in urine show marked differences between the sorbents. While XAD-2 and alkyl-bonded silica extract a wide range of amphiphilic compounds, the Lipidex gel behaves as a solvent of medium polarity, e.g. ethyl acetate.

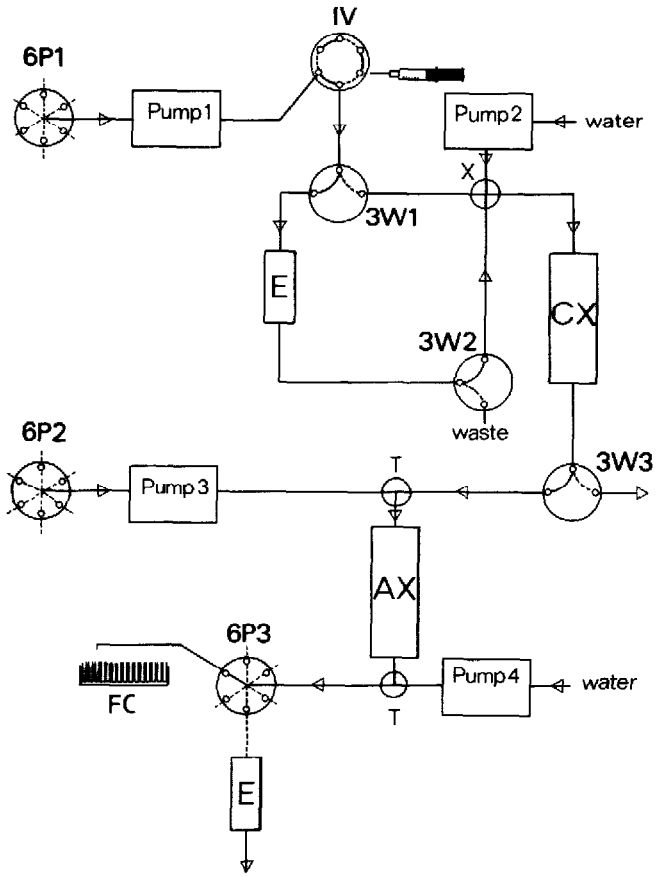

Fig. 1. Configuration of the computerized low-pressure system for automatic sample preparation using lipophilic ion-exchanging derivatives of Sephadex. The stage after injection at which all columns are eluted in series by pump 1 (with addition of water by pump 2) is shown. The components of the system are listed in Table I.

The mechanism of sorption has not been studied, but in practice the water–Lipidex system is equivalent to a reversed-phase liquid–liquid partition system. This is in contrast to the alkyl-bonded silicas, which show additional sorption of quite polar amphiphilic substances, such as peptides [35] and steroid conjugates [17,36], and analogous metabolites of endogenous compounds and xenobiotics [10,37]. This is probably due to polar interactions between these compounds and free silanol groups on the alkyl-bonded silica [38]. A similar interaction apparently does not occur with the hydroxyl groups in and adjacent to the sugar matrix of the Lipidex gel. This may be due to the much higher concentration of alkyl groups in this gel.

Thus, Lipidex 1000 (and 5000) has a higher selectivity for non-polar compounds than octadecylsilica. In extractions of urine, the amount of material sorbed by Lipidex 1000 is only a few per cent of that sorbed by octadecylsilica [29,30,39]. This results in a much higher degree of purification of less polar compounds, and Lipidex gels have been useful sorbents in GC–MS analyses of contraceptive ste-

roids [31,32], unconjugated bile acids [40] and chlorinated aromatic pollutants [41,42] present in aqueous solutions. A partial separation of unmetabolized chlorinated paraffins and their metabolites in bile can be obtained by extraction with Lipidex 1000 [43]. By selection of a suitable washing and elution scheme the extraction is combined with purification. For example, pentachlorophenol in water and urine (after hydrolysis of its glucuronide) is extracted by passage through a small bed of Lipidex 5000, which is washed with aqueous methanol, dried with a nitrogen stream, washed with hexane (which elutes less polar pollutants, e.g. polychlorinated biphenyls, dibenzo-*p*-dioxins and dibenzofurans) and eluted with acetone. The pentachlorophenol in this eluate is sufficiently pure for GC analysis after acetylation [42]. The selectivity for hydrophobic compounds should make Lipidex 5000 a useful alternative to other sorbents for extraction of petroleum products, polyaromatic hydrocarbons and halogenated aromatic pollutants in water. Judging from the studies discussed above, the extracts should contain less of the more polar contaminants than extracts obtained with polymer sorbents and alkyl-bonded silica (see refs. 10,44 and 45).

When more polar compounds are to be analysed, the aqueous washes from the Lipidex gel can be passed through other sorbents. We have found the sequence Lipidex 1000 (or 5000), octadecylsilica, Porapak Q + T (1:1) [46] to be useful when an array of metabolites is to be recovered. When extracted in this way, metabolites of propachlor are sorbed by octadecylsilica [47].

Addition of a non-polar counter-ion may permit extraction of amphiphilic acids as ion-pairs by Lipidex gels. Glycine- and taurine-conjugated, glucuronidated and sulphated bile acids were extracted by Lipidex 1000 in the presence of 0.03 *M* decyltrimethylammonium bromide [30,39]. The structure of the counter-ion was very important; neither the nonyl nor the dodecyl homologue gave satisfactory results. This indicates a strict structural requirement for an interaction with the alkyl chains of the gel. The concentration of counter-ion was also important; it should be as high as possible but below the critical micellar concentration. The mechanism probably involves sorption of an ion-pair formed in the aqueous phase [39]. In the presence of decyltrimethylammonium bromide, the amount of organic material extracted by Lipidex 1000 is greatly increased and similar to the amounts extracted by Amberlite XAD-2 and octadecylsilica [30,39].

In aqueous solutions containing proteins, protein binding counteracts sorbent extraction of analytes. Endogenous compounds may be bound to carrier proteins with very high affinity. In addition, fluids such as plasma and milk contain lipoproteins in which lipid-soluble compounds, depending on their structures, are distributed differently between the lipid core and the surrounding envelope of proteins and polar lipids. This affects their extractability.

Protein binding may be decreased by dilution of the sample, by changes of pH, temperature and ionic composition, and by addition of organic modifiers. Steroids of low and medium polarity bound to carrier proteins or receptors can be extracted with Lipidex 1000 at an elevated temperature [33,48]. In analytical applications the extraction is carried out at 64 °C after dilution of the sample with an equal volume of saline, as originally described with Amberlite XAD-2 as sorbent [25]. If the receptor or carrier proteins are to be studied, the steroid ligand

can be removed by Lipidex 1000 at 45°C without denaturation of the protein [48]. A similar procedure has been described for extraction of fatty acids from albumin [49,50]. At 0–4°C only unbound lipids are extracted [48,50].

Lipids in lipoproteins are poorly or not at all extracted by Lipidex unless the lipoprotein surface is damaged by an added modifier [29,30]. This is similar to when Amberlite XAD-2 [25,27,51,52] or octadecylsilica [27,52,53] are used as sorbents and means that analytes present in the lipoprotein core are not extracted. Dilution with an equal volume of 0.5 *M* triethylammonium sulphate, which allows essentially quantitative extraction of glucuronides and sulphates of steroids [27,52,53] and conjugated bile acids [17,54] in plasma with octadecylsilica at 64°C, does not promote extraction of compounds in the lipoprotein core [27,52]. Addition of pentylamine and methanol to plasma or milk will destroy lipoproteins. If Lipidex 1000 is present in the mixture the lipids will be slowly transferred to the gel phase [41]. However, reactions occur between pentylamine and esterified fatty acids.

Formic acid may be used to destroy lipoproteins. A valuable method for extraction of total lipids and non-polar chlorinated aromatic pollutants in milk consists of incubation of 10 ml each of milk and formic acid with 5 g of Lipidex 5000 at 35°C [55]. After 2.5 h the mixture is transferred to a chromatographic column, which can be washed and eluted to yield fractions of polar compounds, pollutants and lipids. An important aspect of this procedure is that the more polar compounds are not lost but may be recovered by extraction with octadecylsilica or more polar sorbents, while the non-polar pollutants can be purified by adsorption chromatography for multicomponent analysis of chlorinated pesticides and polychlorinated biphenyls, dibenzo-*p*-dioxins and dibenzofurans by GC–MS. Recoveries are 80–90%, and levels of pg/g lipid can be measured [55]. It is very likely that the same principle of extraction can be used on plasma.

Addition of 0.03 *M* decyltrimethylammonium bromide to plasma not only permits the extraction of conjugated bile acids as ion-pairs but also promotes the extraction of lipids [30,39]. However, a quantitative study of different lipid classes has not yet been carried out.

Analysis of tissue samples is likely to assume an increasing importance. Solvent extraction is the first step in conventional methods. However, it is also possible to solubilize tissue samples by chemical or enzymic means, thereby producing aqueous solutions that can be subjected to solid-phase extraction. Lipidex gels have been used in combinations with solvent extraction or detergent solubilization for extraction of steroids, bile acids and xenobiotics in tissues and faeces. One aim has been to bring the samples onto sorbent beds as soon as possible so that the subsequent purification and subfractionation can be carried out in a similar way as for biological fluids. The high capacity of the gels is an important property since large amounts of lipids usually have to be coextracted to ensure quantitative recovery of all analytes of interest.

A method for analysis of metabolic profiles of sterols and bile acids in faeces [40] is based on sorption of lipid-soluble compounds, obtained by solvent extraction and suspended in water, onto a bed of Lipidex 1000. The aqueous washings from this bed are extracted with octadecylsilica. Elution of the two complemen-

tary sorbents gives a quantitative yield of a wide range of non-polar and polar lipids, sterols and bile acids which can be subfractionated and analysed by GC and GC–MS [40]. The same principles were used for extraction of the very complex mixture of metabolites of [^{14}C]benzo[*a*]pyrene in rat faeces [56]. In this method, the primary solvent extract is dissolved in hexane–2-propanol (2:1), Lipidex 1000 is added and the solvents are evaporated. The gel is then slurried in water and poured into a chromatography column for subsequent washing and elution. An analogous procedure was used to transfer testicular lipids and steroids from a solvent extract into Lipidex 1000 [57]. When the gel has been poured into the column, an octadecylsilica cartridge is attached at the end. With an appropriate washing and elution scheme a purified extract is obtained with over 90% recovery of unconjugated steroids [57]. The method formed the basis for GC–MS analysis of twelve testicular steroids at levels between 20 pg and 100 ng per gram tissue [58]. However, clean-up by HPLC prior to GC–MS is necessary.

Analogous methods should be useful for other types of lipid-soluble compounds in tissue. Δ^1-Tetrahydrocannabinol in human fat biopsies is an example [59]. The tissue is homogenized in hexane–2-propanol and centrifuged, and Lipidex 5000 is added to the supernatant. Solvents are removed under a stream of nitrogen, and the gel is transferred to a column and eluted with aqueous methanol, leaving lipids in the gel. The effluent is diluted with water so that the Δ^1-tetrahydrocannabinol can be sorbed on octadecylsilica, from which it is eluted with a small volume of hexane. Derivatization followed by HPLC permits GC–MS analysis of low levels of unlabelled and ^{2}H-labelled Δ^1-tetrahydrocannabinol 4 weeks after smoking of one cigarette containing the labelled drug [59].

Many solvents used in analyses of lipid-soluble compounds are hazardous. A way to decrease their use is to extract tissue samples with aqueous detergent solutions. Lipidex gels can then be added and the solution is diluted to below the critical micellar concentration of the detergent. Lipid-soluble compounds are sorbed by the gel, which can be transferred to a column for washing and elution. This principle was combined with ion-pairing (see above) for extraction and purification of bile acids in rat liver for FAB-MS analysis [60]. The drawback is contamination of the sample with detergent or counter-ion. However, the method deserves further studies with other classes of compounds that do not require ion-pairing for extraction.

Group fractionation on lipophilic gels. Multicomponent analyses and analyses of metabolic profiles, individual compounds at low levels and isotopic composition of these compounds can rarely be performed without preliminary group fractionation with removal of interfering compounds. The interference may be mass of material that overloads the analytical system or compounds whose properties in the chromatographic and detection systems result in lack of specificity. It is an obvious aim to make analytical procedures simple, and methods containing initial subfraction steps are often termed complicated and time-consuming. When MS has to be used to ensure specificity the methods are considered expensive. Although the on-line HPLC methods are rapidly improving, they are usually limited to the analysis of a few compounds at a time. Taking bile acids as an example, urine from healthy subjects and patients may contain 40–50 bile acids with dif-

ferent steroid structures, each occurring in one or several forms as unconjugated or conjugated with glycine, taurine, sulphuric acid, glucuronic acid, glucose, N-acetylglucosamine (see refs. 61 and 62). In several cases, conjugation can occur at different sites and double conjugates can be present. Thus, there is a potential occurrence of 280–350 bile acids in urine, not including all other metabolites, e.g. of steroids, with similar properties. It is obvious that a single on-line chromatographic method cannot be used to analyse more than a few components of such a mixture. Thus, the need for analysis of complex patterns of metabolites should be distinguished from that of analysis of limited numbers of compounds, and group fractionation procedures are required in the former case.

Group separations on lipophilic gels have been based on polarity, charge or presence of specific groups in the analytes. Sephadex LH-20 and Lipidex 1000 or 5000 have been used in normal-phase (straight-phase) systems for separation of steroids and bile acid esters into groups according to number and nature of the oxygen substituents (see refs. 13 and 17). In most cases the polar bonded silica sorbents now available are likely to be easier to use and give better separations. However, bile acid glycosides are conveniently separated from the much larger amounts of unconjugated bile acids in urine by chromatography of the methyl esters on Lipidex 5000 in hexane–chloroform (eluting unconjugated bile acid esters) followed by methanol (eluting the glycosides) [62]. Lipidex 5000 is also a very useful sorbent in hexane for rapid (3 min) removal of reagents and polar contaminants after derivatization with trimethylsilylating reagents [18,31,51,53].

Lipidex 1000 and 5000 are versatile in that they permit normal-phase separations in hexane–chloroform, molecular sieving in chloroform or chloroform–methanol, for example, and reversed-phase partition in methanol [5]. The last is amplified by addition of water with or without 10–20% chloroform. Small reversed-phase column beds are very useful as filters to remove non-polar lipids and cholesterol from initial sorbent or solvent extracts when more polar compounds are to be analysed (e.g. refs. 25,31,53,57 and 58).

Although alkyl-bonded silicas offer much better separation efficiencies, the combination of high capacity and low efficiency can make Lipidex gel beds preferable for initial group separation of crude biological extracts. A rapid group isolation of phospholipids in extracts of tissues and plasma can be obtained on a column of Lipidex 5000 in methanol–ethylene chloride (4:1). The phospholipids pass through the column while other lipid classes are retained [63–65]. Curstedt has also utilized Lipidex 5000 for other purposes in phospholipid analysis and in combination with lipophilic ion-exchangers (see refs. 65 and 66). Several HPLC methods have been described for separation of molecular species of phospholipids, but the initial sample preparation and group isolation is still most conveniently performed using Lipidex 5000.

Ion-exchanging lipophilic gels have been extensively used for group separation of metabolites prior to GC, HPLC and MS (for reviews see refs. 13–17). Of the ion exchangers synthesized (see ref. 14) three are presently used in different applications in our laboratory: diethylaminohydroxypropyl Sephadex LH-20 (Lipidex-DEAP) [8,9], triethylaminohydroxypropyl Sephadex (TEAP-LH-20) [18,66] and sulphohydroxypropyl Sephadex LH-20 (SP-LH-20) [67]. These ful-

fil the needs with the compound classes presently studied. Previously, alkylated ion exchangers were used to permit a combination of ion exchange and reversed-phase separation in the same column [31,51,67], but it is more flexible to perform the two types of separation on separate gel beds.

Lipidex-DEAP has been extensively used to separate metabolites of steroids (see refs, 13–16), bile acids (see ref. 17) and bile alcohols (see ref. 17) into groups according to the mode of conjugation. Recent studies show similar separations of metabolites of xenobiotics [43,47]. Simultaneous separate analysis of unconjugated and conjugated forms of a compound is important since the metabolic origin and fates of the different forms may differ. A recent study of the effects of alcohol-induced changes of the hepatic redox state on the redox couples androstene-3,17-dione–testosterone and estrone–estradiol in plasma showed that only the sulphated and glucuronidated forms of the estrogens were affected. Conjugated estradiol increased 3–14-fold while unconjugated estradiol was unchanged [68]. Previous studies did not consider the need for group separation of conjugates and the results were contradictory. Since the levels of several of the steroids are in the picomolar range, and since each steroid has to be determined in three forms, the analytical method will obviously be time-consuming and require a specificity given only by GC–MS. The need for group separation of conjugates is also illustrated by a recent study of bile acids in urine [62,69]. When combined with HPLC, GC and GC–MS, two new groups of conjugates were detected: glucosides and N-acetylglucosaminides.

Lipidex-DEAP is usually used in acetate form in 70% aqueous methanol or ethanol [8,9,17,40]. While this ensures mild conditions and low risks for chemical transformations of the sample, there is often a need for a stronger ion exchanger and a better solvent for lipid-soluble compounds. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was used in acetate form in 90% methanol for the same purposes as Lipidex-DEAP and to separate groups of bile acid conjugates prior to HPLC [70,71]. This ion exchanger has been extensively used by Japanese workers in steroid and bile acid analysis (see refs. 17,70–74).

In this laboratory TEAP-LH-20 has been used when a stronger, more lipophilic anion exchanger is needed. Phenolic compounds are sorbed by the hydroxide form of TEAP-LH-20 but not by the carbonate form or Lipidex-DEAP [51,75]. This forms the basis for enrichment of phenolic compounds such as estrogens, lignans, isoflavanes and isoflavones from biological materials prior to GC and GC–MS analysis (see refs. 14, 31, 33, 51–53 and 75–82). Methanol can be used as solvent with or without addition of water or chloroform. The phenolic compounds are displaced by addition of carbon dioxide to the solvent. A disadvantage with TEAP-LH-20 in hydroxide form is that it may catalyse transesterification of lipids and transformations of alkali-labile compounds.

TEAP-LH-20 can be used both in aqueous methanol and in mixtures that are good solvents for naturally occurring lipids. It is often combined with the use of SP-LH-20 as cation exchanger [33,53,67,75,78,83]. There are many examples of its use in analyses of steroids (e.g. refs. 18, 31, 33, 51–53, 75, 77, 78 and 84), vitamin D conjugates [85–87], bile acids [88,89] and xenobiotics [47,56]. When

the lipid content is high, chloroform–methanol–water (6:7:1, v/v/v) will dissolve the samples and permit ion exchange [56].

The choice of ion exchangers in a purification scheme is governed by the nature of the compounds to be analysed and the solubility of the sample constituents. A general scheme utilizes a sequence of three columns: neutral sorbent, cation exchanger and anion exchanger. The three columns are first eluted in sequency with neutral solvent and the ion exchangers are then eluted separately. In analyses of bile acids and steroids, the cation exchanger has usually served to remove contaminants and compounds interfering with separations on Lipidex-DEAP [8,9,17]. Depending on the selectivity of the neutral sorbent and when TEAP-LH-20 or PHP-LH-20 are used, a cation exchanger may not be required [54,70–74,89]. When cationic compounds are to be analysed it is obviously necessary and SP-LH-20 has been used for isolation of basic drugs [90] and conjugated metabolites carrying an amino group, e.g. glutathione and cysteine conjugates [43,47,56].

SP-LH-20 has also been used for selective isolation of steroids with an ethynyl group and steroids and bile acids carrying oxo groups. Ethynyl steroids are sorbed from a solution in aqueous methanol by the ion exchanger in silver form [31,32,77,78,84] and are displaced by ethyne in methanol [32,77,78]. Combined with group separation of conjugates, this enrichment procedure permits detailed analysis of metabolites of ethynylic contraceptive steroids in milk, plasma and urine [77,78,84]. Extraction by a silver-sorbent has recently been employed in an elegant on-line HPLC-method for analysis of ethynyl steroids in urine [91]. Although this method is simple and rapid, the limit of detection is an order of magnitude higher than that of the GC–MS method [31,77,78], the applicability to plasma and milk is not known, and the composition of conjugated forms of the steroids is not determined. Thus, the design of a method is governed by the purpose of the analysis.

Steroids and bile acids carrying an oxo group can be selectively isolated by conversion into oximes, which are then sorbed by SP-LH-20 in methanol [19,79,88,89,92]. Biologically active androgens, progestagens and corticosteroids carry a carbonyl group at C-3, and the method permits enrichment of these steroids prior to GC–MS. The combination with HPLC remains to be established. High sensitivity can be obtained as demonstrated by Gaskell et al. [79], who combined immunosorbent extraction with enrichment of the oxime for GC–MS measurements of testosterone in saliva from women at a sensitivity of 2 pg ml^{-1}.

Some ketonic bile acids and sterols have been implicated as promoters in colon carcinogenesis. They occur in mixtures with large amounts of other bile acids and sterols and have to be enriched by isolation of the oximes prior to analysis by GC and GC–MS [88,92]. In this case it is important to use TEAP-LH-20 [88] and not Lipidex-DEAP [92] for initial separation of sterols and bile acids, and the oximes of each group of compounds can then be enriched and separately analysed [88].

This section has reviewed the use of lipophilic gels for group separation of analytes. Lipophilic ion exchangers also provide a general purification prior to analysis by GC and GC–MS. When conjugated metabolites cannot be analyzed

directly, the compounds liberated by hydrolysis often have a different charge or acidity and can be selectively purified by a second passage through the ion exchanger. Derivatives used in GC and GC–MS analyses are frequently sensitive to hydrolysis catalysed by acids in the biological sample. This interference can be markedly reduced by ion-exchange purification.

Automated sample preparation

One reason for the design of gel column–filter systems for sample preparation is the possibility of automation. Although this has been pointed out previously (e.g. refs. 13 and 14), an automated system has not been developed until now. In contrast, column-switching methods have been extensively used for automated analyses by HPLC, particularly of xenobiotics and their metabolites in biological as well as environmental samples (see refs. 10, 37, 91 and 93–96). However, there are some differences between the two types of system. The HPLC systems are intended for one-line high-resolution analysis under high pressures. This puts demands on the components and makes the complete systems expensive. The lipophilic gel system is intended for extraction and group fractionation of samples that are too complex, contain too large amounts of interfering compounds, or are too unknown for on-line analysis. Instead, each fraction has to be separately analysed by a high-resolution method, often requiring MS for confirmation or determination of structures or mass selectivity for specificity. At present, a low-pressure system is sufficient for this initial fractionation and the demands on components are less stringent, making the equipment less expensive.

The configuration of the system is shown in Fig. 1. It was made to imitate manual procedures for sample preparation discussed above and consists of extraction (E), cation-exchanging (CX) and anion-exchanging (AX) column beds. By appropriate choice of sorbents and ion-exchanging gels, the same separations should be possible as with the manual procedures. The system shown in Fig. 1 was made to permit preparation of urine samples for analyses of metabolic profiles of steroids, bile acids and xenobiotics.

The initial extraction is with a bed of octadecylsilica. This is contained in a piece of PTFE tubing between two pieces of PTFE gauze held in place by stainless-steel endpieces. The latter are made to fit tightly in the PTFE tubing, and a clamp on the outside of the tubing provides leak-free connection. The urine sample, 10–20 ml, is injected manually with a syringe in the injection valve, followed by 10 ml of water, all at a flow-rate of ca. 10 ml min^{-1}, the effluent going to waste. It would be possible to automate the injection and washing of the sorbent, but this has not been considered important and would increase the cost of the system. However, the extraction cannot be carried out at an elevated temperature in the present system. Since this is important in several cases, e.g. with plasma, milk or tissue extracts, the extraction column should be replaced by a jacketed one.

When the analytes have been sorbed and inorganic and polar organic material has been washed out, the computer program is started. It controls the pump speeds and valve settings for choice of solvents and direction of flow.

The octadecylsilica is usually eluted with 9 ml of methanol (2—2.5 ml min^{-1}) but chloroform–methanol is needed for non-polar lipids (not present in urine).

In that case, more lipophilic ion exchangers have to be used which are compatible with chloroform–methanol–water mixtures (see ref. 56). In the present examples the ion exchangers are used in 70% aqueous methanol. The system has a pump (pump 2 in Fig. 1) that can adjust the water content of the solvent from the extraction column to the desired level.

The flow (using Pumps 1 and 2) is first directed through the CX and AX columns in series (Figs. 1 and 2). In analyses of urine, the commercially available SP-Sephadex C-25 in hydrogen form can be used as cation exchanger (see refs. 18, 31, 40 and 56). It is contained in an Omnifit column (which is simple, relatively inexpensive and can be obtained in suitable dimensions) in which PTFE gauze is used instead of porous discs or steel frits at the column ends. The discs and frits are clogged by the gel beads, which change volume when the solvent is changed. However, the volume changes have no other consequences for the operation of the system, since changes in dead volumes can be accepted when high plate numbers are not required.

The effluent from the AX column is collected as the neutral fraction. Following switching of valves 3W1, 3W2 and 3W3, Pump 3 starts to elute the AX column (2.5 ml min^{-1}) and pump 1 the CX column (4.5 ml min^{-1}) (Figs. 1 and 2). The AX column is first eluted with solvent alone, and this effluent goes to the neutral fraction. Then the solvents are changed to elute acids of increasing strength, the choice of displacers being determined by the nature of the problem studied. Fig. 3 shows the result of a group separation of ^{14}C-labelled unconjugated, glycine- and taurine-conjugated bile acids added to 10 ml of urine and carried through the system. For purpose of illustration, the fraction collector was controlled to change fractions at regular intervals; the computer program permits any time schedule for this process. The same separation and recoveries (more than 90%) are obtained as with a manual procedure [9] but the time required is much shorter (Fig. 2). The AX column is finally stripped with an alkaline solvent and regenerated to acetate form at a flow-rate of 4.5 ml min^{-1} (Fig. 2).

During the elution of the neutral fraction from the AX column, the CX column

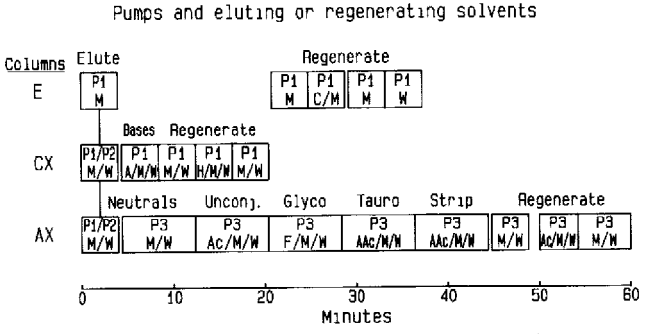

Fig. 2. Event schedule in the automated extraction and subfractionation of unconjugated, glycine-conjugated and taurine-conjugated bile acids shown in Fig. 3. For column and pump abbreviations see Fig. 1. M = Methanol; W = water; M/W = 70% aqueous methanol; C/M = chloroform–methanol (1:1, v/v); A, ammonia (0.3 *M*); H, hydrochloric acid (0.1 *M*); Ac, acetic acid (0.05 *M* during elution of unconjugated bile acids and 0.2 *M* during regeneration); F, formic acid (0.25 *M*); AAc, ammonia–acetic acid, first pH 6 then pH 9.4 (acetate 0.3 *M*)

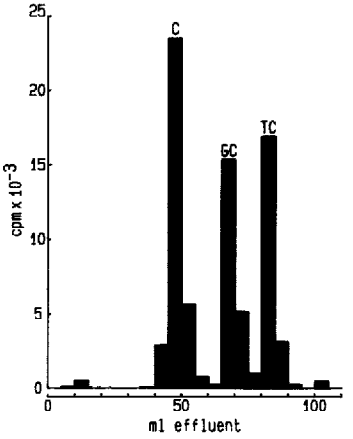

Fig. 3. Separation of ^{14}C-labelled cholic (C), glycocholic (GC) and taurocholic (TC) acids added to urine and carried through the automated system. The schedule of events in this extraction–subfractionation is shown in Fig. 2.

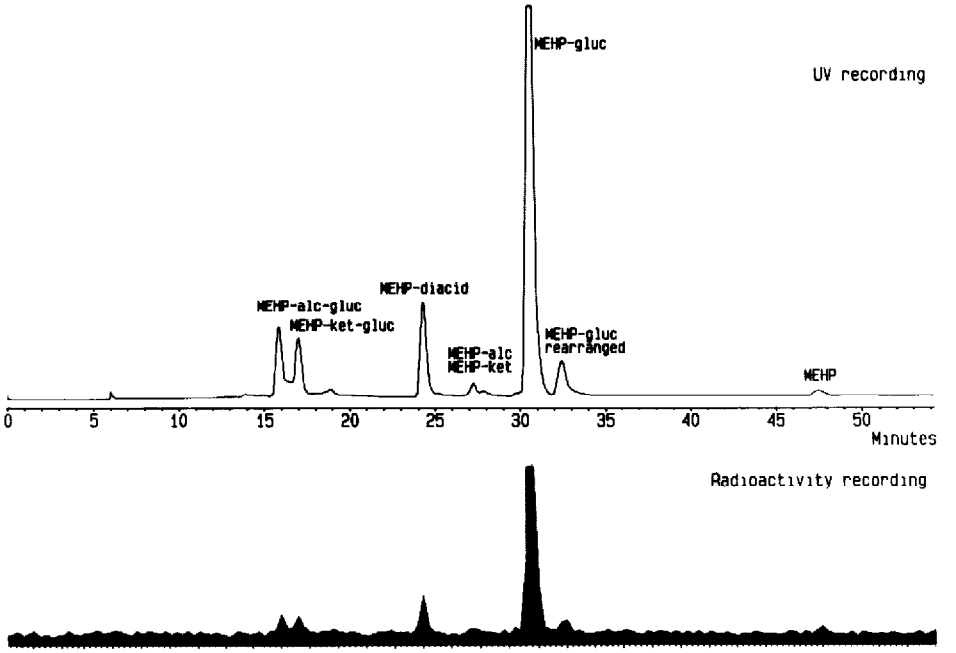

Fig. 4. HPLC of metabolites appearing in the formic acid fraction (cf. event schedule shown in Fig. 2) in the automated processing of a urine sample from a guinea pig given an oral dose of ^{14}C-labelled mono(2-ethylhexyl) phthalate (MEHP). The elution of UV-absorbing material (254 nm, upper curve) and ^{14}C (lower tracing) are shown.

is eluted with ammonia in the solvent (Fig. 2). The effluent appears through the waste outlet of valve 3W3 and is collected in one fraction, which is manually removed. This fraction contains positively charged metabolites, e.g. glutathione and cysteine conjugates (cf. refs. 14, 43, 47 and 56). Drugs and drug metabolites

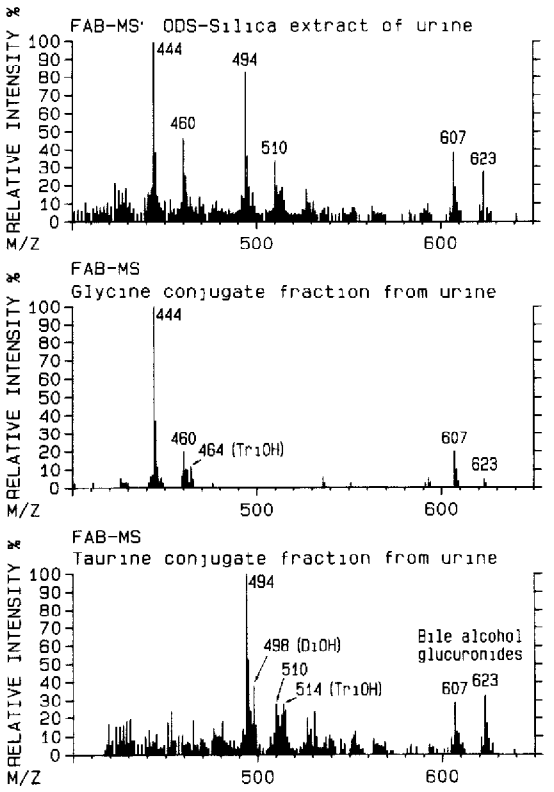

Fig. 5. Upper mass range of the negative-ion FAB mass spectra of bile acids in urine from an infant with cholestatic liver disease [97]. Spectra of the total extract (top) and of the glycine (middle) and taurine (bottom) conjugate fractions from a separation on a Lipidex-DEAP column are shown. The major components have been identified as 7α-hydroxy-3-oxo-4-cholenoic acid and 7α,12α-dihydroxy-3-oxo-4-cholenoic acid excreted as their glycine (*m/z* 444 and 460) and taurine (*m/z* 494 and 510) conjugates. The quasimolecular ions at *m/e* 464, 498 and 514 originate from conjugated dihydroxy- and trihydroxycholanoic acids and the ions at *m/z* 607 and 623 are due to glucuronides of unsaturated ketonic bile alcohols with three and four hydroxy groups.

have been isolated in this way by manual procedures for analysis by GC–MS [90]. The CX column is then washed with solvent and regenerated into the hydrogen form with continued use of pump 1.

Following switching of valve 3W1, pump 1 is used for washing and regeneration of the extraction column (Figs. 1 and 2).

The rate-limiting step in the procedure is the separation on the AX column. This is larger than the CX column because of the predominance of acids in biological fluids. The AX column is eluted stepwise for collection of groups of compounds for profile analyses by HPLC, GC, GC–MS or FAB-MS. Fig. 4 shows an HPLC analysis of metabolites of MEHP eluted in the formic acid fraction from the AX column during the automated preparation of a sample of urine from a guinea pig given [^{14}C]MEHP. Fig. 5 shows negative ion spectra obtained in FAB-MS analyses of urine from an infant with severe cholestatic liver disease [97]. The complete separation of glycine- from taurine-conjugated bile acids on the

AX column is evident. The eluents used in this case (ammonium acetate buffers pH 5.0 and 6.6, respectively, in 70% aqueous ethanol [9]) do not permit isolation of bile alcohol glucuronides, which are acids of intermediate strength, as a separate group.

The material in the effluent from the AX column can be collected in two ways, either in the fraction collector or by solid-phase extraction on column beds identical with that used for the sample extraction. In the latter case the effluent has to be diluted with water to a concentration of methanol that permits sorption. This is done with pump 4, which is started at the desired times to deliver ca. 9 ml min^{-1} of water through a T-connection after the AX column. At the same time the flow-rate from pump 3 is reduced to 1 ml min^{-1} so that the flow-rate through the extraction column is ca. 10 ml min^{-1}. The extraction columns are connected to a six-position valve (6P3, Fig. 1) and only five fractions can be collected in the present system. However, this is sufficient for routine analyses of metabolic profiles of bile acids and steroids by GC. The elution of material sorbed by the extraction columns is manual and is done in reverse direction. Volumes between 200 and 500 μl are sufficient to elute bile acids used in model studies, and recoveries through the entire system are ca. 90% when fractions are collected by sorption on octadecylsilica.

CONCLUSIONS

Lipophilic neutral and ion-exchanging gels are useful in various applications where complex mixtures and metabolic patterns in biological materials are to be analysed. Further development of automated systems should lead to faster and simpler preparation procedures also for larger samples and with possibilities for on-line connection to high-resolution chromatography and MS.

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