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Note

Lipophilic ion exchangers for group separation of conjugated metabolites of xenobiotics

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Xenobiotics undergo a variety of metabolic reactions in the mammalian organism. In many cases the metabolic end products are compounds coupled to glucuronic or sulphuric acid or to amino acids in thioether or amide linkage. Analysis of the mixtures of metabolites involves extraction and chromatographic fractionation prior to identification and quantitation. Usually differences in charge and acidity between metabolites are not utilized in the isolation scheme, and the mixture of metabolites is often hydrolysed first to remove conjugating substituents. This results in loss of information regarding individual conjugated metabolites, specificity of the conjugating enzymes and further metabolic transformation of conjugated compounds.

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The metabolism of xenobiotics shows several analogies with the metabolism of endogenous compounds such as bile acids and hormonal steroids. Groups of metabolites are formed differing in charge and acidity. In order to separate these groups, lipophilic ion exchangers have been synthesized that can be used in organic solvents and that give little or no non-ionic interaction with amphiphilic compounds [1—4]. Methods for the analysis of metabolic profiles of steroids and bile acids have included group fractionation on these ion exchangers prior to gas chromatography—mass spectrometry [3—9].

Because of the physico-chemical analogies between metabolites of less polar xenobiotics and endogenous steroids, we have investigated the use of lipophilic ion exchangers for group separation of metabolites of xenobiotics. Propachlor (2-chloro-N-isopropylacetanilide) was selected as a model compound as its metabolism has been well characterized [10] and is typical of many chlorinated aromatic pollutants.

EXPERIMENTAL

Solvents were of analytical-reagent grade and distilled in an all-glass apparatus. [^{14}C]Propachlor (2-chloro-N-isopropyl-[1-^{14}C]acetanilide) and its conjugated metabolites (cysteine and glutathione conjugates) were compounds used in previous investigations [10]. A germ-free rat (250 g), reared by the methods of Gustafsson [11, 12], was given a single oral dose of [^{14}C]propachlor (1.0 μCi, 2.5 mg) and urine was collected for 48 h.

Lipidex 1000 and Lipidex-DEAP were from Packard (Downers Grove, IL, U.S.A.). Other lipophilic ion exchangers were synthesized as described previously: TEAP-LH-20 (triethylaminohydroxypropyl Sephadex LH-20) [8]; SP-LH-20 (sulphohydroxypropyl Sephadex LH-20) [4]; SPHA-LH-20 (hydroxyalkylated SP-LH-20) [4]. All the lipophilic gels were washed before use [13]. Lipidex-DEAP was used in the acetate form [5], TEAP-LH-20 in the hydroxide form [8] and SP-LH-20 and SPHA-LH-20 in the acid form [4]. SP-Sephadex was from Pharmacia Fine Chemicals (Uppsala, Sweden) and was used in acid form.

Beds of the ion exchangers were prepared in 70% methanol in glass columns of I.D. about 4 mm [3, 8]. Column heights were 40 or 80 mm except for Lipidex-DEAP, which was 250 mm. The solutions of acid, base or buffer used for elution were all made in 70% aqueous methanol.

Beds of Lipidex 1000 were prepared in methanol and were washed with water prior to use [14]. A glass column of 8—10 mm I.D., equipped with a PTFE stopcock, was used. The bed volume was about 2—4 ml. Extraction experiments were made with solutions of the conjugated metabolites in water or in a 0.3 *M* phosphate buffer (pH 7) containing 0.03 *M* decyltrimethylammonium bromide as ion-pairing agent [15]. Flow-rates were about 1 ml/min.

Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.) and were washed with methanol and water prior to use [16].

RESULTS AND DISCUSSION

Extraction

Two methods for extraction of the glutathione and cysteine conjugates of propachlor from aqueous solution were studied. Lipidex 1000 did not retain these metabolites from water or a buffered solution containing 0.03 *M* decyltrimethylammonium bromide as ion-pairing agent. As conjugated bile acids and their sulphates are extracted in the presence of decyltrimethylammonium ions [15, 17], endogenous compounds of this type can be separated from S-conjugated metabolites of propachlor and similar xenobiotics by filtration through Lipidex 1000.

Sep-Pak C_{18} cartridges quantitatively extracted the two conjugated metabolites of propachlor from water with or without additon of decyltrimethylammonium ions. The conjugates were recovered by elution of the cartridge with methanol.

Cation exchange

Both conjugates were sorbed from aqueous methanol by sulphonic acid derivatives of cross-linked dextran. The lipophilic SP-LH-20 and its less polar derivative SPHA-LH-20 were equally effective, while some loss in the neutral fraction was seen with the more hydrophilic SP-Sephadex. Sorption from 70% methanol or 0.1 *M* acetic acid in 70% methanol was more effective than sorption from 30% methanol. The conjugates were quantitatively eluted with 0.3 *M* ammonia in 70% methanol. The results obtained with SP-LH-20 are shown in Fig. 1. The radioactivity not sorbed by the column represents impurities and not leakage of the labelled conjugates, as shown by rechromatography of this material. No attempts were made to separate the two conjugates on the cation exchangers.

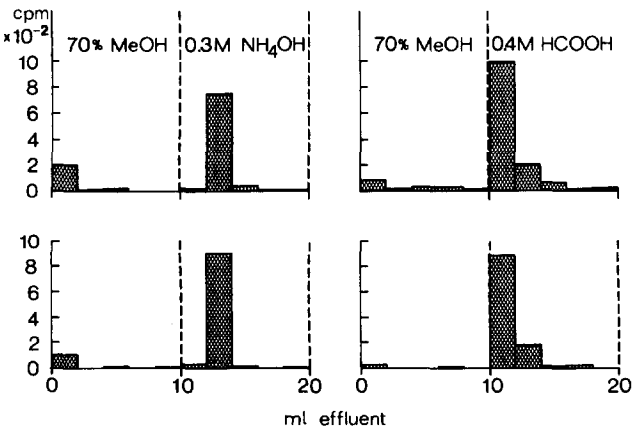

Fig. 1. Chromatography of the cysteine (lower panels) and glutathione (upper panels conjugated metabolites of propachlor on SP-LH-20 (left panels) and TEAP-LH-20 (right panels). Columns: about 80 × 4 mm I.D. All solvents were 70% with respect to methanol.

Anion exchange

Both conjugates were sorbed from aqueous methanol by the lipophilic strong anion exchanger TEAP-LH-20 in base form (Fig. 1). They were quantitatively eluted with 0.4 *M* formic acid in 70% methanol, which will also elute monoglucuronides of aromatic and aliphatic alcohols [5, 8]. Further separation studies were made with the weaker anion exchanger Lipidex-DEAP, which is less likely to produce artifacts with alkali-labile compounds. However, TEAP-LH-20 in base form can be used when phenolic unconjugated metabolites have to be isolated as a separate group [3, 8].

Lipidex-DEAP was used in the acetate form. The glutathione conjugated metabolite was sorbed by this ion exchanger, while the cysteine conjugate was not taken up from either 70% methanol or 0.01 *M* acetic acid, sodium acetate or ammonia in 70% methanol (Fig. 2). The glutathione conjugate was not eluted by 0.25 *M* acetic acid in 70% methanol but appeared after elution with 2—3 column volumes of 0.25 *M* formic acid in 70% methanol. This is about the same position as that of monoglucuronides of neutral and phenolic steroids [5]. Thus, the cysteine and glutathione conjugates can be separated in this sytem, while a glucuronide with no other charged substituents would mix with the glutathione conjugate. However, separation of glucuronides from glutathione conjugates can be readily achieved on the cation exchanger. Further, the system can probably be refined by variations of eluting electrolyte and concentration of water in the solvent. This is illustrated by the group separation of monoglucuronides of neutral from those of phenolic steroids which can be achieved on DEAE-Sephadex when non-aqueous methanol is used as solvent [18].

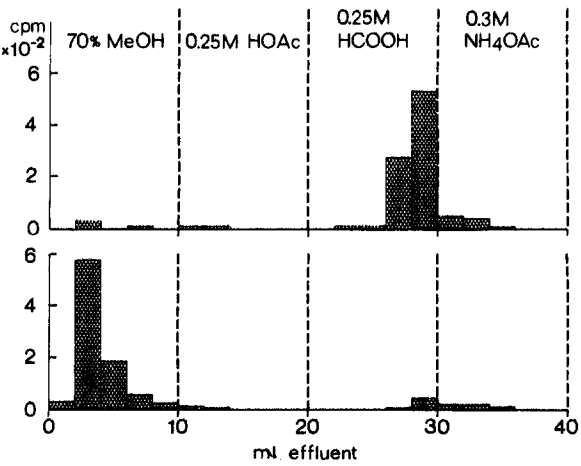

Fig. 2. Chromatography of the cysteine (lower chromatogram) and glutathione (upper chromatogram) conjugated metabolites of propachlor on Lipidex-DEAP in acetate form. Column: 250 × 4 mm I.D. Solvents were 70% with respect to methanol. The radioactivity appearing with formic acid and ammonium acetate in the lower chromatogram represents an impurity.

Fractionation of urine from a germ-free rat given ^{14}C propachlor

Based on the studies of reference compounds, the scheme shown in Fig. 3 was used for extraction and fractionation of urine from a germ-free rat given [^{14}C]propachlor. The yields of radioactivity in different fractions are shown in Table I. The pH of the effluent from SP-LH-20 was routinely checked and adjusted to about 7 with sodium hydroxide before application to Lipidex-DEAP to avoid losses of weak acids in the neutral fraction.

It may be noted that with the distribution of radioactivity found, it was not necessary to evaporate solvents at any point until the final fractions had been collected from Lipidex-DEAP. The volume of urine was 3 ml and the fractions containing more than 90% of the radioactivity were 4 ml, i.e., a volume increase of little practical importance. The capacity of the system has not been determined; however, in work with steroids and bile acids it is possible to process at least 20 ml of urine on columns of the size used. The limiting factor is the amount of organic ions in the sample in relation to the capacity of the ion exchangers.

The distribution of radioactivity in the different fractions permits the following tentative conclusions: (1) all metabolites are polar, as they are not extracted by Lipidex 1000; (2) they do not seem to contain basic substituents, as they are not sorbed by SP-LH-20; (3) they are acidic, as they are sorbed by Lipidex-DEAP; (4) they are stronger acids than glucuronic acid or glutathione conjugates.

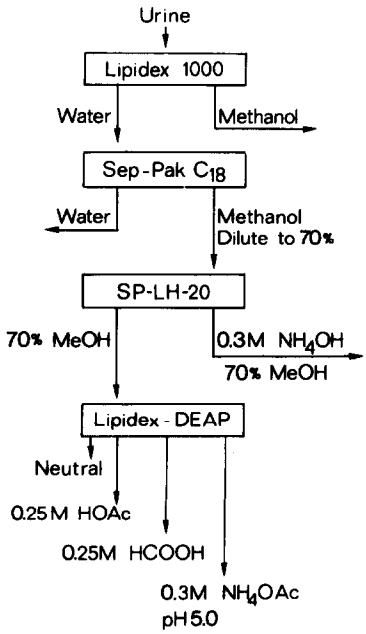

Fig. 3. Proposed scheme for group fractionation of metabolites of xenobiotics in urine. Metabolites eluted in methanol fractions indicated by an arrow at right angles can be subfractionated on the appropriate ion exchanger(s) after evaporation of the solvent. Alternative extraction procedures have to be tested for metabolites not sorbed by Lipidex 1000 or Sep-Pak.

TABLE I

DISTRIBUTION OF RADIOACTIVITY BETWEEN FRACTIONS IN THE SEQUENTIAL EXTRACTION AND GROUP SEPARATION OF METABOLITES IN URINE FROM A GERM-FREE RAT GIVEN [^{14}C]PROPACHLOR

Fractionation step	Fraction	Per cent of radioactivity
Extraction with Lipidex 1000	Water effluent	99.5
	Methanol eluate	0.5
Extraction with Sep-Pak C_{18}	Water effluent	1.6
	Methanol eluate	98.4
Cation exchange on SP-LH-20	70% methanol	99.8
	0.3 *M* ammonia	0.2
Anion exchange on Lipidex-DEAP	70% methanol, 0.25 *M* acetic acid, 0.25 *M* formic acid	0.2
	0.3 *M* ammonium acetate: pH 5.0	95.5
	pH 6.0	1.1

The material in the fractions containing more than 90% of the radioactivity was treated with *n*-butanol—hydrochloric acid as described previously [19]. Gas chromatography—mass spectrometry, carried out on an LKB 2091 instrument, showed that by far the most predominant compounds was the butyl ester of the N-acetylcysteine conjugated metabolite. This was expected from previous characterization of metabolic pathways of propachlor in the germ-free rat [10].

Our study indicates that an extraction and group fractionation procedure of the type described can simplify the purification and analysis of mixtures of metabolites of several types of xenobiotics. While the model case studied is simple and gives only one fraction of metabolite(s), it is evident that the presence of other types of metabolites would have been detected if present. Cysteine and glutathione conjugates would have been sorbed by SP-LH-20 and appeared in the 0.3 *M* ammonia fraction, from which they could be separated on Lipidex-DEAP. Metabolites with a phenolic hydroxyl group as the only acidic substituent would have appeared in the neutral fraction from Lipidex-DEAP. Further purification of this group may be achieved on TEAP-LH-20 [3]. A monoglucuronide would have been eluted with 0.25 *M* formic acid from Lipidex-DEAP [5, 8] i.e., separated from the mercapturic acid. A monosulphate would also be separated from this conjugate, being eluted with acetate buffer pH about 6 [5]. Double conjugates with several acidic groups would also require higher pH for elution [5, 6].

The systems described are intended for group fractionation. Thus, separation of components of different polarity within the groups is negligible. Individual compounds in the fractions may be separated by high-performance liquid chromatography or gas chromatography and analysed by mass spectrometry after suitable derivatization, before or after hydrolysis of conjugates.

It is a disadvantage that non-volatile buffers sometimes have to be used for elution from ion exchangers. These have to be removed by redissolving the sample in water and extracting with Sep-Pak C_{18} cartridges. Advantages of the systems are the high capacity and inertness of the lipophilic ion exchangers, absence of irreversible adsorption and possibilities to select suitable solvents.

Finally, it should be pointed out that similar principles may be applied to analysis of metabolites in plasma, bile and milk. The main differences lie in the extraction procedures, which are influenced by protein binding and presence of lipids. Modified extraction procedures which can be used for plasma and bile have been described for different types of steroids [3, 14, 20, 21].

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REFERENCES

1 J. Ellingboe, B. Almé and J. Sjövall, Acta Chem. Scand., 24 (1970) 463.
2 B. Almé and E. Nyström, J. Chromatogr., 59 (1971) 45.
3 M. Axelson and J. Sjövall, J. Steroid Biochem., 8 (1977) 683.
4 M. Axelson and J. Sjövall, J. Chromatogr., 186 (1979) 725.
5 K.D.R. Setchell, B. Almé, M. Axelson and J. Sjövall, J. Steroid Biochem., 7 (1976) 615.
6 B. Almé, A. Bremmelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
7 J. Sjövall and M. Axelson, J. Steroid Biochem., 11 (1979) 129.
8 M. Axelson, B.-L. Sahlberg and J. Sjövall, J. Chromatogr., 224 (1981) 355.
9 J. Sjövall and M. Axelson, Vitam. Horm., 39 (1982) 31.
10 J.E. Bakke, J.-Å. Gustafsson and B.E. Gustafsson, Science, 210 (1980) 433.
11 B.E. Gustafsson, Acta Pathol. Microbiol. Scand., Suppl., 73 (1948) 1.
12 B.E. Gustafsson, Ann. N.Y. Acad. Sci., 78 (1959) 17.
13 M. Axelson and J. Sjövall, J. Chromatogr., 126 (1976) 705.
14 A. Dyfverman and J. Sjövall, Anal. Lett., B11 (1978) 485.
15 A. Dyfverman and J. Sjövall, in G. Paumgartner, A. Stiehl and W. Gerok (Editors), Biological Effects of Bile Acids, MTP Press, Lancaster, 1979, p. 281.
16 C.H.L. Shackleton and J.O. Whitney, Clin. Chim. Acta, 107 (1980) 231.
17 A. Dyfverman and J. Sjövall, Anal. Biochem., in press.
18 T. Fotsis, H. Adlercreutz, P. Järvenpää, K.D.R. Setchell, M. Axelson and J. Sjövall, J. Steroid Biochem., 14 (1981) 457.
19 J.E. Bakke, J. Rafter, G.L. Larsen, J.-Å. Gustafsson and B.E. Gustafsson, Drug Metab. Dispos., 9 (1981) 525.
20 M. Axelson and B.-L. Sahlberg, Anal. Lett., 14 (1982) 771.
21 M. Axelson and B.-L. Sahlberg, J. Steroid Biochem., 18 (1983) 313.