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PREPARATIVE REVERSED-PHASE CHROMATOGRAPHY OF POLAR AND NON-POLAR METABOLITES ON COLUMNS PACKED WITH MICRONIZED XAD-2 RESIN

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SUMMARY

Coarse-grade XAD-2 resin has been micronized (medium particle size 12 μm) and used as a support for preparative high-resolution liquid chromatography. By equilibration with single-phase mixtures of water, a lower aliphatic alcohol and a hydrophobic solvent a reversed-phase partition system is formed *in situ*. This chromatographic technique is characterized by a large capacity, a high power of resolution and a high degree of selectivity and reproducibility, and versatile application in the isolation of polar and non-polar drug metabolites or naturally occurring compounds from complex mixtures is possible. The examples given relate particularly to the separation of water-soluble, non-extractable compounds, such as glucuronides or other conjugates, and amino acids on large-diameter columns (up to 2.5 cm).

INTRODUCTION

In studies on the biotransformation of xenobiotics, the lack of versatile and efficient methods for the isolation of metabolites is obvious. With the methods available, such as thin-layer, paper, liquid and gas chromatography, it is often difficult to obtain sufficient amounts of pure material. Moreover, most of these techniques are not applicable to polar, non-extractable metabolites or conjugates.

This paper reports on a liquid chromatographic system for the separation of complex mixtures on a preparative scale. Chromatography is carried out in a reversed-phase mode and is therefore specially suitable for polar compounds. A micronized styrene–divinylbenzene copolymer (Amberlite XAD-2 resin) serves as a non-polar stationary phase. The polar mobile phase contains a small amount of a lipophilic solvent that is preferentially taken up by the resin and forms a stationary liquid phase *in situ*.

EXPERIMENTAL

Preparation of micronized XAD-2 resin

A 4.5-kg amount of coarse-grade Amberlite XAD-2 resin (35–50 mesh; Rohm

& Haas, Philadelphia, Pa., U.S.A.) was washed consecutively with approximately 5 × 10 l of water, 2 × 5 l of 0.1 *N* hydrochloric acid, 5 × 10 l of water, 5 × 5 l of ethanol, 5 × 5 l of acetone and 5 × 10 l of water in order to remove soluble additives and impurities. The resin was dried *in vacuo* (100 mmHg) at 100° for 40 h and was then ground in a pinned mill (Kolloplex). The material obtained, which had an average particle size of about 60 μm, was further micronized in an air-jet mill (Type TX, Trost Equipment Corp.).

In order to remove "fines", the resin was suspended batchwise in acetone (weight to volume ratio = 1:15) and allowed to stand for 10 h at room temperature. The procedure was repeated, the supernatant being discarded each time. After the adhering solvent had been sucked off, the resin was first air-dried at ambient temperature and then exhaustively dried *in vacuo* (100 mmHg) at 100° for 24 h. The overall yield was 2.5 kg. Coulter-counter analysis revealed the following particle-size distribution: $d_{p10} = 8\,\mu$m, $d_{p50} = 12\,\mu$m, $d_{p90} = 16\,\mu$m. A photograph of the micronized resin is shown in Fig. 1.

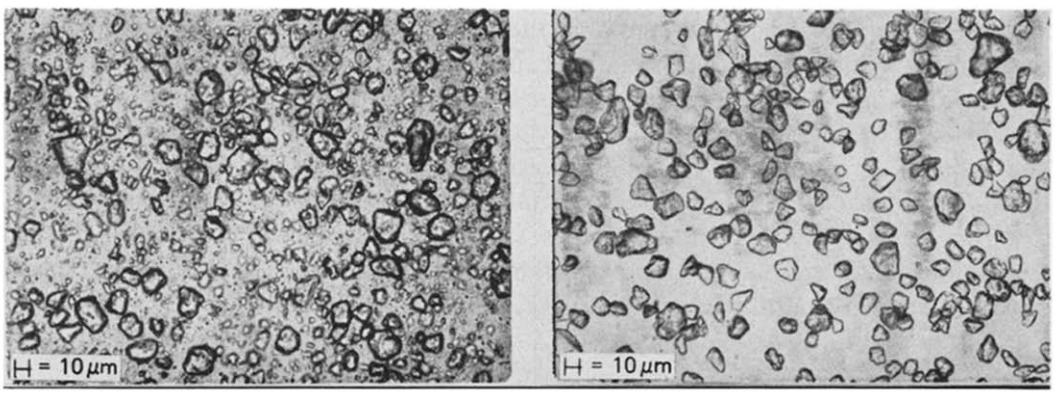

Fig. 1. Micronized XAD-2 resin before (left) and after (right) sedimentation in acetone.

Chromatographic equipment and packing of columns

A modular liquid chromatograph was used that consisted of a pump (Chromatronix CMP-2), a glass column with a diameter of either 12.7 or 25.4 mm (Chromatronix LC, 13 × ½ in. or 23 × 1 in.) and a fraction collector (LKB Ultrorac 7000). Monitoring was effected by means of a radioactivity flow cell (Berthold BF 5026; lithium glass scintillator) or a UV detector with variable wavelength (Cecil CE 212) and a recorder (W + W 1100).

A wet filling technique was employed for packing the columns. For the smaller of the two column sizes (13 × ½ in.) 14.5 g of micronized XAD-2 resin were suspended in 70 ml of a 1:1 mixture of water and methanol. The suspension, after having been degassed by radiation for 3 min in an ultrasonic bath at 25 kHz, was poured into the column tube, to which a second column of the same diameter (length 23 in.) had been connected as a reservoir. The system was immediately pressurized with nitrogen (20 bar) via the top plunger. The suspension medium was allowed to flow out through the bottom plunger, which had been fitted with a 10-μm pore membrane. Sedimentation was completed in about 5 min. The pressure was released before the

surface of the column bed had become dry. After the reservoir had been disconnected and the excess of XAD-2 resin removed, the upper plunger was re-inserted.

For the larger column size (23 × 1 in.), 100 g of XAD-2 resin and 470 ml of methanol–water (1:1) were taken. The nitrogen pressure applied was 7 bar and the time required for sedimentation was 1 h.

Chromatographic solvents and equilibrium of columns

The packed columns were equilibrated and developed with homogeneous, *i.e.*, single-phase, mixtures of volatile solvents. The major components were methanol and water in various volume ratios. A non-polar solvent (toluene or 1,2-dichloroethane) was added as a third component, the amount being such that saturation of the ternary system was avoided. In the examples given below, the chromatographic solvents also contained small amounts of an organic or inorganic acid. It is important to note that with large columns or with solvent mixtures containing less than 1% of toluene or 1,2-dichloroethane, care must be taken to achieve complete equilibrium.

The following chromatographic solvent systems (CS) were used:

CS 1: Methanol–water–toluene–acetic acid (60:40:0.5:1);
CS 2: Methanol–water–1,2-dichloroethane–acetic acid (85:9:5:1);
CS 3: Methanol–water–1,2-dichloroethane–conc. HCl (28:70:1:1);
CS 4: Methanol–water–acetic acid (40:60:1) (toluene saturated);
CS 5: Methanol–water–toluene–acetic acid (60:40:1:1);
CS 6: Methanol–water–toluene–acetic acid (64:34:1:1);
CS 7: Methanol–water–toluene–acetic acid (70:26:3:1).

RESULTS

The chromatographic columns described above have been used successfully in our laboratories for the separation of metabolites of numerous chemically different drugs. The following examples illustrate the usefulness and versatility of the method.

Fig. 2 shows the preparative separation of an impure fraction of urinary glucuronides excreted by volunteers after oral administration of ^{14}C-labelled sulphinpyrazone. A single run with a 50 × 2.54 cm column resulted in complete resolution of two closely related conjugates, *viz.*, the C(4)-glucuronides of sulphinpyrazone and of the corresponding sulphone[1]. These compounds were obtained in amounts of 515 and 24 ng, respectively.

The chromatographic system also displays high selectivity for non-conjugated, lipophilic compounds. In Fig. 3 the separation of an artificial mixture of phenylbutazone and four of its metabolites[2,3] is depicted. These metabolites are derived from the drug by oxidation at different positions of the molecule. The compounds are eluted in order of decreasing polarity, small differences resulting in different elution volumes (see the γ-hydroxy and γ-keto derivatives).

The next example refers to baclofen, a derivative of γ-aminobutyric acid[4]. For metabolic studies in man a radioactive preparation of the drug was purified by reversed-phase chromatography on XAD-2 (Fig. 4). The contaminants of the

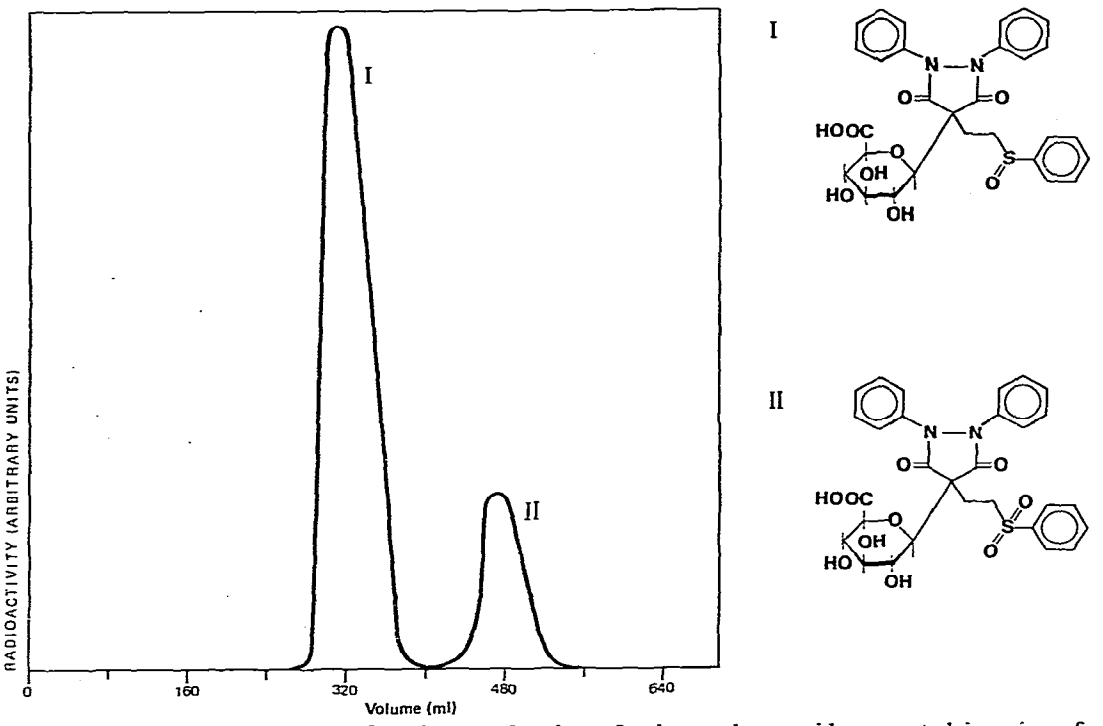

Fig. 2. Preparative separation of an impure fraction of urinary glucuronides excreted in urine of volunteers dosed with [^{14}C]sulphinpyrazone on micronized XAD-2. I = C(4)-glucuronide of sulphinpyrazone (515 mg); II = C(4)-glucuronide of the corresponding sulphone (24 mg). Eluent: methanol–water–toluene–acetic acid (60:40:0.5:1) (CS 1). Column size: 50 × 2.54 cm. Flow-rate: 2 ml/min.

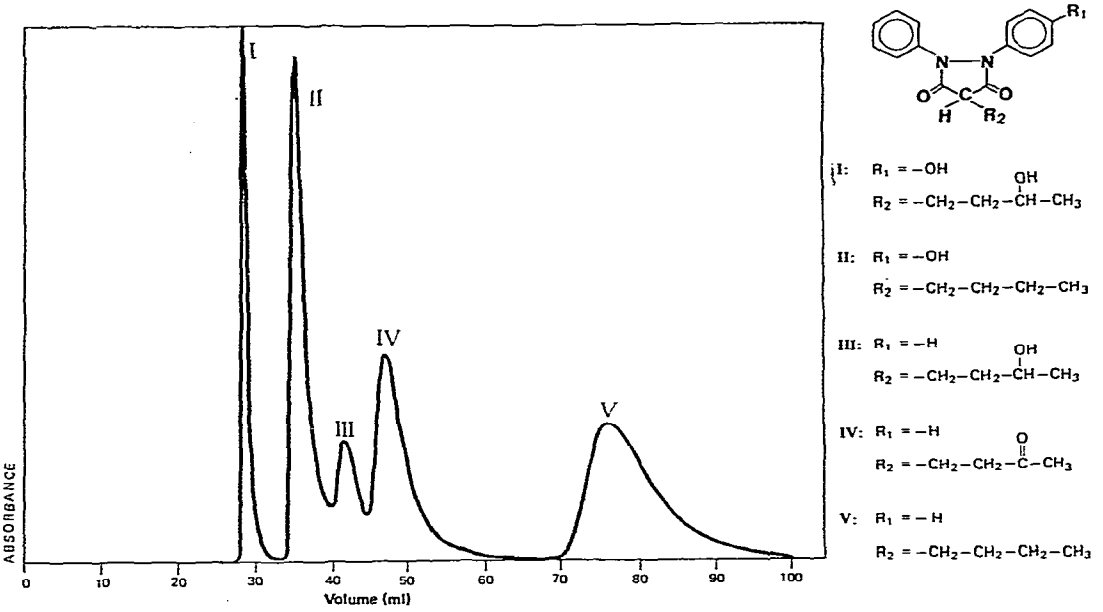

Fig. 3. Separation of a mixture of *p,γ*-dihydroxyphenylbutazone (I), oxyphenbutazone (II), *γ*-hydroxyphenylbutazone (III), *γ*-ketophenylbutazone (IV) and phenylbutazone (V) in milligram amounts on micronized XAD-2. Eluent: methanol–water–1,2-dichloroethane–acetic acid (85:9:5:1) (CS 2). Column size: 30 × 1.27 cm. Flow-rate: 2 ml/min.

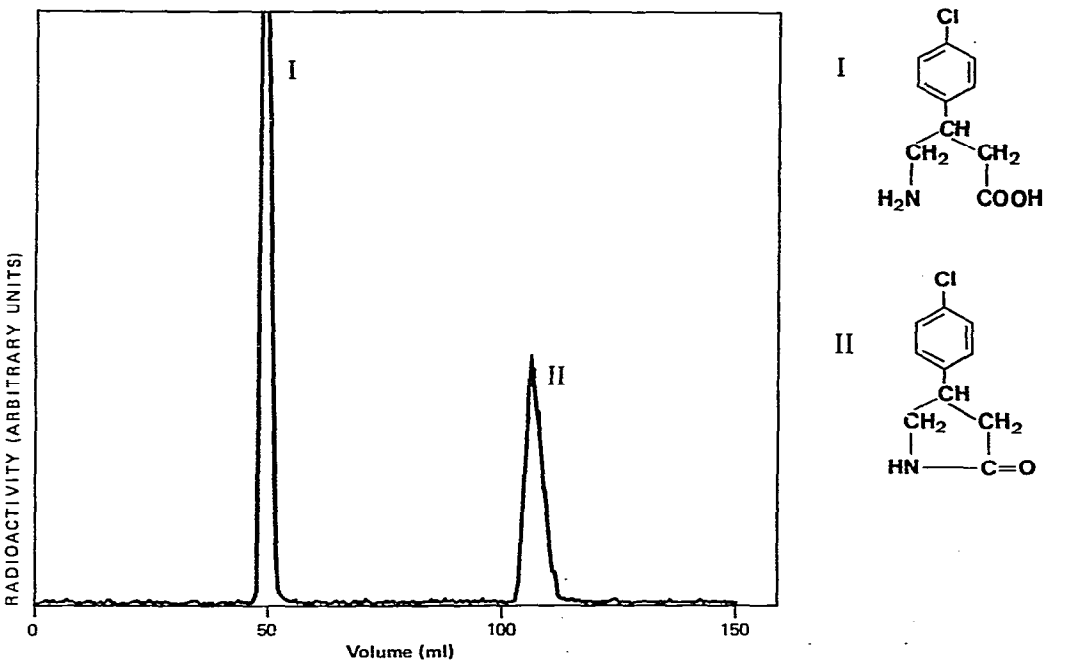

Fig. 4. Separation of a 0.1-mg mixture of ^{14}C-labelled baclofen (I) and its lactam (II) on micronized XAD-2. Eluent: methanol–water–1,2-dichloroethane–conc. HCl (28:70:1:1) (CS 3). Column size: 30 × 1.27 cm. Flow-rate: 1 ml/min.

amino acid were easily removed, which was not possible with other methods of purification, *e.g.*, recrystallization.

Fig. 5 illustrates the excellent reproducibility and high capacity of the reversed-phase system. The upper and lower chromatograms represent two independent runs on samples of the same metabolite mixture. This mixture contained hydrophilic, non-extractable products derived from biotransformation of ^{14}C-labelled clomipramine in man[5]. It also contained large amounts of non-radioactive material. The total amount applied on the column (50 × 2.54 cm) was 800 mg, only 1 mg of that being ^{14}C-labelled substances. Nevertheless, highly enriched metabolite fractions were obtained.

On account of its reproducibility and resolution, the chromatographic system described can be used successfully to record patterns of radioactive metabolites in body fluids. In many instances the biological sample can be chromatographed as such without prepurification. An example is given in Fig. 6, where the patterns of biliary and urinary metabolites reflect species-dependent differences in the biotransformation of diclofenac[6]. In these separations 0.5–1.0 ml of bile or urine was injected on to a 30 × 1.27 cm column.

DISCUSSION

Styrene–divinylbenzene copolymers, such as XAD-2 resin, display a pref-

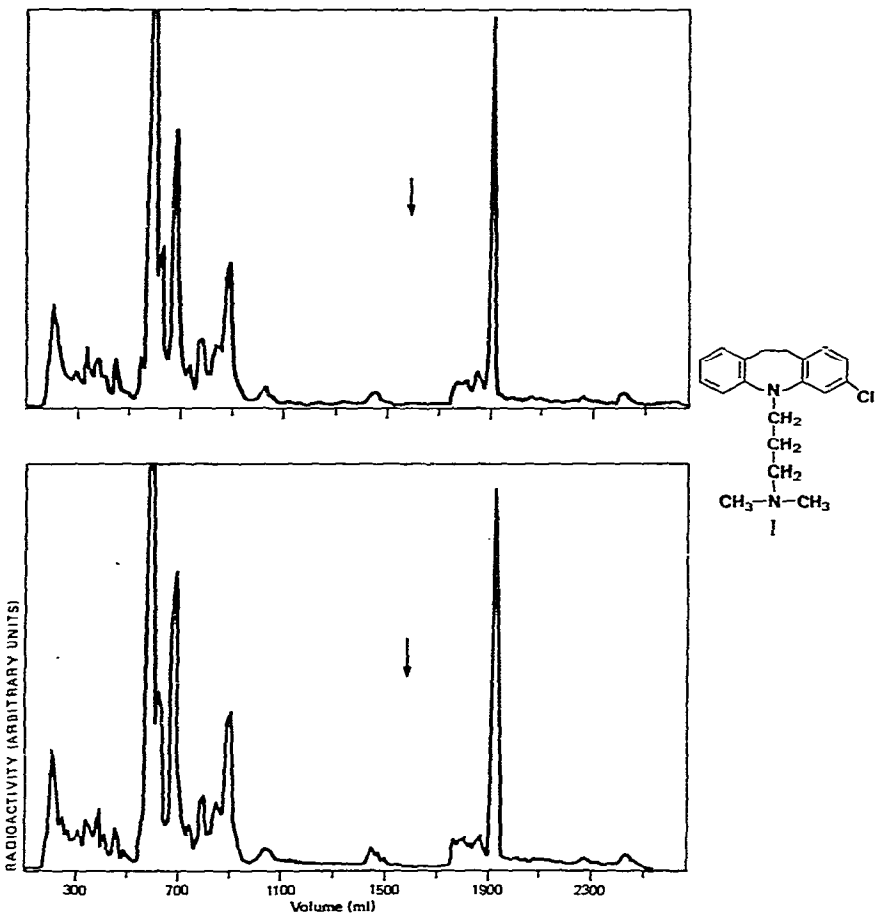

Fig. 5. Two independent chromatographic runs of a crude fraction (total weight 800 mg) containing hydrophilic, non-extractable biotransformation products (1 mg) of [^{14}C]clomipramine (I) in man. Support: micronized XAD-2. Eluent: methanol–water–acetic acid (40:60:1), saturated with toluene (CS 4); at the beginning of each chromatogram, the eluent was changed to methanol–water–acetic acid–toluene (60:40:1:1) (CS 5) at V_E = 1580 ml (indicated by the arrows). Column size: 50 × 2.54 cm. Flow-rate: 2 ml/min.

erential affinity for organic molecules and are therefore widely used to extract drugs, metabolites, steroids and other biologically important compounds from aqueous media[7–10]. When the resins are employed as supports in chromatographic columns, separation into individual components is possible. Published methods have described, for instance, semi-preparative separations of corrinoids[11] and analytical separations of organic acids[12,13].

The technique described here makes use of XAD-2 resin for high-resolution liquid chromatography on a preparative scale. Micronization of the support to a mean particle size of 12 μm, and the slurry packing employed, lead to highly efficient columns. The diameters of the columns used in our laboratories range from about 1 to 5 cm.

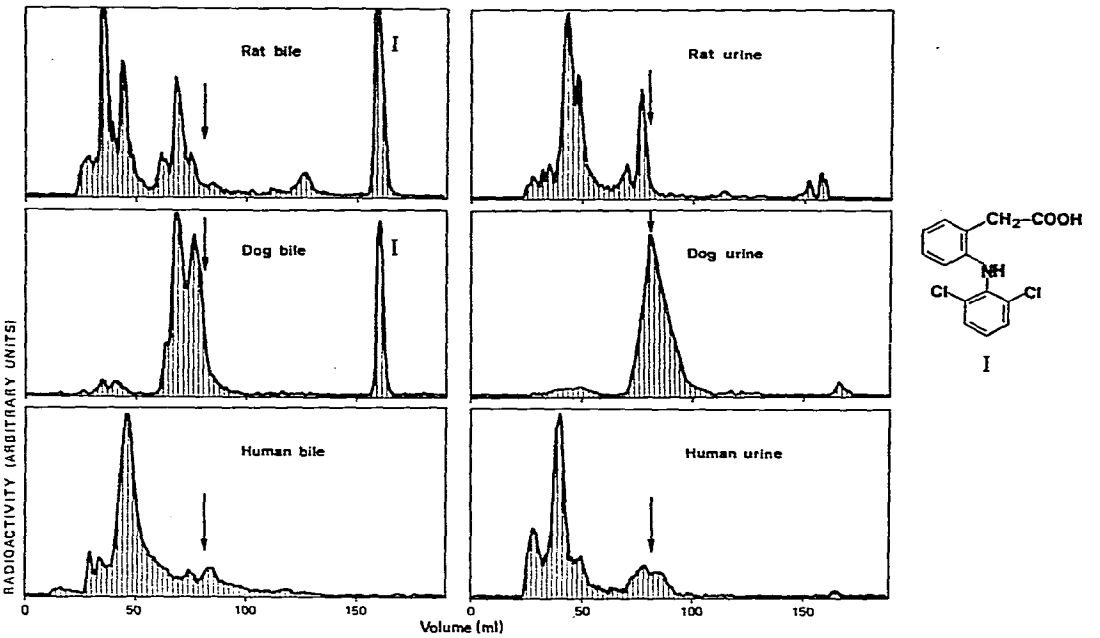

Fig. 6. Metabolite pattern in bile and urine of rat, dog and man following administration of [^{14}C]-diclofenac (I); 0.5–1.0 ml of bile or urine was injected without pre-purification on to the column. Support: micronized XAD-2. Eluent: methanol–water–toluene–acetic acid; the volume ratio was 64:34:1:1 (CS 6) at the beginning of each chromatogram and was changed to 70:26:3:1 (CS 7) at V_E = 80 ml (indicated by the arrows). Column size: 30 × 1.27 cm. Flow-rate: 2 ml/min.

The solvent systems consist of three essential components, *viz.*, water, a lower aliphatic alcohol and a hydrophobic solvent, such as 1,2-dichloroethane or toluene. When the column is equilibrated, the hydrophobic component of the mixture builds up a stationary liquid phase on the XAD-2 support, which results in reversed-phase partition chromatography. The solvent system is a single-phase mixture well below the point of saturation. Thus, the usual variations in ambient temperature do not adversely affect the chromatographic performance.

In the examples given above (Figs. 2–6), the content of water in the solvent systems varies widely, being 70% for baclofen, a strongly polar amino acid, and 9% for phenylbutazone, a lipophilic acidic compound. In general, it is possible to find optimal chromatographic conditions for a given compound simply by changing the ratio of the components in the solvent mixture. As regards the hydrophobic component, different solvents are available, so that the selectivity can be further modified. When compounds with greatly differing polarities are present in the same sample, gradient elution is feasible without disturbance of the reversed-phase system.

Chromatography on XAD-2 resin offers many advantages in metabolic work, particularly for the preparative separation of polar, non-extractable compounds. With a 50 × 2.54 cm column, crude samples of up to 1 g can be separated (Fig. 5), and there are no difficulties in dissolving such a sample in a small volume of the chromatographic solvent. All components of the solvent are easily volatile and, therefore, work-up of the eluted fractions is simple. In ion-exchange chromatography, in con-

trast, one usually obtains effluents containing non-volatile electrolytes. The chemical inertness and physical stability of the XAD-2 resin permits the repeated use of the same column; in our laboratories more than 100 runs have been performed without loss of efficiency. Contaminated support can easily be regenerated in the column by washing with lipophilic solvents and re-equilibrating with the chromatographic solvent system.

On account of its wide variability, the separation system can be applied to most metabolites encountered in biotransformation of drugs. In rare cases, extremely polar products may elute with the solvent front, even when the eluent mixture contains the highest feasable percentage of water (*ca.* 80%). The addition of small amounts of an electrolyte, *e.g.*, 1% sodium chloride solution, leads to better chromatographic properties. However, the advantage of a simple work-up is then lost.

In conclusion, preparative reversed-phase partition chromatography on XAD-2 resin is a versatile technique characterized by a large capacity and high resolution, selectivity and reproducibility. It can thus be used successfully to separate drug metabolites or naturally occurring compounds from complex mixtures.

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