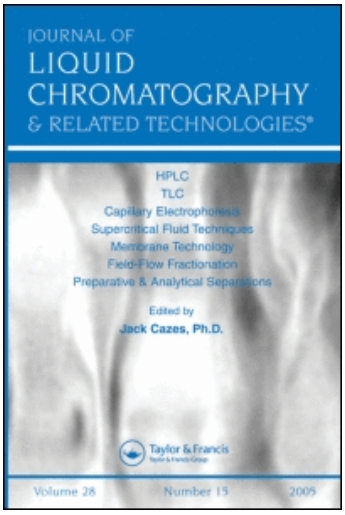

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PREPARATIVE HPLC FOR THE FACILE ISOLATION OF DRUG GLUCURONIDE CONJUGATES FROM CRUDE EXTRACTS OF URINE

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ABSTRACT

Reverse-phase preparative HPLC has been used to advantage for the isolation of crystalline quantities of drug glucuronide conjugates from crude urinary extracts. Following chronic administration of large doses of diazepam, levorphanol and hydroxyethylflurazepam to dogs, urine was collected and the water soluble drug conjugates adsorbed on a column of Amberlite XAD-2 resin. In each instance elution with methanol and solvent evaporation yielded a crude oil (approx. 3 g) which was chromatographed in one portion on either PrepPAK 500 C_{18} (Waters) or Magnum 40 ODS-3 (Whatman) columns using aqueous methanol solvent systems. A greater than 90% purification was achieved in this single initial chromatographic step. Employing a combination of subsequent semi-preparative HPLC steps on either C_{18} or silica gel columns, milligram quantities of the glucuronides of oxazepam, levorphanol and hydroxyethylflurazepam were isolated. The isolation procedures provide a general approach for obtaining milligram quantities of intact drug conjugates which may otherwise be difficult to obtain by chemical synthesis. Such conjugates can be used as authentic standards in the quantitation of certain drug metabolites in biological media during pharmacokinetic/biopharmaceutic studies.

INTRODUCTION

For many drugs and/or their metabolites conjugation with glucuronic acid to yield water soluble products represents a major

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pathway in their biotransformation and elimination from the body. Alcohols and phenols form ether-type glucuronides, aliphatic and aromatic acids form ester linkages while amines and thiols form N- and S-glucuronides, respectively. Although glucuronides are primarily excreted in the urine, the conjugates may also be excreted via the bile. Since glucuronides are more hydrophilic and usually more acidic than the parent drug they can less easily permeate cell membranes and generally have little, if any, pharmacologic activity compared to the parent drug.

However, although glucuronides may represent the end products in the metabolism of certain drugs in some instances their quantitation in either blood and/or urine provide the only feasible approach for evaluating the pharmacokinetics and/or bioavailability of the parent drug (1). For example a drug may undergo such extensive "first-pass" metabolism on oral administration that the plasma and urine concentrations of the intact drug are non-quantifiable with available methodologies while the glucuronide conjugate may be present in one or both media in high concentrations. Since the highly polar water soluble conjugates are usually difficult to extract into organic solvents without at the same time extracting large amounts of interfering biological substances, the most usual approach has been to cleave the conjugate by hydrolysis with β-glucuronidase and extract the drug moiety into a relatively non-polar solvent for subsequent quantitation.

A more simple approach is offered using reverse-phase HPLC whereby urine samples containing the unhydrolyzed conjugate can be

injected directly onto the column. However, such an approach requires the availability of the pure glucuronide as a chromatographic reference standard in the generation of calibration curves for quantitative purposes.

The objective of the present study was to investigate the utility of preparative HPLC in conjunction with both semi-preparative and analytical HPLC as a relatively simple means of obtaining milligram quantities of drug conjugates which could subsequently be used as analytical standards and thereby avoid certain difficult and time consuming chemical syntheses of such conjugates. The isolation of the glucuronides of oxazepam, levorphanol and hydroxyethylflurazepam, which were required for quantitative analytical purposes, were chosen to develop the chromatographic procedures.

MATERIALS and METHODS

HPLC instrumentation

Preparative chromatography was carried out using a Waters 500A preparative liquid chromatograph (Waters Associates, Milford, MA 01757) attached to an LDC DuoMonitor (LDC, Riviera Beach, Fla. 33404). Semi-preparative and analytical HPLC was performed with an LDC dual pump with a Model 1601 gradient master and a Hitachi variable wave length UV detector.

The types of columns used during the various chromatographies are noted in the text of the experimental sections.

Physico-chemical analysis methods

Proton NMR spectra were obtained with a Varian XL-200 spectrometer (Varian Associates, Palo Alto, Cal.) operating in the Fourier-transform mode.

Low resolution mass spectra (MS) were obtained with a Varian MAT CH5 instrument at 70 eV by direct insection probe. Liquid chromatography-mass spectrometry (LC-MS) was carried out using a Finnigan 1015 MS equipped with a high speed pumping system and a Hewlett-Packard direct liquid insertion (DLI) probe. Glucuronide conjugates were methylated with diazomethane prior to LC-MS.

Drug administration

Diazepam, levorphanol tartrate and hydroxyethylflurazepam were administered orally in solution to beagle dogs (11-15 kg body weight) in doses of 10, 10 and 50 mg/kg, respectively, for five consecutive days. The animals were housed in metabolic cages, allowed food and water ad lib and their urine collected for 7 days. Each separate urine pool was stored at 4° to await analysis.

Extraction of urine

Each urine pool (4-6 liters) was adjusted to pH9 with concentrated ammonium hydroxide and allowed to stand at room temperature overnight. The fine precipitate which formed was removed by filtration following the addition of ca. 20 g/liter of Celite 545 (Johns-Manville) to aid in the filtration process. The clear yellow filtrate was then allowed to flow under gravity through a

1.5 liter bed of Amberlite XAD-2 resin (Rohm & Haas Corp. Philadelphia, PA) in water contained in a 120x 6 cm (i.d.) column equipped with a coarse fritted-glass disc. The resin was then washed with 5 liters of water and any excess water remaining in the resin bed removed with suction through the column outlet. The adsorbed drug conjugates were then eluted from the resin with 3 x 1 liter portions of methanol. The combined methanolic eluates were evaporated *in vacuo* at 50° to yield a dark brown oil which was reconstituted in 100 ml of water, adjusted to pH9 with ammonium hydroxide and extracted twice with 2 volumes of ether to remove non-polar components. The aqueous phase was evaporated *in vacuo* to an oil by azeotropic distillation with an equal volume of n-butanol. The extract was stored at 4° to await preparative HPLC.

Monitoring preparative HPLC with analytical HPLC

Prior to any preparative isolation procedures small aliquots of each XAD-2 extract were used to develop a rapid analytical HPLC procedure for monitoring the elution profile of each conjugate from the preparative and semi-preparative columns. Reverse-phase chromatography with a Whatman Partisil-5 ODS-3 RAC column (10 cm x 9.4 mm i.d.) was used for monitoring purposes. Aliquots (10-50 µl) of fractions collected from the preparative columns were injected onto the analytical column using a WISP (Waters) automatic injector system. The solvent system was selected such that each fraction could be monitored in about 5 min.

Isolation of S-(+)-oxazepam glucuronide

The urine extract (3g) was dissolved in water (200 ml), filtered through a 1μ filter and applied to two PrepPAK 500 C_{18} cartridges, linked in series, through the pump of the Waters Prep 500 chromatographic unit. With UV detection at 254 nm the columns were eluted at 100 ml/min with a 1 liter step gradient starting with methanol:water:acetic acid (40:60:1) as shown in Figure 1. While the bulk of the chromogens eluted in fractions 1-10, the fractions (200 ml each) comprising the major peak (12-16) were pooled and evaporated *in vacuo* to 450 mg of an oil which was rechromatographed on four μBondapak C_{18} (Waters) columns (7.8 mm x 30 cm), linked in series, using a gradient of 20% methanol in 0.025M NaH_2PO_4 to 100% methanol. Elution was monitored with analytical HPLC, the main peak (fractions 95-122) evaporated *in vacuo* and the solid residue desalted on a column of sephadex LH-20 (Pharmacia) in methanol:water (7:3) to yield a pale yellow oil weighing 40 mg. The oil was rechromatographed on an M-9 Partisil (Whatman) column using chloroform:methanol:acetic acid (80:20:1) at a flow rate of 4 ml/min and fractions collected each min. A yellow chromagen eluted in fraction 4 and oxazepam glucuronide in fractions 6-9. Solvent evaporation yielded 26 mg of a white amorphous powder which could not be crystallized.

The NMR, MS, LC-MS (methyl ester) and UV spectra were consistent with the proposed structure and its HPLC characteristics in

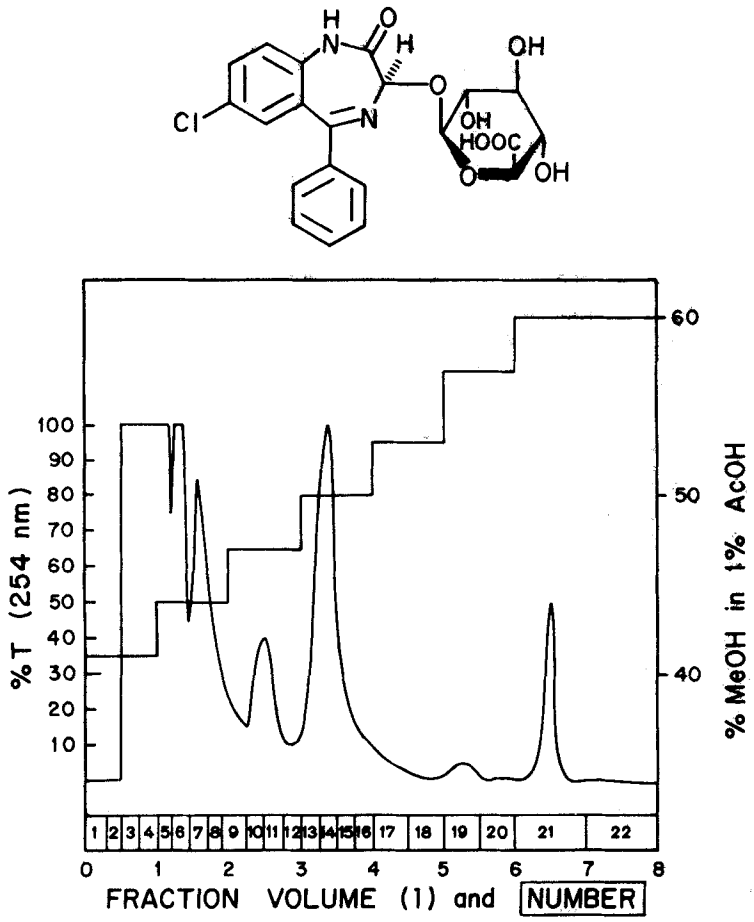

Figure 1. Structure of oxazepam glucuronide and the initial preparative chromatogram obtained using the Prep PAK 500 C_{18} column during its isolation from a crude extract of urine.

agreement with the findings of Ruelius et al. (2) for S-(+)-oxazepam glucuronide.

Isolation of levorphanol glucuronide

One-half of the urinary extract (1.6 g) was dissolved in water, filtered and applied to two PrepPak 500 C_{18} columns as

previously described. With detection at 280 nm, elution was carried out as shown in Figure 2 with a gradient of methanol in 0.01 M NaH_2PO_4 and 200 ml fractions collected at a flow rate of 100 ml/min. The main peak (fractions 26-30) was concentrated in vacuo and desalted on Diaion HP-20 (100-200 mesh: Mitsubishi Chemical Industries, NY, NY 10017) packed in a 24 x 1/2 in. stainless steel column. The sample, dissolved in 300 ml of water, was pumped onto the column, washed with water (100 ml) and eluted with methanol. The fractions containing the desired conjugate, as determined by analytical HPLC, were pooled and concentrated to give a light-brown solid (145 mg). The solid was dissolved in water and chromatographed on an M-9 ODS-2 (Whatman) 50 cm column again using a gradient of methanol in 0.01M NaH_2PO_4 at a flow rate of 2 ml/min. The major peak (280 nm) which eluted was concentrated and desalted on Diaion HP-20 as before and the product crystallized from methanol to yield 32 mg of levorphanol glucuronide mp 216-219°. The NMR, MS, UV and IR spectra were consistent with the proposed structure.

Isolation of hydroxyethylflurazepam glucuronide

The oily urine extract (3.5 g) was dissolved in 200 ml of water, filtered and applied to a 48 mm x 50 cm M-40 ODS-3 column (Whatman). Elution was carried out with an isocratic system of methanol:water:acetic acid (50:50:1) at a flow rate of 50 ml/min and 55 ml fractions collected. The desired metabolite was located by analytical HPLC (fractions 38-56), concentrated to 580 mg of an

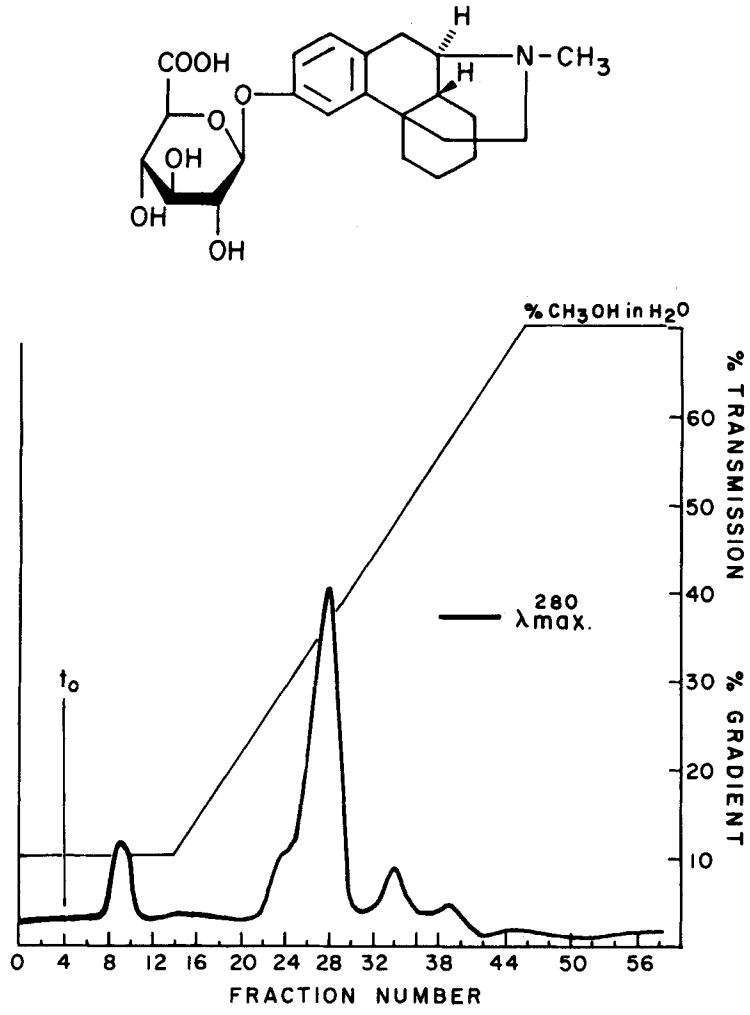

Figure 2. Structure of levorphanol glucuronide and the initial preparative chromatogram obtained using the Prep PAK 500 C_{18} column during its isolation from a crude extract of urine.

oily solid and rechromatographed on the same M-40 column using an isocratic system of methanol:acetonitrile:water:acetic acid (40:10:50:1) with detection at 254 nm. Concentration of the peak fractions yielded 150 mg of a yellow oil which was rechromatographed on an M-9 Partisil-10 column (Whatman) using an exponential gradient from 50% chloroform:hexane (6:4) to 100% chloroform:methanol:H_2O:acetic acid (90:10:1:1). The peak fractions were concentrated to an aqueous solution and lyophilized to give 46 mg of hydroxyethylflurazepam glucuronide as a white powder. Attempts at crystallization were unsuccessful. The NMR, LC-MS (methyl ester) and IR spectra were consistent with the proposed structure (Figure 3).

RESULTS AND DISCUSSION

The present studies demonstrate the utility of preparative HPLC in the isolation of water soluble glucuronide conjugates from crude urine extracts. In particular, preparative HPLC has the necessary capacity to handle such extracts in a single chromatographic run and to provide >90% purification in a single chromatographic step. Other approaches have involved the use of large ion exchange columns (2) or by partition column chromatography on celite (3). Both of the latter procedures are time consuming and have limited capacity in handling large crude extracts.

Two types of preparative C_{18} columns were evaluated; the Waters Prep PAK 500 C_{18} cartridge and the Whatman Magnum-40 ODS-3.

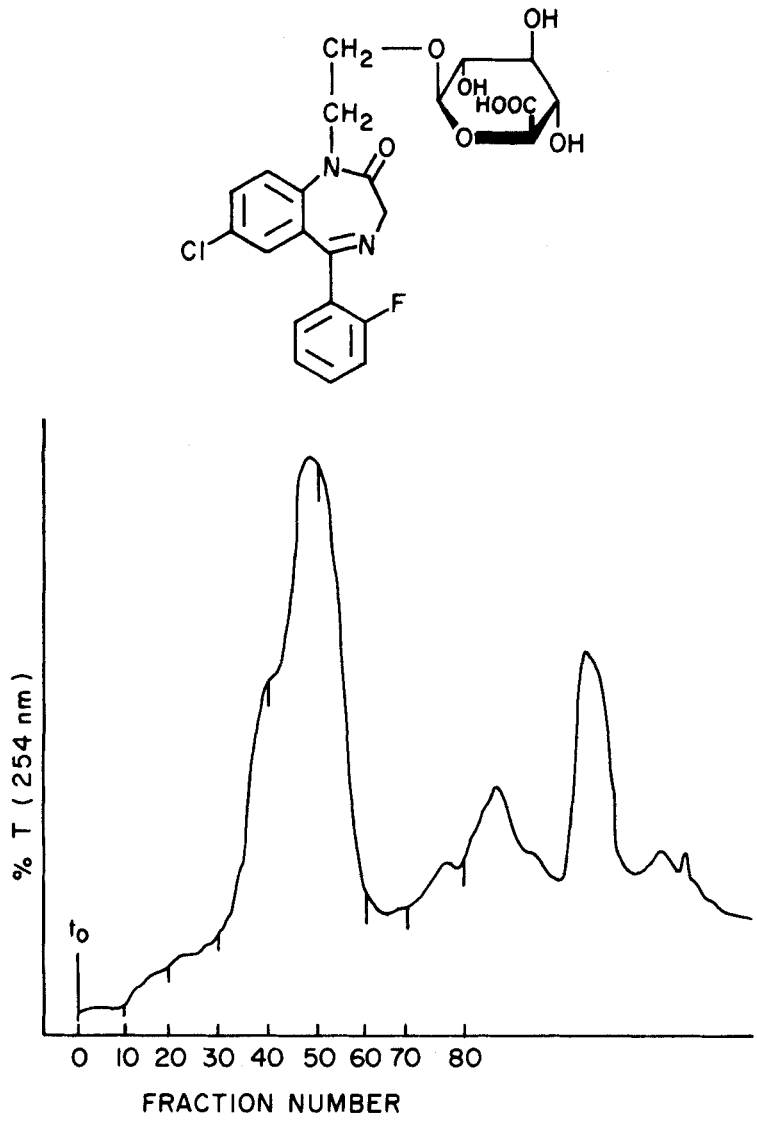

Figure 3. Structure of hydroxyethylflurazepam glucuronide and the chromatogram obtained on a preparative M-40 ODS-3 column during its isolation from a crude extract of urine.

Although satisfactory results were obtained with both types of columns, it has generally been noted, during both the present and other unpublished studies, that a single Magnum-40 column affords better resolution than two PrepPAK 500 columns linked in series, while their capacities are similar. The crude XAD-2 resin extracts were applied to the C_{18} columns as solutions in 200-300 ml of water. In this manner the extracts were evenly adsorbed to the top of the column prior to starting chromatography with the methanol gradient. In some instances it may be necessary to initially dissolve the crude extract in a small volume of methanol prior to dilution with water in order to achieve complete dissolution of the extract.

Following the initial chromatographic step, it now appears, in retrospect, that rechromatography on a Magnum-40 column using a different solvent system is the direction of choice since at this point the glucuronide is still grossly contaminated with urinary pigments and other interfering substances. The latter approach was used in the case of hydroxyethylflurazepam glucuronide. Subsequent chromatography can then be carried out on semi-preparative C_{18} or silica gel columns. One problem noted was that reverse phase chromatography was usually unsatisfactory as a final chromatographic purification step in completely removing urinary chromogens contaminating the glucuronides. For this reason, except in the case of levorphanol glucuronide which crystallized readily, the final chromatographic step was carried out on a silica

gel column which adequately resolves the interfering chromogens. Desalting of fractions containing buffering salts, as in the case of levorphanol glucuronide, was best achieved by HPLC on columns packed with Diaion HP-20 (100-200 mesh) which is similar in nature to XAD-2 as a high porosity copolymer of styrene and divinylbenzene. The procedure is rapid and also offers an advantage over open-column desalting on Sephadex LH-20 (oxazepam glucuronide) or XAD-2 in that a methanol gradient may be readily applied during the elution process which in certain instances affords additional chromatographic purification.

Ruelius et al. (2) have previously reported the isolation of the diastereoisomeric glucuronides of oxazepam from swine urine following chronic administration of large daily doses (50 mg/kg) of oxazepam to the animals. These investigators employed a combination of open-column ion exchange and adsorption chromatography for the isolation of the S(+)-and R(-)-isomers which was considerably more time consuming than the present HPLC procedure. More recently, while the present work was in progress, Seideman et al. (4) isolated S(+)-oxazepam glucuronide from dog urine using HPLC and a similar approach to that used in the present studies. However, it is not apparent as to whether single or multiple chromatographic runs were used at the initial purification step since the column employed has lower capacity than the large preparative columns used here.

In conclusion, preparative HPLC provides a convenient approach for the isolation of milligram quantities of water soluble conjugates from crude urinary extracts. With suitable choice of columns and solvent systems analytically pure material can be obtained in as few as three separate chromatographic runs.

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