

High Performance Liquid Chromatography Coupled with Radioactivity Detection: A Powerful Tool for Determining Drug Metabolite Profiles in Biological Fluids

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Radioactivity Flow-Through Detector, Coupling of HPLC with Continuous Radioactivity Detection, Drug Metabolism, High Performance Liquid Chromatography

High performance liquid chromatography coupled with continuous radioactivity detection represents an advancement in drug metabolism research. Using radioactive substances labelled in biologically stable positions, all metabolites can be specifically detected by radioactivity measurement. Thus no clean-up of biological fluids is required prior to HPLC. This can prevent artefact formation from unstable metabolites, reduces recovery problems and facilitates quantitation. Separation of highly polar and unpolar metabolites is possible in a single chromatographic run using gradient elution and reversed phase materials. This technique is also well-suited for preparative isolation and purification of metabolites for subsequent structure elucidation.

Various metabolite profiles of drugs labelled with carbon-14 or tritium are shown. Metabolites of the following drugs are presented: norfenefrine, etozolin, thymoxamine, naloxone, and levobunolol. We review the general methodology and report our experience with this technique. In principle, this technique may be useful for all biological systems in which tracer techniques are applied.

Introduction

Metabolic investigations are essential in the development of new drugs. Knowledge of biotransformation is required for a comparative evaluation of pharmacological and toxicological data. The importance of phase II metabolites (*e.g.* glucuronic acid or sulphuric acid conjugates) is becoming increasingly recognized so that describing unconjugated metabolites or cleavage products alone is no longer sufficient.

The investigation of metabolite profiles, particularly clean-up and purification for structural elucidation of metabolites is complicated by several problems. The substances of interest are normally present in small quantities and low concentrations. Many endogenous compounds, several of which are present in large quantities, may interfere. The metabolites may be unstable. Their polarities cover a great range.

These problems can largely be solved by using HPLC coupled with continuous radioactivity detection. The methodology has been known for more

than ten years [1–8], however, the main advantage is not consistently made use of. Biological samples can be injected directly onto the HPLC column without prior clean-up due to the specificity of the detector [9–11]. To encourage other investigators to make use of the potential advantages of this methodology, we will discuss our experience in this report, part of which has already been published [3, 5, 12–16].

First the general methodology will be presented, followed by the specific method used. Then several metabolite patterns and metabolite structures will be shown and the purification of metabolites for structural elucidation will be discussed.

Of course, this technique is not restricted to drug metabolism research in animals and man. In principle, it can be used for all biological systems in which tracer techniques are applied, *e.g.* biosynthesis studies [26], microbiological transformations in biotechnology, separation of labelled proteins [27] and nucleotides [28].

General Methodology

HPLC

Drug metabolites are usually more polar than the parent substance, and polarity ranges from very polar to unpolar. HPLC is the method of choice for

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chromatographic separation of these compounds. The reversed phase column combined with gradient elution is ideally suited for such separation problems. Selectivity can be increased by choosing multidimensional conditions, *e.g.* separation with different pH, packing material, eluant, temperature. The specificity of the radioactivity detector makes it possible to inject biological fluids directly onto the column.

Radioactivity detection

Tritium and carbon-14 are the most commonly used radioactive isotopes in drug research. Radioactivity detection of the column eluates can be made discontinuously or continuously in a flow-through cell. In our opinion, discontinuous detection is only suitable in cases where low concentrations of radioactivity are present with no possibility of enrichment.

In continuous radioactivity detection, the measurements in the homogenous phase are differentiated from those in the heterogenous phase. In the homogenous phase, a scintillator solution is added to the column eluate, while during the measurement in the heterogenous phase, the scintillator is present in the flow-through cell and is surrounded by the column eluate.

Measurement in homogenous phase

The measurement in homogenous phase is important mainly for the analysis of ^3H -labelled substances. The lower detection limit is much better than with glass scintillators. With mixing ratios eluent: scintillator cocktail of 1:1 to 1:4, static counting efficiencies of 20–50% are possible [8, 17]. Another advantage is the avoidance of adsorption on the scintillator surface which is often seen by use of glass scintillators. Because of the short light ways in the cell, quenching is not as important as if measured in scintillation vials, but may be significant with high eluate content [8, 18]; computer programs are available for quench correction.

An additional pulseless pump for the scintillation cocktails is needed for measurement in homogenous phase. In cases where the separated substances will be used for further investigations, *e.g.* structure elucidation, splitting techniques are indicated. In the case of very low radioactivity, a splitter controller can be used which can be driven by an internal standard or automatically to admit only radioactive fractions

to the cell. The radioactive zones remain in the cell until the next radioactive peak is eluted. In this case, the counting time depends on the time between radioactive peaks. A nearly static measurement is possible. Unmixable liquids can be inserted before and after a radioactive substance to avoid peak broadening, and the regions of interest can be stored in a large loop. When the chromatogram is finished, the stored eluate is pumped back through the detector and so the counting time can be regulated by the radioactivity in the eluate [20].

Measurement in heterogenous phase

For the flow-through cell, glass scintillators are normally used, (*e.g.* Cer-activated Lithium glass) or Yttrium silicate. Europium-activated CaF₂ scintillators in plastic or organic substances such as anthracene are of minor importance. Using optimal crystal diameter and cell dimensions, the static counting efficiency of glass scintillators and Yttrium silicate range from 30–70% for ^{14}C [18, 19, 21, 22], and from 0.6–6% for tritium [8, 18, 22].

The main advantage of glass scintillators is a potential use of the eluate for further investigations. This is important for structure elucidation of substances from biological fluids *e.g.* urine, plasma, and bile as well as plant extracts. Due to low quenching [21], glass scintillators are well suited for quantitative determinations.

Normally no major adsorption problems occur with glass scintillators, but adsorption may be more frequent using Yttrium silicate. Scintillators can be washed with alkaline detergents or acids. Sometimes adsorption can be avoided by silylation of the scintillator surface.

The cell volumes normally used range from 25 to 500 μl for glass scintillators [18, 21] and from 100 to 2000 μl for the homogenous phase method [18]. These relatively large dead volumes, however, do not result in remarkable peak broadening, if the peak volumes are larger than 1 ml.

Data collection and quantitative evaluation

Formerly the data from the radioactivity detector were collected by multichannel analyzer, but now microcomputers are used for this purpose. They have the advantage of being more flexible and user friendly. The number of data points per time unit (*e.g.* the time resolution) depends on chromatographic condi-

tions and storage space available. The chromatogram can be observed "live" on a CRT and may be recorded simultaneously on a strip chart recorder. Sophisticated software enables various control functions (*e.g.* controlling a fraction collector), quench correction, data storage on disc, data evaluation during the run and reevaluation of stored data.

Data evaluation is done according to the user's requirements. For metabolite profiles the user needs a report which lists the counts for individual peaks and the whole chromatogram as well as the background to calculate percentages of each peak on the total radioactivity. For comparative studies, chromatograms can be depicted in a 3-dimensional plot.

Simultaneously, the microcomputer can collect the data of a second radioactivity channel *e.g.* for double-labelled compounds as well as the data of UV- and/or fluorescence detectors.

Materials and Methods

Labelled substances

[¹⁴C]norfenefrine, [¹⁴C]etozolin, [¹⁴C]thymoxamine and [³H]naloxone were synthesized in-house. [³H]levobunolol was obtained from Warner-Lambert/Parke Davis, Ann Arbor, Michigan. Positions of the label are indicated in the corresponding figures. Radiochemical purity was > 98% in all cases.

Animal and human studies

Radioactivity dosage was 20 to 100 µCi/kg in animals and 100 µCi per subject. All substances were administered orally in adequate dosages/specific activities. Urine, bile, and plasma samples were deep frozen during or immediately after collection.

HPLC and radioactivity measurements

HPLC separations were carried out by means of a Varian 5000 using reversed phase columns (50 × 0.46 cm) packed with µ-Bondapak C-18, 10 µm (Waters) in our laboratory if not otherwise specified. A precolumn (4 × 0.46 cm) filled with the same material was installed for the protection of the separation column. For UV-detection an LC 55 photometer (Perkin-Elmer) was used. Radioactivity was measured with radioactivity detector LB 5025 or LB 503 (Berthold, Wildbad), with 100 or 300 µl

cells, filled with cer-activated lithium glass (Koch-Light) or HS-scintillator (Berthold, Wildbad). For the measurements in the homogenous phase, scintillation cocktail Rialuma (Baker) was used. For data evaluation multichannel analyzer BS 27 (Berthold-Silena) with magnetic band station was used. The analog signals of the radioactivity and UV detectors and the gradient were registered on a 3-channel (Rikadenki) or 4-channel (Linseis) recorder. Application of the samples to the column was made using a loop injector (Rheodyn) with a 2 ml dosage loop.

The urine, bile, and plasma samples to be injected were adjusted to the required pH and filtered over a membrane filter (Sartorius). For analytical runs the volumes were 1–5 ml; the radioactivity amounts injected were 100,000 to 1 Mio dpm for urine and bile and 5,000–20,000 dpm for plasma. For preparative runs up to 1 l urine was pumped onto reversed phase-columns (16 mm i.d.).

Results and Discussion

The applicability of the system HPLC radioactivity detection in metabolite research is demonstrated on the basis of drugs which were investigated in our laboratory.

Metabolite profiles in urine, bile, and plasma

[¹⁴C]Norfenefrine

The sympathomimetic norfenefrine is a strong hydrophylic substance and, like its metabolites, immediately eluted from the C-18 column, even in the presence of buffer solution. For this reason, analyses were conducted using tetrabutylammonia ions as counter ions. The parent substance was still eluted early, but the metabolites could be separated with the aid of gradient elution (buffer vs acetonitrile).

The metabolite profiles of norfenefrine in rats, dogs, cats, and man are given in Fig. 1. The following metabolic reactions were observed: glucuronic acid and sulphate conjugation of norfenefrine, as well as oxidative deamination in which 3-hydroxymandelic acid and 3-hydroxyphenylglycole were formed. The last two metabolites mentioned were also subject to conjugation reactions [12].

Norfenefrine is a racemate. The two enantiomers of 3-hydroxymandelic acid formed in the organism could be separated on a reversed phase column using

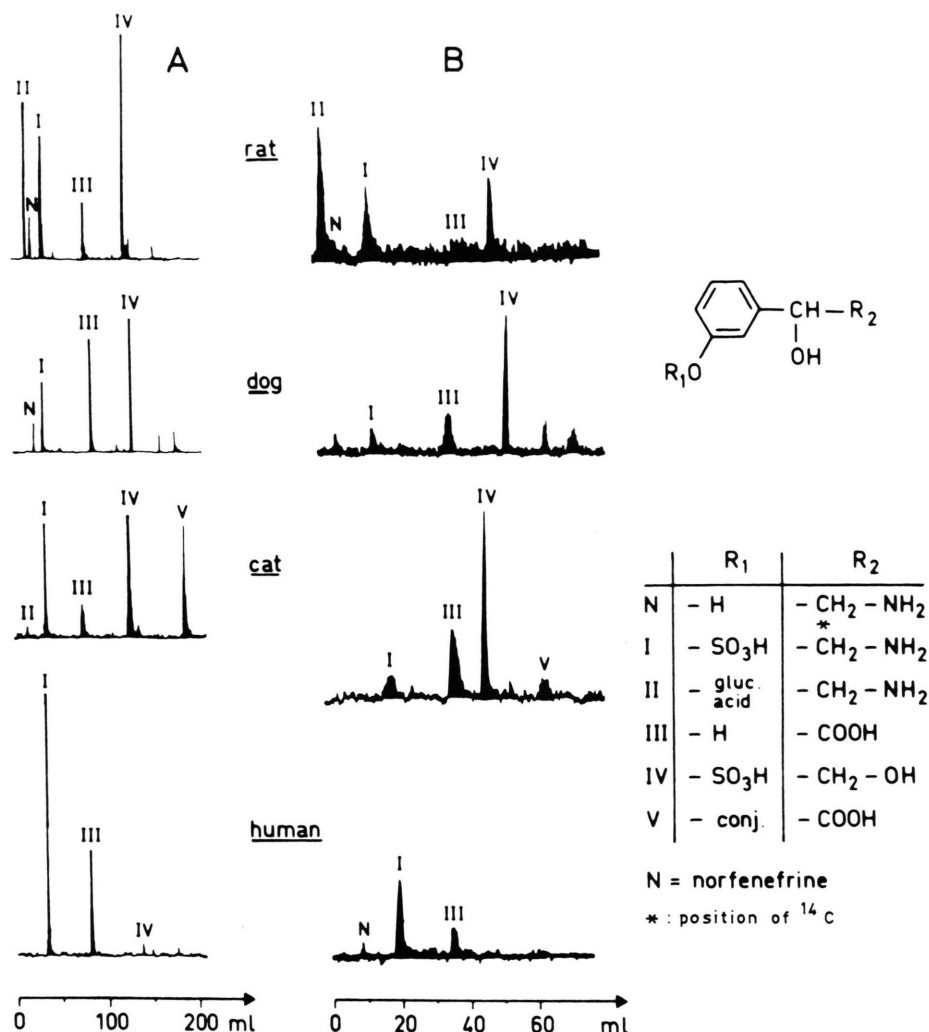


Fig. 1. Metabolite profiles in 0–24 h urine (A) and 2 h plasma (B) following oral administration of [¹⁴C]norfenefrine to rats, dogs, cats, and humans.

Column: μ Bondapak C-18 (50 \times 0.46 cm).

Gradient: Aqueous solution of 0.2% tetrabutyl ammonium hydrogen sulphate, and 0.1% ammonium acetate vs acetonitrile.

L-phenylalanine and copper sulphate solution as eluents [23].

Due to the strong polarity and relative instability of some of the substances described, a differentiated investigation of the metabolism using other methods was not possible.

Fig. 1 shows the separation of the metabolites in urine (A) and plasma (B). The profiles are qualitatively comparable. In plasma, the radioactivity con-

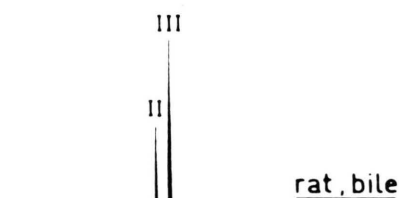
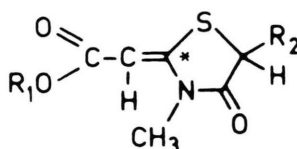
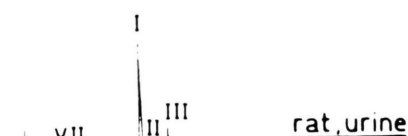
centrations are in the range of a few nCi/ml, so that analysis could only be made near the sensitivity limits of the radioactivity detector. In order to achieve maximum sensitivity, the flow was reduced to 0.2 ml \times min⁻¹. The time duration of the radioactivity in the cell is thereby increased resulting in a better signal/noise ratio.

The human plasma sample (1.4 ml) contained 8.400 dpm. Due to the high load of the column with

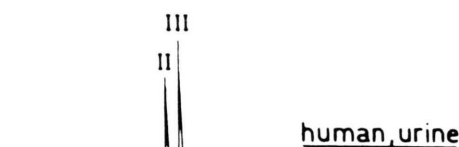
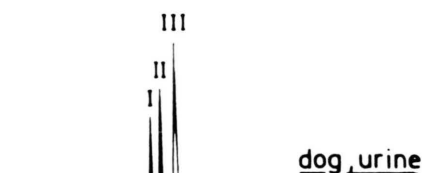
large amounts of plasma, the reproducibility of the retention volumes was reduced. Therefore the metabolites were assigned to the pattern obtained in subsequent runs after addition of authentic unlabelled substances to the plasma.

[¹⁴C]Etozolin

The studies on the metabolism of the diuretic etozolin give an impressive example of the efficiency and power of the HPLC radioactivity detection system.



	R ₁	R ₂
Et	-C ₂ H ₅	-N ₁
I	-H	-N ₁
II	gluc. acid	-N ₁
III	gluc. acid	-N ₁
IV	-C ₂ H ₅	-NH-(CH ₂) ₄ -COOH
V	-C ₂ H ₅	-N ₁
VI	-H	-N ₁
VII	-H	-N ₁



Et = etosolin

* : position of ¹⁴C

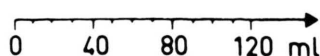


Fig. 2. Metabolite profiles in 0–24 h urine and 0–8 h bile following oral administration of [¹⁴C]etozolin to rats, dogs, and humans.

Column: LiChrosorb RP-8 (25 × 0.46 cm).

Gradient: Aqueous solution of 0.5% diethylaminoethylamine adjusted with formic acid to pH 7.5 vs acetonitrile.

The metabolite profiles of urine and bile (Fig. 2) show seven compounds, some of which were very unstable. Slight changes to alkaline or acidic pH caused artefact formations due to ring opening of the thiazolidinone ring. In earlier studies several artefacts appeared during extraction, clean-up with Amberlite-XAD-2 chromatography and enzyme incubations. Origination of metabolite profiles was only made possible when using the HPLC radioactivity detection system, since no clean-up was necessary. Separation and enrichment was achieved under most careful conditions [5].

Etozolin is a racemate. Its main metabolite (I), which is formed by enzymatic cleavage of the ester bond, is esterified in the organism with β -D-glucuronic acid to the diastereomeric glucuronic acid conjugates II and III. Although these metabolites are highly unstable and racemize easily, it was possible to get a quantitative and qualitative description of their occurrences in urine using the HPLC radioactivity detection system [5].

[¹⁴C]Thymoxamine

Thymoxamine is an α -blocking drug. The metabolite profiles in urine following oral administration of [¹⁴C]thymoxamine to rats, dogs, cats, and humans (Fig. 3) show large differences among the species [16]. The metabolites which occurred were almost exclusively glucuronic and sulfuric acid conjugates. An interspecies comparison of the metabolite patterns using standard methods like enzyme incubation, extraction and thin-layer chromatography would have been complicated for this substance, since the sulphate conjugates could not be cleaved with arylsulphatases of different sources (helix pomatia, Boehringer Mannheim and limpets, Sigma). On the other hand, it was relatively easy using the HPLC radioactivity detection method, since the conjugates as such could be quantitatively determined in a single run.

Up to a few years ago, unchanged conjugates were rather disregarded in metabolite studies, since they seemed uninteresting from a pharmacological point of view. However, it has now been recognized that they can indeed show pharmacological activity. The sulphate conjugates (III) and (IV) are examples [24].

[³H]Naloxone

Fig. 4 shows the metabolite profiles of the opiate antagonist naloxone in urine and bile following oral

administration to rats, dogs, and humans. Biotransformation reactions are reduction of the carbonyl group, hydroxylation or splitting off the allyl group and conjugate formation [13].

All analyses shown were made in the homogenous phase, since the [³H]concentration in humans was relatively low, concentration procedures were to be avoided and optimal comparability was to be attained.

For the enrichment and purification of metabolites to define structure, we made the radioactivity measurements in the heterogenous phase in order to avoid unnecessary loss of substance.

[³H]Levobunolol

Levobunolol is a potent noncardioselective β -blocker. The metabolite profiles in urine following oral administration to mice, rats, dogs, and humans are given in Fig. 5. The radioactivity was measured in homogenous phase by means of the splitting technique. There are great differences among species [14], a fact which is relevant for the comparative interpretation of pharmacological and toxicological data.

In previous investigations using TLC and hydrolysis of conjugates, quantitative data on metabolite composition could not be given and the main metabolite III was not detected in man. However, metabolite III has the same affinity to the β_1 -receptor as levobunolol and the dihydro metabolite II [25].

In our experiments more than 95% of the urinary radioactivity could be described by identified metabolites.

Preparative work: Enrichment and purification of metabolites

Although MS-, IR-, or NMR-investigations can be done at the low μ g-level, normally 50 to 300 μ g are required for structure elucidation. In most cases preparative isolation of metabolites is therefore required.

Principally the preparative isolation from urine can be done by columns which have the same quality and length as the analytical columns but their diameter has to be related to the urine volume and the amount of substances. In some cases a concentration step may be advantageous prior to separation: urine is pumped onto the RP-column and after washing with water the metabolite mixture is eluted with a

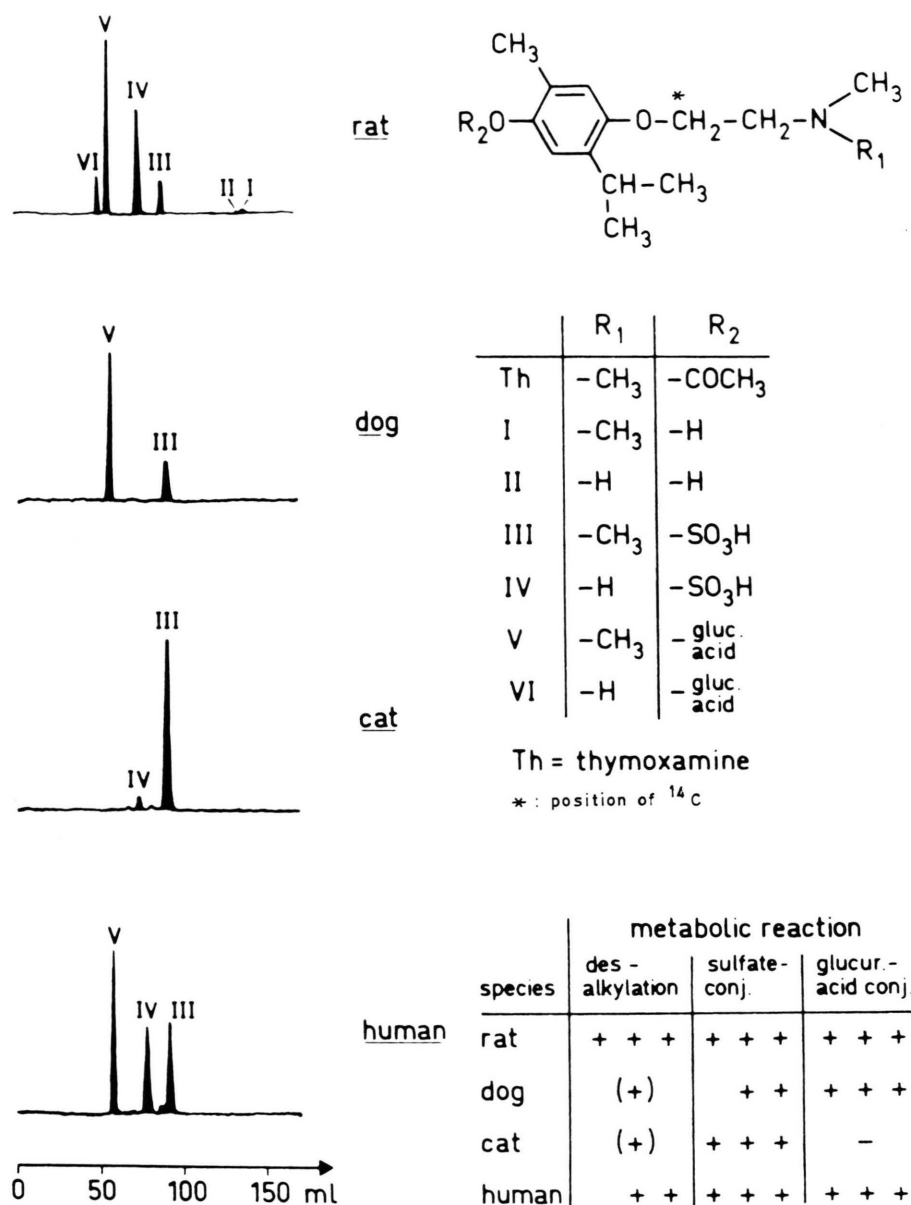


Fig. 3. Metabolite profiles in 0–8 h urine following oral administration of [¹⁴C]thymoxamine to rats, dogs, cats, and humans.

Column: LiChrosorb RP-18 (25 × 0.46 cm).

Gradient: Aqueous solution of 0.5% diethylaminoethylamine adjusted to pH 6.7 with formic acid vs. acetonitrile.

small volume of methanol. Ion exchange chromatography may be used for group separation to obtain groups of glucuronides, sulphates, unconjugates.

For purification of the separated metabolites several different chromatographic runs are necessary

due to the multitude of endogenous compounds by varying stationary phase, eluent, pH, temperature. Usually we needed about five purification steps. Simultaneous UV detection (one wave length or whole spectrum) is very useful to control success of

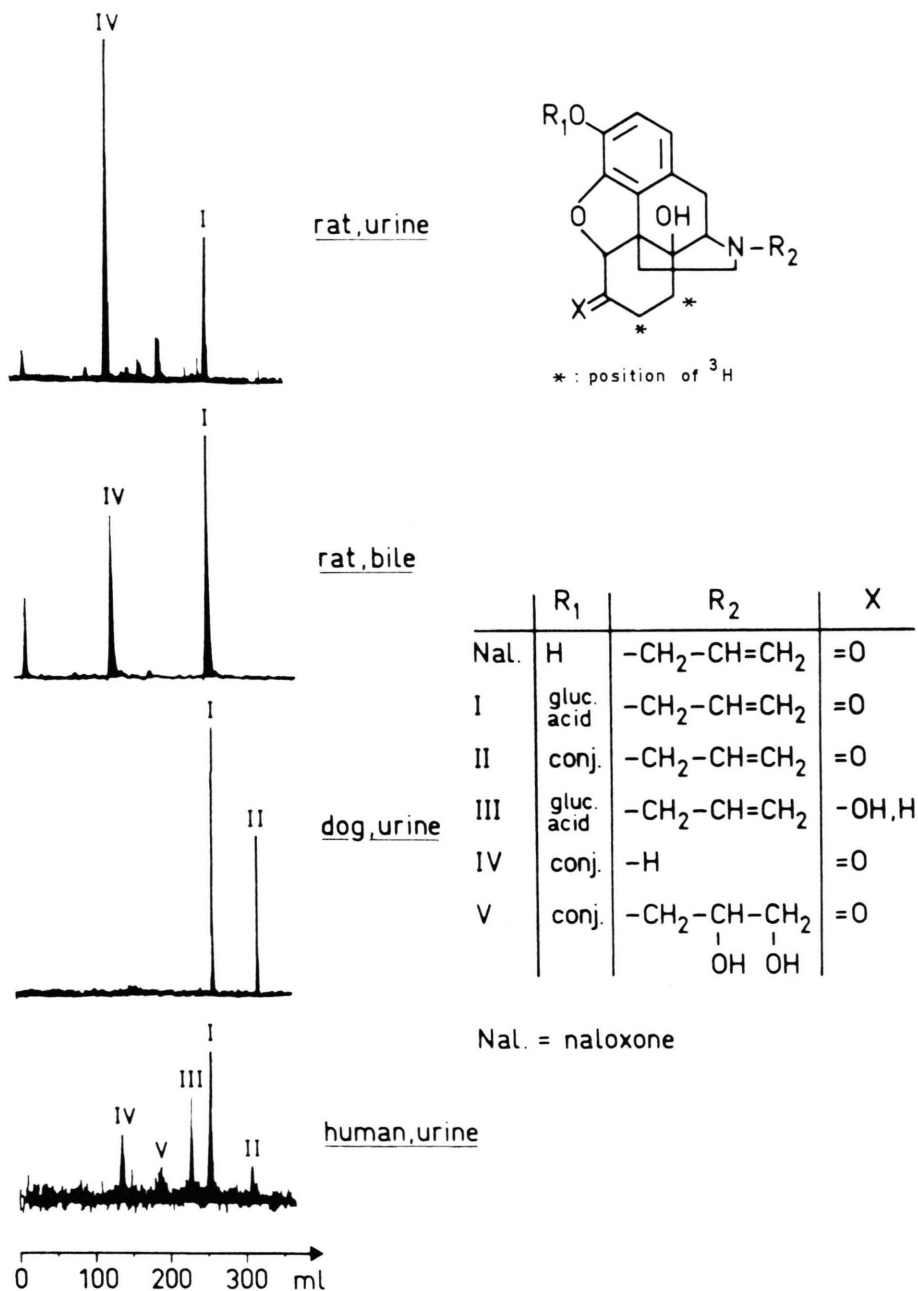


Fig. 4. Metabolite profiles in 0–24 h urine and 0–8 h bile following oral administration of [^3H]naloxone to rats, dogs, and humans.

Column: μ -Bondapak C-18 (50 \times 0.46 cm).

Gradient: Aqueous solution of 0.5% ammonium acetate, 0.5% N,N-dimethylethylenediamine (pH 8.3) vs acetonitrile.

purification. The last purification step includes the separation from salts. A photo diode array detector may give important information on the structures.

In principle, the technique described can of course be applied also for isolation of non-radioactive substances from biological material.

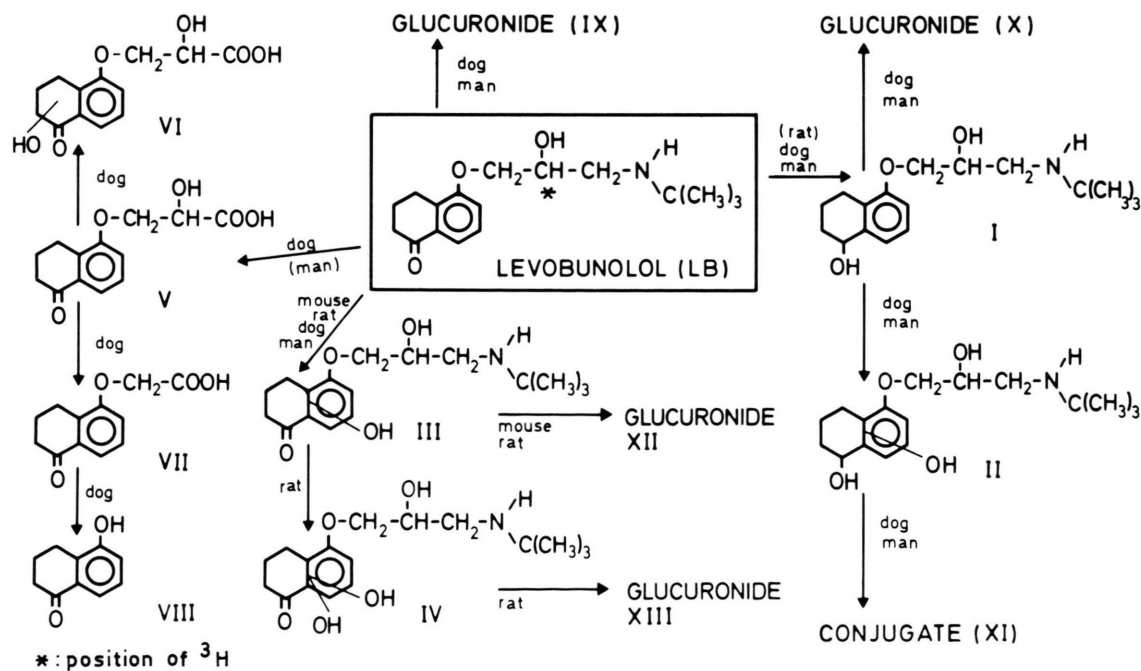
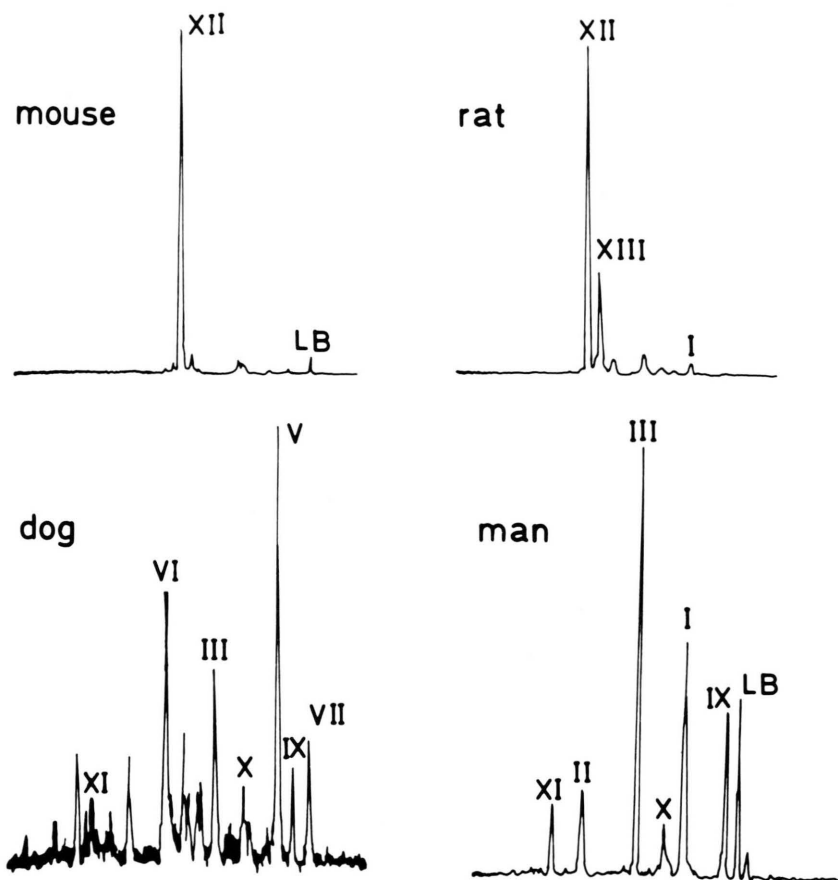


Fig. 5. Metabolite profiles in 0–24 h urine following oral administration of $[^3\text{H}]$ levobunolol to mice, rats, dogs, and humans.

Column: μ -Bondapak C-18 (50 \times 0.46 cm).

Gradient: 0.1 M KH_2PO_4 (pH 3) vs acetonitrile.

In Fig. 6 the procedure for isolation and purification of the levobunolol metabolites is shown in experimental detail as an example. Starting from 600 ml urine six metabolites (I, II, III, IX, X, XI) and the parent drug (LB) were isolated *via* five steps

for subsequent structure identification by mass spectrometry. In the first step 10 μ m filling material was used according to the needed high capacity. Separation from endogenous compounds was achieved in steps 2 to 5 using finer filling material, variation of

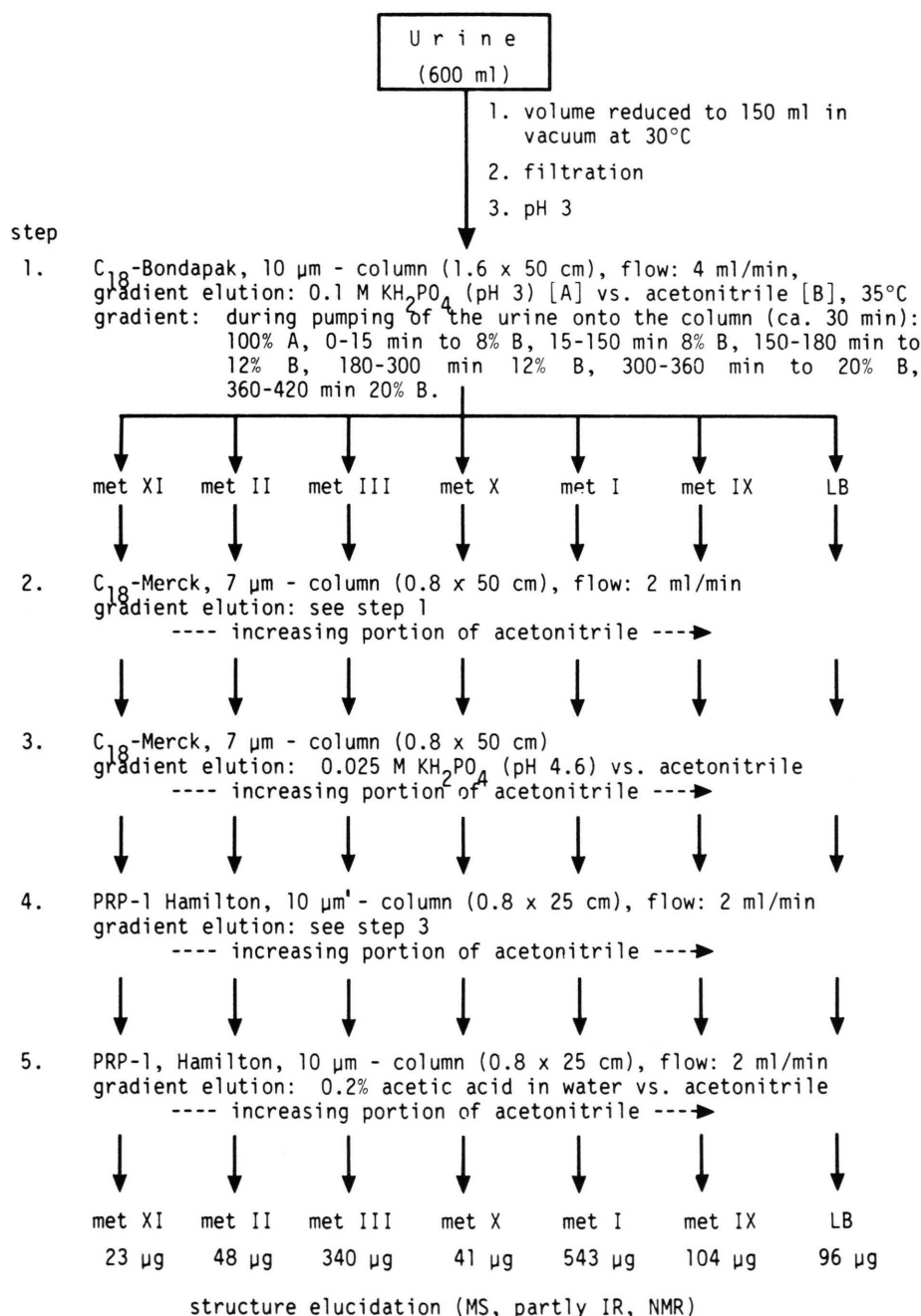


Fig. 6. Procedure for isolation and purification of levobunolol metabolites from human urine.

pH and change of filling material. Progress of purification was controlled by simultaneous UV and fluorescence detection.

After structural elucidation an aliquot of the individual metabolite should be used to determine reten-

tion times under analytical conditions. This is needed to detect possible artefact formation during purification or even thereafter. Furthermore it may serve the final identification of corresponding peaks in the metabolite profile.

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