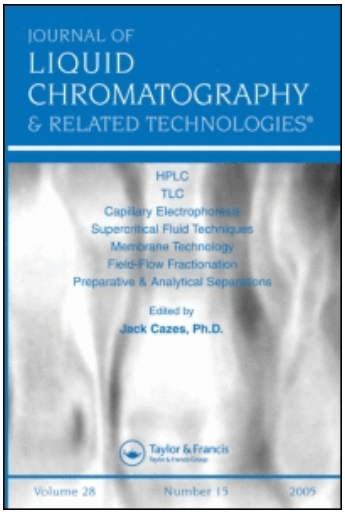

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The Use of Thin-Layer Chromatography in Experimental Xenobiology

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THE USE OF THIN-LAYER CHROMATOGRAPHY IN EXPERIMENTAL XENOBIOLOGY

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ABSTRACT

Studies of the metabolism and disposition of most drugs or other xenobiotics share one common feature: the need for an analytical method to measure the xenobiotic and/or its biotransformation products in biosamples. Although chromatographic methods such as GC and HPLC are important and preferred by some laboratories, initial investigations of new xenobiotic entities are often conducted using radiolabeled compounds, and, in such instances, TLC assay methods are frequently selected.

TLC is utilized in xenobiotic metabolism and disposition studies to evaluate the radiochemical purity of labeled xenobiotics, the extent of metabolism, the profile of metabolites in blood and excreta, the concentrations of the xenobiotic and specific metabolites in blood and other biosamples, the stability of the xenobiotic and its metabolites in biosamples, and the relative behavior of metabolites and reference compounds. Preparative TLC is frequently used to isolate specific metabolites. Thin-layer radiochromatography (TLRC) has been utilized to study the pharmacokinetics of drugs and other xenobiotics in man and in animals. Especially where radiolabeled compounds are used, TLC provides an attractive chromatographic alternative to GC and HPLC in research in experimental xenobiology.

INTRODUCTION

Drug metabolism is a relatively new and rapidly growing field. Because it is such a young discipline, the term "drug

metabolism" may convey varying shades of meaning even to different scientists conducting research in the field. This ambiguity is compounded by the fact that the term is sometimes used to refer to the field as a whole and sometimes is used to refer to the more limited area of drug biotransformation. Because of the lack of a universally accepted definition of "drug metabolism" and the need for a better designation for the field, the term "drug disposition" came into use during the 1970's. To insure that both *in vitro* and *in vivo* studies would be included, the term "drug metabolism and disposition" was suggested as a further refinement. Although these newer terms were useful in helping to describe the new discipline, these terms do not adequately reflect the fact that compounds other than drugs are also studied. The term "xenobiotic" (from the Greek XENOS for foreign and BIOS for life) has come into widespread use in recent years to designate an exogenous or foreign substance that is introduced into a biological system. Examples of classes of xenobiotics include antifungal agents, antiparasitic agents, antiinflammatory agents (non-steroidal), antimicrobial agents, antitumor and other anti-proliferative agents, antiviral agents, cardiovascular drugs, chemicals, contrast agents and other diagnostic agents, drugs affecting the central nervous system, drugs affecting renal function, environmental pollutants, herbicides, orally or parenterally administered hormones (insulin, steroids, etc.), local anesthetics, radiodiagnostic agents and vitamins. Thus a term is needed to describe the discipline which investigates the biological fate of

xenobiotics. The name which I have proposed for this discipline, and which I hope will come into widespread use during the 1980's, is "xenobiology". The term xenobiology will be used throughout this article, as will the term "xenobiologic", which will be used adjectivally to mean "of or relating to xenobiology or xenobiotics".

Xenobiologic studies include evaluation of factors related to the introduction of a xenobiotic into, distribution through, biochemical, chemical and physicochemical interactions in, and elimination from biological systems. These studies are usually conducted to help understand, explain and ultimately to predict the pharmacologic, toxicologic, biochemical or other consequences of introducing xenobiotics into biosystems. For drugs, such studies probe aspects of safety and efficacy and may help explain phenomena such as non-linear dose responses and differences in pharmacodynamics or toxicity among the species studied. Xenobiologic studies may be carried out in live intact animals (in vivo), using isolated organ systems (in situ), or using single cell preparations or subcellular fractions (in vitro). In vivo studies are conducted to evaluate processes such as absorption, distribution, biotransformation, and excretion. In situ studies may involve isolated organs such as intestine, liver, or kidney and may be carried out to study absorption or uptake, active transport or secretion, biotransformation or excretion. In vitro studies are useful for studying protein binding, and for investigating enzyme systems. Studies of enzyme systems may probe

heterogeneity of the enzymes, as well as effects of genetic and nutritional factors, sex, strain, age and species differences on substrate specificity, inducibility, and cofactor requirements. In addition, reaction rates may be determined, biotransformation profiles and pathways may be evaluated, and reactive intermediates may be detected and trapped.

All these studies have one common denominator. They entail qualitative or quantitative measurements of the compounds under investigation and/or their biotransformation products. The success or failure of many xenobiologic studies rests on the ability to establish and utilize appropriate analytical methodology. As the frontiers of our field are pushed back, more sophisticated and sensitive analytical methods and techniques are being developed. As in many fields, keeping up with the "state-of-the-art" is a continuing struggle.

Selecting the Analytical Method

Laboratories involved in xenobiologic research have a tendency to develop their own unique character, and often specialize in either radioassay or chemical assay methodology. Furthermore, there is a tendency to use one chromatographic method much more extensively than others. Thus, some laboratories use predominately gas chromatographic (GC) assays, some prefer high performance liquid chromatographic (HPLC), and others thin-layer chromatographic (TLC) assays. Consequently, the views and approaches discussed in this article will not represent those of all xenobiologic research laboratories. Many factors affect the delicate decisions

involved in the selection of the analytical method to be used most extensively in a given laboratory. Some investigators choose one method over another because of experience obtained in early professional training. Others may seek out methods for which the instrumentation and procedures involved are simple to operate, especially when technicians with minimal professional training may be responsible for running the assays. In most laboratories it is necessary to consider the cost of purchasing equipment, and the ultimate cost of each assay.

When all the possible factors are carefully weighed, it is apparent that, for many applications, TLC is an extremely useful analytical method. In many laboratories conducting research on xenobiotics, TLC is an extremely important, even ubiquitous, analytical tool which represents state-of-the-art technology. In laboratories such as my own, in which radiolabeled compounds have been extensively utilized to study most of the xenobiotics of interest, TLC is usually the chromatographic method of choice. Of course, the use of TLC in xenobiology is in no way restricted to studies of radiolabeled molecules, and the study of radiolabeled compounds is not limited to TLC methods.

Applications of Thin-Layer Chromatography

Applications of TLC in xenobiologic studies may begin as early as the synthesis of the radiolabeled xenobiotic. Purification, structure confirmation, and determination of chemical and radiochemical purity may all be carried out with the aid of TLC. Autoradiography (or radioautography) is a method by which radio-

activity from weak β-emitters such as ^{3}H, ^{14}C or ^{35}S in two-dimensional specimens (e.g., TLC plates, electrophoretic gels, or tissue slices) is detected after exposure to appropriate photographic emulsions. Commercial film is usually used, and enhancement of sensitivity is often obtained by using low temperature, long exposure times, and scintillator sprays. Figure 1 illustrates how an autoradiogram of a thin-layer plate helps ascertain radiochemical purity. This example was chosen to illustrate the utility of the method for detecting major and minor impurities with excellent resolution; in this case the compound was of high purity when synthesized, but underwent significant degradation on long term storage. Autoradiograms provide excellent resolution and sensitivity for qualitative, semiquantitative, or even quantitative analyses. This technique is usually augmented by zonal analysis ("scraping and counting") of the TLC plates to provide better quantitation of radioactive components.

Xenobiologic studies can be divided into several stages including: introduction of the xenobiotic into the biosystem, collection of appropriate samples, analysis for the xenobiotic and/or its metabolites in biosamples, and suitable manipulation and evaluation of the data obtained. TLC may be employed in a multiplicity of applications in the analytical phase of xenobiologic studies; some such applications include determination of the radiochemical purity of xenobiotics in the formulations administered, generation of biotransformation profiles (i.e., characterizing metabolites by their chromatographic behavior),

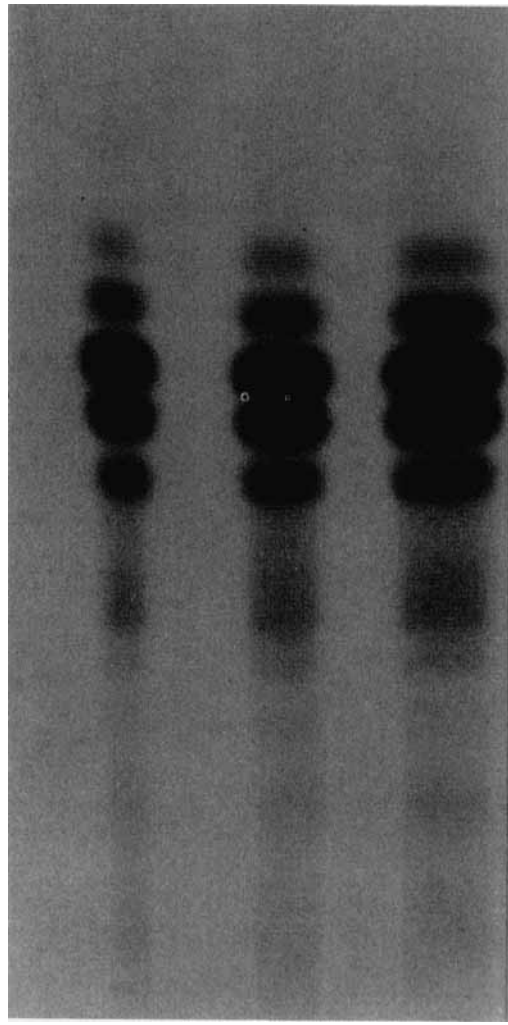


FIGURE 1: Autoradiogram showing multiple impurities of a degraded sample. The original ^{14}C-labeled compound is the third spot from the top. The chromatogram shows the same sample chromatographed at different concentrations.

quantitation of xenobiotics and/or specific biotransformation products in biosamples, and purification, isolation and tentative identification of biotransformation products.

The use of TLC coupled with detection of radiolabeled substances on the thin-layer chromatographic plates (by radioscanning, non-scanning ionization detectors, autoradiography or zonal analysis) is sometimes referred to as thin-layer radiochromatography or TLRC. The biotransformations of a benzothiazine, SQ 11,579, were studied by TLRC by Lan and coworkers in vitro and in vivo (1). The molecule, which possesses antiinflammatory activity, was extensively metabolized. TLRC was utilized to obtain the metabolite profile shown in Figure 2. The separation of all the non-conjugated and deconjugated (i.e., aglycone) metabolites by TLC using a single solvent system demonstrates the great value of TLC in such studies. After further studies of this compound, most of the metabolites were identified. These metabolites, as shown in Figure 3, were the result of many different biotransformations including N-oxidation, N-demethylation, aromatic hydroxylation, sulfoxidation, and conjugation with glucuronic acid.

In the laboratories of Levine (2), studies of the dye molecule N,N-dimethyl-4-aminobenzene (DAB) was conducted in rats. Since DAB and its metabolites are highly colored, and are separable by TLC, they provide an excellent example of the utility of TLC in the study of non-radiolabeled xenobiotics. Figure 4 shows an actual photograph of a thin-layer chromatogram of an extract of a

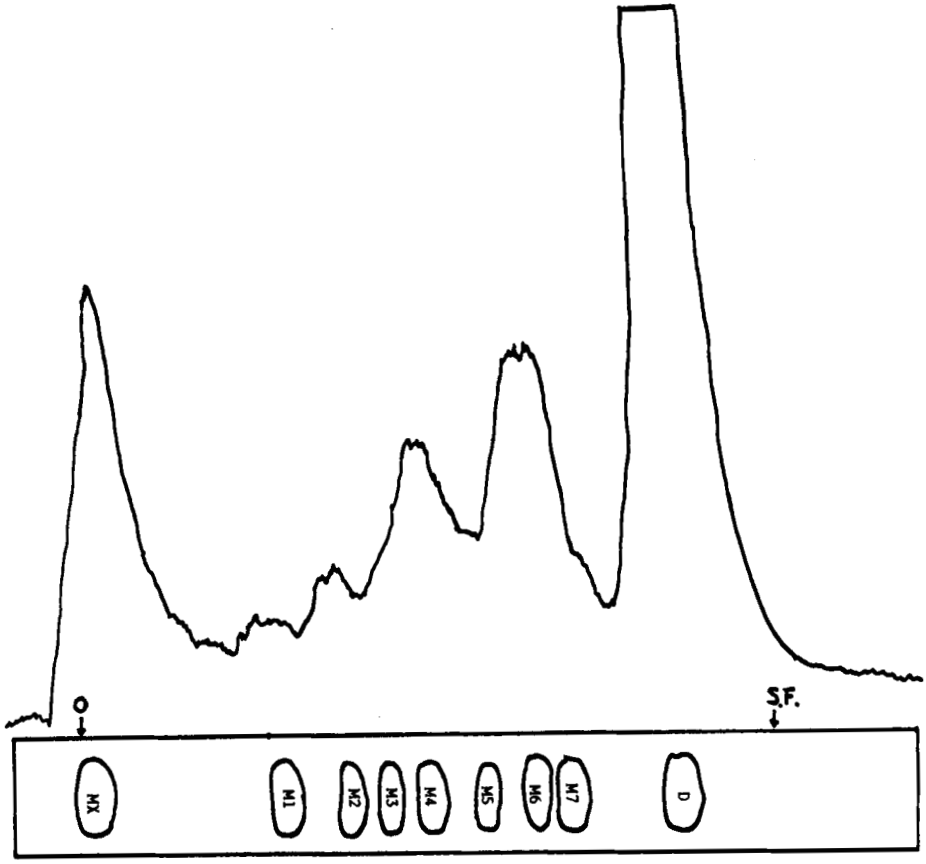
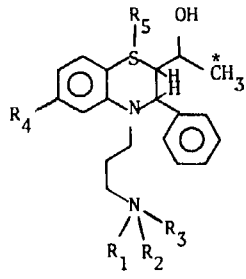


FIGURE 2: TLRC biotransformation profile of SQ 11,579-^{14}C in β-glucuronidase/sulfatase treated monkey urine.

rat bile sample obtained after administration of DAB. In this particular study radiolabeled compound was actually employed, but a radioassay was not needed to characterize the metabolites by TLC. Many of the metabolites visible in the thin-layer chromatogram have been identified; their structures were confirmed to be those



*Site of ^{14}C-label

Compound	R_1	R_2	R_3	R_4	R_5	Biotransformation(s)
SQ 11,579(D)	CH_3	CH_3	-	H	-	-
M1	H	H	-	H	0	S-oxidation N-demethylation
M2	CH_3	CH_3	0	-	-	N-oxidation
M3	CH_3	H	-	-	0	S-oxidation N-demethylation
M4	CH_3	CH_3	-	OH	-	aromatic hydroxylation
M5	H	H	-	H	-	N-demethylation
M6	CH_3	H	-	H	-	N-demethylation
M7	CH_3	CH_3	-	H	0	S-oxidation
MX	Structure(s) unknown					

FIGURE 3: Structures of SQ 11,579 and its aglycone metabolites.

of the various demethylated and aromatic hydroxylated metabolites suggested by the comparisons of TLC R_f values with the known standards.

Obviously, relatively few of the compounds under investigation will be visible on TLC plates without special visualization procedures. Other methods for detecting and/or quantitating non-radiolabeled xenobiotics and their metabolites on TLC plates may make use of ultraviolet absorption or fluorescence of the compounds

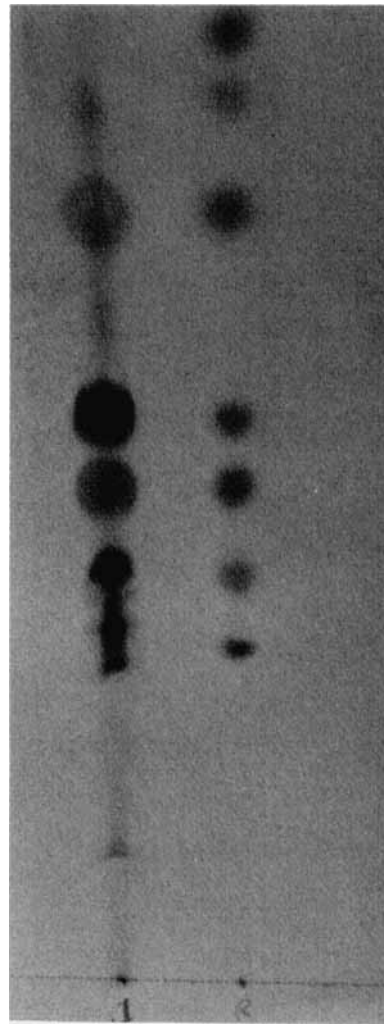


FIGURE 4: Photograph of a thin-layer chromatogram showing DAB and its metabolites in an extract of rat bile (left channel) and DAB and other reference standards (right channel). The references, in order from highest to lowest R_f values, were N,N-dimethyl-4-aminobenzene (DAB), the monodesmethyl analog, the didesmethyl analog, monohydroxy (4') DAB, the monohydroxy (4') monodesmethyl analog, the monohydroxy (4') didesmethyl analog, and the N-acetylated monohydroxylated (4') didesmethyl analog.

themselves or may involve visualization after formation of appropriate derivatives, either during the sample purification or on the TLC plate. Microbiologically active compounds may be detected (and quantitated) using TLC bioautography. TLC bioautography is a technique somewhat analogous to TLC autoradiography. In TLC bioautography, the surface of the TLC plate is placed in contact with a nutrient medium (e.g., agar) containing specific strain(s) of microbes for an appropriate time period (e.g., overnight), and zones of inhibition are measured. By using suitable reference compounds and dilution techniques, the parent compound, and those metabolites which exhibit antimicrobial activity, can be quantitated with good sensitivity and specificity.

Perhaps the most important aspect of most xenobiologic studies is the quantitation of the parent compound in biological samples. In most cases this entity is primarily responsible for the production of any therapeutic or toxic effects. Several years ago when I was at McNeil Laboratories, some of my former coworkers and I wanted to measure the time course of the anti-inflammatory drug, tolmetin, and one of its known metabolites, MCPA (Figure 5), in the plasma of individual rats and mice. This was accomplished using ^{14}C-labeled tolmetin of relatively high specific radioactivity and TLRC techniques. Assays were carried out on sequential plasma microsamples from individual rats and mice (3). Knowledge of the TLRC metabolite profile in urine permitted development of the simplified zonal analysis of plasma samples as illustrated in Figure 6.

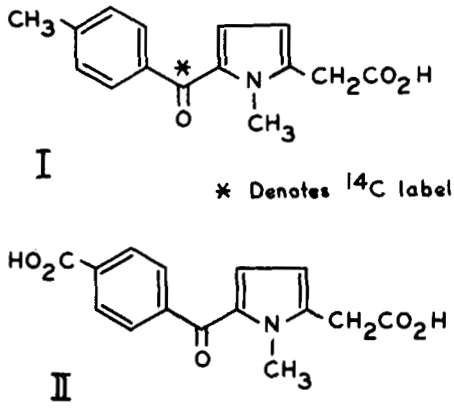


FIGURE 5: The structures of tolmetin-^{14}C (I) and its metabolite MCPA (II).

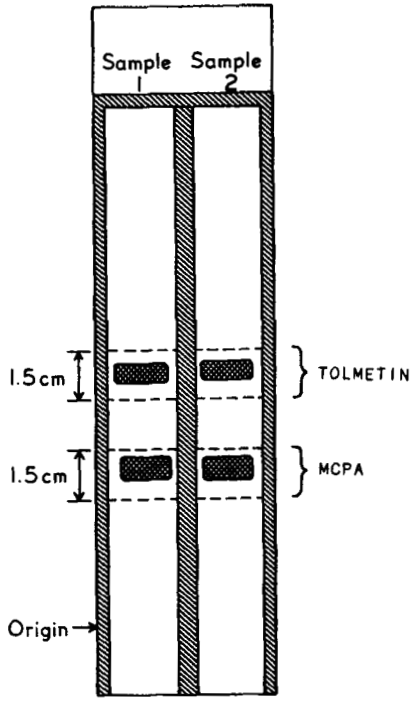


FIGURE 6: Illustration of the TLRC zonal analysis of tolmetin and its metabolite, MCPA.

Figure 7 shows the plasma level time course of tolmetin and the MCPA metabolite in individual male and female mice on rectilinear plots. The same data, in the form of semilogarithmic plots, are frequently useful in determining pharmacokinetic half-life ($t_{\frac{1}{2}}$) values. The rectilinear plots shown are useful for accurately representing relative concentrations; in this example concentrations of tolmetin and MCPA are shown. At each time point studied, less than 200 microliters of blood was drawn from

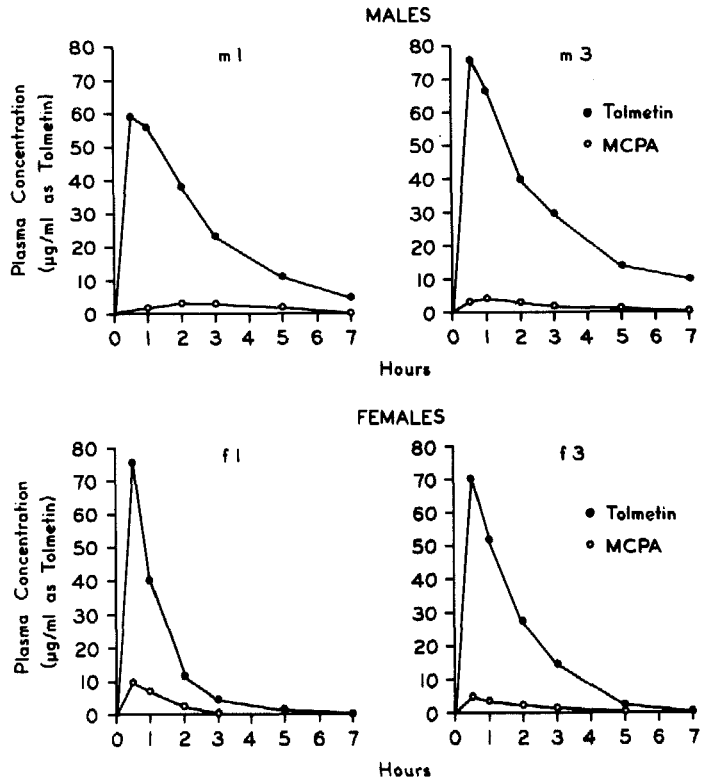
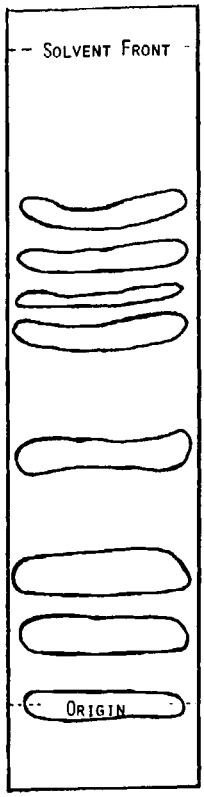


FIGURE 7: Levels of tolmetin (•—•) and its metabolite MCPA (o—o) in the plasma of individual mice, as determined by TLRC.

each animal, and plasma assays were conducted using a total of only about 50 microliters. It was possible to simultaneously obtain the plasma level curves for tolmetin and MCPA in individual mice using plasma microsamples because the TLRC assay was so straightforward and provided excellent sensitivity. This example is particularly noteworthy because it represents the first time that such blood or plasma concentration vs time curves were obtained for a specific xenobiotic in individual mice; each mouse weighed approximately 20 to 25 grams.

The utility of TLC in obtaining biotransformation profiles, and ultimately in elucidating biotransformation pathways, is well illustrated by studies carried out by Kripalani and coworkers (4) on a substituted pyrazolopyridine, SQ 65,396, which is an inhibitor of cyclic AMP phosphodiesterase. ^{14}C-labeled SQ 65,396 was studied in rats, dogs, monkeys, and humans. The solvent system used was selected because it was capable of separating eight radioactive components present in the β-glucuronidase-treated urine of monkeys following oral administration of SQ 65,396-^{14}C (Figure 8). The structure of these metabolites was elucidated (Figure 9), and it was found that the TLC solvent system separated metabolites resulting from ester hydrolysis, N-deethylation of the pyrazole ring, α-hydroxylation of the N-butylamino side chain, and N-dealkylation of the butyl side chain. (No unchanged SQ 65,396 was detected.)

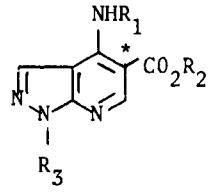
Squibb is currently developing captopril (Figure 10), a novel orally active inhibitor of angiotensin-converting enzyme



Metabolite	Radioactivity in Urine (%)
M7	3.9
M6	2.6
M5	1.5
M4	3.0
M3	22.2
M2	32.8
M1	26.0
M0	7.9

FIGURE 8: TLRC separation of metabolites of SQ 65,396-^{14}C in the β-glucuronidase/sulfatase treated urine of monkeys.

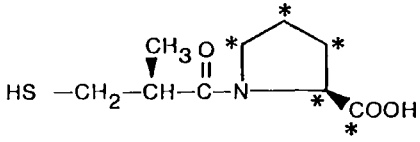
(ACE) for the treatment of hypertension and congestive heart failure. TLRC has been used extensively in our studies of captopril, and provides several examples of our current state-of-the-art utilization of TLC. Captopril is chemically unstable in biological samples and requires chemical stabilization or derivatization in freshly obtained biosamples. Thus far, derivatization of captopril with N-ethylmaleimide (NEM) has proven to be the best method of



*Site of ^{14}C label

Compound	R_1	R_2	R_3
SQ 65,396	$CH_2CH_2CH_2CH_3$	CH_2CH_3	CH_2CH_3
M7	$CH_2CH_2CH_2CH_3$	H	CH_2CH_3
M6	$CH_2CH_2\underset{\displaystyle OH}{\overset{|}{C}}HCH_3$	CH_2CH_3	CH_2CH_3
M5	H	H	CH_2CH_3
M4	$CH_2CH_2\underset{\displaystyle OH}{\overset{|}{C}}HCH_3$	H	CH_2CH_3
M3	$CH_2CH_2CH_2CH_3$	H	H
M2	H	H	H
M1	$CH_2CH_2\underset{\displaystyle OH}{\overset{|}{C}}HCH_3$	H	H
MO	Structure(s) unknown		

FIGURE 9: The structures of SQ 65,396-^{14}C and its metabolites.



* Denotes sites of ^{14}C labels.

FIGURE 10: The structure of captopril-^{14}C.

treating samples. NEM forms an adduct with captopril and blocks the SH group from further oxidation. Because of the chemical and physicochemical properties of captopril, it is neither readily separated from nor distinguished from endogenous compounds, even after derivatization with NEM. Consequently it has proven difficult to devise a chemical assay for captopril in biosamples in general, and blood in particular. At present the only chemical assay for captopril in blood is a sophisticated gas chromatographic/mass spectrometric assay which involves the use of a selected ion monitor and a computerized data system. Few would doubt that the GC/MS assay represents state-of-the-art technology. Interestingly, a simple zonal profile TLRC assay (5) was developed prior to the GC/MS assay. The TLRC assay was very much the equal of the GC/MS assay in sensitivity and specificity and showed less assay variability when comparisons were made for clinical samples obtained after 100 mg (50 μCi) doses. In animal studies, where captopril of high specific radioactivity could be administered, the TLRC assay was superior to the GC/MS assay in sensitivity. Not surprisingly, the TLRC assay for captopril was extensively used in animal studies and was used in clinical studies where administration of radiolabeled captopril could be justified. It would seem appropriate to also consider this assay one which represents the current state-of-the-art, since not only does it equal or surpass the GC/MS assay for captopril in all respects, but it also provides for the simultaneous quantitation of the disulfide metabolite of captopril and of all other metabolites collectively.

TLRC has also played an important role in the generation of biotransformation profiles for captopril in biosamples. The examples cited will be for human urine following a single oral 100 mg dose of ^{14}C-labeled captopril (6). TLC was not the only analytical method employed to obtain such profiles, and Figure 11 shows both a TLC radioscan and an HPLC profile obtained for human urine. The HPLC profile was obtained using a reverse phase gradient elution technique on a Waters HPLC system equipped with a radioactivity detector. The results obtained by the two methods were qualitatively and quantitatively comparable, but the TLRC procedure was far simpler to carry out than the HPLC gradient

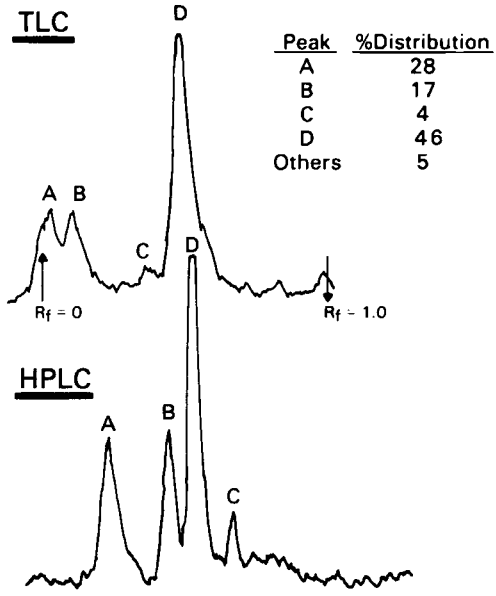


FIGURE 11: HPLC and TLRC biotransformation profile radioscans of captopril-^{14}C (as the NEM derivative) and its metabolites in human urine.

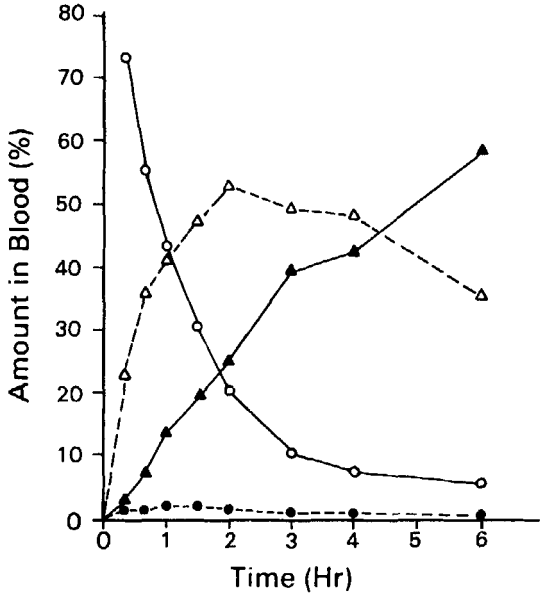


FIGURE 12: Percent distribution of captopril-^{14}C (o—o), captopril disulfide dimer (o---o), other extractable metabolites (Δ----Δ), and protein-bound metabolites (▲—▲) in human blood (in vitro incubation).

elution procedure. The resolution on the TLC plate was better than suggested by the radioscan, and excellent quantitation was accomplished by zonal analysis.

TLRC was also utilized in the isolation and identification of captopril-^{14}C and its metabolites in biosamples. Preparative TLRC was utilized in conjunction with other techniques for isolating captopril and its metabolites from human urine, and both TLC R_f and HPLC retention time values helped identify captopril (as its NEM derivative) and its metabolites (captopril disulfide, captopril-cysteine mixed disulfide, etc.).

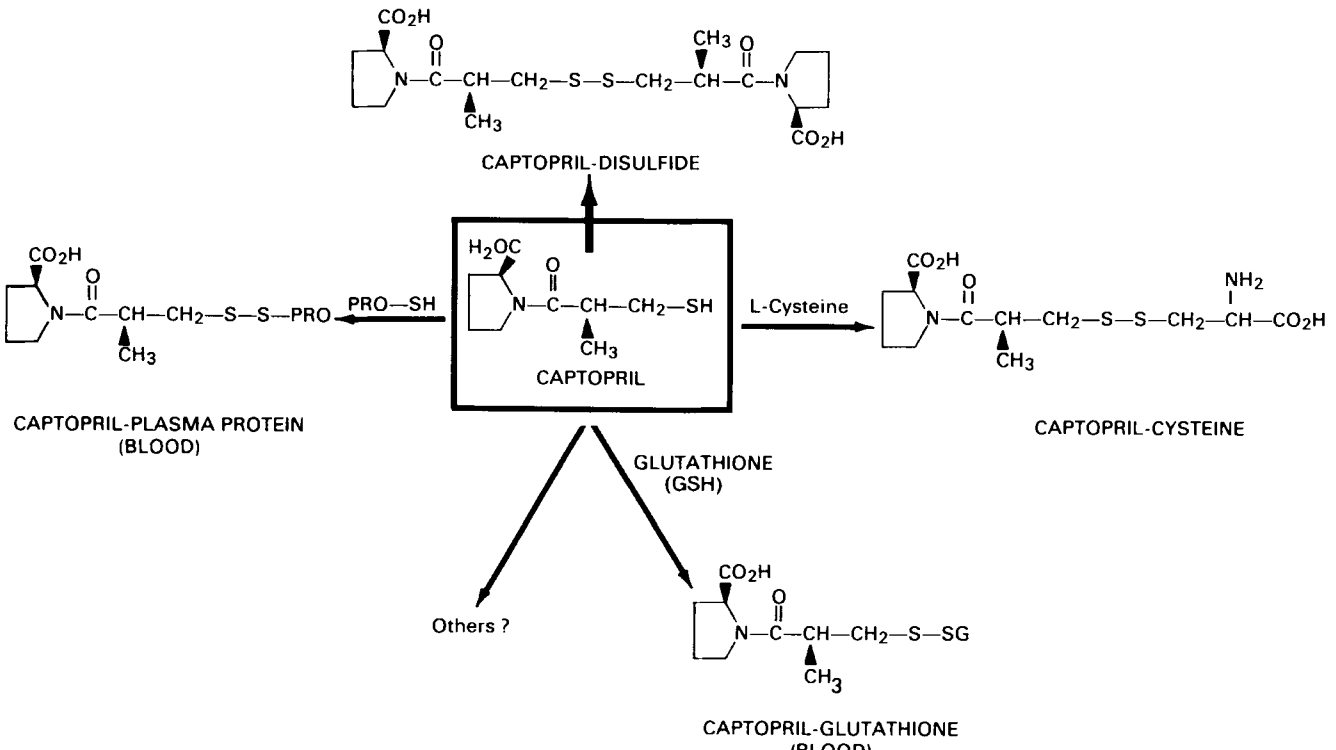


FIGURE 13: Scheme summarizing the overall biotransformation products of captopril-^{14}C found in human blood (*in vitro*) and urine (*in vivo*).

^{14}C-Labeled captopril was used for *in vitro* studies of human blood. A combination of radioassays, extractions and TLRC analyses for radiolabeled captopril, captopril disulfide, and other extractable metabolites (collectively) were used to study the dynamics of what was occurring (Figure 12). Note that three of the four curves, i.e., those for captopril, captopril disulfide and other extractable metabolites, were generated using a single TLRC assay.

Figure 13 shows a generalized biotransformation scheme for captopril in man. It represents a composite of *in vivo* and *in vitro* studies, and was generated by extensive utilization of analytical and preparative TLRC, as previously described. It would have been extraordinarily difficult, if not impossible, to generate this scheme within the same time frame without the extensive use of TLC.

It is hoped that the examples chosen, and the discussion presented have provided some insight into the extent to which TLC may be utilized in the investigation into the metabolism not only of drugs, but of xenobiotics in general.

ACKNOWLEDGEMENTS

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tories, and permitting its inclusion in this review. The editorial assistance of Ms. H. M. Zarsky is gratefully acknowledged.

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