# Metabolite Analysis, Isolation and Purity Assessment Using Various Liquid Chromatographic Techniques Combined With Radioactivity Detection

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## Abstract

**During the discovery of metabolic routes of a drug candidate, radioactively labeled substances are administered. This study reports the multidimensional application of over-pressured layer chromatography (OPLC) and high-performance liquid chromatography (HPLC) coupled with online or off-line nondestructive radioactivity detection methods in metabolism studies. Among these methods, digital autoradiography and flow-cell radioactivity detectors (RD) using solid scintillators are used. In this study, the hyphenation of OPLC with RD is reported. The application of the OPLC–RD technique is demonstrated on a metabolism study as well as the multidimensional chromatographic selectivity using normal-phase OPLC for the separation in the first dimension, followed by reversed-phase HPLC–RD, which provide additional selectivity to the separation. Information regarding the identity of radiolabeled metabolites and data obtained from spectroscopic methods could be advantageously used during structure elucidation.**

## Introduction

Discovery of the metabolic pathways of an investigational drug in animals and man is a complex process. Quantitation and isolation of drug metabolites in different biological matrices require special instrumentation, clean-up techniques, and a strategy combining available analytical and preparative methods according to their advantages (1–7).

For in vivo or in vitro biotransformation investigations, drugs labeled radioactively using $^{3}H$ or $^{14}C$ isotopes provide the possibility to track and quantitatively analyze the metabolites in complex biological matrices using separation techniques that are coupled to radioactive detection methods (8–10). Although mass spectrometry (MS) can be powerfully used for quantitative analysis and structure elucidation, its application is difficult in the quantitative assay of an unknown metabolite because MS responses strongly depend on structure-related ionization efficiency. In contrast to MS, radioactively labeled compounds always have the same response on a radioactivity detection system. Although a higher level of radioactivity provides a higher response, practical reasons dictate that the radioactive dose should be reduced to the lowest amount that provides adequate sensitivity. This also leads to the requirement of the use of instrumentation that is capable of detecting the low intensity of radioactivity.

For the investigation of a metabolic pattern, sample preparation and separation methods have to be developed that provide adequate sensitivity and selectivity for forthcoming quantitative assay and spectroscopic studies for structure elucidation. In most cases, the initial steps of analysis include sample preparation and chromatographic separation. For the monitoring of these activities, liquid scintillation counting (LSC) could be used (11).

During the optimization of separation method, column and layer chromatographic techniques with gradient and isocratic elution on reversed- and normal-phase sorbents are most commonly evaluated and the separation scheme is established. During the analysis of complex biological matrices (such as feces or tissues) the necessary purification of the analyte usually could not be achieved using one separation technique. In such cases, a combination of analytical methods with different selectivities should be used, thus providing a further dimension of increased selectivity.

The hyphenation of various separation techniques with selective or specific detection methods is an often required and emphasized objective during the development of analytical techniques for analytes that are present in complex matrices and at low concentrations. Hyphenated techniques include online methods that are capable of detecting compounds emerging from the separation system (in real time) and off-line techniques that collect defined aliquots of the sample fractions and the detection takes place separated in space and time. Online hyphenated methods include MS and Fourier-transform infrared spectrometry (FTIR) coupled to gas chromatography and overpressure layer chromatography (OPLC) (12). Successful applications include the hyphenation of high-performance liquid chromatography (HPLC) to atomic absorption spectrometry, inductively coupled plasma–MS and nuclear magnetic resonance spectroscopy (NMR).

Thin layer chromatography (TLC) with its simplicity for the

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separation of nonvolatile compounds has also been successfully coupled to a range of spectroscopic (FTIR, MS, and NMR) (13–15) and other detection techniques, such as flame ionization detection (16).

OPLC is a recently developed, versatile, economic, and instrumental layer chromatographic method. It allows the development of the chromatogram to be the length of the plate or overrun using isocratic or gradient elution. Sample application is performed as spots (up to 18) or to increase sample load in semipreparative separations as band. In most cases method development and transfer of existing TLC method could be easily performed using the OPLC approach.

In previous reports, various off-line and online radioactivity detection methods have been described. In situ detection of spots originating from labeled compounds were performed by digital autoradiography (DAR). Spots eluted or removed from the plate were analyzed for radioactivity by LSC and structure elucidation was performed by MS and NMR (17–21). An off-line approach to isolate metabolites by micro-HPLC used fraction collection onto the wells of specially prepared microplates coated with solid scintillator, which allowed 12 samples to be simultaneously counted at the same time (10).

In this study, the recently developed technique of online hyphenation of OPLC and radioactivity detection was presented during a metabolism study of a $^{14}$C-labeled investigational drug. In this technique, a radioactivity detector equipped with solid scintillator was coupled to the outlet of a personal OPLC system. This setup enabled the continuous monitoring of the effluent from the OPLC separation as well as fraction collection. After elution, the high-resolution layer was examined by DAR to prove the absence of radioactive spots, thus avoiding the loss of metabolites (23). Strongly retained, noneluted components can be detected on the plate by DAR. Despite good intrinsic separation power of OPLC, the method could not be exploited because of a relatively high sample load. Thus, a second chromatographic separation using gradient HPLC with radioactivity detectors (RD) was performed for purity assessment and final isolation of metabolite fractions.

## Experimental

### Chemicals

All reagents used were analytical reagent grade. All solvents used for the OPLC and HPLC separation were gradient grade (Merck, Darmstadt, Germany). Purified water was obtained inhouse by the means of Milli-Q laboratory water supply system (Millipore, Milford, MA).

### Materials and samples

The $^{14}$C-labeled investigational compound was synthesized at the Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences. Healthy male volunteers participated in the in vivo metabolism study. Urine, feces, and plasma samples were collected for two weeks.

Sample preparation was carried out by solid-phase extraction (SPE) using Merck LiChrolut C18 SPE cartridges (500 mg, 3 mL).

OPLC separation was performed on a fine-particle HTSorb flat sorbent bed sealed on four sides with aluminum-backed silica gel of high-performance thin layer chromatographic quality, a size of 20 x 20 cm, and a layer thickness of 0.20 mm (BIONISIS-OPLC, Le Plessis Robinson, France). Before each run the layer was prewashed with a step-wise gradient of acetonitrile and water to eliminate the impurities adsorbed from air and a fraction of binder.

HPLC separation was performed on a LiChroCART, 250-4 Purospher, RP-18 (5-mm) HPLC column, equipped with a LiChroCART 4-4 Purospher RP-18 5-mm endcapped guard column (Merck, Darmstadt, Germany).

### Instrumentation

The OPLC separation was carried out using the OPLC 50 instrument (BIONISIS-OPLC) (24–27). The instrument included a separation chamber and liquid-delivery system. The cassette containing the analytical or preparative layer was inserted into the chamber. External pressure (5 MPa), eluent volume, and flow rate were set and development time was calculated automatically. Pressurization, development, and depressurization were controlled during the automatic separation process.

Digital autoradiography was performed with an EG & G Berthold (Wildbad, Germany) LB 287 autoradiograph equipped with a Compaq computer that used a 20- x 20-cm position-sensitive, 600- x 600-element multiwire proportional chamber with 1200-V positive polarity of high-voltage for the determination of the $^{14}$C-radiolabeled compounds (26). Measurements were performed using an argon–methane mixture (9:1, v/v) as the counting gas, which was saturated with methylal (formaldehyde-dimethyl-acetal, Merck) vapor at 3.4°C and at a flow rate of 5 mL/min (22). The DAR system was controlled by WinDAR software version 1.09. The run time was 3 and 4 h for the detection of separated urinary metabolites and for checking the layer after overrun, respectively.

HPLC analysis was performed on a Hewlett-Packard 1090 Series II/M liquid chromatograph equipped with autosampler, diode array detector and column thermostat (Waldbronn, Germany). The chromatographic separation was performed by gradient elution using 15mM ammonium acetate (pH 2.75) as mobile phase A and acetonitrile as mobile phase B at a flow rate of 0.95 mL/min . The gradient profile included: 5% B for 3 min, 5–17% B for 12 min, 17–30 % B for 10 min, 30–60 % B for 15 min, 60–100 % B for 2 min, and it was held at 100% B at 8 min. The total gradient time was 70 min.

A Berthold LB 506-C-1 radioactive detector equipped with YG-150 U4D type detector cell (150-mL volume) with a solid scintillator was used for the online detection and isolation of radioactive compounds after HPLC and online OPLC separation. For data acquisition, Gina-Star interface and radiochromatography software version 2.03 was used (Raytest Isotopenmessgeräte, Staubenhardt, Germany).

### Sample preparation

Urine samples (0–24 h) were purified and concentrated by reversed-phase (RP)-SPE. The sample preparation column was preconditioned with 2 mL methanol and 2 mL water. Ten-milliliter aliquots of urine and plasma samples were mixed with 1 mL of 0.3% (w/v) ammonium acetate solution. Then the aliquots were centrifuged (3000 min$^{-1}$, 15 min, 4°C) and applied to the car-

tridge. The cartridge was washed with 2 mL water and eluted with 3 mL methanol consisting 2% (v/v) acetic acid. The methanolic solution was evaporated to approximately 20 mL. Then it was diluted with 1:1 (v/v) methanol–15mM ammonium acetate solution (pH 2.8) to give a 200-mL solution. In some cases, more than one parallel extraction was performed from the same urine sample (10-mL aliquots each) and the extracts were combined.

The extracted metabolite solutions were applied onto the layer by an automatic TLC sampler (Linomat, CAMAG, Muttenz, Switzerland). For off-line OPLC–DAR analysis the extracted urine fraction and $^{14}$C-labeled drug standard were streaked. For online OPLC–RD separation the urine extract corresponding to 10 mL of urine was applied onto the silica layer as a 16-cm band.

For HPLC–RD separation, 30 mL of urine of the same fraction was extracted, evaporated under $N_2$ stream, diluted in the mobile phase, and injected into the HPLC instrument.

## Results and Discussion

The flowchart of the laboratory phase of the metabolism study is depicted in Figure 1. The first step of metabolite isolation and purification was the sample cleanup performed by SPE using octadecyl-modified silica cartridges. The recovery of the metabolites was determined by LSC using 10-mL aliquots of the extract. As a first step of metabolite purification, OPLC hyphenated with RD was used. Samples were applied off-line to the OPLC layer and the metabolites were separated using fine-particle silica gel adsorbent using continuous development. The OPLC operation parameters are listed in Table I. The metabolite fractions were collected on the basis of online radioactivity detection of the OPLC effluent. This assured a more convenient method to obtain clean metabolite fractions than the off-line method reported earlier. Compounds not eluted from the layer could be detected by DAR and, if necessary, a modification in the mobile phase composition

**Table I. Operating Conditions of Online OPLC–RD Separation**

| Parameter | Value |
|---|---|
| Eluent composition | Mixtures of 1-butanol-acetic acid-water (e.g., 4:1:1, v/v/v) |
| Development time (min) | 160 |
| External pressure (MPa) | 5 |
| Flow rate (µL/min) | 250 |
| Sample volume (µL) | 200 |
| Start position (mm) | 25 |
| Number of fractions | 40 |
| Volume of fractions (µL) | 500–1000* |

* Depending on actual peak start/end.

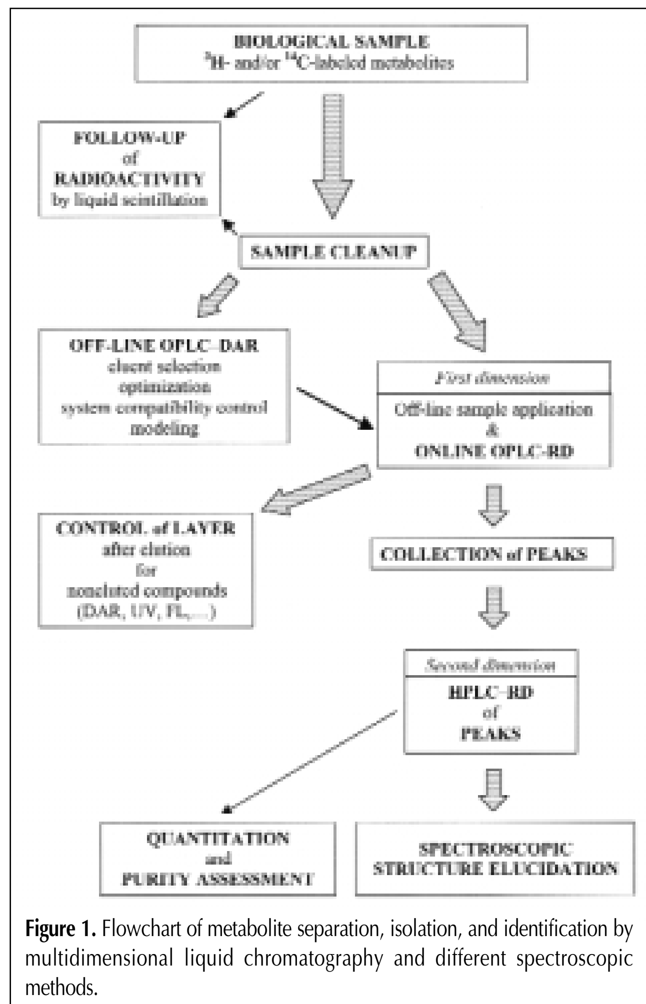


**Figure 1.** Flowchart of metabolite separation, isolation, and identification by multidimensional liquid chromatography and different spectroscopic methods.

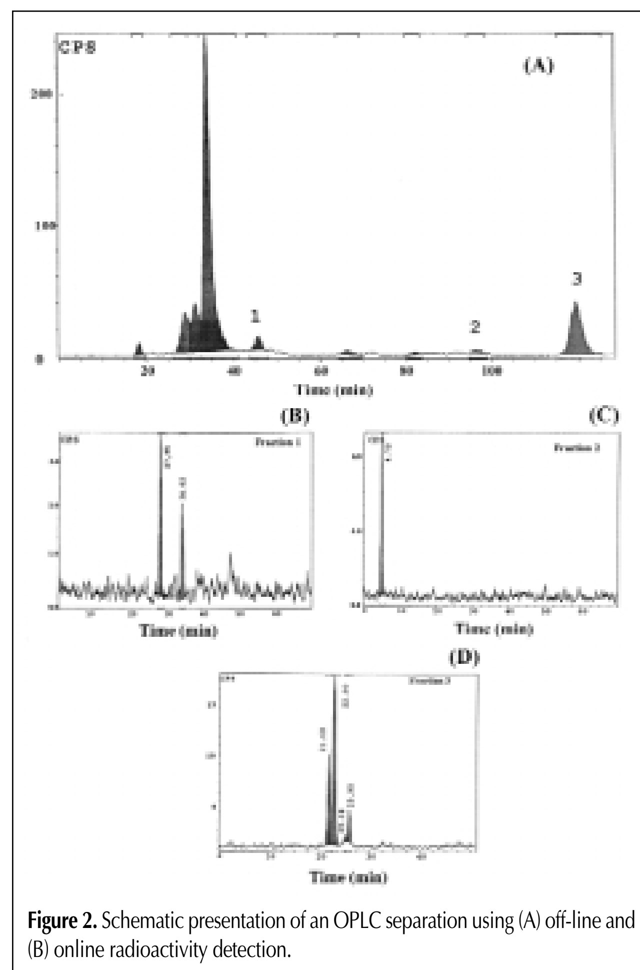


**Figure 2.** Schematic presentation of an OPLC separation using (A) off-line and (B) online radioactivity detection.

could be performed for their isolation.

Based on the data in Table I, the estimated run time for a compound having a capacity factor of 7 is approximately 160 min, which is in good agreement with those measured (Figure 2).

Online OPLC separation of the urine sample extracts as a first step of purification has distinct advantages. Because relatively high sample load could be allowed by applying the sample as a single band across the plate, the limit of detection could be improved and the amount of the isolated metabolite could be increased. The metabolites are separated and purified from several endogenous components, which represented a significant portion of sample weight. With greatly reduced sample load, the resolution of gradient HPLC coupled with RD in the second step of metabolite purification could be fully exploited.

Figure 3 illustrates the scheme of OPLC separation using off-line and online radioactivity detection. When parallel analysis is performed, samples and standards can be separated by off-line OPLC–DAR by allowing the development to reach the length of the plate, thus all of the sample components remained on the plate and could be detected. In case of continuous development (overrun conditions), the eluted compounds were detected online by RD. This approach was suitable for the isolation of metabolites by applying one sample per plate. After elution, the strongly retained components could be detected by off-line methods, which included DAR, derivatization, densitometry (visible, UV, or fluorescence), and FTIR.

Figure 4A shows the off-line OPLC–RD and 4B shows the online OPLC–DAR chromatograms of the 0–24-h urine sample after the administration of a $^{14}$C-labeled investigational drug. Spots detected on the layer by DAR were associated with the peaks of the online chromatogram. The marked peak was also analyzed by RP-HPLC–RD for further purification of the isolated metabo-

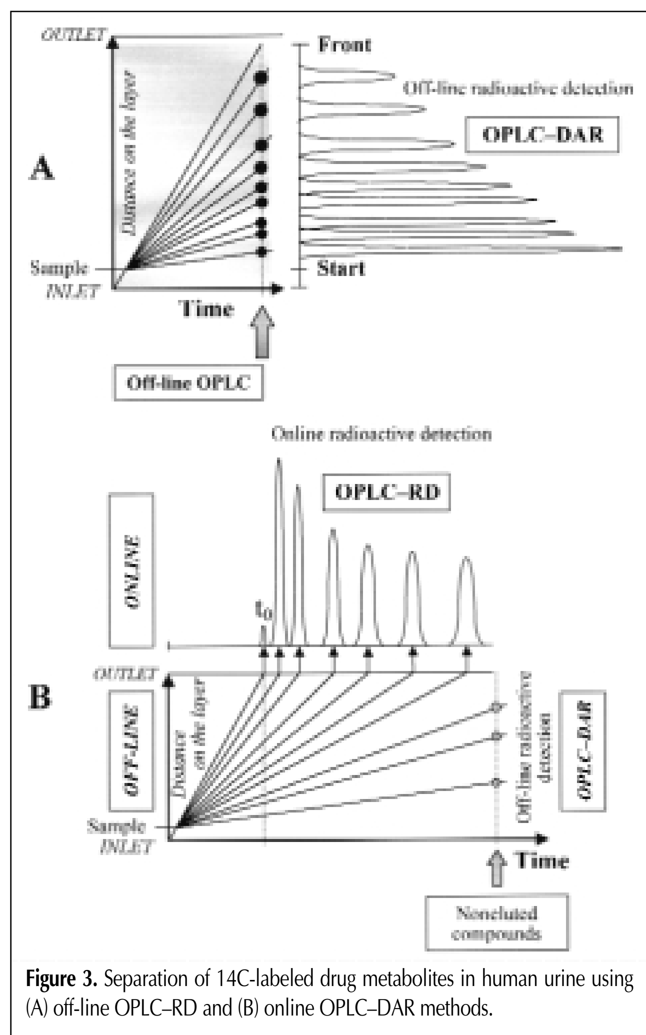


**Figure 3.** Separation of 14C-labeled drug metabolites in human urine using (A) off-line OPLC–RD and (B) online OPLC–DAR methods.

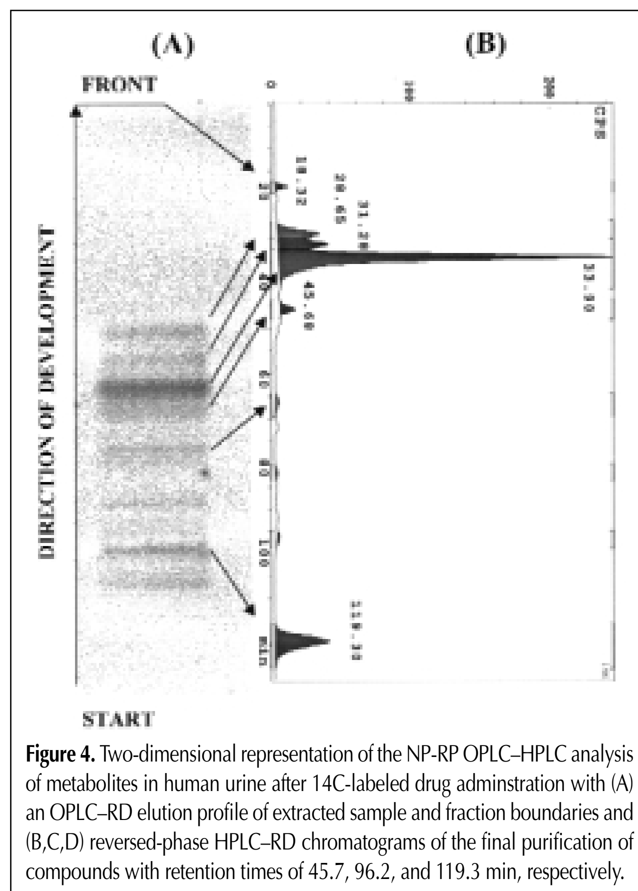


**Figure 4.** Two-dimensional representation of the NP-RP OPLC–HPLC analysis of metabolites in human urine after 14C-labeled drug adminstration with (A) an OPLC–RD elution profile of extracted sample and fraction boundaries and (B,C,D) reversed-phase HPLC–RD chromatograms of the final purification of compounds with retention times of 45.7, 96.2, and 119.3 min, respectively.

**Table II. Applicability of Hyphenated Radioactivity Detection Techniques in Metabolism Research**

| Feature | OPLC–DAR | OPLC–RD | HPLC–RD |
|---|---|---|---|
| Operation mode | off-line | Online | Online |
| Sample purity requirement | Low | Low/medium | Medium |
| Sample load | Low | Low/medium | Medium (high) |
| Sample number/run | 12–18 band/spot | 1 (band) | 1 |
| Possibility of fast fingerprinting of metabolites/metabolite conjugates | Yes | No | No |
| Suitability for isolation for structure elucidation | Yes | Yes | Yes |
| Cost of sorbent/sample | Low | Medium | Low |
| Cost of mobile phase/sample | trace | medium | medium (high) |
| Assessment of recovery from sorbent | Easy (direct) | Easy (indirect) | Difficult (indirect) |

lite fraction. Purity of the collected peak was also tested by HPLC–RD.

Data from the described experiments were arranged in a two-dimensional representation (Figure 2). In this depiction, each dimension represents a different chromatographic selectivity. In our case, the first dimension was online OPLC–RD on silica gel sorbent (Figure 2A), and Figures 2B, 2C, and 2D display the chromatogram of fractions with retention times of 45.7, 96.2, and 119.3 min, respectively, in an RP-HPLC–RD system. It could be seen in Figure 2B and 2D that the corresponding fractions (fraction 1 and 3 in Figure 2A, respectively) contain more than one chemical entity, therefore further fractionation was necessary to obtain homogeneous metabolites. This was carried out by HPLC–RD. From Figure 2C, we concluded that for fraction 2, no further fractionation was necessary.

During the separation process, metabolites are concentrated and purified because they are separated from emerging endogenous matrix components and also related substances (such as another metabolite or parent drug). The aim of the purification process is to reduce the chemical background and obtain an essentially homogeneous chemical entity that could be submitted even to structure analysis method without preceding separation technique. As an example, NMR analysis is often carried out off-line because of the time requirement and relatively high amount of sample required. Inhomogeneous samples often inhibit the proper evaluation of spectra or other analytical signals that result from the off-line analysis of isolated fractions containing metabolites.

Table II summarizes the applicability of online (OPLC–RD and HPLC–RD) and off-line (OPLC–DAR) radioactivity detection techniques from the view of metabolite research. In Table III compares the features, advantages, and drawbacks of the three techniques.

## Conclusions

OPLC hyphenated with RD offers the selectivity of RD combined with the good separation efficiency of OPLC, which is of paramount importance in the initial steps of metabolite isolation. After sample preconcentration and application to the plate, selectivity of the system can be optimized. The process of optimization can be easily followed by DAR, which applicable as detection technique for performing the simultaneous analysis of multiple samples on a single plate. For the isolation of separated sample constituents, the sample extract is applied to the plate as a single band and metabolites of interest are eluted and detected using online RD. Noneluting compounds are detectable by DAR and, if necessary, can be removed mechanically with the sorbent and eluted with a strong eluent (27).

The isolated metabolite fractions is further purified by different high-efficiency liquid phase separation methods, such as HPLC coupled with RD. The purity of such isolated metabolites is usually appropriate for spectroscopic structure elucidation.

The recently developed OPLC–RD method, along with the previously reported OPLC–DAR and HPLC-RD techniques, creates a multidimensional, rapid, economic, and effective separation system, which can be advantageously applied in metabolism research.

**Table III. Comparison of Various Chromatographic Techniques for Separation and Detection of Radioactive Metabolites**

| Feature | OPLC–DAR | OPLC–RD | HPLC–RD |
|---|---|---|---|
| Sensitivity | Excellent | Good | Good |
| Speed | Good | Good | Excellent |
| Resolution | Fair | Good | Excellent |
| Linearity, range | Excellent | Good | Good |
| Quantitative evaluation | Excellent | Excellent | Excellent |
| Detection limit | Excellent | Good | Good |
| Sample capacity | Fair | Good | Good |
| Cost of instrumentation | Fair | Excellent | Fair |
| Operation cost | Excellent | Good | Good |

## Acknowledgement

The authors thank Rozália Haász for her skillful assistance.

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Manuscript accepted July, 30 2002.