

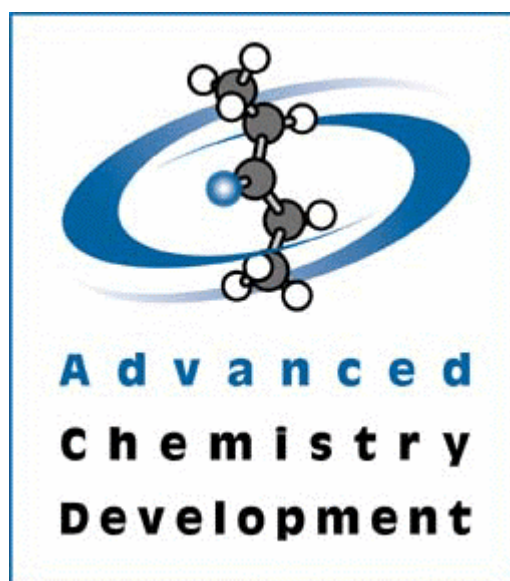
# ACD/LC Simulator & ACD/ChromManager

Version 5.0 for Windows

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Technical Note

*HPLC Method Development*



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# HPLC Method Development

Eduard Kolovanov and Marina Surnina

*Advanced Chemistry Development Ltd., Russian Office, 6 Bakuleva Street, Moscow 117513, Russia*

Michael McBrien

*Advanced Chemistry Development Inc., 90 Adelaide Street West, Suite 702, Toronto, Ontario, M5H 3V9*

## **Abstract**

The combination of searchable database and retention time prediction constitutes a powerful aid to method development. Chromatographic databases of successful separations, no matter how extensive, rarely contain the exact mixture you would like to separate. In spite of this, such databases still contain valuable chemical information that can allow you to propose good initial methods to try. For example, you can select a method based on functional group similarities to your mixture. Better yet, ACD/Labs predictions allow you to simulate in minutes how your compounds would behave under this method. Together, ACD/Labs database and prediction software can dramatically speed up method development by allowing you to make chemically intelligent choices.

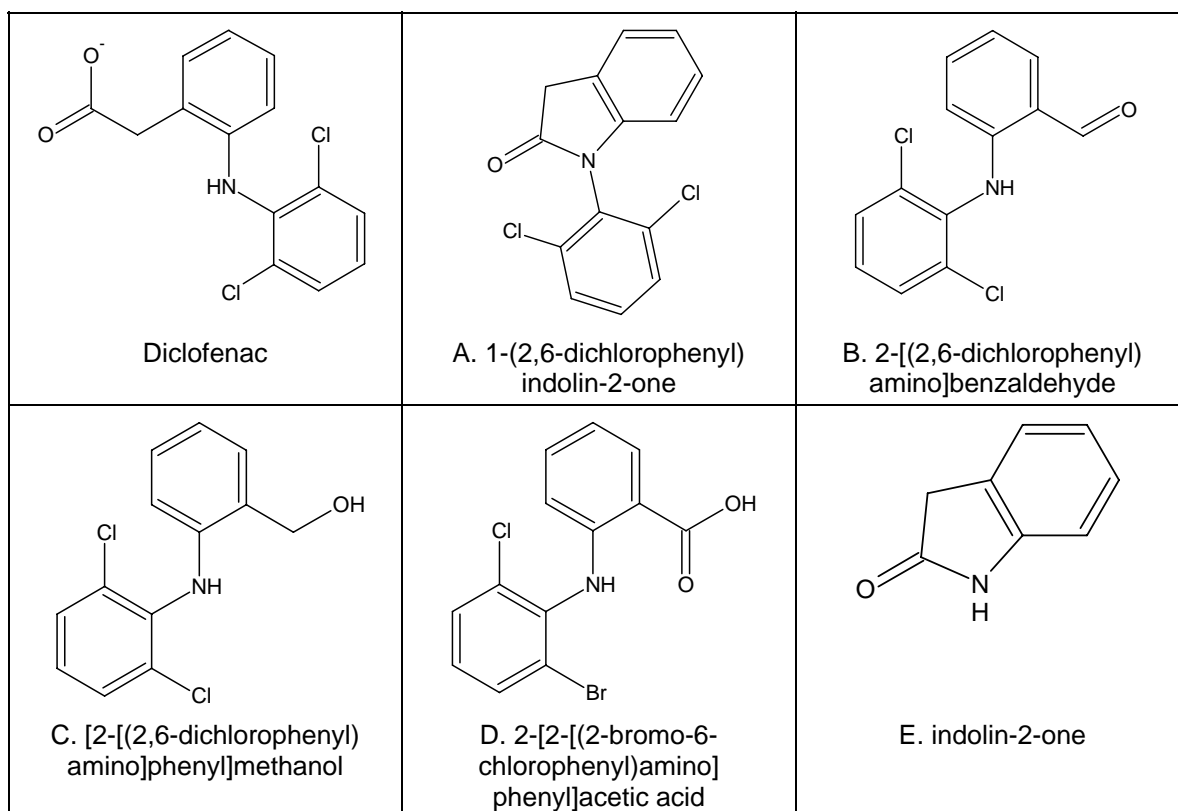
## **Introduction**

How can you leverage information for a few chemical compounds to maximize returns by reducing method development time?

A great deal of HPLC method development is still done by trial and error, and you can save time by reducing the amount of guesswork. ACD/Labs' software uses stored chemical information and fundamental properties predictions to guide your selection toward a viable separation method on your first tries. The procedure is outlined below.

**Important** To carry out the following method development tutorial, you **MUST** have copies of ACD/LC Simulator and ACD/ChromManager installed in the same ACD/Labs software folder.

As a working example, we are going to use our software to find the appropriate conditions for the optimal separation in time of the well-known analgesic, diclofenac, from its impurities that are shown in Figure 1.



**Figure 1. Structures for a mixture of diclofenac and related impurities.**

**Tip** These structures can be taken from **diclofenac.sk2** file that is placed in the ACD/Labs examples folder.

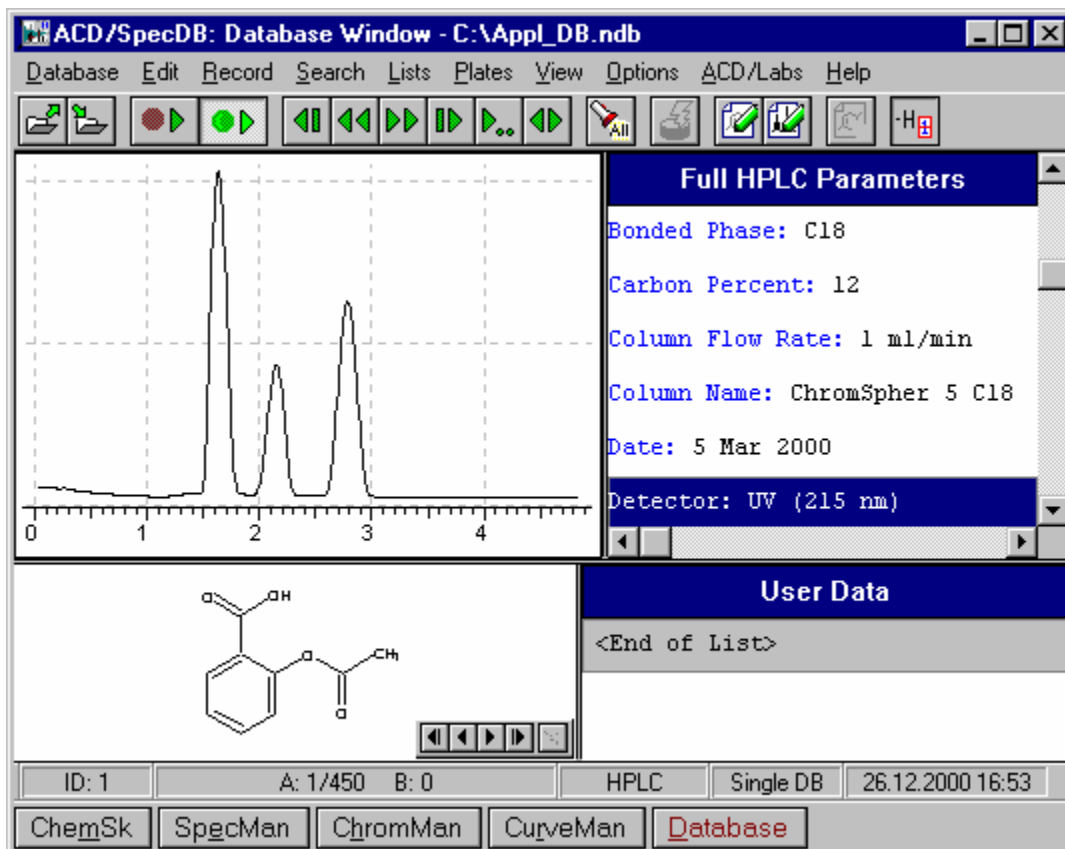
To follow this technical note, we recommend that you stick to the example and databases provided. This will allow you to gain experience with the procedure before trying it with your own databases and proprietary compounds. This method development technique is so simple and intuitive, that you will readily be able to apply it to your own compounds and databases.

This document assumes that you are familiar with ACD/ChemSketch and with using our program interface. The interface was designed to be as intuitive as possible, and you should be able to follow without too much difficulty. But if you require a step-by-step tutorial that carefully walks you through each step, this is also available on request.

## Searching the Applications Database

In this section, we are going to search the ACD/Chromatography Applications Database (**appl\_db.ndb**) to find records containing the structure of diclofenac (or a part of it) in order to find separation methods that could be applied for the proposed mixture.

1. On the Window Switching Bar of ACD/ChromManager, click **Database** Database to switch to the Database Window.
2. On the **File** menu, click **Open**, and in the **Open Document** dialog box, browse through the directories to the location of the **appl\_db.ndb** file. Click **OK**. The database will open on the first record.

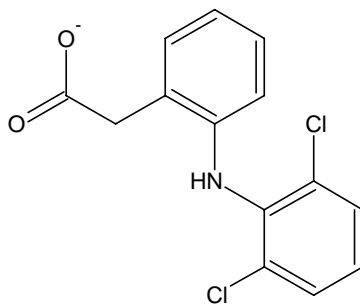


**Figure 2. Database window open on the first record of the HPLC application database.**


**Note** The number of records in the ACD/Labs applications database is constantly being updated. On the status bar of Figure 2, you see 450 records, but this is subject to change. The total number of records for **appl\_db.ndb** may be bigger in your case.

3. On the Windows Switching bar, click **ChemSketch** ChemSk to switch to the structure drawing area.

4. In the ChemSketch workspace, draw diclofenac as shown in Figure 3, or look it up in ACD/Dictionary.




**Figure 3. Diclofenac anionic structure (the metal cation should not be used in the calculation).**

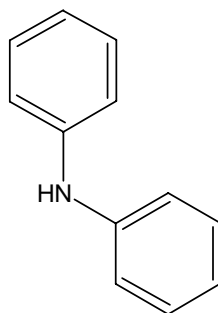
5. On the Windows Switching bar, click the **Sub-structure Search** button  to search the open database for this structure. Because there is no record containing this particular structure in the current database, the program gives you the following message: "No hits found for your query."

**Note** If you have more than one structure in the ChemSketch Window, select diclofenac prior to searching.


On the Structure toolbar, click the **Select/Move** button , and then click next to the desired structure.

6. If you had used a fragment common to most compounds in your mixture, it is reasonable to assume that more hits could have resulted from the search. So we are going to search according to a fragment of diclofenac that is also found in some of the impurities. In Figure 4, we see that *N,N*-diphenylamine can be obtained from diclofenac or from impurities A, B, C, and D in Figure 1.

In the ChemSketch window, click the **Delete** button , and then click off the ring substituents of diclofenac until you are left with the following. A greater number of "hits" in the database might be found,




**Figure 4. *N,N*-diphenylamine**

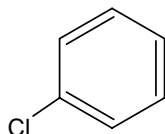
7. On the Windows Switching bar, click the **Sub-structure Search** button . The program compares the fragment against all records in the Chromatography Applications Database and informs you that 57 hits were found for this query.

**Tip** Multiple databases can be searched. You can search your own database at the same time you are searching the ACD/Chromatography Applications Database. On the **Search** menu, click **Define Multiple DB List**, and define the list of databases to be searched. For more details, refer to the ACD/SpecDB User's Guide.

Although looking through 57 hits is better than looking through 450 chromatograms, it would be helpful to reduce the number even more. A careful look at the diclofenac mixture in Figure 1 shows that impurities A, B, C, and D all share a chlorine substituted phenyl ring.

8. Return to the ChemSketch Window, and then delete the –NH-Ph groups so that only benzene remains.

9. On the Atoms Toolbar, click  and then add a chlorine group to the benzene ring.



**Figure 5. Chlorobenzene**

10. Click on the **Sub-structure Search** button.

11. The message “19 hits found for your query” appears. This is a manageable number, and we can now examine each method individually by applying your own judgment on the likely suitability of the proposed methods.

## ***Evaluating the Recovered Method***

Once the database has been narrowed down to just a few chromatograms, you are going to evaluate the separations according to other criteria. For example, how similar are the compounds to those of your mixture, and do you have a similar column on hand? The sequence of operations below assumes that we have narrowed our choices to methods described the file name ***a0936.esp*** and ***a0806r.esp***.

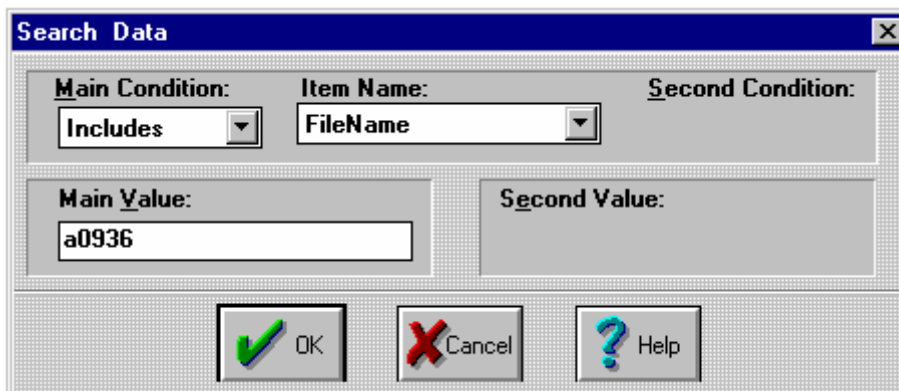
### **Developing Method**

On the **Lists** menu, click **Duplicate**, so that both list A and B contain 19 records found in the previous search. This will allow us to keep a copy of the original search results. (For more details on the operations with search lists refer to the ACD/SpecDB User's Guide.)

### Searching Database for the Required File

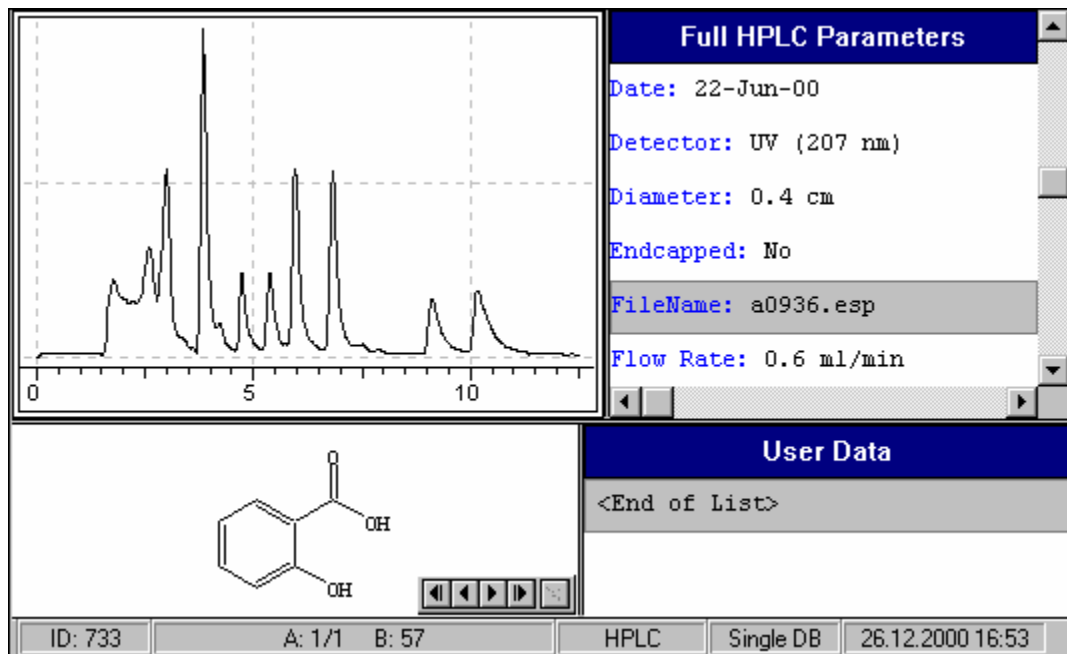
To find the record described above

1. On the **Search** menu (Database Window), click **Parameter**. The **Search Data** dialog box will appear.
2. In the **Main Condition** box, select **Includes**, and in the **Item Name** drop-down list, select the **FileName**. Then type **a0936** in the **Main Value** box (see Figure 6).



**Figure 6. Search Data dialog box with correct settings for this example.**

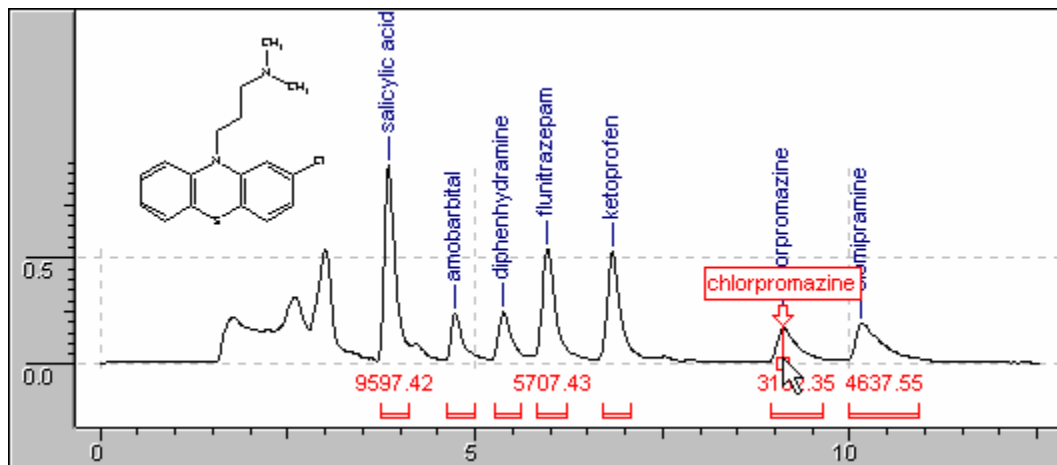
3. After you click **OK**, the program switches to the specified file.



**Figure 7. Results of database search for Filename a0936.esp.**

### Viewing Conditions of Separation

1. Double-click the chromatogram to transfer the file to the Chromatogram Window. You can now view all the assignments and other associated data.



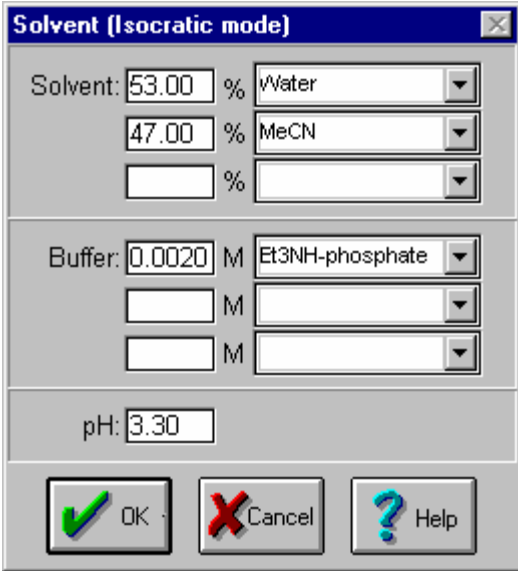
**Figure 8. Chromatogram Window view of the a0936.esp file.**

From the **Edit** menu, click **HPLC Parameters** to view the column parameters.

Column parameters:	
Column name: LiChrospher 5 RP18	Observed t0: 1.78 min
Length: 15.00 cm	Observed pressure: 720.00 psi
Diameter: 0.40 cm	at a flow rate of: 0.60 ml/min
Particle size: 5.00 microns	Endcapped: No
Pore diameter: nm	% Carbon: 21.40
Plate number:	Bonded phase: C18

**Figure 9. Column parameter area cutout for method a0936.esp.**

- Under the **Elution Data** area, click the **Solvents** button . In the dialog box that appears, you can see the solvents data.



The dialog box titled "Solvent (Isocratic mode)" contains the following fields and controls:

- Solvent:** Three rows of percentage and solvent name. The first row shows 53.00% vWater, the second shows 47.00% MeCN, and the third is empty.
- Buffer:** Three rows of molarity and buffer name. The first row shows 0.0020 M Et3NH-phosphate, the second and third are empty.
- pH:** A single text field containing the value 3.30.
- Buttons:** OK (with a green checkmark), Cancel (with a red X), and Help (with a blue question mark).

**Figure 10. Solvent data dialog box for file a0936.esp (from the HPLC Parameters dialog box).**

Note that these separation conditions resemble closely those of the *European Pharmacopoeia for Sodium Diclofenac, 3<sup>rd</sup> edition [1002] (1)*:


“The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with end-capped *octylsilyl silica gel for chromatography R* (5  $\mu\text{m}$ ),
- as mobile phase at a flow rate of 1 ml per minute a mixture of 34 volumes of a mixture of equal volumes of a 1 g/l solution of *phosphoric acid R* and a 1.6 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 and 66 volumes of *methanol R*,
- as detector a spectrophotometer set at 254 nm.”

- Click **OK** to close this dialog box.

### Optimizing Chromatogram

Now you can predict the retention time values for your target compounds (diclofenac and its impurities) in this condition. For this example, let's assume you want to set up a reversed phase separation.

- On the General toolbar, click the **Optimize Chromatogram** button .
- The program asks whether you want to optimize chromatogram in the Prediction mode—click **OK**. This will transfer the structures, retention times, peak width (W), dead time ( $t_0$ ), pH, etc. to ACD/LC Simulator.
- In the **Data Input (Prediction)** dialog box, select the **Reversed Phase** option in the **Type of Chromatography** box, and then click the **Calculate Predicting Equation** button



Calculate Predicting Equation

. After the calculation is finished, the program displays the prediction results

```
Calculations have been successfully completed.
The following prediction equation has been obtained:
Log(Retention time, sec) = 0.063(± 0.041)·LogD + 8.4e-3(±
1.4e-3)·MR + 1.62(± 0.083)
n = 7, R = 0.9530, StD = 0.080
Training set:
1, 2, 3, 4, 5, 6, 7
Special parameters:
Reversed Phase
pH = 3.30
t0 = 1.78
```

where  $n$  = the number of structures used to calculate prediction equation:

$R$  = the correlation coefficient;

$StD$  = standard deviation.

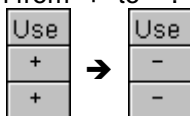
The calculated prediction equation generally more reliable

- With greater number of structures (no fewer than 3–4);
- When  $R$  is close to 1; and
- When the  $StD$  value is close to 0.

The calculated prediction equation is used to extrapolate retention times for our target compounds, and uses property calculations based on their chemical structures.

**Tip** To print the results, press ALT+PRT SCR (Print Screen) then paste the picture in any application (e.g., MS Word or Paint Shop), and on the File menu, choose the **Print** command.

**Tip** To exclude the structure from calculation, in the **Data Input (Prediction)** dialog box, change the **Use** column from “+” to “-.”



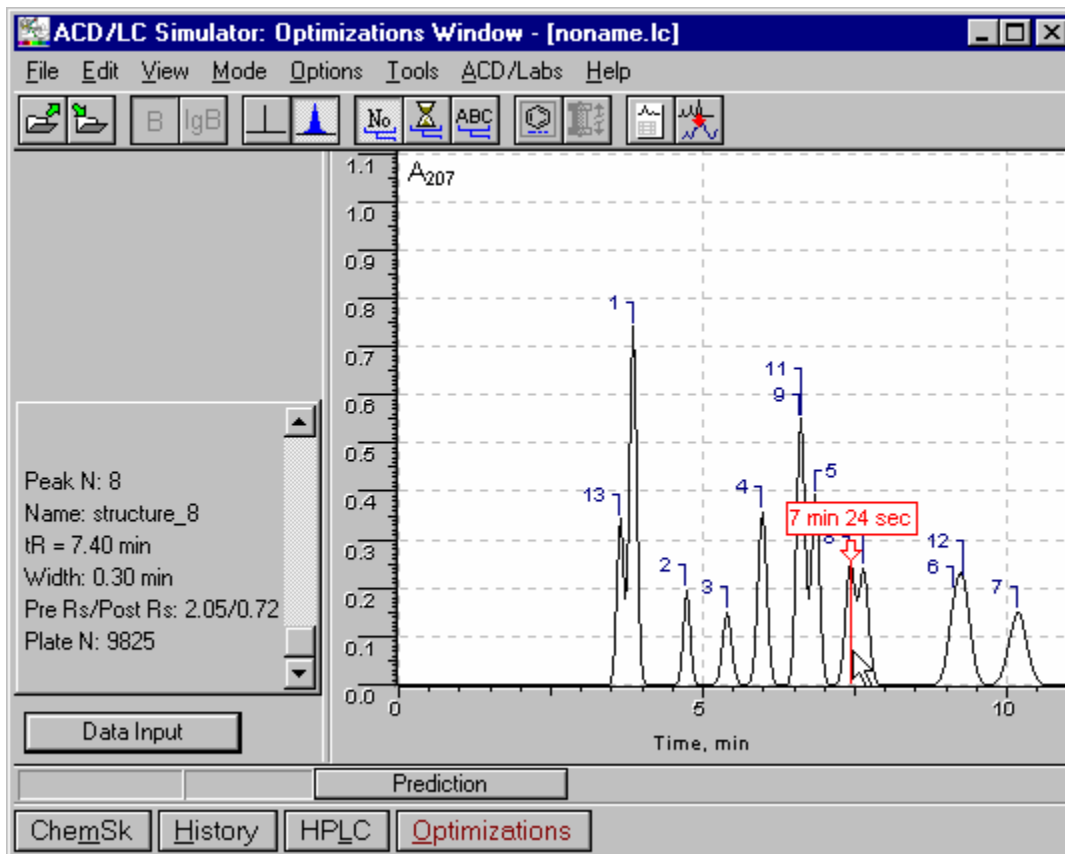
4. Click **OK** to close the results window, and then click **OK** again to close in the **Data Input (Prediction)** dialog box.

### **Predicting New Structures**

After calculating the predicting equation, we want to predict the retention times for our diclofenac sample with its impurities.

1. Return to the ChemSketch Window, and draw diclofenac and its impurities as shown in Figure 1. Or, from the Examples folder open **diclofenac.sk2**, where the required structures have already been drawn for you.

2. Select the structures one at a time, and click the **Optimizations** button  on the Window Switching bar. LC Simulator will predict the retention time for each compound using the prediction equation. Repeat until all the compounds in the sample mixture have been calculated.



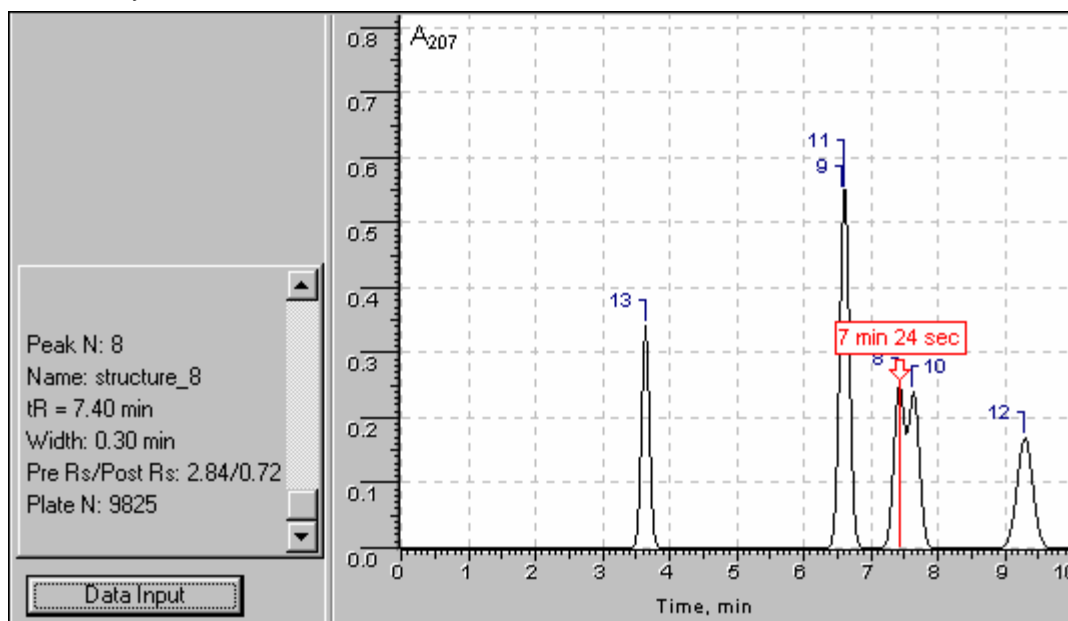
**Figure 11. LC simulation windows showing peaks for the original method's compounds and for the proposed diclofenac sample mixture.**

3. To focus just on the peaks belonging to diclofenac and its impurities, click the **Data Input** button . In the **Data Input (Prediction)** dialog box, set the setting from "+" to "-" in the **View** column to hide the experimental structures.

No.	Struc	Use	View	Peak Name	tR (exp)	tR (calc)
1	YES	+	-	salicylic acid	3.84	3.65
2	YES	+	-	amobarbital	4.72	4.70
3	YES	+	-	diphenhydramine	5.38	5.98
4	YES	+	-	flunitrazepam	5.96	7.10
5	YES	+	-	ketoprofen	6.83	6.68
6	YES	+	-	chlorpromazine	9.11	8.63
7	YES	+	-	clomipramine	10.15	8.63
8	YES	N/A	+	structure_8		7.40
9	YES	N/A	+	structure_9		6.58
10	YES	N/A	+	structure_10		7.62

**Figure 12. Data Input dialog box set to hide the experimental training peaks.**

- Click **OK** and the chromatogram will be redrawn to show just the new sample compounds of interest to you.



**Figure 13. Predicted chromatogram for just diclofenac and its impurities based on the a0936.esp separation conditions and column parameters.**

Figure 13 shows that peak 8 (diclofenac) and peak 10 (impurity B) are poorly resolved. The fact that peaks for impurities 9 and 11 are poorly resolved is of little concern here because we gave ourselves the simple goal of isolating diclofenac from its impurities. If separating peak 9 and 11 had been important, it might be prudent to try to find a different method for this separation.

**Note** The resolution of the selected peak with the previous and following peaks is shown on the Chromatogram Parameters panel (e.g., for the currently selected peak on the picture—Pre Rs/Post Rs: 2.84/0.72.)

## Refining Existing Method

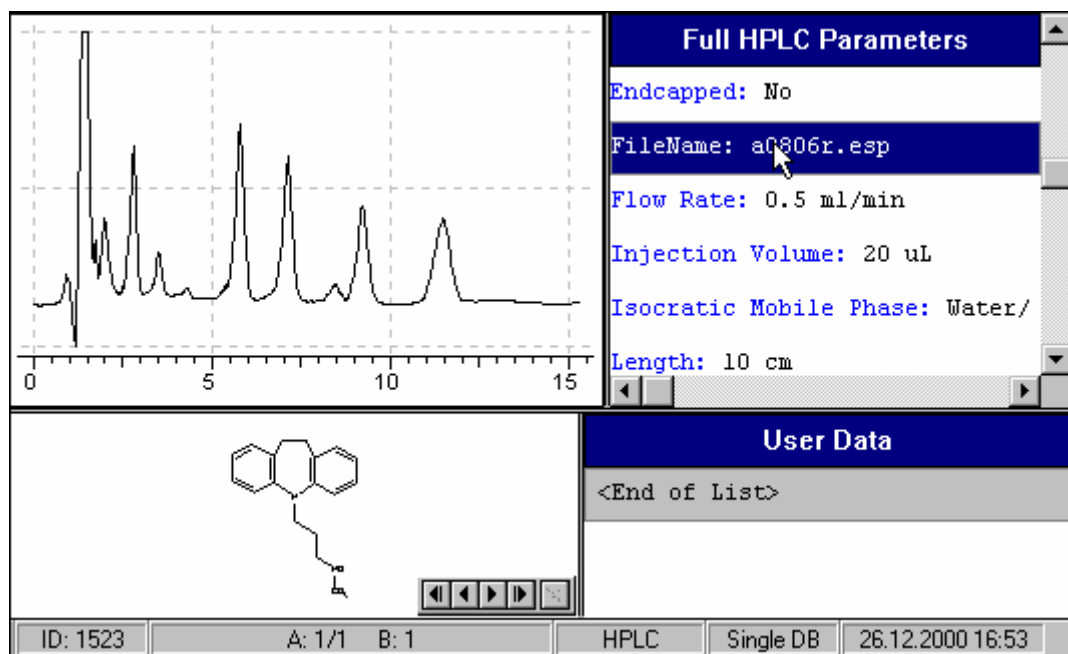
The objective of the previous section was to provide a viable starting point for the separation. Keep in mind that if the method is not entirely satisfactory, that you can refine it. At this stage, it will be necessary to make a judgment call based on your chromatographic experience. But instead of going at it blind, you have a chromatogram simulation to guide you.

Now let us try a different method from the HPLC application database, using different column parameters.

### Searching Database for the Required File

First, we are going to load the 19 records found in the original search into the active list (List **A**):

1. Switch to the Database Window of ACD/SpecDB by using the **ACD/Labs** menu or by pressing **SHIFT+ESC** on the keyboard.
2. From the **Lists** menu, click **Switch** to interchange the contents of lists A and B.
3. On the **Search** menu (Database Window), click **Parameter** to open the **Search Data** dialog box. In the **Main Value** box, type **a0806r** and click **OK**. The program switches to the specified file.



**Figure 14. Results of database search for FileName a0806r.esp.**

### Viewing Conditions of Separation

1. Double-click the chromatogram to transfer the information to the Chromatogram Window.

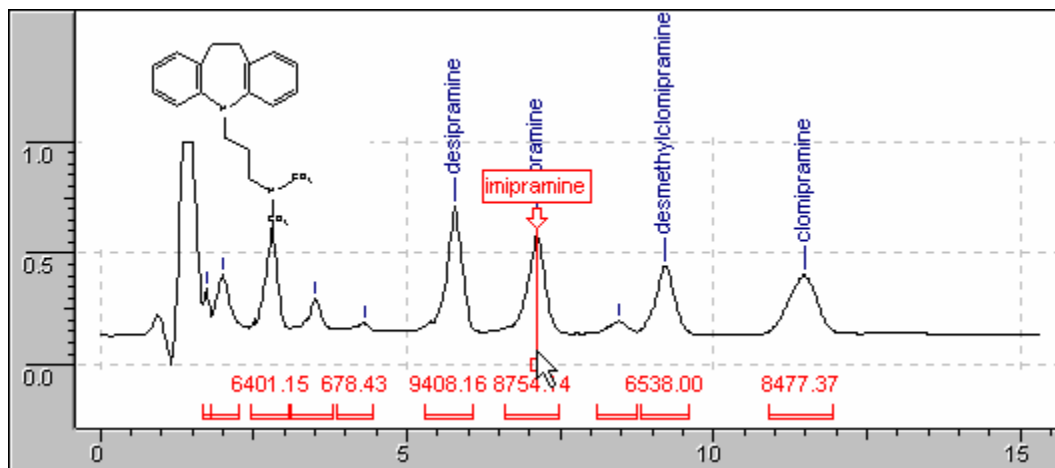


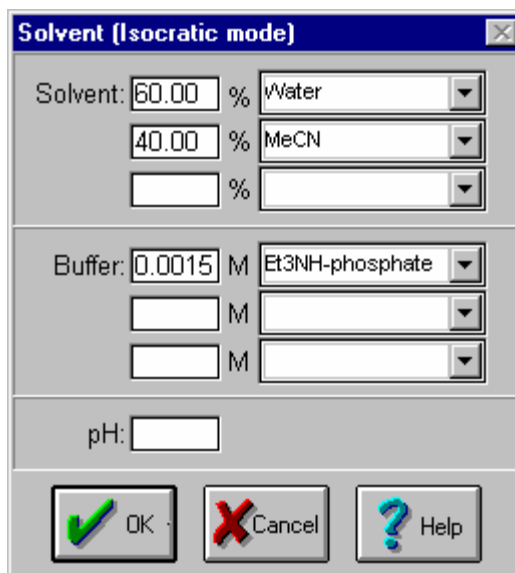
Figure 16. Chromatogram Window view of the a0806r.esp file.

2. On the **Edit** menu, click **HPLC Parameters**. Note that the column parameters differ from those of the previous example.

Column parameters:	
Column name: ChromSpher 5 B	Observed t0: 1.16 min
Length: 10.00 cm	Observed pressure: 570.00 psi
Diameter: 3.00 cm	at a flow rate of: 0.50 ml/min
Particle size: 5.00 microns	Endcapped: No
Pore diameter: nm	% Carbon: 8.30
Plate number:	Bonded phase: ODS

Figure 17. Column parameters for method a0806r in the HPLC application database.

- Under the **Elution Data** area, click the **Solvents** button and examine the solvent data.



**Figure 18. Solvent data dialog box for a0806r.esp file (from the HPLC Parameters dialog box).**

- Click **OK** to close the dialog box.

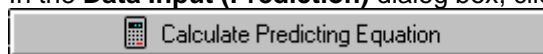
### Optimizing Chromatogram

Lets predict the retention time values for diclofenac and its impurities under these new conditions.

- On the General toolbar, click the **Optimize Chromatogram** button. Click **OK** when to optimize in Prediction mode. The structures and retention times will be transferred to LC Simulator.

**Note** The program will ask you to save the results of previous calculations. For the current example, skip this step.

- In the **Data Input (Prediction)** dialog box, click **Calculate Prediction Equation**



After the calculation, the program will display the following prediction results.

```

Calculations have been successfully completed.
The following prediction equation has been obtained:
Log(Retention time, sec) = 0.016(± 1.1e-3)·LogD + 6.6e-3(±
3.7e-5)·MW + 0.66(± 0.014)
n = 4, R = 1.0000, StD = 7.4e-4
Training set:
6, 7, 9, 10
Special parameters:
Reversed Phase
pH = 7.00
t0 = 1.16

```

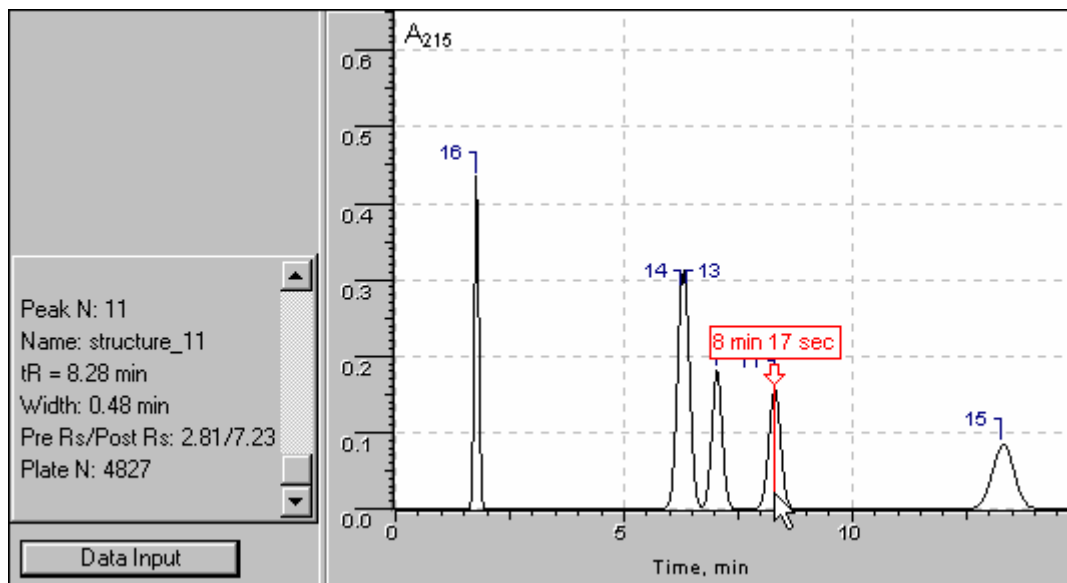
The number of structures used for calculation is 4, the correlation coefficient is 1, and standard deviation is very close to 0. The calculated prediction equation thus shows an

excellent fit. This equation will be used to extrapolate retention times of our target compounds based on their chemical structure properties.

3. Click **OK** to close the dialog boxes.

### **Predicting the Structures' Chromatogram**

1. Switch to the ChemSketch Window and draw diclofenac and its impurities, or open **diclofenac.sk2**.
2. Select structures one at a time, and click the **Optimizations** button on the Window Switching bar. LC Simulator will predict the retention time for each compound.
3. To focus solely on peaks belonging to diclofenac and its impurities, click the **Data Input** button and in the **Data Input (Prediction)** dialog box, set the **View** column settings from + to – for the experimental structures. Click **OK** to close the dialog box.




**Figure 19. Predicted chromatogram for just diclofenac and its impurities based on the a0806r.esp separation conditions and column parameters.**

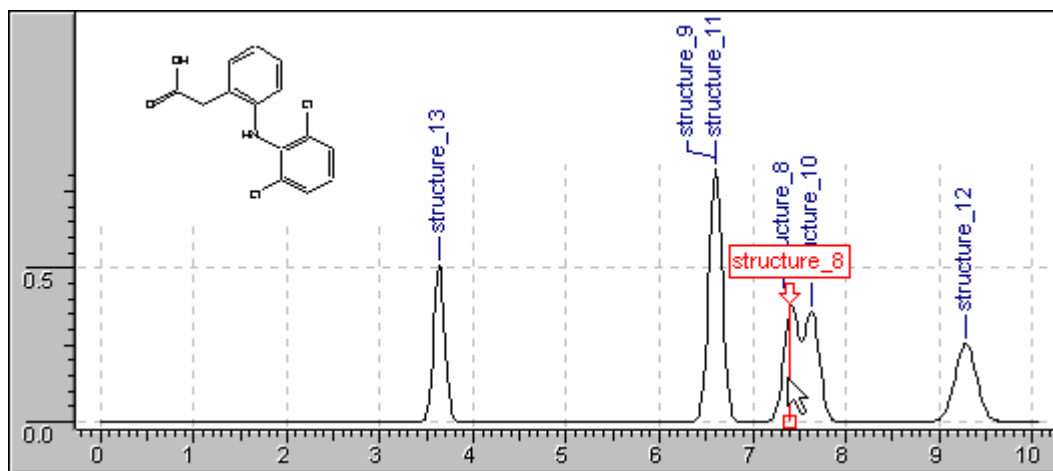
The picture shows us that diclofenac (11) is perfectly separated from its impurities (Pre Rs/Post Rs: 2.81/7.23.)

## Saving Results

Once you have achieved a satisfactory resolution, the last step is to archive the method for later retrieval. The chromatography data predicted on the basis of similar structures can be saved, updated to the user database or printed and then used for comparison with the experimental data.

To archive the chromatogram you should first transfer it to ACD/ChromManager.

- Click the **Copy Chromatogram to ChromManager** button  on the Top toolbar to switch to ACD/ChromManager (see Figure 20).




**Figure 20. Predicted chromatogram of diclofenac and its impurities using method a0806r.esp that was exported to ChromManager.**

To save the results on disk

- On the **File** menu, click **Save**.
- OR—
- You can also export the file to ASCII (.txt) and NetCDF (\*.nc, \*.cdf) formats by means of **Export...** command from the **File** menu.

To update the chromatogram to the database

- First open or create a user database, switch to ACD/ChromManager, and then click the **Update Database** button  on the General toolbar. For more details see the ACD/SpecDB User's Guide.

**Tip** User data fields are shown in the right-aligned Parameters sub-window corner of the Database Window. You can define your own fields, and you can semi-automate the introduction of these fields using ACD/Data Forms Manager (for more information on creating database forms refer to the separate guide.)

## Conclusion

No matter how big we make the ACD/Labs applications database, this is nothing compared to the results you will get if you create your own database. A database built from your work and that of your colleagues will greatly increase the number of relevant experiments to draw from. This allows you to build on previous successes and avoids reinventing experiments that had already been worked out before. For the two or three seconds that it takes to put a separation in a database, you can save days of work later on.

The combination of a searchable database and retention time prediction is a truly powerful method development tool. The database is an “anchor to reality” and while it is unlikely to contain your unique mixture it does give you relevant information for your new method development. The predictions, based on chemical functional groups, give you a very good first approximation of how the method will work out. Together, these two tools dramatically speed up your method development time by avoiding methods that are not suited to your needs, and by allowing you to do refinements before the first run.

## References

1. European Pharmacopeia 3rd Edition,