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A STUDY OF AN EMPIRICAL EQUATION FOR THE EVALUATION OF SEPARATION EFFICIENCY IN CHROMATOGRAPHY

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ABSTRACT

An empirical equation has been developed to evaluate chromatographic separation efficiency. This equation can be expressed as the product of a "separation" term, a "capacity" term and an "alignment between peaks" term. It can be shown that this equation is insensitive to other column or separation parameters. Its sensitivity depends only upon the resolution between peaks. This in turn allows the linking of the equation with any column or separation parameter during the optimization process as a response function. The utility of this equation has been extensively tested with the aid of computer-simulated overlapping peaks. This method is applicable to any separation technique that is based upon separation of compounds due to differences in partitioning between the stationary and mobile phases (column chromatography, HPLC, counter-current distribution, etc.). This equation can also be used to evaluate the quantity of each separated or overlapped compound giving a true picture of separation efficiency.

INTRODUCTION

Due to today's increasingly stringent requirements for pure pharmaceutical, agricultural and industrial chemicals, an increased burden has been placed upon the analytical chemist. As such, one must not only choose the best separation technique (column chromatography, HPLC, counter-current chromatography, molecular exclusion, etc.), but also optimize all parameters in order to achieve the maximum resolution of the main constituent of a mixture. In order to obtain a suitable chromatographic solvent, the novice must rely on solvent systems reported in literature for the separation of similar types of compounds and use trial-and-error techniques to arrive at a suitable solvent composition. An experienced analytical chemist may select better solvent combinations, but still may not have optimized conditions so to achieve the best resolution. Regardless of whether a novice or an expert separation chemist is confronted with a separation problem, the question inevitably arises as to what criteria one uses in assessing separation efficiency.

The literature contains a number of more or less complicated equations[1-5] attempting to describe column efficiency in terms of resolution between peaks and involving one or more separation parameters such as N (theoretic plate number), R_S (column resolution), a (column selectivity) and k' (capacity factor) among others. Said[6] first developed an equation for separation of a pair of compounds (Said's recovery index) for Gaussian peaks that is not restricted to elution chromatography systems. Unfortunately this method is useful only for binary systems that afford a Guassian distribution of peaks. This is not adequate for most real world separations. What is needed is a mathematical expression by which to evaluate the efficiency of the separation of any number of components with any peak shape, using any separation process. This expression should require only experimental data and empirical coefficients. The pioneering work in this area had been done by Rony,[7-9] Giddings[10] and De Clerk.[11]

Numerous functions describing the chromatographic response function (CRF) have been derived and some tested by Wegscheider.[5] Most of these methods are based on the assumption that, if the composition of a mixture is known (which is often not the case), the only problem remaining is to optimize the parameters of the equation describing the resolution of the mixture. A number of different approaches to finding an adequate solution to, and refinement of, these equations have also been described.[12] Still, all of these equations suffer from an interdependence of the separation parameters themselves, and thus prohibit generalization of a separation response function.

A problem often encountered by the separation chemist during the analysis of a complex mixture is the determination of the number and quantity of each compound in the sample. Still pronounced complete peak overlap is common during the routine HPLC separation and spectral analysis of extracts obtained from natural products. With the advent of today's UV/visible liquid chromatography diode-array detectors, and lineshape simulation programs, reliable estimates of the number of compounds contained within overlapping peaks, their retention times and relative abundance, can be at least estimated. Such information can subsequently be introduced into the equation described below, which assesses the separation efficiency for the system used as a numerical value between 0 and 1. When linked with other separation parameters (solvent composition, column dimensions, flow rate, etc.), it can provide the chromatographer with a method of optimizing separation leading to improved resolution of the overlapping peaks.

EXPERIMENTAL

Programs were written for the calculation of separation efficiency using GWBASIC v.3.22., (Microsoft corp.). Overlap calculations and testing of the equations was done on an IBM PS/2 and Macintosh Quadra 650 computer. Polynomial curve fitting was done using Cricket Graph v.1.3 and all drawings were generated with either Microsoft Excel, v5.0 (Microsoft corp.) or SuperPaint v.2.0 (Silicon Beach Software) software on a Macintosh computer.

THEORY

The three factors should be considered for the evaluation of the separation efficiency: peak overlap, quantity of the material under the peak and peak apex distribution.

The first factor, peak overlap or separation factor, evaluates how well peaks are separated from each other, thus evaluates their mutual overlap (E1).

The second factor evaluates the quantity of pure and overlapped compound(s) under the peak or part of it (E2). This factor measures mass distribution during separation. For instance we might have a severe overlap, but the mass of the compound(s) under the overlap is small compared to the total quantity of the separated material. Therefore the contribution to the separation efficiency will be small, and vice versa.

The ideal separation has all peaks evenly distributed throughout the chromatogram. The third factor (A) evaluates the peak distribution compared to ideal, and is designated as a allignment between peaks. The impact of this factor to the product of E1 and E2 is significant in cases where some peaks are grouped in a chromatogram and others are far apart. This indicates a poor solvent or column selection, wrong gradient profile, etc.

The separation efficiency (E) can be then expressed as the product of a "separation" term (E1), a "capacity" term (E2) and an "alignment between peaks" term (A), *i.e.*:

$$E = E1 * E2 * A * 100 \qquad (1)$$

E1 is a "separation" factor expressed as the number of fractions containing only one compound ($T_{(1)}$) in total number of fractions ($T_{(n)}$) containing "n" compounds. C is the highest number of compounds present in any fraction and *b* is an empirical coefficient which will be explained later. The separation is then defined as:

$$E1 = \frac{\left(\sum T_{(1)}\right)^{b}}{\sum_{n=1}^{C}\left[n*\left(\sum T_{(n)}\right)^{b}\right]} \qquad (2)$$

E2 is the "capacity" factor which evaluates the quantity of material in each fraction ($W_{(n)}$) containing the same number of compounds (n). W_t represents total quantity of material present. The E2 is actually a percentage of a compound in a mixture divided by the number of compounds (n) present in a fraction. *a* is an empirical coefficient which will be explained later, therefore E2 is defined as:

$$E2 = \sum_{n=1}^{C} \frac{W_{(n)}}{n^{a} * W_t} \qquad (3)$$

The quantity may be expressed as the area under a curve or the mass of the fractions as long as it is consistent throught the calculations.

Combining E1 and E2 and multiplication by 100 (for conversion to percentage), then rearranging defines E' which represents the separation efficiency based on the number of compounds under a peak with its relative amount. The exponents "a" and "b" represents empirical coefficients. Their values will be determined later.

$$E' = \frac{100 * \left(\sum T_{(1)}\right)^{b} * \sum_{n=1}^{C} \frac{W_{(n)}}{n^{a} * W_{t}}}{\sum_{n=1}^{C} \left[n * \left(\sum T_{(n)}\right)^{b} \right]} \qquad (4)$$

The alignment factor[13] (A) which keeps track of the separation between peaks and tries to separate them as far as possible, may be expressed as:

$$A = \frac{\prod_{i=1}^{P-1} \left[Rt_{(i+1)} - Rt_{(i)} \right]}{\left[\frac{Rt_{(max)} - Rt_{(min)}}{P-1} \right]^{(P-1)}} \qquad (5)$$

Where $Rt_{(i)}$ is the retention time for an individual peak or fraction at its apex. $Rt_{(max)}$ is the peak with maximal retention time or fraction containing last compound, $Rt_{(min)}$ is the peak with shortest retention time or fraction number containing the first compound, and P is number of peaks.

Thus the final equation for E, with alignment incorporated becomes E= E' * A, *i.e.*

$$E = \frac{100*\left(\sum T_{(1)}\right)^{b} * \left[\sum_{n=1}^{C} \frac{W_{(n)}}{n^{a} * W_t}\right] * \left\{\prod_{i=1}^{P-1}\left[Rt_{(i+1)} - Rt_{(i)}\right]\right\}}{\left\{\sum_{n=1}^{C}\left[n*\left(\sum T_{(n)}\right)^{b}\right]\right\} * \left[\frac{Rt_{(max)} - Rt_{(min)}}{P-1}\right]^{(P-1)}} \qquad (6)$$

The only necessary data required for input into equations (4) or (6) are: the number of peaks (P) and their positions [$Rt_{(i)}$], the total number of compounds (C) contained under each peak ($T_{(n)}$), and the weight of each fraction $W_{(n)}$ (or the area under the peaks).

As will be demonstrated, the function is nondimensional, and as such is insensitive to the shape of the peak, type of separation, column size, flow, and quantity of material separated. Subsequently, E takes into account the peak width and could be affected by k' and the selectivity, a. Equation (6) does not require approximations that are necessary for Purnell's[14] or Knox's[15] methods. Nor does it suffer from column parameter factors or other methods of separation. Thus, equation (6) leads to a general solution of the optimization problem. The functions E, E', E1, E2 are fitted in the range between 0 and 1 (0 represents no separation and 1 base line resolution between two or more peaks) near or on baseline.

RESULTS AND DISCUSSION

To test equations, hypothetical test mixtures were generated by computer simulation using a BASIC program that contained all the necessary peaks and separation between peak parameters.

Example of Estimation of Exponent "b" for E1:

A test matrix was generated which represents the number of compounds (1 and n) in fractions (1-12) for 13 separations. This matrix represents 13 separations as follows: the separation 1 represents 12 fractions of peak each

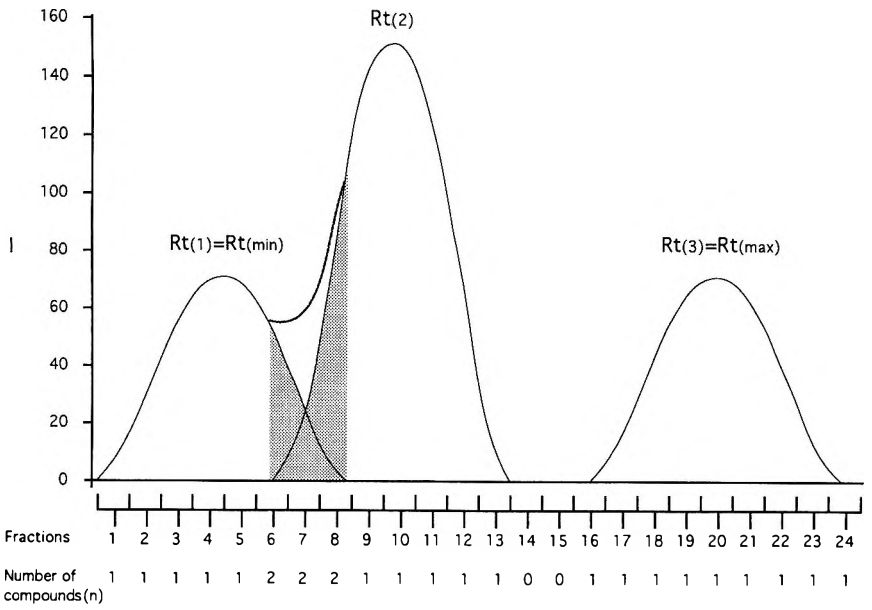

Figure 1. Partial overlap of two peaks

Table 1

Validity of Equation (1)

Separation #	Fractions 1	2	3	4	5	6	7	8	9	10	11	12
1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	n
3	1	1	1	1	1	1	1	1	1	1	n	n
4	1	1	1	1	1	1	1	1	1	n	n	n
5	1	1	1	1	1	1	1	1	n	n	n	n
6	1	1	1	1	1	1	1	n	n	n	n	n
7	1	1	1	1	1	1	n	n	n	n	n	n
8	1	1	1	1	1	n	n	n	n	n	n	n
9	1	1	1	1	n	n	n	n	n	n	n	n
10	1	1	1	n	n	n	n	n	n	n	n	n
11	1	1	n	n	n	n	n	n	n	n	n	n
12	1	n	n	n	n	n	n	n	n	n	n	n
13	n	n	n	n	n	n	n	n	n	n	n	n

contains one compound; in separation 2, the fractions 1 to 11 contains one compound and fraction 12 contain n compounds, in separation 3 the fractions 11 and 12 contain n compounds; and so on. The last separation (13) is a single peak that contains n compounds (total overlap).

To prove validity of equation (1), substituting the number of compounds as 1 (separation 1) [n=1, $T_{(1)}$= 12, $T_{(n)}=T_{(1)}=12$] in equation (1) for any value of b, E1=1; if the number of compounds under the peak is ∞ [n=∞, $T_{(1)}=0$, $T_{(n)}=\infty$] for any value of b, E1=0 or in our example for separation 13, if n=2 then $T_{(1)}=0$, $T_{(2)}=13$ for any value of b, E1=0 (Table 1).

The equation E1 actually counts fractions (time increments) with only one compound and compare them with the number of fractions with two or more compounds. This might be illustrated as in Figure 1.

The number of fractions with 1 compound (n=1) is 19 [$T_{(1)}=19$]
The number of fractions with 2 compounds (n=2) is 3 [$T_{(2)}=3$]
C=2 (maximal number of compounds in single fraction)

In this case $E1 = \frac{19^b}{1*19^b + 2*3^b}$

if b=1.5 then E1=0.89 (89%) (disregarding E2 and A)

in more complex example where three compounds overlap (Figure 2) we have:

The number of fractions with 1 compound (n=1) is 13 [$T_{(1)}=13$]
The number of fractions with 2 compounds (n=2) is 6 [$T_{(2)}=6$]
The number of fractions with 3 compounds (n=3) is 3 [$T_{(3)}=3$]
C=3 (maximal number of compounds in single fraction)

in this case $E1 = \frac{13^b}{1*13^b + 2*6^b + 3*3^b}$

if b=1.5 then E1=0.51 (51%) (disregarding E2 and A)

In order to determine the best value of exponent "b", the exponent "b" was varied over a range between 0.5 to 2.0, and the number of compounds (n) was taken as 2 to simplify presentation. The calculation can be expanded to any number of compounds.

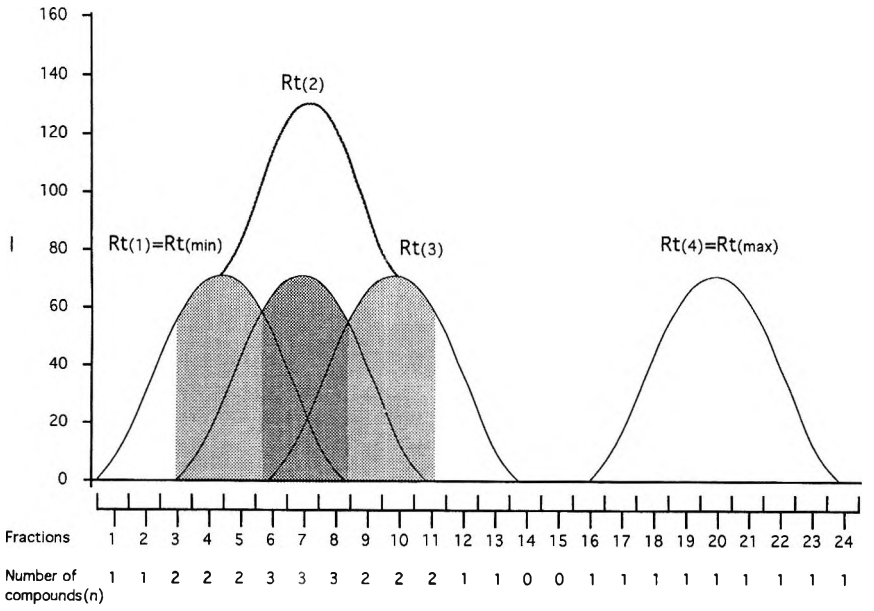

Figure 2. Three peak overlap.

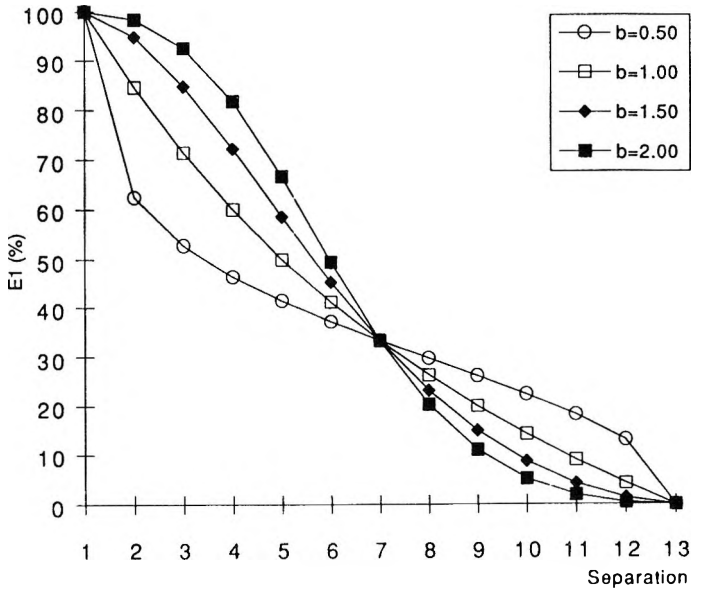

Figure 3. Plot of data for E1 with differing exponent "b"; n=2

By examination of the Figure 3, "b" lies between 1 and 2. For overlap of 11/12, E1=84.6% (b=1), resembling big drop of 15.4%. With the same value of b, for the overlap of 11/12, the E1=4.35%. For the value of b=2 and overlap of 11/12, the E1=98.4% that is close to 1, and for overlap of 11/12, the E1=0.41%, close to 0. The exponent "b" of 1.5 was chosen as algebraic mean to describe presented set of data for separation efficiency. This choice of "b" exhibits a slow drop in the beginning, a faster drop in the middle and again a slower drop at the end, thus resembling sigmoidal shape.

Example of Estimation of Exponent "a" in E2:

To test equation E2, an initial set of data was obtained from a randomly chosen real peak from a real HPLC separation. From this data, a polynomial equation was determined by non weighted least square curve fitting which described the peak. The factor x^5 is so small and does not have significant impact on fit.

The polynomial equation for the peak was determined to be:

$$y= 2.6279 - 3.3561\ x + 1.2608\ x^2 - 6.3247 \bullet 10^{-2}x^3 + 8.5469 \bullet 10^{-4}x^4$$

Applying this polynomial equation, new hypothetical sets of data could be generated which simulated conditions ranging from total overlap of two peaks to complete resolution of the peaks. This data could then be used in subsequent calculations of various E2 values for each set of conditions.

The details of this procedure are as follows:

The peak was divided into 12 sections to simplify calculations and the area under each section of the peak determined by integration of polynom:

$$P = \int_{x1}^{x2} \left(2.6279 - 3.3561x + 1.2608x^2 - 6.3247*10^{-2}x^3 + 8.5469*10^{-4}x^4\right)dx$$

For estimation of the exponent "a" in E2, conditions involving complete overlap to complete resolution of two peaks were performed in stages. First, the peak was overlapped with one identical sized peak and E2 calculated. Then the second peak was shifted one place to the right and E2 again calculated. This procedure was repeated until both peaks were fully resolved (13 steps).

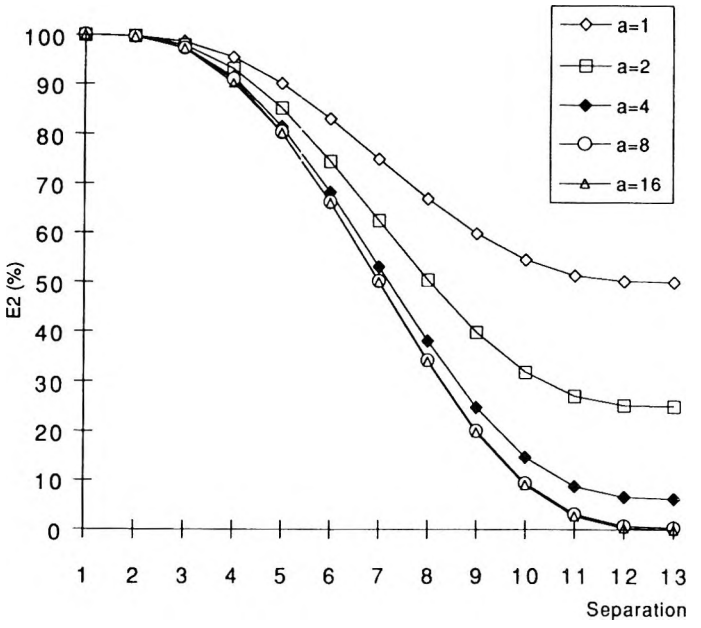

Figure 4. Plot of data for E2 with differing exponents "a".

The calculation of E2 was done for different exponents of "a" (n=2) and results are shown in Figure 4.

For equation (3) the possible value for exponent "a" could be 8 when used by itself, because for total overlapping of peaks if a=8, then E2 is close to zero. We can see that E2 $\rightarrow$ 0 (total overlap), when a $\rightarrow \infty$. With the value of 16 for "a" the E2 is 10^{-5}.

The function was further tested using computer generated peaks by assessing separation between two peaks with greatly differing areas (ratios varying between 1:1 to 1:32). Assuming that both peaks have the same width, the average drop is 1.87% for 50% overlap to 0.07% for 9% overlap in the range of 1:1 to 1:32 of the relative area ratio. From the results obtained apparently response is not affected with size of peaks, but only with their mutual overlapping.

The Testing of Equation E' (4):

Equation (4) was calculated using b=1.5 with differing exponents of "a".

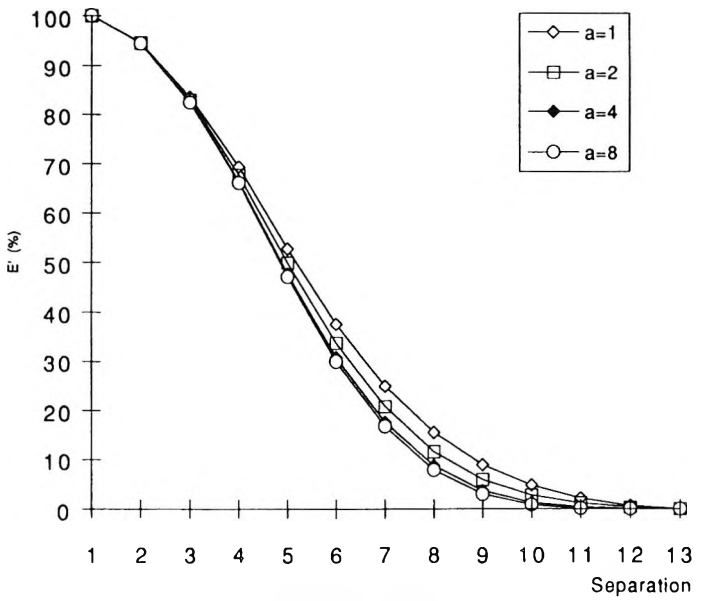

Figure 5. Plot of data for E', b=1.5 with differing exponents of "a".

Table 2

Description of Symbols

E, E1, E2	separation of efficiency
A	alignment
$T_{(1)}$	number of fractions which contain only one compound (regardless of Rf)
n	number of compounds in each fraction
$T_{(n)}$	number of fractions (test tubes or ΔRt etc.) with "n" compounds
C	total number of compounds
Wt	total weight or area under peaks of all fractions after separation
$W_{(n)}$	weight of each fraction or group of fractions (or area) with same n
a, b	empirical coefficients
$Rt_{(i)}$	retention time for individual peak
$Rt_{(max)}$	peak with maximal retention time
$Rt_{(min)}$	peak with minimal retention time
P	number of peaks

From Figure 5, it is evident that equation (4) is not very sensitive to the value of exponent "a".

An important characteristic of E2 and consequently E' and E is that it is not necessary to know the ratio of compounds in overlapping parts of a peak, but only the total quantity of this part of a peak, and of course, the number of compounds contained in the overlapped peak. If we do not know the number of compounds under overlaying peaks, an estimation will serve well. Later during optimization other compounds may emerge from the overlapped peaks. Misjudged numbers of compounds under a peak causes only a small error.

CONCLUSION

Equation (4) and, consequently, equation (6), is independent of conventional chromatography column parameters (column width or length, stationary phase composition, mobile phase composition, elution time, flow, etc.). This independence allows one to link this equation to the any optimization method (e.g. Simplex[16]) as a response function in which one or more parameters are optimized at the time (length of column, quantity of sample, composition of mobile phase, etc.). An account can be taken of all peaks present or only peaks of interest (for example, the best resolution of valuable material from impurities) during optimization process. The results can, however, be generalized to a case involving "n" compounds. The only prerequisite parameters for the calculation of separation efficiency necessary to enter in equation are $Rt_{(i)}$, $T_{(n)}$, C, P, and $W_{(n)}$. The values for factors a and b from above analysis are a=2, b=1.5 .

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CONTAMINANT DETERMINATION IN COLD ROLLING OIL USING HPSEC WITH RI AND PDA DETECTION

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ABSTRACT

A size exclusion liquid chromography (HPSEC) method using refractive index (RI) and photodiode array (PDA) detectors and a BASIC calculation program was developed for high molecular weight contaminant determination in the C_{15} linear paraffin-based cold rolling oil. Eight laboratory standards were analyzed to determine the new HPSEC/RI calibration factor for the C_{15} linear paraffin rolling oil. Three contaminants and thirteen standard solutions were evaluated to establish calibration curves. The HPSEC/RI/PDA method will quantitatively determine the concentrations of polyisobutylene-based hydrodynamic backup bearing lubricant, mist-applied extreme pressure gear oil for the workroll bearing and antiwear hydraulic oil in the cold rolling oil. Deviations for backup bearing lubricant, workroll bearing lubricant and hydraulic oil are approximately 3%, 4% and 7% of the contaminant concentration, respectively.

The BASIC calculation program can assist operators in reducing calculation error and improving speed and accuracy for the HPSEC method.

INTRODUCTION

Two types of lubricants are used in the cold rolling mills to produce aluminum sheet and foil. The cold rolling oil, which is composed of a hydrocarbon base oil and long chain, polar friction reducing additive, provides lubrication and cooling to the workroll and the aluminum sheet. The mechanical lubes, such as hydraulic fluid, bearing greases, and gear oils, are used to provide lubrication to the mechanical parts of the mill system. During the rolling process, the mechanical lubes of the mill system have the potential to contaminate the cold rolling oil through accidents or leaks. The contaminated cold rolling oil tends to generate "smut" and thermal stain on the sheet, decrease the rolling speed, and cause slippage of the workroll.

In practice, zero contaminants in the rolling oil is almost impossible to achieve in the plant. Determining the level of high molecular weight contaminants (tramp oil) in the rolling oils and providing this valuable information to the mill supervisor are a challenge to the plant's QA laboratory. Traditionally, infrared spectroscopy (IR) and viscosity measurement are most commonly used by the QA laboratory for a quick contaminant determination; however, both techniques have accuracy, sensitivity and characterization problems. Most of the time, IR analysis is very limited due the functional similarity of the rolling oil and mechanical lubes. The viscosity test cannot provide an accurate result for the contaminated rolling oil because it cannot distinguish between the type of mechanical lubricant contaminant and because the viscosities of the potential contaminants can cover a wide range (about 32 - 7,750 cSt @ 40°C).

High performance size exclusion chromatography (HPSEC), which is also known as gel permeation chromatography (GPC), has been well developed[1] and widely used in the polymer, oil, paper, and other industries for characterization and separation of polymeric materials[2], asphalts[3], cellulosics[4], pectins, chelate complex[5] and proteins[6]. Actually, size exclusion or gel permeation is a form of liquid chromatography in which the solute molecules are separated as a result of their diffusion into solvent-filled polymer matrixes in the column packing. Normally, these porous column matrices are formed by heavily cross-linked polymer chains. Large molecules can be excluded from the porous matrices of the packing due to their physical size in the mobile phase. Therefore, the large

Table 1

Chemical Composition of Standard Solution

Standard	Backup Bearing Lubricant (%)	Hydraulic Oil (%)	Workroll Bearing Lubricant (%)
	1	6	0 0
2	0	6	0
3	0	0	6
4	3	3	0
5	3	0	3
6	0	3	3
7	2	2	2
8	1	5	0
9	5	1	0
10	3	2	0
11	3	2	1
12	3	2	2
13	3	2	4

Table 2

SEC Calibration Factor for C_{15} Linear Paraffin SEC/RI Standards

Backup Bearing Lubricant (%)	Hydraulic Oil (%)	Workroll Bearing Lubricant (%)	Additive (%)	SEC/RI Time Slicing Data (%)	SEC Calibration Factor
-	-	-	8	0.80	-
6	-	-	-	16.98	0.35
-	6	-	-	12.66	0.47
-	-	6	-	15.15	0.40
3	3	-	-	14.56	0.41
3	-	3	-	15.0	0.40
-	3	3	-	13.58	0.44
2	2	2	-	14.48	0.41

Notes: 1. All standards were prepared using C_{15} linear paraffin as base oil.
2. Virgin C_{15} linear paraffin base oil was used as the baseline standard.

solute molecules are eluted from the GPC column before the smaller molecules, which have the opportunity to permeate into the porous, solvent-filled matrix. The order of elution from the GPC column will always be the largest molecules first, the smallest last[7].

Two of the most commonly used packing materials, Styragel™ and Poragel™, are highly cross-linked, polystyrene-divinyl benzene type polymers. They are hydrophobic and allow the chemist to separate molecular weight ranges from somewhere around 100 to about 50,000,000. There is a rough way to determine what pore size columns will be applicable for a given application. The 100 Å columns are used for compounds that have molecular weight of <700. The 500 Å gels are used to separate molecular weights between 12,000 and 700 or lower.

The highly viscous mechanical lubes have much higher molecular weight or size than the cold rolling oil. Therefore, HPSEC is a better choice for quantitative and qualitative determination of mechanical lubes in the cold rolling oil. An HPSEC method using UV and RI detection was developed by B. L. Riddle, et al..[8] in 1984. The method was able to demonstrate the capability of HPSEC for mechanical lube determination in cold rolling oil.

Since then, the method has been modified several times in our laboratory due to improvement of the GPC instrument and changes in column technology, the data handing software and the detection system. The modified HPSEC not only meets the challenge from the complexity of the cold rolling oil but also provides more specific and accurate information to the mill supervisor.

The modified HPSEC method[9] using refractive index (RI) and photodiode array (PDA) detectors and a BASIC program was developed for high molecular weight contaminants determination in the C_{15} linear paraffin rolling oil. Three major contaminants from the cold rolling mill were evaluated using HPSEC with RI and PDA detectors to determine if the HPSEC method can quantitatively and qualitatively detect these contaminants in the C_{15} linear paraffin rolling oil. These contaminants are polyisobutylene-based hydrodynamic bearing lubricant, an extreme pressure petroleum-based gear oil used to mist lubricate the mill's antifriction workroll bearing and a petroleum-based hydraulic fluid.

Thirteen standard solutions with various concentrations of the contaminants in the C_{15} linear paraffin base oil were prepared. Chemical composition of the standard solutions are listed in Table 1. The backup bearing

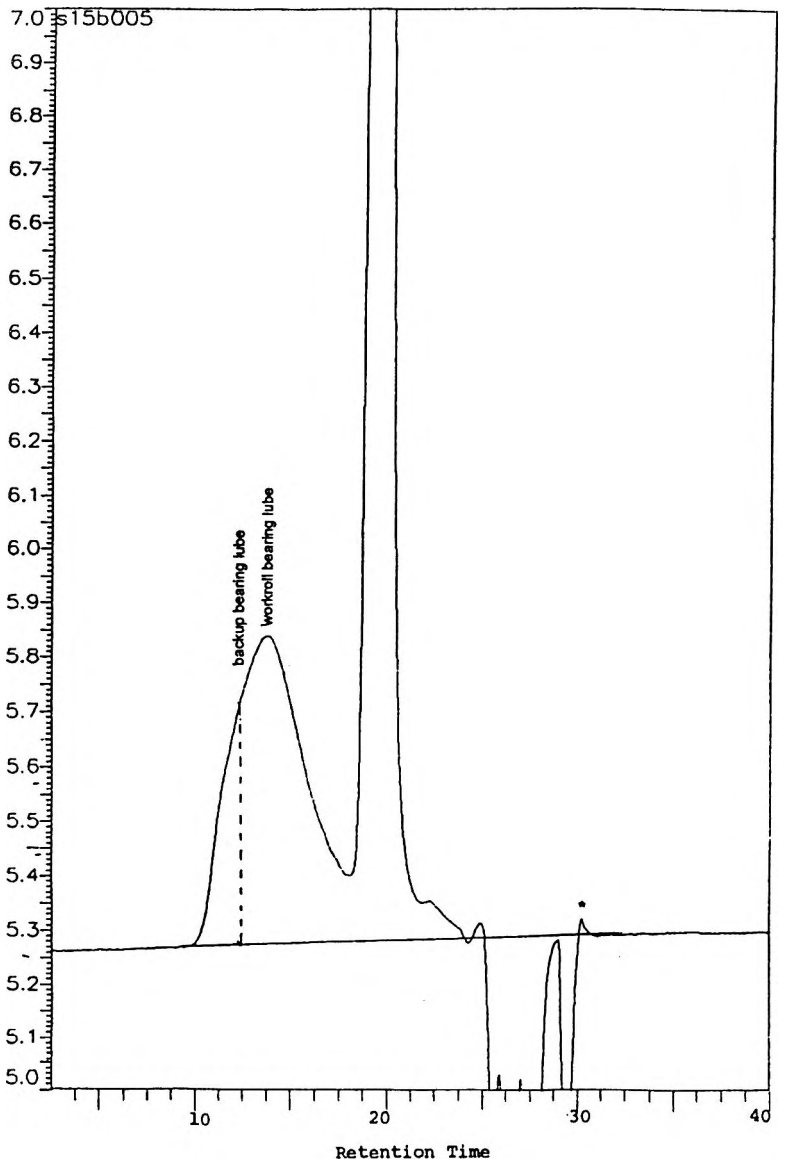


Figure 1. GPC/RI chromatogram of 3% backup bearing lube and 3% workroll bearing lube in C_{15} linear paraffin oil.

lubricant standard used in the study is a mixture of polyisobutylene H-95 and H-25 in a 1:2 ratio.

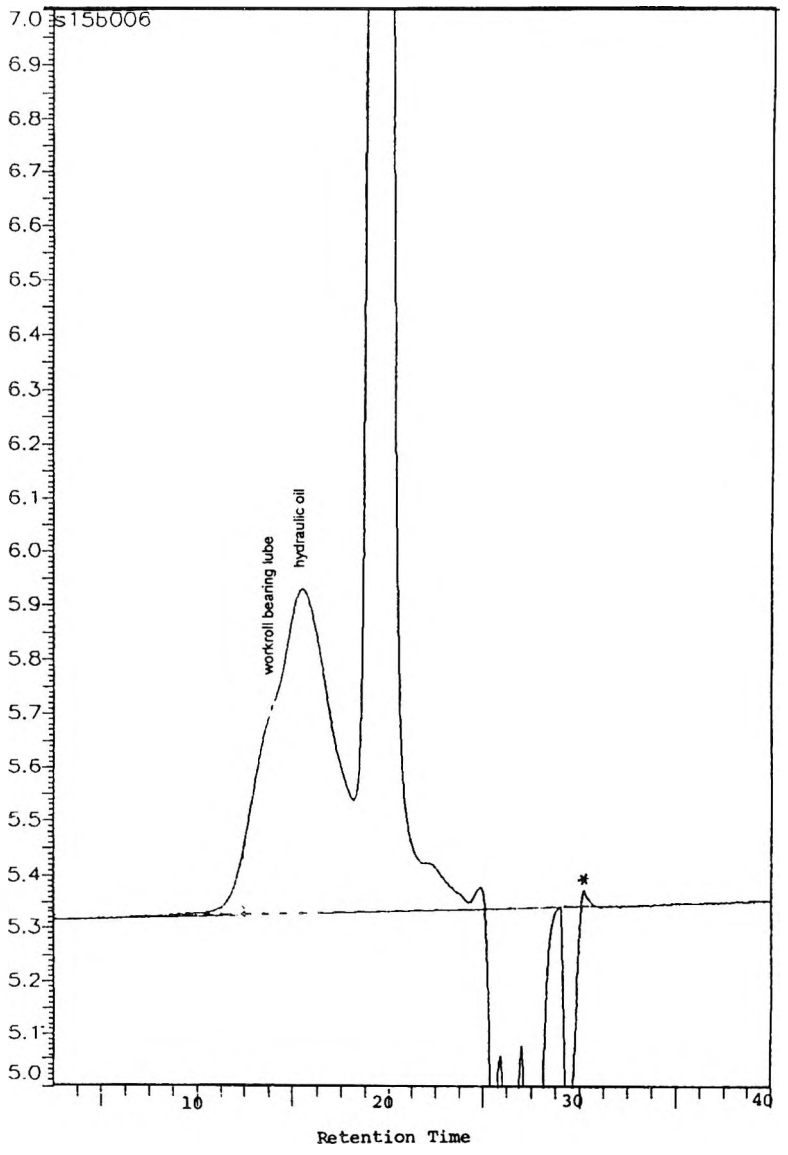


Figure 2. GPC/RI chromatogram of 3% workroll bearing lube and 3% hydraulic oil in C_{15} linear paraffin oil.

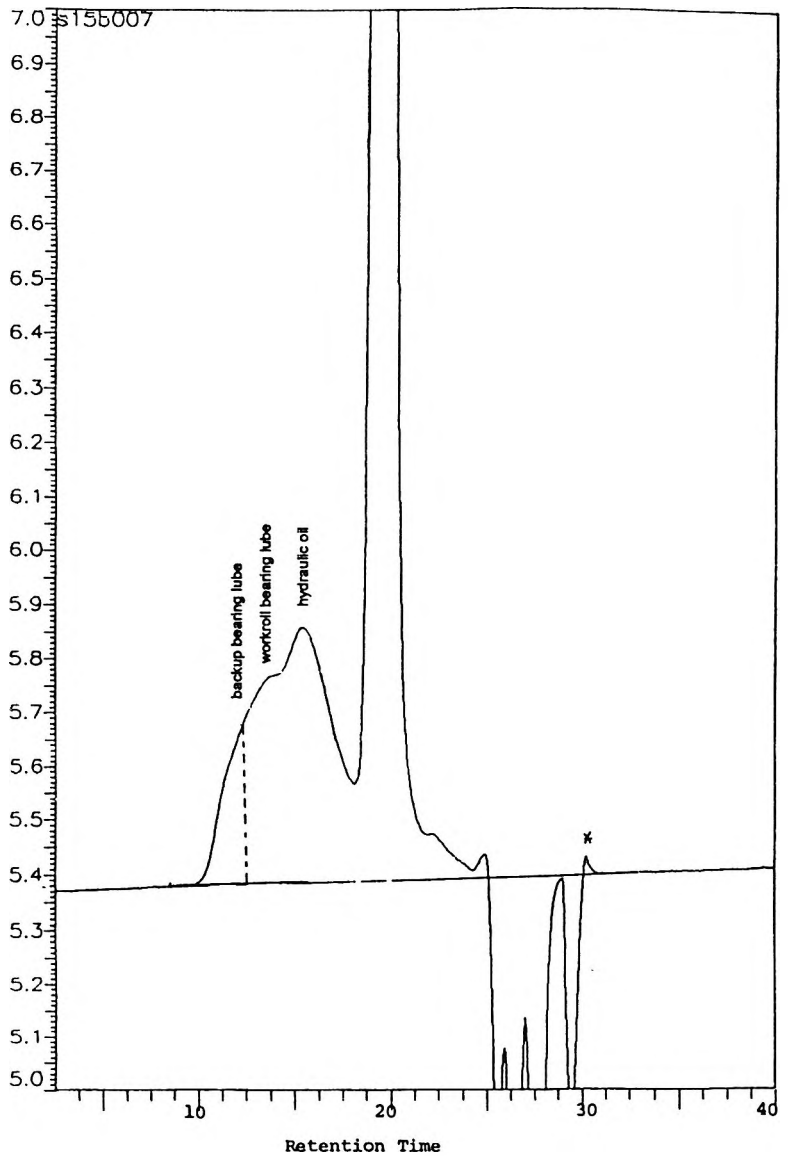


Figure 3. GPC/RI chromatogram of 2% backup bearing lube, 2% workroll bearing and 2% hydraulic oil in C_{15} linear paraffin oil.

EXPERIMENTAL

Chromatographic Conditions

GPC Column:	500, 100 and 100 Å Ultrastyragel columns from Millipore Co.
Mobile Phase:	Tetrahydrofuran (THF) w/o preservative
Flow Rate:	1.0 ml/minute
Injection Volume:	250 μl
Sample Concentration:	2% (wt/vol) in THF
Detectors:	▪ refractive index (RI) detector at 32X ▪ UV or photodiode array(PDA) detector @ 280 nm

RESULTS AND DISCUSSION

Determination of RI Response Factor

Linear paraffin base oils for cold rolling are highly refined and have a significantly narrower carbon number distribution than conventional middle distillate products, such as mineral seal oil. According to our experience, the C_{15} linear paraffin shows a very different response to the RI detector than mineral seal oil. It was not necessary to calibrate GPC/RI results for mineral seal oil due to the similar nature of mineral seal oil and the base oils used in mechanical lubricants. However, it was necessary to determine calibration factors for the C_{15} linear paraffin to calculate the actual percentage of high molecular contaminants. Eight laboratory standards were used for the evaluation. Calibration factors, calculated contaminant concentration and percentages of contaminants from a time slicing program are listed in Table 2.

The average calibration factor calculated for the HPSEC/RI and the C_{15} linear paraffin rolling oil is 0.40. The actual percentage of high molecular weight contaminants used in the C_{15} linear paraffin rolling should be calculated by multiplying the HPSEC/RI time slicing data of the oil by 0.40. Another factor we should consider is the concentration of additive in cold rolling oil. Normally, a control standard is analyzed along with used oil sample for baseline correction. As the time slicing data in Table 1 show, the baseline of 8% additive in the C_{15} linear paraffin oil increases 0.8% when the virgin C_{15} linear paraffin oil without the additive was used as the control standard. It suggests that the amount of additive will affect the time slicing result when the

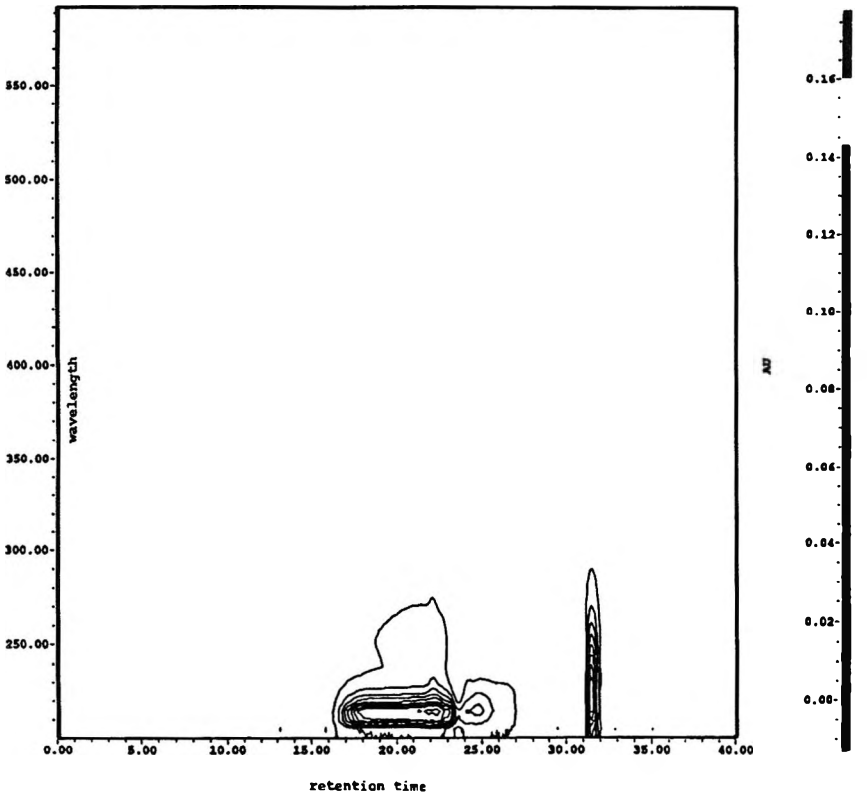


Figure 4. PDA contour plot of 6% backup bearing lube in C_{15} linear paraffin oil.

additive concentration changes. The change of baseline for the additive is about 10% of additive concentration (%) in the C_{15} linear paraffin rolling oil. For the future HPSEC/RI analysis, 8% additive in the C_{15} linear paraffin will be used as the baseline standard for cold rolling oil analysis.

SEC/RI and SEC/PDA Analysis of Standard Solution

Initially the HPSEC/RI method was conducted to investigate the contaminants in laboratory-prepared standard rolling oils. As the RI chromatograms in Fig. 1-3 show, the chromatographic separation between backup bearing lubricant / workroll bearing lubricant, workroll bearing

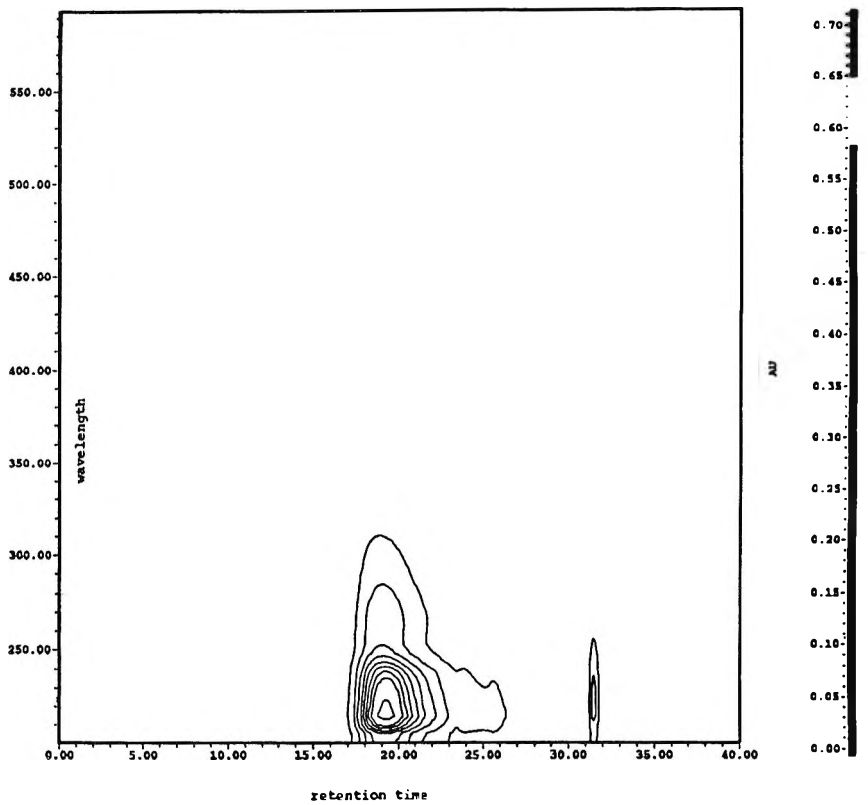


Figure 5. PDA contour plot of 6% workroll bearing lube in C_{15} linear paraffin oil.

lubricant/hydraulic oil and backup bearing lubricant/workroll bearing lubricant/hydraulic oil are very poor. The results suggest that HPSEC/RI alone is not a good method to quantitate the contaminants in rolling oil. However, the PDA or UV detector can help us solve the quantitation problem.

The PDA contour plots of backup bearing lubricant, workroll bearing lubricant and hydraulic oil are shown in Fig. 4-6. The PDA data reveal that all contaminants have UV absorbencies below 270 nm. Workroll bearing lubricant is the only contaminant that has a UV absorbance above 300 nm. The PDA evaluation suggests that (1) UV detection at 280 nm can be used to differentiate workroll bearing lubricant and hydraulic oil contaminant without a interference from backup bearing lubricant, and (2) UV wavelength of 300 nm can be used to analyze workroll bearing lubricant without the interferences from backup

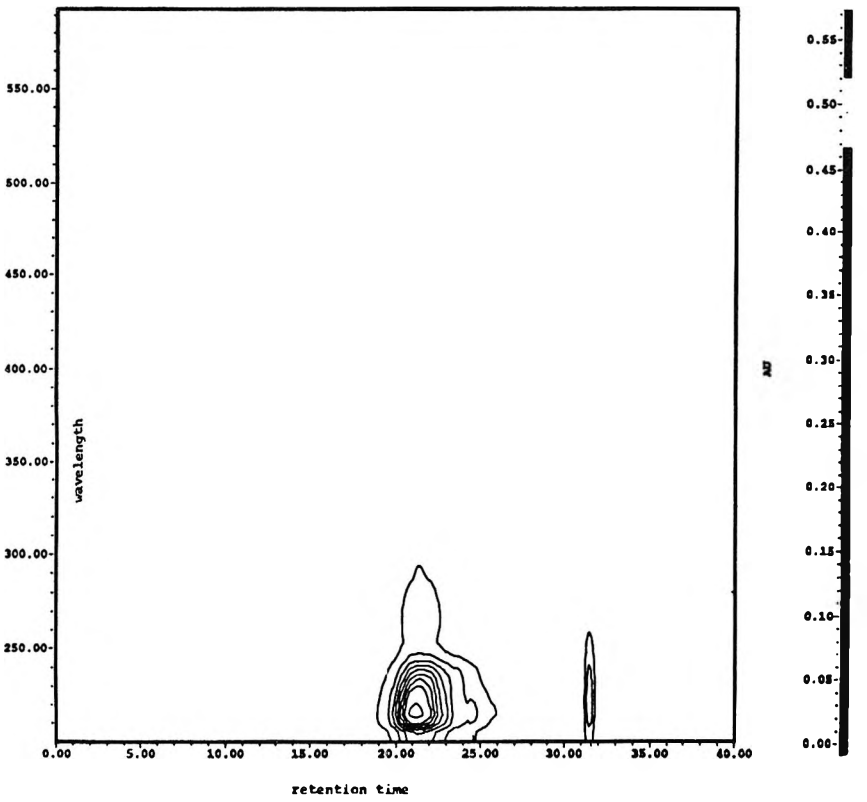


Figure 6. PDA contour plot of 6% hydraulic oil in C_{15} linear paraffin oil.

bearing lubricant and hydraulic fluid. A fair separation was observed in the GPC/UV chromatogram of workroll bearing lubricant and hydraulic fluid (Fig. 7). As the GPC/UV chromatogram in Fig. 8 show, the UV absorbance at 280 nm for backup bearing lubricant is very small compared to that of workroll bearing lubricant and hydraulic fluid (Fig. 9 and 10).

The PDA detector at 280 nm was used for workroll bearing lubricant and hydraulic oil determination. The concentration of backup bearing lubricant was determined using RI detection data. In order to avoid the possible retention time deviation of each run, the location of backup bearing lubricant, workroll bearing lubricant and hydraulic oil peaks are important. Listed below is the way to determine the location for each peak.

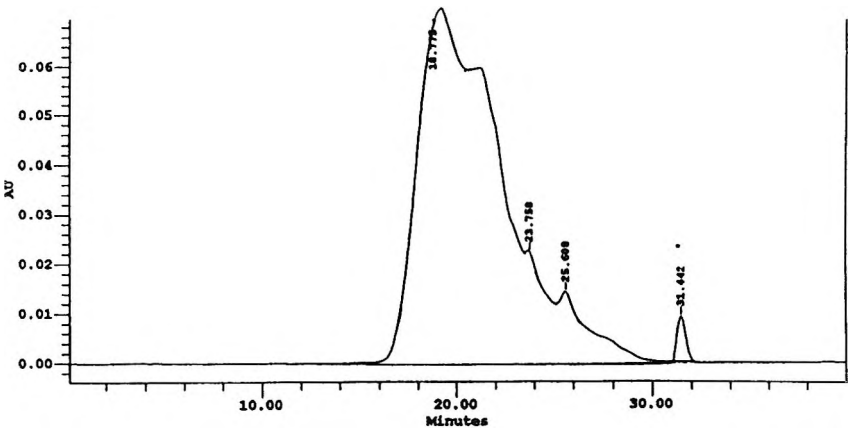


Figure 7. GPC/UV chromatogram of 3% hydraulic oil and 3% workroll bearing lube in C_{15} linear paraffin oil.

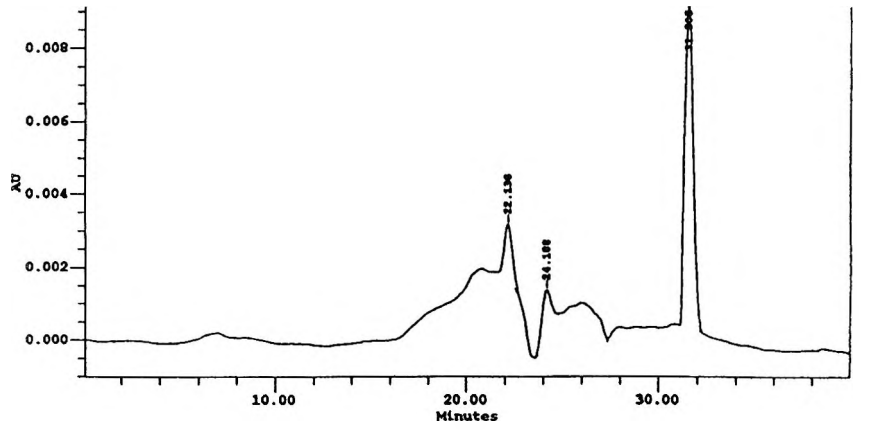


Figure 8. GPC/UV chromatogram of 6% backup bearing lube in C_{15} linear paraffin oil.

Table 3

Measured Peak Height of Contaminants in Standard Solution

Standard	Detector	Backup bearing lubricant	Workroll bearing lubricant	Hydraulic oil
1	RI	0.710	(0.370)*	(0.200)*
	UV	0.001	(0.001)*	(0.002)*
2	RI	(0.01)*	(0.08)*	0.775
	UV	0	(0.01)*	0.066
3	RI	(0.200)	0.750	(0.360)*
	UV	0	0.135	(0.052)*
4	RI	0.355	-	0.500
	UV	-	-	0.0345
5	RI	0.450	0.570	-
	UV	-	0.064	-
6	RI	-	0.39	0.600
	UV	-	0.072	0.060
7	RI	0.300	0.380	0.470
	UV	-	0.045	0.038
8	RI	0.120	-	0.660
	UV	-	-	0.055
9	RI	0.575	-	0.320
	UV	-	-	0.0124
10	RI	0.350	-	0.370
	UV	-	-	0.023
11	RI	0.380	0.360	0.435
	UV	-	0.025	0.031
12	RI	0.280	0.390	0.475
	UV	-	0.047	0.040
13	RI	0.350	0.615	0.630
	UV	-	0.084	0.055

*Data shown in ( ) are the peak height interference to adjacent peaks.

backup bearing lubricant: 8.1 cm from the marked internal peak in HPSEC/RI chromatogram.

workroll bearing lubricant: 5.0 cm from the marked internal peak in HPSEC/UV chromatogram.

hydraulic oil: 4.2 cm from the marked internal peak in HPSEC/UV chromatogram.

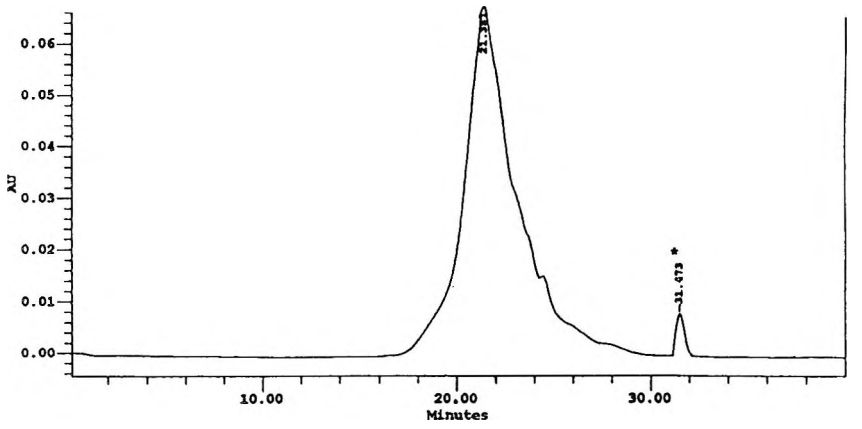


Figure 9. GPC/UV chromatogram of 6% hydraulic oil in C_{15} linear paraffin oil.

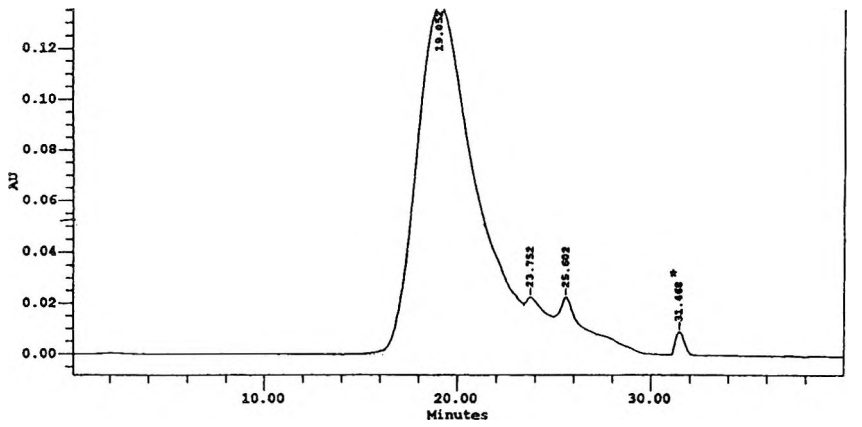


Figure 10. GPC/UV chromatogram of 6% workroll bearing lube in C_{15} linear paraffin oil.

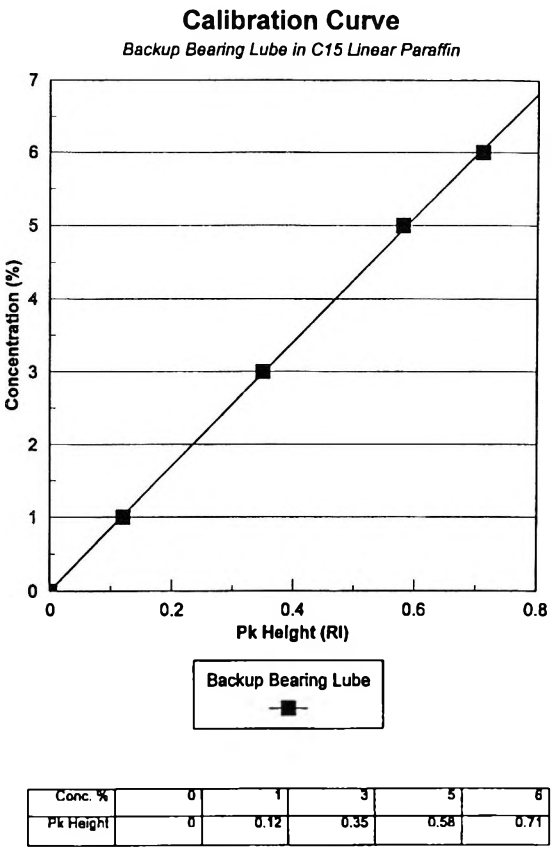


Figure 11. Calibration curve of backup bearing lube in C_{15} linear paraffin oil.

Thirteen standard solutions were analyzed using the HPSEC/RI/PDA method. The UV and RI peak height data for the standard solutions are outlined in Table 3. Calibration curves and formulations for backup bearing lubricant, workroll bearing lubricant and hydraulic oil are shown in Fig. 11-13.

As the data in Table 3 show, peak interferences among the contaminants are present. To improve the method accuracy, these peak interferences should be considered in the concentration calculation. Due to the complexity of the HPSEC/RI/PDA method, a BASIC program was written to reduce operator error and improve the speed and accuracy of the analysis. The BASIC program (Appendix A) developed for the HPSEC/RI/PDA method is attached for your

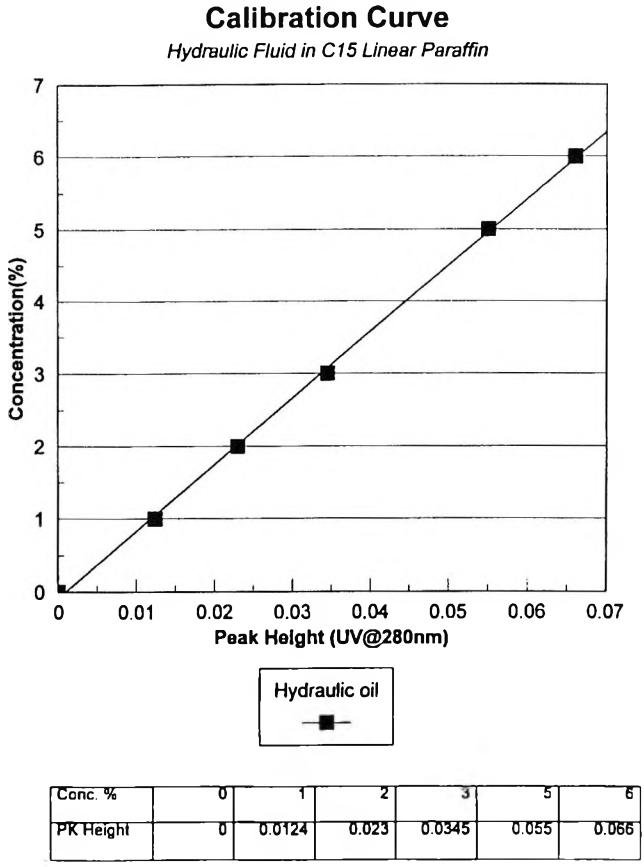
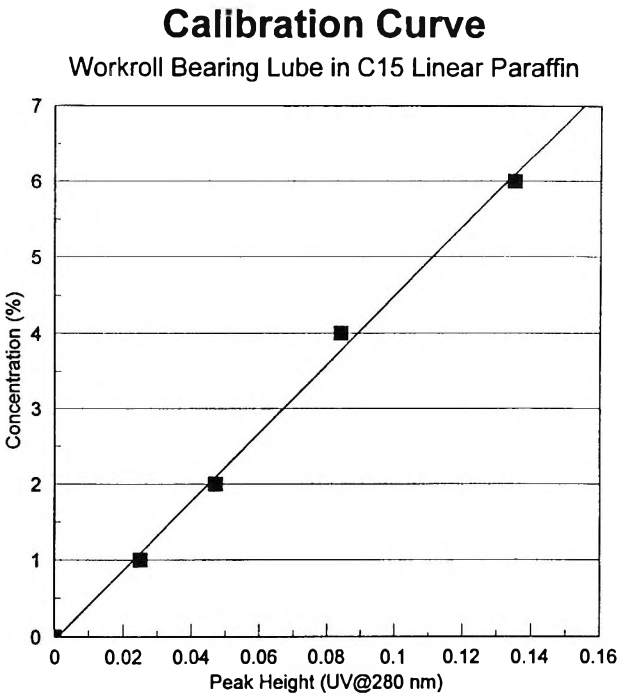


Figure 12. Calibration curve of hydraulic oil in C_{15} linear paraffin oil.

information. Five standard solutions were used to test the accuracy of the calibration method. Their actual and calculated data are listed in Table 4.

The comparative concentrations for backup bearing lubricant, workroll bearing lubricant and hydraulic oil shown in Table 4 indicate that the HPSEC/RI/PDA method is a feasible analytical method for the study. The deviations for backup bearing lubricant, workroll bearing lubricant and hydraulic oil are about 3%, 4% and 7% of the contaminant concentration, respectively.



Conc. %	0	1	2	4	6
Pk Height	0	0.025	0.047	0.084	0.135

Figure 13. Calibration curve of workroll bearing lube in C_{15} linear paraffin oil.

Analysis of Used C_{15} Linear Paraffin Rolling Oil

A used cold rolling oil (12/03/93) was selected as an example to demonstrate the HPSEC/RI/PDA method. Peak heights measured for backup bearing lubricant, workroll bearing lubricant and hydraulic oil are shown in Fig. 14 and 15. Peak heights and calculated concentrations of backup bearing lubricant, workroll bearing lubricant and hydraulic oil are listed in Table 5.

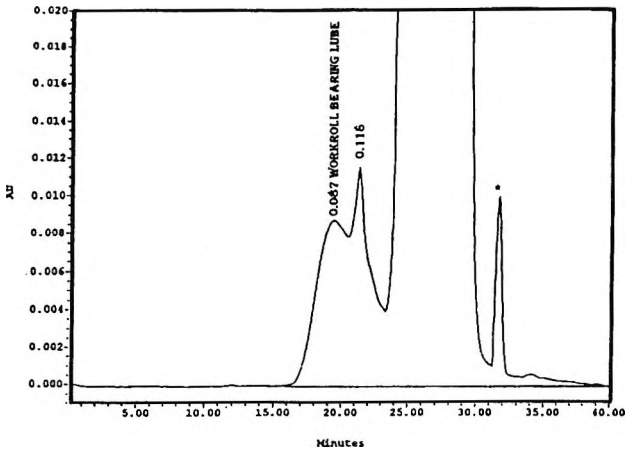


Figure 14. GPC/UV chromatogram of used cold rolling oil.

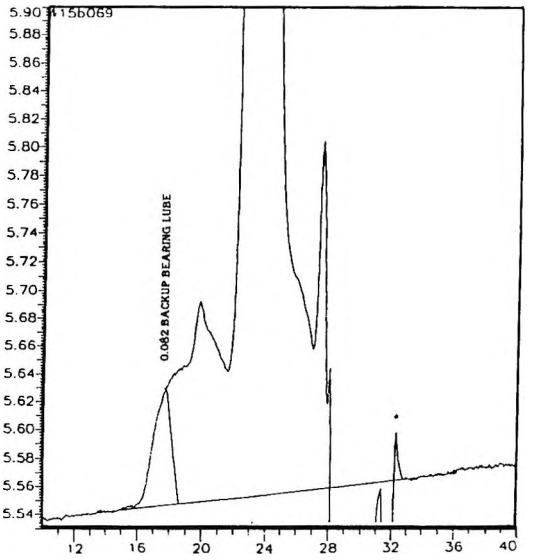


Figure 15. GPC/RI chromatogram of used cold rolling oil.

The total concentration of contaminants calculated by the time slicing program and peak height measurement are 1.46% and 1.56%, respectively. The time slicing data of the oil is used to ensure the accuracy of the HPSEC method. If there are other contaminants which have very intensive UV absorbencies present in the used oil, the total contamination calculated from peak height will

be greater than the time slicing data. The total contamination difference between time slicing and peak height results should be less than 20% of the time slicing data based on our experience.

Table 4

Comparative Data of Contaminants from HPSEC/RI/PDA Method

Standard Solution	Backup Bearing Lubricant (%)		Workroll Bearing Lubricant (%)		Hydraulic Oil (%)	
	Actual	Calc.	Actual	Calc.	Actual	Calc.
7	2.0	1.91	2.0	1.84	2.0	2.25
10	3.0	3.14	0.0	-0.25	2.0	2.11
11	3.0	3.07	1.0	0.96	2.0	1.97
12	3.0	2.91	2.0	2.02	2.0	2.08
13	3.0	2.99	4.0	3.80	2.0	2.21
Avg Dev:	0.08		0.11		0.14	

Table 5

Used C_{15} Linear Paraffin Cold Rolling Oil (12/03/93)

Contaminant	Backup Bearing Lubricant	Workroll Bearing Lubricant	Hydraulic Oil
Peak Height	0.082	0.087	0.118
Calc. Conc.	0.52%	0.27%	0.63%

CONCLUSION

The SEC/RI/PDA method can be used to determine the high molecular weight contaminants in any cold rolling oil. However, RI response factors for the rolling oil and the contaminants should be determined. Standard hydraulic oil, gear oil and bearing oil should be investigated as well for each of the rolling oils to quantitatively and qualitatively determine the amounts of the

high molecular weight contaminants. A BASIC calculation program was written to reduce the operator error and to improve speed and accuracy of the HPSEC method. Since peak height is used for contaminant determination, the injection volume and concentration of oil sample are critical. Sample preparation using wt/wt or wt/vol concentration and a fixed volume sample loop are strongly recommended for the HPSEC method.

A UV detector can be used to replace the photodiode array (PDA) detector for the HPSEC study. Based on the PDA evaluation, a UV detector @ 280 nm can be used to differentiate workroll bearing lubricant and hydraulic oil contaminant without an interference from backup bearing lubricant. The UV wavelength @ 300 nm can be used to analyze workroll bearing lubricant without the interferences from backup bearing lubricant and hydraulic oil.

ACKNOWLEDGMENT

Discussions and comments from Mr. Carroll D. Davis, Supervisor of the Chemistry Section, and Mr. Barry. L. Riddle, Supervisor of the Lubrication and Surface Technology Section, are gratefully acknowledged.

APPENDIX A

```
1 REM COLD ROLLING OIL CONTAMINANT ANALYSIS
5 DIM x(20)
10 DIM y(10)
15 DIM z(10)
20 PRINT: PRINT TAB(6);"** COLD ROLLING OIL CONTAMINANT
ANALYSIS **"
30 PRINT : PRINT TAB(15); "*** by C. B. HUANG, 12/02/93 ***"
40 PRINT : PRINT : INPUT "enter DATE of Cold Rolling Oil Sample,
**/**/**"; D$
50 PRINT : INPUT "enter UV Peak Height of Workroll Bearing Lube"; x(1)
55 PRINT : INPUT "enter UV Peak Height of Hydraulic Oil"; x(2)
60 PRINT : INPUT "enter RI peak height of Backup Bearing Lube"; x(3)
62 PRINT : INPUT "enter the Total %Contamination calculated by time slicing
program"; z(4)
65 LET y(1)=45.4789 * x(1)-.04687
70 LET x(4)=x(2)-y(1)*.008
75 LET y(2)=91.8231*x(4)-.0881
80 LET y(3)=8.62785*x(3)-.0543
```

```
82 IF y(1)/y(3)>.7 THEN GOTO 140
90 LET x(11) = x(1) - y(2) * .0017-y(3) * .0002
95 LET x(12) = x(2) - y(1) * .008 - y(3) * .0003
100 LET x(13) = x(3) - y(1) * .03 - y(2) * .0017
110 LET y(1) = 45.4789 * x(11) - .04687
120 LET y(2) = 91.8231 * x(12) - .0881
130 LET y(3) = 8.62785 * x(13) - .0543
140 LET z(1) = y(1) + y(2) + y(3)
150 LET z(5) = z(4) / z(1)
160 LET y(1) = z(5) * y(1)
170 LET y(2) = z(5) * y(2)
180 LET y(3) = z(5) * y(3)
200 LET z(1) = INT(z(1) * 100 + .55) / 100
210 LET y(1) = INT(y(1) * 100 + .55) / 100
220 LET y(2) = INT(y(2) * 100 + .55) / 100
230 LET y(3) = INT(y(3) * 100 + .55) / 100
235 INPUT "press 'retur' when printer is ready"; A$
240 LPRINT : LPRINT ; LPRINT "*** *** *** *** *** *** *** *** *** ***
*** ***"
250 LPRINT : LPRINT "Date of Cold Rolling Oil ** "; D$; " **"
260 LPRINT : LPRINT " % Backup Bearing Lube = "; y(3)
270 LPRINT : LPRINT " % Workroll Bearing Lube = "; y(1)
280 LPRINT : LPRINT " % Hydraulic Oil = ";
290 LPRINT : LPRINT " % Total Contamination by Time Slicing = "; z(4)
300 LPRINT : LPRINT " % Total Contamination by Peak Height = "; z(1)
310 Input "Do you want to calculate another sample? (y/n)"; A$
320 IF A$ = "y" THEN 40
400 END
```

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HPLC SEPARATION OF CEPHALOTAXINE, HARRINGTONINE AND HOMO-HARRINGTONINE FROM CALLUS AND ROOT CULTURES OF *CEPHALOTAXUS HARRINGTONIA*

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ABSTRACT

A simple extraction and analytical protocol was developed to assay cephalotaxine, harringtonine, and homoharringtonine from callus and root cultures of *Cephalotaxus harringtonia*. The process involves extraction by methanol followed by partitioning between 0.5% ammonium hydroxide and chloroform. The chloroform fraction recovered greater than 90% of all three alkaloids. This fraction was concentrated to dryness, resuspended in methanol and analyzed by high pressure liquid chromatography using a UV detector.

Peaks corresponding to all three alkaloids were identified by comparing their retention times with authentic standards and their identity confirmed by fast atom bombardment spectrometry. The root cultures contained higher levels of harringtonine and

homoharringtonine (2.4 and 3.9 mg/kg dry matter, respectively) compared to the callus cultures; however, the levels of cephalotaxine were comparable (10.2 mg/kg dry matter).

INTRODUCTION

Harringtonine and homoharringtonine are two esters of the alkaloid cephalotaxine, isolated from the *Cephalotaxus* species.[1] Both compounds were selected for preclinical development as new anticancer agents[2] and, to date, have been used in the treatment of different types of leukemia,[3,4] carcinomas,[5,6] and chloroquinine-resistant malaria.[7]

Several studies on the synthesis of cephalotaxine and its structural analogs have been reported recently.[8] However, to date, extraction from plant sources has been the major source of these important alkaloids. The use of callus cultures derived from *C. harringtonia* as an alternative source of these alkaloids has also been reported.[9] Recently, we reported the establishment of fast-growing callus and root cultures of *C. harringtonia*;[10] however, the alkaloid contents of these cultures were not evaluated.

Prior to this report, all the protocols used in the extraction and isolation of these alkaloids from *Cephalotaxus* plant extracts included two or more partitioning steps followed by separation and analysis using counter-current chromatography and/or gas chromatography-mass spectrometry (GC-MS).[1,11,12,13] The analysis of callus cultures reported by Delfel and Rothfus[9] was also performed using the two-step partitioning protocol (chloroform with 2.5% aqueous tartaric acid followed by basification of the aqueous phase with ammonium hydroxide and re-extraction with chloroform) followed by GC-MS analysis, as described above.[1,11] However, a simple, yet efficient extraction and analytical protocol (for separation and identification) that would facilitate the screening of large numbers of plant tissue culture samples has to be developed or modified from existing protocols.[14]

In this report, we present a simple extraction and analytical protocol for characterizing cephalotaxine, harringtonine, and homoharringtonine from callus and root cultures of *C. harringtonia*, via a single-step partitioning process (partitioning between chloroform and 0.5% ammonium hydroxide) followed by high pressure liquid chromatography equipped with a UV detector.

MATERIALS AND METHODS

Plant Tissue Cultures

Callus and root cultures were established as described by us previously.[10] The callus cultures were grown in Magenta vessels and and root cultures were grown in 250 mL flasks. Callus cultures and root cultures were harvested during the log growth phase (four to five weeks and three to four weeks, respectively) and after being left to "age" without being subcultured (ten to twelve weeks and eight to ten weeks old, respectively). They were lyophilized and stored in a desiccator at room temperature until the time of analysis. All samples ranged between one to five grams, on a dry weight basis.

UV Spectra

Cephalotaxine, harringtonine, and homoharringtonine were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) for use as authentic standards, and were resuspended in acetonitrile at a concentration of 250 µg/mL. The UV spectra for all three alkaloids were scanned on a Shimadzu (Model 160U-UV) UV-visual recording spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Extraction

Samples of callus and roots (1 to 5 g) were extracted in methanol (50 to 250 mL) by homogenizing in an Omni-mix homogenizer (Omni International, Waterbury, Connecticut, USA) followed by sonication for five minutes. The extract was concentrated to dryness *in vacuo*, resuspended in 0.5% (v/v) ammonium hydroxide (at a ratio of 10 mL/g of callus or roots), and partitioned with an equal volume of chloroform. For larger volumes, the samples were allowed to stand overnight at 4°C for separation, while smaller volumes (less than 40 mL) were separated by centrifugation (1000*g*). The chloroform fraction was carefully separated and re-partitioned twice with additional 0.5% ammonium hydroxide. Finally, the chloroform fraction was concentrated to dryness under a stream of nitrogen and the residue resuspended in methanol (at the ratio of 1 mL /g of callus or roots).

During the process of developing the extraction protocol, samples fortified with authentic standards were partitioned with either 0.1, 0.5, or 2.0% (v/v) ammonium hydroxide or Milli-Q UF Plus water (Millipore Corp., Bedford,

Massachusetts, USA) and chloroform. This extraction was also compared with the two-step partitioning protocol (chloroform with 2.5% aqueous tartaric acid followed by basification of the aqueous phase with ammonium hydroxide and re-extraction with chloroform) described by Powell et al.[1]

Analytical High Pressure Liquid Chromatography (HPLC)

Instrumentation consisted of a HPLC system equipped with a Waters 600E multisolvent delivery system, a Waters model 700 satellite WISP, and a Waters 484 tunable absorbance detector (all from Millipore Corp., Milford, Massachusetts, USA). Peak areas were calculated with a HP 3394A integrator (Hewlett-Packard Co., Avondale, Pennsylvania, USA).

Separation was performed on a Dynamax 60 Å 8 μm phenyl column (4.6 mm x 250 mm) with a phenyl guard module (Rainin Instrument Co. Inc., Woburn, Massachusetts, USA). A linear gradient was used, starting from 70:15:15 (10 mM ammonium acetate buffer [pH = 4.0] : acetonitrile : methanol) and ending at 55:30:15, in 30 minutes. This ratio was then maintained for the next 30 minutes. The flow rate was held constant at 1.2 mL/min for the entire run. Cephalotaxine, harringtonine, and homoharringtonine were detected by monitoring the absorbance at 291 nm.

The final methanol extract from callus and root samples was filtered through a 0.2 micron nylon filter and used for HPLC analysis.

Identification and Confirmation of the Presence of Cephalotaxine, Harringtonine, and Homoharringtonine

The alkaloids were identified by comparing their HPLC retention times with authentic standards, and also by spiking.

Fractions corresponding to the cephalotaxine, harringtonine, and homoharringtonine peaks were collected from root extracts, concentrated *in vacuo* to remove the organic solvents, and lyophilized to yield a trace residue. This residue was then subjected to fast atom bombardment mass spectrometry along with samples of authentic cephalotaxine, harringtonine, and homoharringtonine, according to a previously published protocol,[15] at the mass spectrometry facility in the Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania, USA.

RESULTS AND DISCUSSION

Cephalotaxine, harringtonine, and homoharringtonine exhibited UV absorbance spectra consisting of a single absorbance peak between 260 and 312 nm, with an absorbance maxima at 289.5, 290.8, and 290.6 nm, respectively. Therefore, the wavelength 291 nm was selected to monitor peaks during HPLC analysis.

Separation of cephalotaxine, harringtonine, and homoharringtonine was achieved, with the peaks eluting after 15.5, 26.1 and 31.9 minutes, respectively (Figure 1). The HPLC protocol was capable of detecting levels of cephalotaxine, harringtonine, and homoharringtonine at amounts as low as five nanograms per injection.

A single partitioning step (partitioning between chloroform and 0.5% ammonium hydroxide) was sufficient to provide a semi-crude extract which contained cephalotaxine, harringtonine, and homoharringtonine, as determined by HPLC (Figure 1). Partitioning with 0.5% (v/v) ammonium hydroxide gave the best recovery of all three alkaloids (greater than 90%), compared to partitioning in 0.1% or 2.0% ammonium hydroxide or Milli-Q water (Table 1). Similar recovery rates were also observed when methylene chloride was substituted for chloroform (data not shown).

Table 1

*** Recovery of Cephalotaxine (CT), Harringtonine (HT), and Homoharringtonine (HHT) following Partitioning with Chloroform and Different Aqueous Phases+**

Aqueous phase	CT (%)	HT (%)	HHT (%)
Milli-Q water	34 ± 2	66 ± 3	68 ± 3
0.1% NH_4OH	64 ± 4	105 ± 5	100 ± 7
0.5% NH_4OH	94 ± 1	108 ± 5	106 ± 3
2.0% NH_4OH	92 ± 2	97 ± 4	95 ± 4

* Recovery is based on the amount of authentic standards that were used to fortify each callus/root sample. Data represents the mean ± SD from three replications.

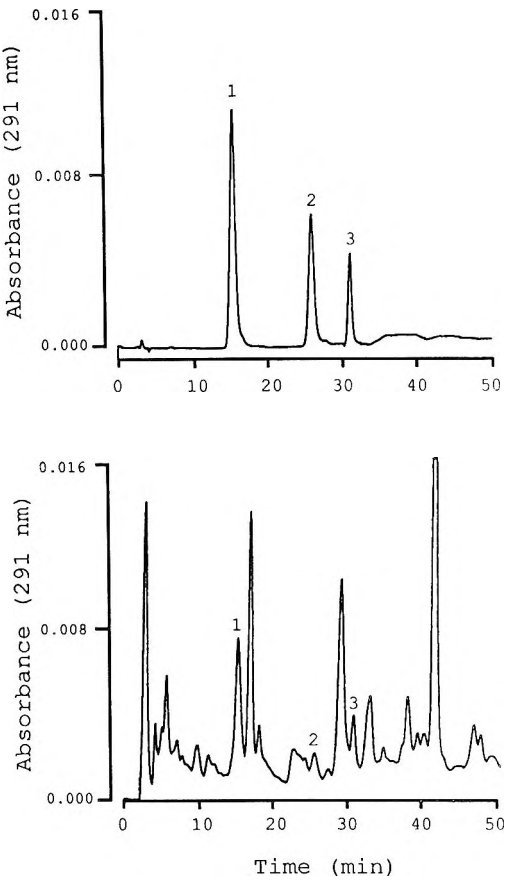

Figure 1. HPLC separation of authentic standards (TOP) and a semi-crude root culture extract following partitioning with 0.5% ammonium hydroxide and chloroform (BOTTOM). Peak number 1 = cephalotaxine, 2 = harringtonine, and 3 = homoharringtonine. Separation was performed on a Dynamax 60 Å 8 µm phenyl column and peaks detected by monitoring absorbance at 291 nm. A linear gradient was used, starting from 70:15:15 (10 mM ammonium acetate buffer [pH = 4.0] : acetonitrile : methanol) and ending at 55:30:15, in 30 minutes. This ratio was then maintained for the next 30 minutes. The flow rate was held constant at 1.2 mL/min during the entire run.

We found that the two-step partitioning protocol (chloroform with 2.5% aqueous tartaric acid, followed by basification of the aqueous phase with ammonium hydroxide and re-extraction with chloroform) described by Powell

et al.[1] gave a recovery of 72% cephalotaxine, 86% harringtonine, and 93% homoharringtonine. Therefore, the single-step partitioning protocol (partitioning between chloroform and 0.5% ammonium hydroxide) described here gave superior recovery of all three alkaloids, compared to the two-step partitioning protocol of Powell et al.[1]

The root cultures and callus cultures that were allowed to "age" contained similar amounts of cephalotaxine (10.2 mg/kg on a dry weight basis). However, the "aged" root cultures had at least two- to three-times more harringtonine and homoharringtonine (2.4 and 3.9 mg/kg on a dry weight basis, respectively) compared to the "aged" callus cultures. These values are two- to five-fold higher than the concentrations found in callus cultures reported initially by Delfel and Rothfus,[9] but comparable with their 1979 report.[16] The young actively growing callus and root cultures contained less than one-tenth the amount of cephalotaxine, harringtonine and homoharringtonine compared to the "aged" cultures (data not shown).

The HPLC peaks corresponding to cephalotaxine, harringtonine, and homoharringtonine exhibited molecular ions $(M + H)^+$ with mass-to-charge ratios of 316, 532, and 546, respectively. These values corresponded to the values of the molecular ions observed from authentic standards of cephalotaxine, harringtonine, and homoharringtonine.

Callus and organ cultures in general, accumulate only a fraction of the levels of the secondary products found in field-grown plants, and therefore, in most cases, the protocols developed for the analysis of whole plants and plant parts cannot be extrapolated to analyze callus and organ cultures.[17] In addition, the metabolites may be stored in different tissues and/or compartments than found in the whole plant, or bound in such a way that they may not be retrieved by conventional extraction methods used with field-grown plants. Therefore, in order to detect the presence of such metabolites, more efficient extraction protocols are needed. Existing extraction protocols could also be simplified due to the absence of very hydrophobic compounds such as oils, waxes, and other complexed cuticular components in callus and organ cultures, otherwise usually associated with plant extracts.

The protocol described in this paper sufficed as the only partitioning step needed to yield a semi-crude extract rich in cephalotaxine, harringtonine, and homoharringtonine from callus and root cultures.

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SEPARATION OF POSITIONAL ISOMERS OF CHLOROPHENOLS BY REVERSE PHASE HPLC

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ABSTRACT

With RP-HPLC, an optimum gradient elution was achieved for separation of 15 chlorophenol positional isomers. Recoveries were satisfactory. The lowest detection limit is 5×10^{-10} g. This method is suitable for determining these substances in environmental samples. The relationship between Capacity Factor (k'), Total Surface Area (TSA), and Molecular Connectivity Index (^{1}x, $^{1}x^{v}$) of chlorophenol congeners was studied, which is reported here for the first time.

INTRODUCTION

Chlorophenols are widely used as intermediates in chemical synthesis of various compounds, and as broad-spectrum biocides and preservatives for leather, glue and some textiles. Thus, chlorophenol congeners have made them ubiquitous contaminants of soil and water. Chlorophenols are highly toxic to man and aquatic organisms; even at very low concentration (less than 1 ng/L) phenols affect the taste and odor of water and fish.[1] In addition, they are also resistant to degradation and tend to bioaccumulate. Therefore, chlorophenols are of great concern in the aquatic environment, and it is necessary to develop a

method for determinating these compounds. Both HPLC-UV and HPLC-EC have succeeded in analyzing chlorophenols. EC detection is more sensitive and selective,[2-4] but it is not suitable for determining all chlorophenols with gradient elution with high sensitivity. This limits the application of EC detection for complete separation of all chlorophenol positional isomers at one injection. UV detection offers the possibility to separate chlorophenols, with a gradient technique, with good resolution, in a short run time. Although several authors reported that they attained a complete separation of chlorophenols using RP-HPLC with gradient elution, there are still some problems with their chromatograms for analysis of real water samples.[3,5,6]

The aims of the present study are (1) to achieve an optimum gradient elution for separating 15 positional isomers of chlorophenol; (2) to develop an optimized method for the determination trace amounts of chlorophenols in water samples; (3) to investigate the relationship between k', TSA and Molecular Connectivity Index.

EXPERIMENTAL

Materials and Reagents

o-Chlorophenol, m-chlorophenol, p-chlorophenol, 2,3-dichlorophenol, 2,5-dichlorophenol, 2,4-dichlorophenol, 3,4-dichlorophenol, 3,5-dichlorophenol, 2,3,6-trichlorophenol, 2,3,4-trichlorophenol, 2,3,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol were obtained from Aldrich Chem. Co., USA. Methanol (HPLC grade), analytical grade glacial acetic acid, butyl acetate and cyclohexane were obtained from Tianjin, China. Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). C_{18} Sep-Pak cartridges (Waters, Milford, MA, USA) were used as solid phase columns.

Instrumentation

The HPLC system (Model 244, Waters) consisted of two LC pumps (Model 510), an injection valve (Model U6K), a UV detector (Model 481), a temperature control system, an automatic gradient controller (Model 680) and a data module (Model 730) for monitoring outputs. Chlorophenols were separated on an analytical reverse phase column which was a 3.9mm i.d. X 150mm Delta Pak C_{18} 300-Å column (Waters). Samples were eluted with a gradient elution program. The effluent was monitored using the UV detector at an analytical wavelength of 280 nm. The column temperature was 35°C.

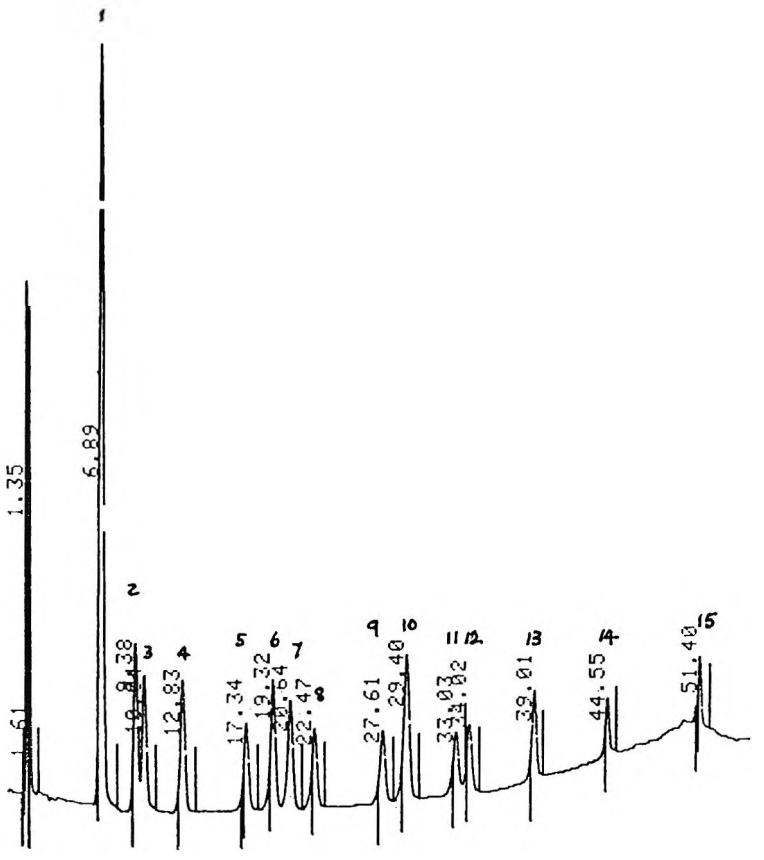

Figure 1. Gradient elution separation of 15 chlorophenols on a Delta C_{18} column using a 50 min linear gradient program as stated in the text. 1: 2-CP; 2: 4-CP; 3: 3-CP; 4: 2,6-DCP; 5: 2,3-DCP; 6: 2,5-DCP; 7: 2,4-DCP; 8: 3,4-DCP; 9: 2,3,6-Tri-CP; 10: 3,5-DCP; 11: 2,3,4-Tri-CP; 12: 2,4,6-Tri-CP; 13: 2,3,5-Tri-CP; 14: 2,3,4,6-Tetra-CP; 15: Penta-CP.

Mobile Phase

Mobile phase A was prepared by mixing 35% methanol and 65% buffer (0.01M sodium acetate + 0.002 M sodium EDTA, pH 4.0). Mobile Phase B was 100% methanol. Prior to use, all the mobile phases were filtered and degassed ultrasonically.

Chlorophenol congeners were weighed accurately and dissolved in an appropriate volume of methanol to prepare a stock solution containing 10^3 μg/mL, and kept at 4°C in the dark. This stock solution was further diluted to obtain working standard solution with a concentration of 10 μg/mL. Using appropriate volumes of this working standard solution, solutions were prepared containing 25, 50, 75, 100, 125, 150 ng/mL of chlorophenol congener mixture, which was used to propare a standard calibration curve.

Sample Preparation

3L water sample was filtered with 0.45 μm filter; 1M NaOH was used to adjust the pH to 11, and subsequently was extracted with 50 mL cyclohexane. The separated organic phase was discarded. The pH of the aqueous phase was adjusted to 3-4 with glacial acetic acid, then the water phase was passed through a C_{18} Sep-Pek for enrichment. Finally, 2 mL of 75% methanol was used to elute chlorophenols from C_{18} cartridge, and the eluate was collected for analysis. Injection volume was 20 μL.

RESULTS AND DISCUSSION

The optimized linear gradient program was as follows:

	Flow Rate (ml/min)	A%	B%
Initial	1.00	100	0
30 min	1.00	75	25
45 min	1.00	46	54
55 min	1.00	46	54

According to these conditions, we obtained a complete separation of 15 positional isomers of chlorophenol. (Figure 1).

The influence of temperature on the chromatographic capacity factor (k') was investigated. Results are shown in Table 1. The capacity factor (k') of chlorophenol isomers decreased with increasing temperature, except for the 2,4,6- and 2,3,4- congeners. It was found that, for temperatures up to 45°C, these two peaks were eluted as a single peak and the resolution was completely

Table 1

Capacity Factor (k') of Chlorophenols at Various Temperatures

No.	Compound	Temperature, °C 25	35	45	γ*
1	2-CP	3.05	2.43	1.95	0.9989
2	4-CP	3.89	3.21	2.57	0.9996
3	3-CP	4.06	3.38	2.73	0.9999
4	2,,6-DCP	4.89	4.16	3.46	0.9999
5	2,3-DCP	5.98	5.17	4.28	0.9985
6	2,5-DCP	6.43	5.60	4.68	0.9984
7	2,4-DCP	6.76	5.90	4.97	0.9987
8	3,4-DCP	7.25	6.32	5.30	0.9985
9	2,3,6-Tri-CP	8.32	7.40	6.38	0.9984
10	3,5-DCP	8.78	7.79	6.68	0.9982
11	2,3,4-Tri-CP	9.03	8.32	7.52	0.9981
12	2,4,6-Tri-CP	9.88	8.87	7.52	0.9939
13	2,3,5-Tri-CP	10.73	9.71	8.57	0.9982
14	2,3,4,6-Tetra-CP	12.30	11.30	10.17	0.9980
15	Penta-CP	14.67	13.65	12.51	0.9982

* Regression Correlation Coefficient.

lost. Therefore, in our experiments, considering the resolution and analysis time, a column temperature of 35°C was selected.

The effect of pH on capacity factor has been reported by Alarcon.[7] In our experiments, the result is consistent with that of Alarcon. Over the range of pH 3.5 - 5, the differences of k' values for the fifteen chlorophenols are significant and a desirable resolution of these positional isomers can be achieved. Thus pH = 4 was selected for this study.

The LDL of fifteen chlorophenol congeners was from 0.5 ng to 5 ng. Within the linear response range of the detector, using 15 μL standard mixture of concentration at 5 ppm, the solution was analyzed six times. The relative standard deviation (RSD) was in the range from 1% to 5% (Table 2).

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Table 2

LDL Precision and Recovery

No.	Compound	LDL(ng)*	RSD(%)	Recovery (X±SD)%** 20μg/L	50 μg/L
1	2-CP	0.5	1	42.5±1.2	51.1±6.5
2	4-CP	2.0	2	50.5±3.3	82.2±5.0
3	3-CP	2.5	2	64.0±4.3	87.1±4.8
4	2,6-DCP	2.5	2	91.6±13.9	89.9±7.6
5	2,3-DCP	5.0	3	95.6±11.7	97.1±5.3
6	2,5-DCP	2.0	2	87.7±12.9	99.3±5.0
7	2,4-DCP	5.0	3	92.1±16.1	97.2±4.4
8	3,4-DCP	5.0	3	97.7±12.3	102.1±3.0
9	2,3,6-TCP	5.0	2	104.4±9.3	98.0±4.0
10	3,5-DCP	2.0	3	91.7±11.4	99.9±4.9
11	2,3,4-TCP	5.0	3	98.8±11.7	101.8±3.6
12	2,4,6-TCP	5.0	3	107.6±16.9	99.8±3.5
13	2,3,5-TCP	5.0	4	95.5±10.7	97.7±4.6
14	2,3,4,6-Tetra-CP	5.0	4	98.2±14.0	96.3±4.5
15	PCP	5.0	5	101.4±10.2	96.5±3.7

* LDL = Lowest Detection Limit evaluated as the amount of chlorophenol that gives a signal two-times greater than the noise level.

** Average of six extractions.

The recoveries are quantitative and greater than 90% for all chlorophenol congeners except for mono-chlorophenol isomers (Table 2). This is because mono-congeners have lower retention on the C_{18} column than other chlorophenols. Thus their recoveries are in the range 42 to 87%, which are increased with higher concentration. This may be due to loss by evaporation during analysis. Therefore the methods proposed is only suitable for quantitative analysis of chlorophenols except for monochlorophenols.

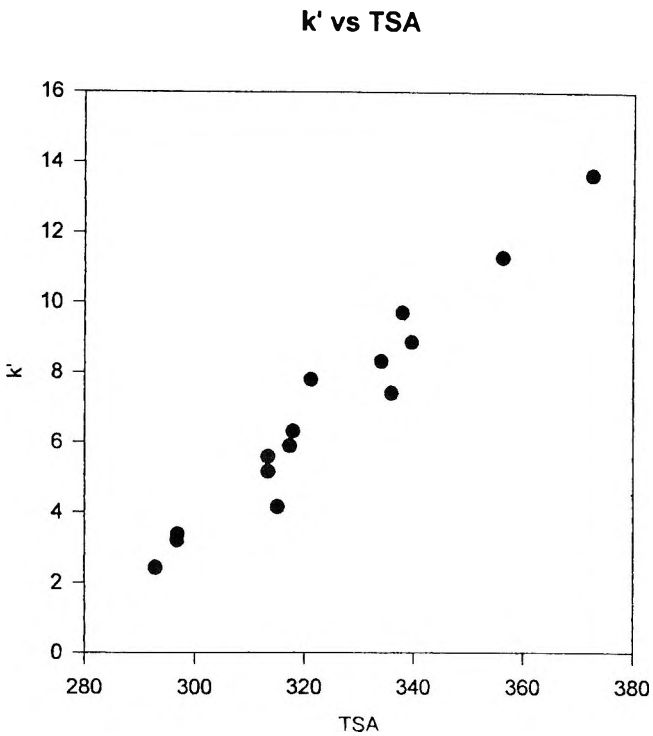

Figure 2. Capacity factor (k') vs total surface area (TSA).

equations are y = 277.1x + 6.82 (r = 0.9767) and y = 2.421 X + 0.1432 (r = 0.9592). There is also good correlation between k' and $^1X^v$ (Y = 2.154x + 0.1655, r = 0.9587). From this observation, it can be predicted that a peak emerged on the chromatogram identifies which chlorophenol congener. When four positional isomers, including 2,6-, 3,5-, 2,3,6- 2,3,5-congeners, are not taken account, the correlation coefficients between k' and those two parameter (TSA and ^1X) reach 0.9983 and 0.9936, respectively. This reveals that some other structural variables other than TSA and ^1X influence the chromatographic behavior of these four isomers. This phenomenon has to be further investigated.

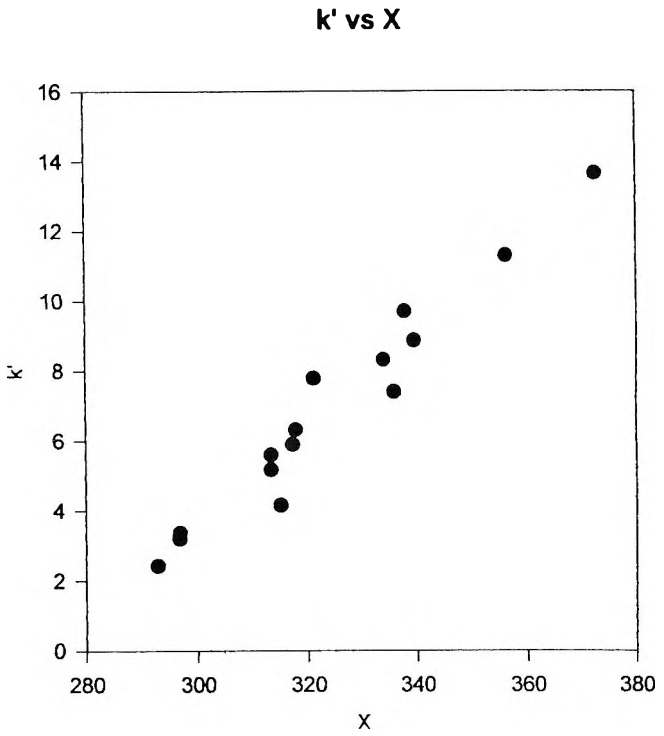

Figure 3. Capacity factor (k') vs molecular connectivity index (^{1}X).

APPLICATION

Four water samples were collected for analysis of chlorophenols. Using the method described previously, mono-, di-, and penta-chlorophenols were determined in these four kinds of water samples (Table 3); addition of standards identified the peaks which eluted, and standard calibration curves were prepared for quantitative analyses. For example, one real water sample analysis is illustrated in Figure 4. Chlorophenols were found in the tap water. This could be due to sterilization with chlorine. Tap water samples were taken early in the morning or in the evening. At these times there was an irritant odor in the tap water. Pentachlorophenol was detected in rain water collected near the main teaching building of Nankai University. This suggests that the the atmosphere above Nankai University was contaminated with pentachlorophenol from a factory nearby, becauce pentachlorophenol can exit as air-particulates in the local atmosphere. Chlorophenols occur in well water.

ERRATUM

H. Guolan, Z. Weihua, Z. Zhiren: "Separation of Positional Isomers of Chlorophenols by Reverse Phase HPLC," J. Liq. Chrom. & Rel. Technol., 19(6), 899-909 (1996).

Figure 3, as originally published, is incorrect. Following is the correct figure.

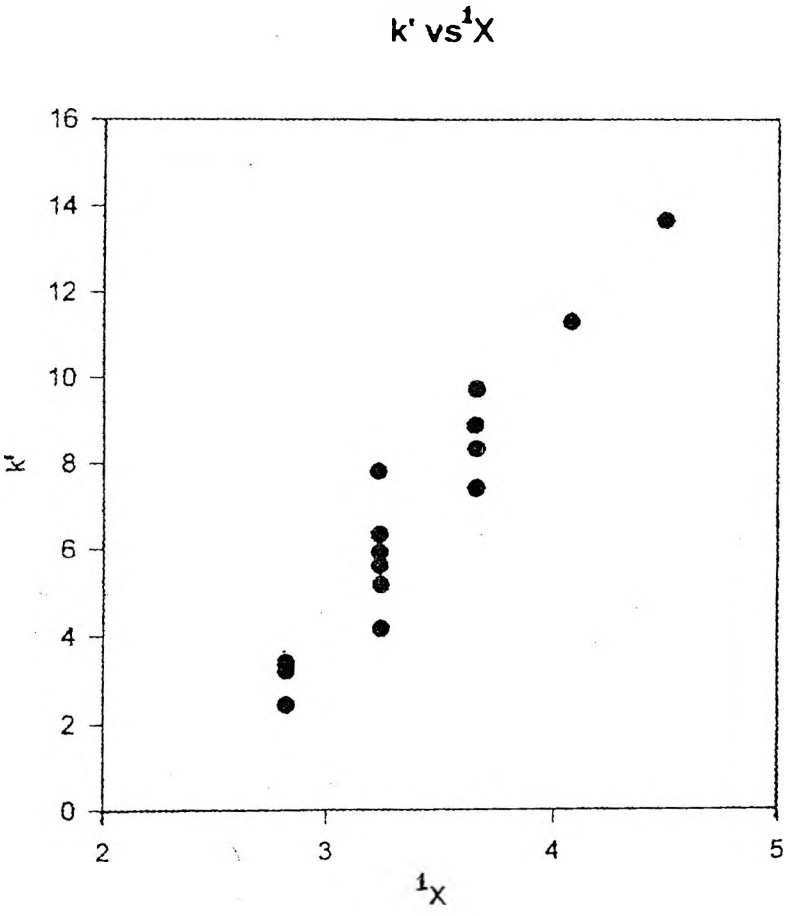

Figure 3. Capacity Factor (k') vs. Molecular Connectivity Index (^1X)

Table 3

Determination of Chlorophenols in Real Water Samples

Sampling Date	Location	Sample	Concentration of Chlorophenols (ppb)*,** PCP	2-CP	2,3-DCP	2,4-DCP	3,5-DCP
5/19/94	Lab at Nankai Univ.	Tap Water	0.40	--	--	0.10	--
11/9/93	Tianjin Paper Mill	Pulp-Bleach Effluent	1.28	--	4.55	--	2.61
5/12/94	Near Main Teaching Bldg. of Nankai Univ.	Rain Water	1.01	--	--	--	--
5/17/94	Tianjin Pesticide Factory	Well Water	1.35	0.05	--	--	--

* Average of two determinations
** -- Not detected

This results, possibly, from contamination by chlorophenol pollutants which were produced during generation processes. These pollutants were drained out with waste water, then permeated through underground soil, eventually leading to the contamination of the well water. Chlorophenol contaminants were also detected in the pulp-bleaching effluent from the paper mill.

This method has the advantages of simplicity, good recovery and replication. In addition, in real sample analysis with complex matrices, it can give a complete separation chromatogram for quantitative analysis .

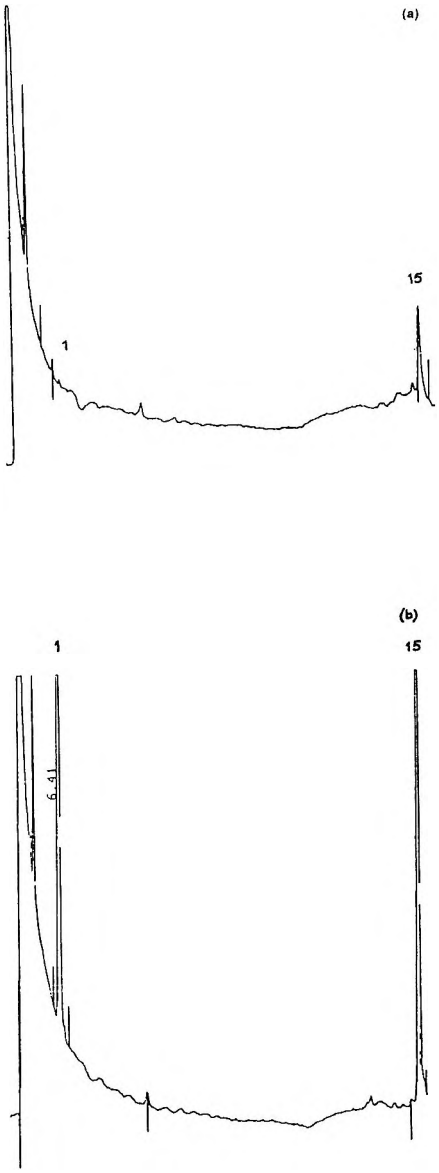

Figure 4. Chromatogram of well water sample on a Delta C_{18} column using a 50 minute linear gradient program as described in the text; (a) Real sample; (b) Real sample + standards. For peak identification, see Table 1.

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SIMULTANEOUS SEPARATION OF PHENYLUREA-, TRIAZINE- AND PHENOXY-ACID HERBICIDES BY REVERSE PHASE ION-INTERACTION HPLC. APPLICATION TO SOIL ANALYSIS

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ABSTRACT

An ion interaction method is presented for the simultaneous separation of herbicides belonging to different structural classes. Phenylurea-, triazine- and phenoxyacid derivatives are considered and the method is applied in the analysis of typical Italian soils characterized by different composition.

INTRODUCTION

Phenoxyacids, urea- and triazine- herbicides are widely used for weed control in agriculture and forestry.[1] Because of their low biodegradability and low volatility, the herbicides and their residuals can easily cumulate in soils and become phytotoxic for sensitive crops cultivated in rotation.

Multiresidual methods for soil analysis are therefore required not only to evaluate source and extent of pollution, but also to learn about possible phytotoxicity problems.

Literature reports different methods for herbicide determination which employ immunoanalytical techniques;[2-4] gas-chromatography with ECD,[5-8] NPD[5,9,10] and MS detection;[11-13] HPLC with UV, diode-array,[8,14-16] fluorimetric[17] or MS detection[18,19] and MS-MS[20] technique. Generally the methods concern the separation of herbicides characterized by similar structure and chemical properties.

This paper presents an ion-interaction HPLC method for the simultaneous separation of herbicides belonging to different functional classes and in particular triazinic, ureic and phenoxyacid pesticides. The method is then applied in analysis of soils.

EXPERIMENTAL

Apparatus

A horizontal mechanic shaker (Shaker 309), a centrifuge ALC 4226 and a Rotovapor Heidolph VV 2000, equipped with a thermal bath, were employed in the extraction process.

The chromatographic analysis was performed with a chromatograph Merck-Hitachi (Tokyo, Japan) Lichrograph model L 6200 with a two channel D 2500 chromato integrator interfaced with a UV-Vis detector L 4200 and with a conductivity detector of the same manufacturer.

A spectrophotometer, Hitachi 150-20, was used for absorbance measurements.

pH values were measured with a Metrohm (Herisau, Switzerland) 654 pH-meter provided with a combined glass calomel electrode.

Chemicals and Reagents

o-Phosphoric acid, acetonitrile, dichloromethane, methanol were analytical grade reagents from Fluka (Buchs, Switzerland).

2,4-D, dichlorprop, 2,4,5-T, diuron, isoproturon, fenuron, 2,4-DB, bromacil, terbumethon, terbutylazine and propazine were from Lab Service Analytica (Anzola dell'Emilia, Bologna, Italy).

Ultrapure water from a Millipore (Milford, MA) MilliQ system was used for the preparation of solutions.

Filters, Merck (Darmstadt, Germany) Anotop 25 Plus (0.22 μm), were employed in the sample preparation.

Chromatographic Conditions

The stationary phase was a reverse phase Phase Separations (Desidee, CLWYD, UK) Spherisorb ODS-2 5S cartridge type column, 250x4.6 mm (5 μm), together with a guard precolumn, Merck (Darmstadt, Germany) LiChrospher 100 RP 18 (5 μm). The experimental mobile phases were 5.0 mmol/L solutions of n-octylammonium o-phosphate prepared in acetonitrile/water at different volume ratios and brought to pH 6.4±0.1 with o-phosphoric acid.

According to proposed models,[21-24] the ion interaction reagent contained in the mobile phase is bound through adsorptive and electrostatic forces onto the surface of the stationary phase, where it gives rise to an electrical double-layer. The interaction properties of the reverse phase packing material are, therefore, modified; the new surface is able to retain anionic and cationic species. About one hour is required for the modification process. Every third day of use, the column is washed (flow-rate 1.0 mL/min) with ultra pure water (20 min), acetonitrile/water 1:1 V/V (20 min) and acetonitrile (10 min).

Dead time was evaluated through conductimetric detection of unretained sodium ion, injected as $NaNO_3$ (15 mg/L).

Retention time reproducibility is within 2% for the same mobile phase preparation and always within 5% for different preparations.

Table 1

Soil Characteristics

Soil	Organic Carbon, %	pH	Clay, %
Fossano	0.91	6.5	10.0
Carpi	0.98	8.0	43.4
Macomer	11.31	5.6	2.9

Preparation of the Standard Solutions

The standard solutions were prepared every 20 days, at concentration of 100.0 mg/L, in acetonitrile from analytical grade standards and stored in brown bottles at 4°C. Working solutions were prepared in water/acetonitrile in the same ratios as in the mobile phase just before the injection into the HPLC system.

Soil Residue Extraction Procedure

An extraction procedure was developed which is able to simultaneously extract from soil all the herbicides investigated. The recovery yields were evaluated for typical Italian soils respectively sampled at Fossano (Piemonte, North-West Italy), Carpi (Emilia, Mid Italy) and Macomer (Sardinia Island, South-West Italy), which are characterized by different compositions (as listed in Table 1).

The method is based on the procedure reported by Meier et al.[25] and modified as follows. Before fortification, the soils were dried to 10% moisture and passed through a 2 mm sieve. 50 g aliquots of soil were then placed into 300 mL screw capped PVC bottles and 2 mL of an aqueous solution of a mixture of the herbicides (5 mg/L each) was added to provide a fortification level of 0.2 mg/Kg. The samples were then added with 50.0 mL of 0.01N NaOH, shaken on a mechanical shaker for 15 min and centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a 300 mL PVC bottle.

Two extractions were performed, each with 50 mL of 0.01 N NaOH and the extracts combined. 10 mL of 1.00 N HCl were then added, the extract centrifuged for 10 min at 4000 rpm and the supernatant transferred to a separatory funnel and shaken three times with portions (50 mL) of dichloromethane. Dichloromethane extracts were dried over anhydrous sodium

sulfate and evaporated to dryness under vacuum on a rotovapor (water bath 30°C). The residue was then dissolved in acetonitrile/water 50/50 V/V and the final volume adjusted to 5.0 mL. The extracts, filtered through 0.22 μm Anotop 25 Plus filters, were analyzed under the HPLC optimized conditions. Further dilutions of the extracts (up to 1/40 V/V) were also performed in order to minimize the matrix effects.

RESULTS

Development of the Method

As previously mentioned, the present study is devoted to the development of a separation method for herbicides belonging to different functional classes, in consideration that: a) commercial formulations often contain mixtures of different herbicides, b) the different and the generally low herbicide biodegradability can induce cumulating processes, which lead to the presence in soils of herbicides of different structure.

Triazinic, ureic and phenoxyacid herbicides are here considered and namely: propazine (2-chloro-4,6-bis(isopropylamine)-s-triazine), terbutylazine (2-*tert*-butylamino-4-chloro-6-ethylamino-1,3,5-triazine), fenuron (N,N-dimethyl-N-phenylurea), isoproturon (N,N-dimethyl-N'-[4-1-methyl-ethyl)phenyl]urea), diuron (N'(3,4-dichlorophenyl)-N,N-dimethylurea), terbumethon (2-*tert*-butylamine-4-ethylamine-6-methoxy-1,3,5-triazine), 2,4-D ((2,4-dichlorophenoxy)acetic acid), dichlorprop ((±)-2-2-(2,4-dichlorophenoxy)propionic acid), bromacil (5-bromo-3-*sec*-butyl-6-methyluracil), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 2,4-DB (4-(2,4-dichlorophenoxy)butyric acid). Their structures suggest the use of ion-interaction chromatographic methods, already developed in this laboratory for the simultaneous separation of anionic species and species which contain protonable nitrogen atoms.[23,24]

The absorbance spectra recorded between 200 and 400 nm for the analytes investigated indicated 228 nm as the wavelength which offers the best average absorbance for all the compounds investigated.

In order to obtain the separation of the largest number of herbicides within reasonable analysis times, the chromatographic conditions of ion-

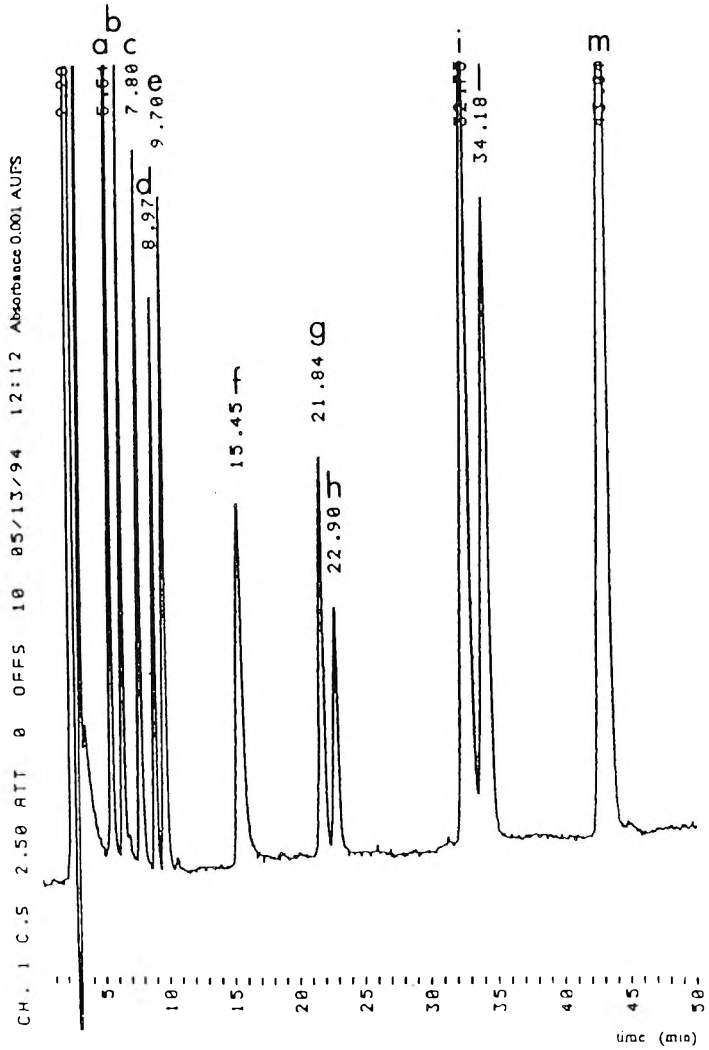

Figure 1. Chromatogram of the standard mixture. a: fenuron (100.0 μg/L), b: 2,4-D (100.0 μg/L), c: dichlorprop (100.0 μg/L), d: bromacil (100.0 μg/L), e: 2,4,5-T (100.0 μg/L), f: 2,4-DB (100.0 μg/L), g: isoproturon (70.0 μg/L), h: diuron (50.0 μg/L), i: terbumethon (100.0 μg/L), l: propazine (50.0 μg/L), m: terbutylazine (100.0 μg/L). Stationary phase: Phase Separations Spherisorb 5S ODS-2 (250x4.6mm; 5 μm); mobile phase: 5.0 mmol/L n-octylamine in water/acetonitrile (65/35) brought to operational pH=6.4 with o-phosphoric acid. Injection volume 100 μL. Flow rate: 1.0 mL/min. Spectrophotometric detection at 228 nm.

interaction reagent (concentration and properties) and organic modifier concentration in the mobile phase were optimized. The concentration of octylammonium o-phosphate (used as the ion interaction reagent) was varied between 1.0 and 10.0 mmol/L with acetonitrile concentrations in the mobile phase ranging between 25 and 40%. A pH value of 6.4 was chosen, which permits the formation of ionized species from both basic (urea and triazine derivatives) and acidic (phenoxyacids) pesticides.

Because, due to their more lipophilic properties, the triazine- derivatives generally show, under these conditions, higher retention with respect to phenoxyacids, gradient elutions were investigated. It is worth mentioning that the use of gradient elution in ion-interaction chromatography is a very controversial point, because some authors advantageously use gradient elutions while others affirm that the gradient elution mode is not suitable. Our results agree with the latter opinion; just when the gradient conditions were imposed, high baseline noise together with uncontrolled and non-reproducible conditions were obtained.

Very likely, when the ion-pair reagent is present in the mobile phase at concentrations which are high enough to assure to the analyte more lipophilic properties but not to induce a surface modification of the stationary phase, gradient elution can be advantageously used. When, on the contrary, as in our conditions, the ion interaction concentration is high enough to cause the dynamic modification of the surface, the increasing concentration of the organic modifier disturbs the conditions of dynamic equilibrium which have been established between the moiety adsorbed onto the stationary phase surface and the mobile phase.

Therefore, the best conditions for the separation of the mixture turned out to be: isocratic elution, 5.0 mmol/L n-octylammonium o-phosphate, acetonitrile concentration 35%, pH=6.4, flow-rate 1.0 mL/min, UV detection at 228 nm.

As an example, Figure 1 shows the separation obtained under these conditions for a mixture containing the following eleven herbicides: fenuron, dichlorprop, 2,4-D, bromacil, 2,4,5-T, isoproturon, diuron, 2,4-DB, terbumethon, propazine and terbutylazine, at concentration of 100.0 μg/L (or less) each.

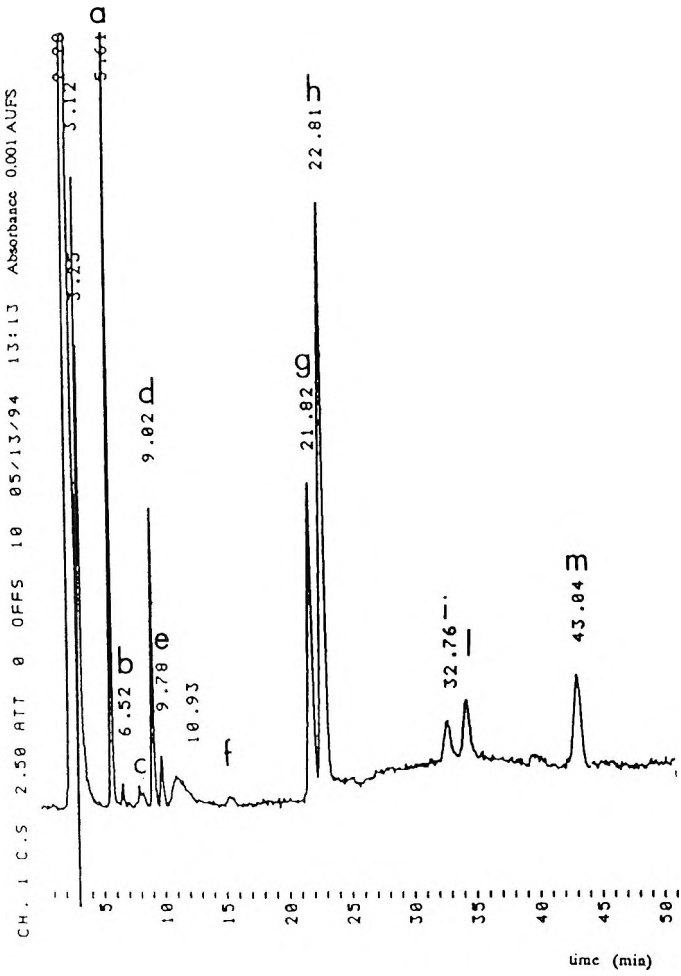

Figure 2. Elution of the standard mixture of Figure 1 in the same chromatographic conditions. Spectrophotometric detection at 254 nm.

Taking into account the different absorbance spectra of the components of the mixture, detection at different wavelengths was also investigated, in order to obtain conditions of partial spectral selectivity and for identification purposes. Figures 2 and 3 show, as an example, chromatograms recorded for the same mixture as in Figure 1 under the same chromatographic conditions, except for the detection wavelengths which, respectively, are 254 and 288 nm. At 254 nm (Figure 2) the urea derivatives and bromacil can easily be resolved

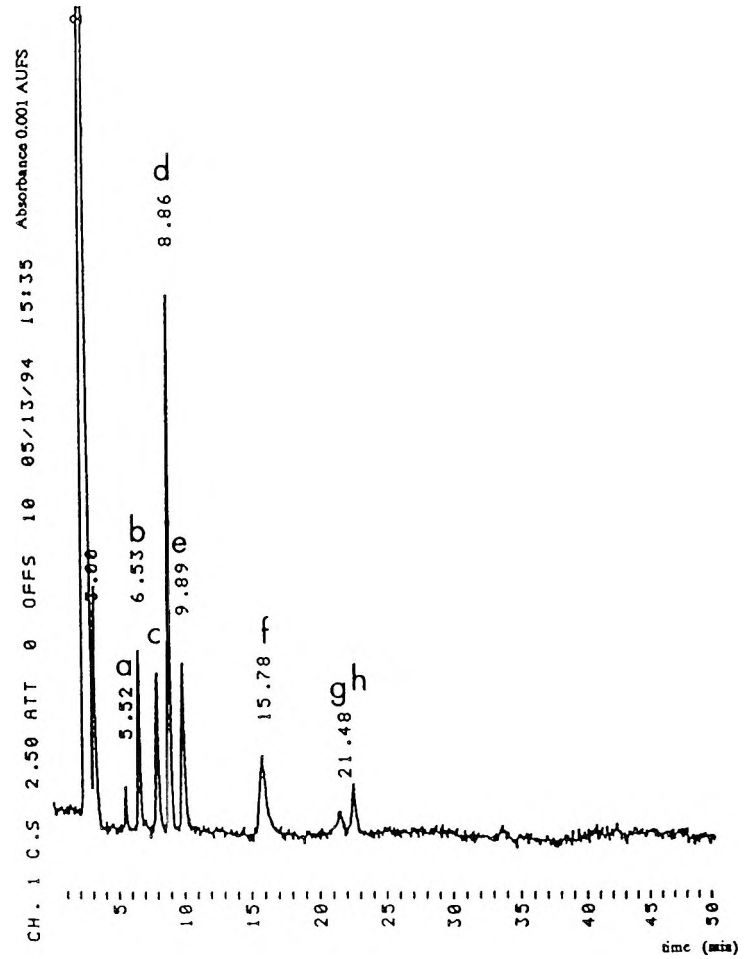

Figure 3. Elution of the standard mixture of Figure 1 in the same chromatographic conditions. Spectrophotometric detection at 288 nm.

from phenoxyacids and triazines which do not absorb significantly and, in turn, at 288 nm (Figure 3), only bromacil is clearly detected because triazine derivatives do not absorb and phenoxyacids and ureic derivatives show a very low absorbance.

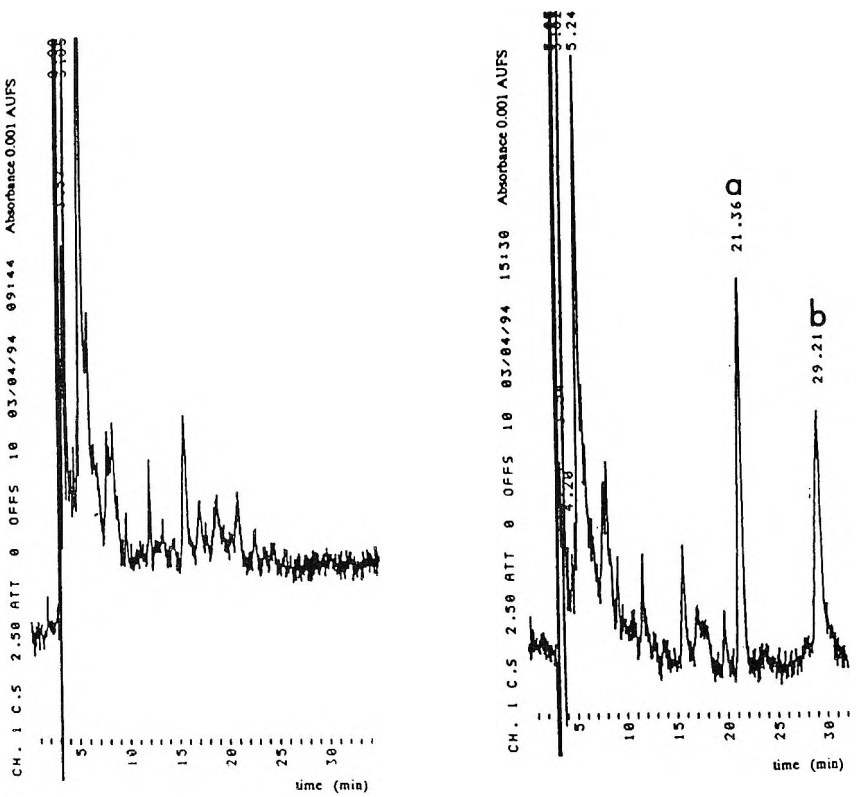

Figure 4. Chromatographic analysis of Fossano soil sample. (A): native sample extracted and diluted to final volume of 100 mL; (B): same treatment on sample added of a: 0.200 mg/Kg of 2,4-D and b: dichlorprop. Stationary phase: Phase Separations Spherisorb 5S ODS-2 (250x4.6mm; 5 μm); mobile phase: 5.0 mmol/L n-octylamine in water/acetonitrile (73/27) brought to operational pH=6.4 with o-phosphoric acid. Injection volume 100 μL. Flow rate: 1.0 mL/min. Spectrophotometric detection at 228 nm.

Application to Soils

The method was then applied for the analysis of three samples of soils representative of the Italian geopedology and, namely, from Fossano (North Italy), Carpi (Mid-Italy) and Macomer (Sardegna Island) and characterized by a different content of organic carbon and clay (see Table 1).

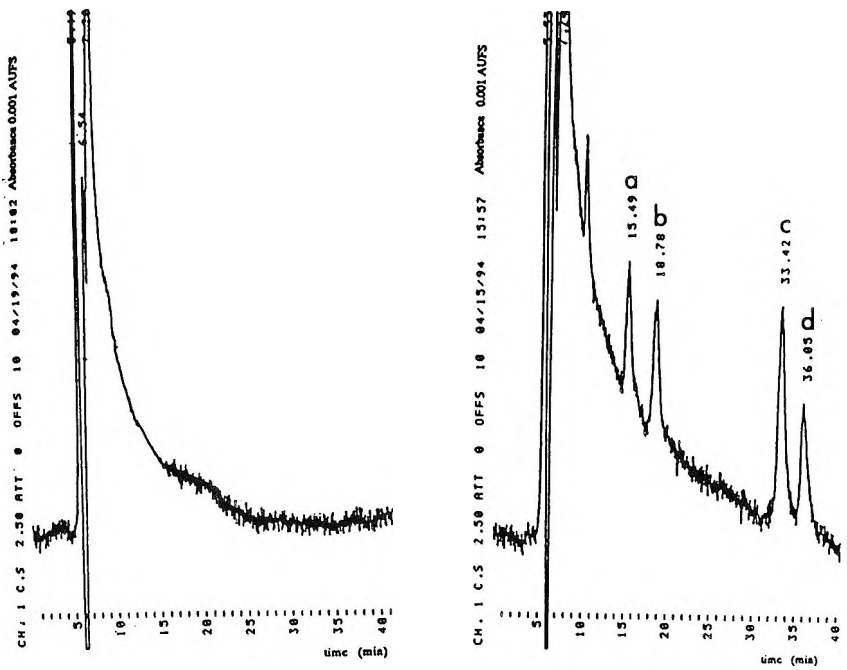

Figure 5. Chromatographic analysis of Macomer soil sample. (A): native sample extracted and diluted to final volume of 200 mL; (B): same treatment on sample added of 0.200 mg/Kg of a: 2,4-D, b: dichlorprop, c: isoproturon and d: diuron. Stationary phase: Phase Separations Spherisorb 5S ODS-2 (250x4.6mm; 5 μm); mobile phase: 5.0 mmol/L n-octylamine in: water/acetonitrile (65/35) brought to operational pH=6.4 with o-phosphoric acid. Injection volume 100 μL. Flow rate: 0.5 mL/min. Spectrophotometric detection at 228 nm.

In order to consider matrix interference as well as the variability of the sample composition, a blank of each soil was extracted according to the described procedure and a series of chromatograms was recorded by sequential dilutions of the extract until acceptable baselines were obtained. Fortunately, the chromatograms showed that the time windows corresponding to the investigated pesticides can be considered free from matrix interference.

As an example, Figure 4 shows (A) the chromatogram recorded for the extract of native Fossano soil and (B) the chromatogram of the extract of the same sample after fortification with 2,4-D and dichlorprop (0.2 mg/Kg each).

Table 2

Pesticide Recoveries

Herbicide	Fortification Level (mg/Kg)	Number of Assays	Mean Recovery, %	S.D. ±
2.4-D	0.20	8	74.8	8.9
Dichlorprop	0.20	7	69.4	10.6
2,4,5-T	0.20	3	63.7	10.2
Isoproturon	0.20	3	57.7	10.1
Diuron	0.20	3	55.0	8.5

Figure 5 shows the chromatograms of the extracts of Macomer soil before (A) and after (B) contamination with 2,4-D, dichlorprop, isoproturon and diuron (0.2 mg/Kg each).

The residue fortification and the extraction steps were performed for all the soils considered and the standard addition method was employed in order to evaluate the percentual recovery yields. All the experiments were repeated three times and acceptable linearities were always obtained (regression correlation factors r^2 always > 0.90).

The results obtained are reported in Table 2 and show that the recovery percent yields range around 64%. The yield is not very high, but these values are of the same order as those obtained by Meier et al.[25] for phenoxyacid herbicides and depend on the complexity of the matrix.

In conclusion, the method presented here permits the simultaneous determination, in soils, of herbicides belonging to different chemical classes and, in particular, of herbicides with cationic properties (like triazine- and phenylurea- derivatives) and anionic (like phenoxyacids) at the natural pH of water and soil.

The method, moreover, can be used for soils of different origin and composition.

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DETERMINATION OF TRANYLCYPROMINE IN URINE AND PHARMACEUTICAL FORMULATION BY HPLC USING SYMMETRY COLUMN

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ABSTRACT

A simple isocratic reversed phase HPLC method for the determination of tranylcypromine sulfate (TCP) is described. The column used is Symmetry C_{18} (Waters Corp., Milford, MA, USA) and the mobile phase consists of 50 mM KH_2PO_4 (pH 4.55):methanol:water (20:10:70). Tranylcypromine sulfate is monitored by U.V. detection at 264 nm. The calibration curve of tranylcypromine sulfate is constructed over the range 25 nmol - 375 nmol/mL with a correlation coefficient 0.999. The method is specific and sensitive with a lower limit of detection 5 nmol/mL, the intra-assay and inter-assay relative standard deviation were below 10%. The validated assay procedure was applied to determine the tranylcypromine sulfate in urine and also to evaluate TCP in pharmaceutical preparation. Detailed methodology and the advantage of this C_{18} reversed phase is presented.

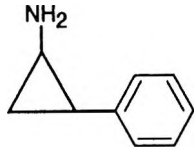

Figure 1: The chemical structure of tranylcypromine (TCP).

INTRODUCTION

Tranylcypromine (TCP) is one of many psychotropic agents which is administered as racemate, chemically known as (±) trans-2-phenylcyclopropylamine (Figure 1). Tranylcypromine is a monoamine oxidase (MAO) inhibitor. It is used as an antidepressant drug, particularly for atypical depression and for the treatment of phobias.[1] It was withdrawn from the market because of severe side effect related to MAO activity such as hypertensive episodes and cerebrovascular accident.[1] In 1964, it was reinstated for limited use.[2]

Baldessarini[1] reported that the antidepressant action of TCP is generally assumed but not definitely shown to be due to MAO inhibition. The same report stated that the maximal oxidase inhibition is produced in few days, whereas, the antidepressant effect takes two to three weeks to develop.[1] It was also proposed that catecholamine uptake inhibition as a possible mechanism for antidepressant effect of TCP.[3]

Furthermore, it is reported that chronic but not acute treatment with low doses of TCP increases extracellular 5-hydroxytryptamine (5-HT) concentration, suggesting that clinical effect of this MAO inhibitor are related to its capacity to enhance serotenergic transmission.[4]

It may be essential to monitor TCP concentration in biological fluids. Herein we describe a simple reliable and rapid HPLC method for the determination of TCP sulfate in biological fluid and pharmaceutical preparation.

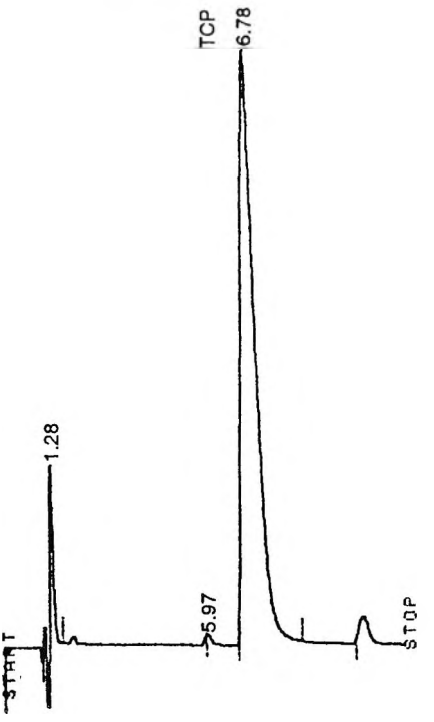

Figure 2: Chromatogram of tranylcypromine. Column:Symmetry C_{18} (150 mm x 3.9 mm I.D., particle size 5 μm); mobile phase; 50 mM KH_2PO_4 (pH 4.55):methanol:water (20:10:70); flow rate: 1 mL/min; chart speed: 0.5 cm/min; temp 23°C; detector UV 264 nm; sensitivity 0.01 aufs; sample quantity: 1 nmol.

MATERIALS AND METHODS

Chromatography

The HPLC system consisted of a Bio-Rad 1350 Solvent Delivery Pump, a Rheodyne Model 7125 Injector, a Waters Lambda Max 481 variable wavelength detector set up at 264 nm and Hewlett-Packard 3394A integrator. The columns used was Symmetry C_{18} reversed phase (150 mm x 3.9 mm i.d. particle size 5 μm) Lot No. T50961 kindly supplied by Dr. Michael E. Swartz, Waters Corp., Milford, MA, USA and Nucleosil C_{18} reversed phase (250 mm x

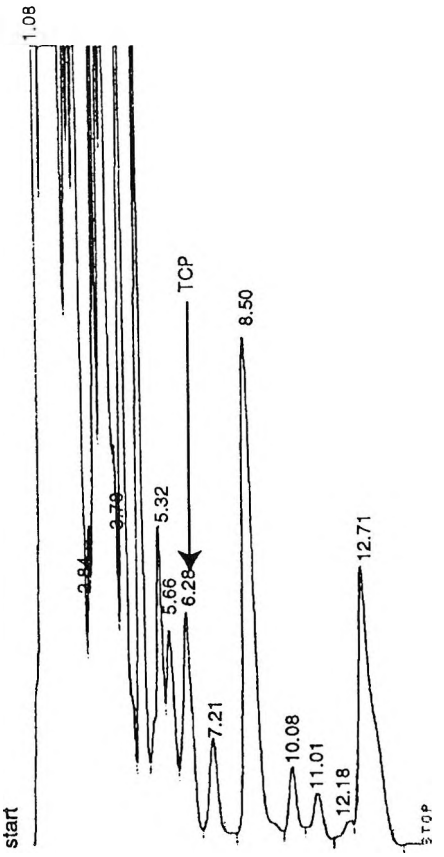

Figure 3: Chromatogram of spiked urine extract. Column:Symmetry C_{18} (150 mm x 3.9 mm I.D., particle size 5 μm); mobile phase; 50 mM KH_2PO_4 (pH 4.55):methanol:water (20:10:70); flow rate: 1 mL/min; chart speed: 0.5 cm/min; temp 23°C; detector UV 264 nm; sensitivity 0.01 aufs.

4.6 i.d., particle size 5 μm), Lot. No. 102H3409 purchased from Sigma-Aldrich, St. Louis, MO, USA. The mobile phase consists of 50 mM KH_2PO_4 (pH 4.55):methanol:water (20:10:70). All other chromatographic conditions are described in figure legends.

Chemicals

Authentic tranylcypromine sulfate, control no. 11605 kindly supplied by Dr. Ober from Rohm Pharma (Darmstadt, Germany). ACS-grade n-octanol, sodium hydroxide, HPLC-grade methanol, potassium phosphate and phosphoric acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). Boric acid was obtained from BDH Chemicals, Ltd., Poole, England.

Sample Preparation and Extraction

Authentic TCP sulfate solution is dissolved in the mobile phase. Urine sample spiked with TCP sulfate and tablet were extracted according to Krugers Dagneaux et al.,[5] with some minor modification.[6]

Following the addition of 1 mL of 0.2 M boric acid/KCl buffer to 1 mL of urine the final solution was brought to pH 8.5 in a glass tube, the contents were extracted with 5 mL of a mixture of 10% n-octanol and 90% n-hexane. The tubes were repeatedly inverted for 2 min and centrifuged at 1500 X g for 15 mins. The organic layer was recovered and further extracted with 4 mL of n-octanol and 1 mL of 50 mM H_3PO_4, in the same manner. Following centrifugation, the organic layer was discarded and 20 μL of aqueous portion was injected onto the HPLC system.

RESULTS AND DISCUSSION

Chromatograms

A typical chromatogram of TCP sulfate is presented in Figure 2, while the chromatogram of spiked urine extract is shown in Figure 3. Figure 4 shows chromatogram of TCP sulfate on Nucleosil C_{18} column under the same chromatographic condition.

Linearity

The calibration curve of the TCP sulfate was constructed over the range 25 nmol-375 nmol/mL with a correlation coefficient 0.999 (n=6). The lower limit of quantitation (LLQ) was 5 nmol/mL.

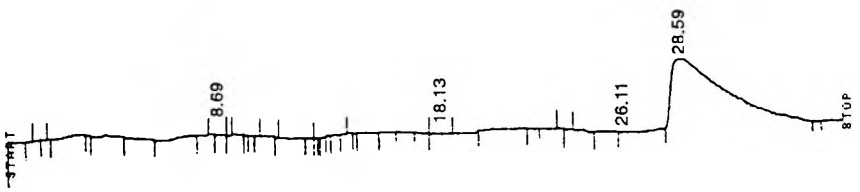

Figure 4: Chromatogram of tranylcypromine authentic sample. Column:Nucleosil C_{18} (250 mm x 4.6 mm I.D., particle size 5 μm); mobile phase; 50 mM KH_2PO_4 (pH 4.55):methanol:water (20:10:70); flow rate: 1 mL/min; chart speed: 0.5 cm/min; temp 23°C; detector UV 264 nm; sensitivity 0.01 aufs; sample quantity: 1 nmol.

Inter and Intra-Assay Accuracy and Precision

The intra-assay accuracy and precision were evaluated by analysing six replicates of two different quality control levels 25 nmol/mL and 250 nmol/mL TCP on the same day. The inter-assay accuracy and precision were determined similarly except that the analysis was carried out several times during one week period. The accuracy of the assay was calculated as the percentage deviation (DEV%) of the mean observed concentration from the nominal concentration of quality control levels. The precision was expressed as the relative standard deviation (RSD%) of the observed concentration from the known concentration of quality control levels. The results were presented in Table 1.

The recovery of TCP is established by analysing a spiked standard solutions of known concentration of TCP onto HPLC system. The recovery is 100%.

To avoid serious cardiovascular crisis due to TCP treatment, a selective, simple and fast method is required to monitor drug level. Accordingly, several analytical methods have been developed to monitor TCP in biological fluid by gas chromatography[7,8] and HPLC with fluorimetric detection.[9] In the investigation reported here, we describe a simple method for TCP determination on Symmetry C_{18} column. The mobile phase used is: 50 mMKH_2PO_4 (pH 4.55): methanol;water (20:10:70). The effluent is monitored at 264 nm. The capacity factor (k') for TCP is 4.15.

It is of interest to mention that the previous analytical methods performed on plasma samples for pharmacokinetic studies of patient on TCP medication indicated that TCP was rapidly absorbed and eliminated.[7,8,9] The mean time to

Table 1

Accuracy and Precision for TCP Assay

Interassay	No.	Mean	DEV%	RSD%
25	6	24.5	2	6.1
100	6	99.0	1	1.2
Intra-Assay				
25	6	23.5	6	7.23
100	6	98.0	2	2.04

peak plasma level (T_{max}) following a 20 mg oral dose was 1.5 hours and the mean elimination half-life ($t_{½}$) was 2.5 hours.[10] Owing to the depression state of these patient it is advisable to monitor TCP medication in urine. The procedure described herein is applied for the assay of TCP in urine (spiked urine) with a recovery of 75%.

TCP sulfate in pharmaceutical preparation; Parnate tablets, 10 mg (Smith Kline and Beecham, Hertfordshire, England) is determined by the same method. The result of the two batches analysed are 9.0 ± 0.25 and 9.0 ± 0.3 respectively (mean ± S.D).

Repeatability and accuracy are important criteria for the quality of a pharmaceutical analysis, and in HPLC, both are influenced by the quality of the column and the packing material. Materials based on high-purity silica provide superior peak symmetry. The USP tailing factor for 1 nmol of TCP sulfate on Symmetry C_{18} is 1.3 while it is 3.8 for Nucleosil C_{18} column under the same chromatographic conditions (Figure 2 and Figure 4). The short retention time of TCP sulfate assay on Symmetry C_{18} column is also an advantage as it saves time and effort.

CONCLUSION

The use of the Symmetry C_{18} column provides a superior peak shape over a comparable reversed phase C_{18} column for this basic drug (pK_a8.2). Accordingly, more accurate quantitation of the active ingredient in its pharmaceutical formulation can be achieved and validated. The method can also be used in TCP therapeutic drug monitoring in depressive patients. The presented HPLC method is simple, accurate, reproducible and rapid.

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DIRECT HPLC SEPARATION OF INDENOLOL ENANTIOMERS USING A CELLULOSE CHIRAL STATIONARY PHASE

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ABSTRACT

Racemic indenolol was resolved into its individual enantiomers by high performance liquid chromatography (HPLC) using a commercially available cellulose tris (3,5-dimethylphenyl carbamate) chiral stationary phase, known as Chiralcel OD. Indenolol contains two positional isomers, giving it a total of four enantiomers. The enantiomeric ratio was validated and peak identification for each enantiomer was established according to their optical rotation sign.

INTRODUCTION

Indenolol and other β-blockers are one drug class of many, whose enantiomers have been analyzed via chiral high performance liquid chromatography (HPLC).[1,2,3] The asymmetric pharmacological actions present in the corresponding enantiomers of a racemic drug have been well documented.[4] Research in chirality has been going on for quite some time but the area is still evolving as pharmaceutical companies and other research establishments channel their efforts into marketing therapeutically important

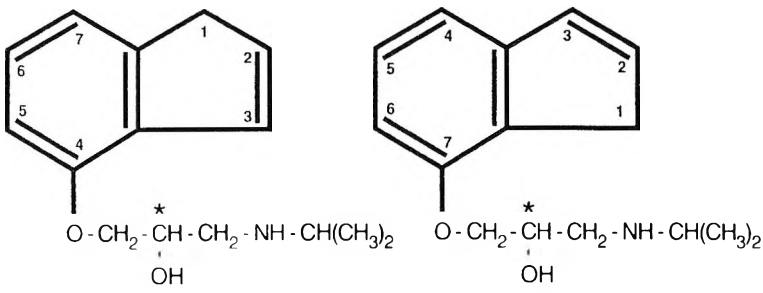

Figure 1. The tautomeric chemical structures of indenolol. (a) 4-indenyloxy and (b) 7-indenyloxy isomers. Asterisk denotes the position of the chiral carbon.

compounds as single enantiomers. Chiral chromatography can achieve two results with the successful separation of a racemate's enantiomers: (1) the determination of optical purity and (2) the obtention of the enantiomeric ratio present in the racemic mixture. The analysis also isolates the respective enantiomers which can then be further studied depending on the interests of the researcher.

This paper describes a direct, simple, HPLC gradient method for the enantiomeric separation of racemic indenolol, 1-(inden-4(or 7)-yloxy)-3-isopropylamino-2-propanol. Indenolol is a non-selective β-adrenoceptor antagonist that consists of two positional isomers, whose chemical structures are shown in Figure 1. The racemic drug exists as a tautomeric mixture of the 7- and 4- indenyloxy isomers in the ratio of 2:1. There are a total of four enantiomers and the method outlined later achieves baseline separation throughout most of the chromatographic run. The enantiomeric ratio was verified by determining the elution order (peak identification) and comparing the areas under each peak to establish the composition of the racemic mixture.

EXPERIMENTAL

Chemicals

Racemic indenolol hydrochloride (lot # PUP-A504) was a generous gift from Yamanouchi Pharmaceutical Corporation (Tokyo, Japan). HPLC grade organic solvents and reagent grade diethylamine were purchased from Fisher Scientific (Springfield, NJ, USA).

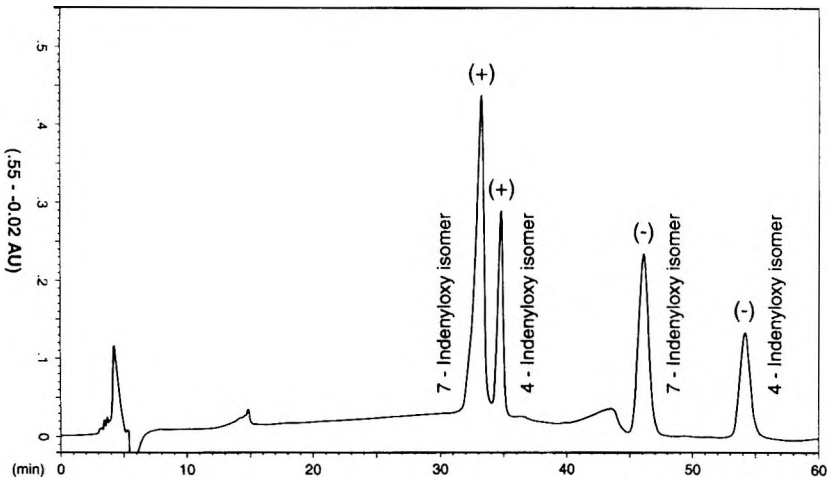

Figure 2. HPLC enantiomeric separation of racemic indenolol hydrochloride. Column: Chiralcel OD (25 cm x 4.6 mm id); Mobile Phase: Solvent A- hexane : ethanol : diethylamine, 99:1:0.2 (v/v/v); Solvent B- ethanol : diethylamine, 100:0.2 (v/v); Linear Gradient Profile: 0 min, 100% A; 20 min, 100% A; 60 min, 80% A, 20% B; Flow Rate: 1 mL/min; Initial Pressure: 198 PSI; Temperature: 23°C; Chart Speed: 0.5 cm/min; Detection: UV at 250 nm; Sample Quantity: 10 nmoles.

Method

A stock solution of indenolol hydrochloride was prepared in hexane:ethanol, 1:1 (v/v), containing 1.5% diethylamine, of which 20 mL was injected for analysis using a Waters (Milford, Massachussetts, USA) HPLC system comprising a 600E multisolvent delivery pump, a multivolume U6K injector, a 5200 printer/plotter and a 990+ photodiode array detector with a NEC Powermate 2 computer module. The cellulose tris (3,5-dimethylphenyl carbamate), Chiralcel OD column (lot # 50-20-30318, 25 cm x 4.6 mm i.d., 10 μm particle size, coated on silica gel) was purchased from J.T. Baker, Inc. (Phillipsburg, NJ, USA). The analysis was done using a linear gradient mode with a run time of 60 minutes. The mobile phases were solvent A-hexane: ethanol:diethylamine, 99:1:0.2 (v/v/v) and solvent B-ethanol:diethylamine, 100:0.2 (v/v). The gradient composition was 100% A at 0 time, 100% A at 20 minutes, and 80% A, 20% B at 60 minutes. The flow rate was held constant at 1 mL/min. The wavelength of detection was 250 nm. All other chromatographic conditions are described in Figure 2.

Peak identification was established using the Shodex OR-1 optical rotation detector (JM Sciences, NY, USA), with the same chromatographic conditions as described above. Capacity factors (k`) were calculated using the equation $k` = (V_r - V_o)/V_o$, where V_r is the elution volume and V_o is the void volume. The separation factor (α) was calculated using the equation $\alpha = k`_2 / k`_1$, where $k`_2$ and $k`_1$ are the capacity factors for the second and first eluted peaks.

RESULTS AND DISCUSSION

Published reports[5,6] have described HPLC analyses of β-blocking agents with more than one chiral center, such as racemic nadolol, and achieved the separation of its four enantiomers. However, prior to injecting the sample onto the chromatographic system, a derivatization step was necessary. The present method, described above, does not require derivatization and, once the compound is dissolved in the mobile phase, the sample is directly injected onto the column.

The enantiomeric separation of racemic indenolol is shown in Figure 2. At the time of writing this manuscript, the authors could not determine the absolute configuration of the individual enantiomers. Thus, the chromatographic peaks were identified according to their optical rotation sign (results not shown). Referring to Figure 2, the first 2 eluting enantiomers carry the (+)-dextrorotatory sign while the latter 2 are both (-)-levorotatory. Furthermore, the enantiomers can be grouped under the isomer which they belong to. The manufacturer states that the composition of racemic indenolol is a tautomeric mixture of the 7- and 4-indenyloxy isomers in the ratio of 2:1. Quantitation of the peaks in Figure 2 by obtaining the area under each peak via integration gave the following results: The ratio of the total percent area of the first (+) and third (-) eluting enantiomers over that of the second (+) and fourth (-) eluting enantiomers was 1.97. This confirms the manufacturer's composition of racemic indenolol and concludes that the first and third eluting peaks are the corresponding enantiomers for the 7-indenyloxy isomer, and the second and fourth eluting peaks represent the enantiomers for the 4-indenyloxy isomer.

The chromatographic parameters calculated for the indenolol enantiomers are summarized in Table 1. Both the 4- and 7-indenyloxy isomers have the (+)-dextrorotatory enantiomer eluting first (lower capacity factor). Each isomeric pair of enantiomers is well separated, indicated by the high α values. When one looks at the chromatographic resolution in Figure 2, for those enantiomers of the same optical rotation sign, the (-)-levorotatory enantiomers are well

Table 1

Chromatographic Parameters, Capacity (k`) and Separation (α) Factors for the Indenolol Enantiomers.

Isomer	k_1`	k_2`	α
4-indenyloxy	8.36 (+)	13.73 (-)	1.64
7-indenyloxy	7.93 (+)	11.50 (-)	1.45

separated, whereas, the (+)- dextrorotatory enantiomers have similar k` values (Table 1). The isocratic mode (results not shown) did not separate the four individual enantiomers. A gradual introduction of the organic solvent (ethanol) was necessary to separate the first two enantiomers, but resulted in longer retention times for the last two eluting peaks.

Less polar organic modifiers such as 2-propanol or branched chain alcohols may improve the resolution of the first two eluting peaks but they may also cause a much prolonged run time which would require reoptimization of the gradient profile. This may prove difficult to do since the run time in the above analysis is 55 minutes and various attempts to improve the resolution of the first two eluting peaks could be limited by this parameter.

CONCLUSION

In this paper, the isomeric ratio of the two positional isomers present in racemic indenolol was verified by chromatographing the individual enantiomers via HPLC using cellulose tris (3,5-dimethylphenyl carbamate) chiral stationary phase, Chiralcel OD. Gradient elution was necessary to separate all four enantiomers of indenolol and peak identification was established according to the optical rotation sign. Although the above analysis may not be suitable for laboratories that require quick results, the assay can be used by quality control laboratories and for those who are interested in pursuing research with chiral drugs.

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A NEW RAPID AND ECONOMICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH ELECTROCHEMICAL DETECTION FOR THE DETERMINATION OF ETOPOSIDE (VP-16) IN HUMAN PLASMA SAMPLES

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ABSTRACT

A high performance liquid chromatographic assay with electrochemical detection has been developed for the determination of etoposide in human plasma samples. 2-acetamidophenol was used as internal standard and extraction was performed with ethyl ether-dichloromethane (2:1) in a single step. Analyses were carried out on a Nova Pak C_8 column using methanol-75 mM acetate buffer (45:55) as mobile phase. The effluent from the column was monitorized at +800 mV against Ag/AgCl and the resulting current was registered. The assay was linear within the range of 0.02-4 μg/mL, coefficients of variation being lower than 10%. Detection limit was 5 ng/mL. The

method is rapid and economical. Therefore, it is suitable for routine monitoring of etoposide pharmacokinetics after administration of low oral doses according to recent therapeutic schemes.

INTRODUCTION

Etoposide (VP-16) is an antitumor agent derived from epipodophyllotoxin which is widely used in the treatment of several malignancies.[1,2] Although a number of methods have been described for the quantitation of etoposide concentrations in human fluids by high performance liquid chromatography (HPLC), they present some disadvantages. Procedures using spectrophotometric[3-6] or fluorescence[7] detection exhibit low sensitivity. Therefore, plasma concentrations can be below detection limits, specially when etoposide is administered according to recently reported regimens consisting of low oral doses.[8,9] Electrochemical detection has shown to be an adequate alternative to improve sensitivity. Notwithstanding, the available methodologies require a time-consuming extraction procedure[10] long run times,[11,12] or expensive on-line equipment.[13] Moreover, the reagents used in these methods are expensive. Hence, such procedures do not appear to be suitable for routine monitoring of etoposide pharmacokinetics in clinical practice.

The purpose of the present work was to develop an improved HPLC assay with electrochemical detection for the quantitation of etoposide plasma concentrations in patients receiving low oral doses. The procedure appears to be simple, specific and sensitive, as well as economical. Hence it can be used in routine monitoring of etoposide clinical pharmacokinetics.

EXPERIMENTAL

Materials

Etoposide was kindly provided by Bristol-Myers-Squibb de México (Mexico City). 2-acetamidophenol, which was used as internal standard, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, chromatographic grade, was obtained from Merck (Darmstadt, Germany). High quality deionized water, employed for solution preparation, was obtained using a Milli Q Reagent Water System (Continental Water Systems, El Paso, TX, USA). All other reagents were of analytical grade.

Solutions

Stock solutions (1 mg/mL) of etoposide and of the internal standard were prepared in methanol. Standard etoposide solutions, used for the preparation of calibration curves ranging from 0.1 to 20 μg/mL, were prepared by appropriate dilution with methanol. A standard solution of 2-acetamidophenol, at a fixed concentration of 0.5 μg/mL, was prepared by dilution of the stock solution with deionized water. Standard solutions were stored at -20°C.

Sample Preparation

1 mL plasma samples (unknown samples, drug free plasma or plasma samples containing known etoposide amounts) were placed in 15 mL conical glass tubes and spiked with 0.1 μg (200 μL of a 0.5 μg/mL solution) of 2-acetamidophenol, the internal standard. After addition of 4 mL of a 0.5 M NaH_2PO_4, pH 7.2, solution, samples were extracted with 5 mL of a mixture of ethyl ether and dichloromethane (2:1 v/v) by vortexing at maximal speed for 1 min. The two layers were separated by centrifugation at 4500 RPM for 5 min, the upper organic layer was transferred to a clean tube and the solvent was evaporated to dryness at 45°C under a gentle nitrogen stream. The dry residue was redissolved in 200 μl of mobile phase (see below) and 100 μl aliquots were injected into the chromatographic system.

Chromatographic Conditions

The chromatographic system consisted of a M-45 solvent delivery system (Waters Assoc., Milford, MA, USA), a 100-μL loop injector (Rheodyne, Cotati, CA, USA), an electrochemical transducer coupled to a LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) and a Servogor 120 recorder (Norma Goerz Instruments GmbH, Neudorf, Austria). Analyses were carried out on a 150 x 3.9 mm I.D. Nova-Pak C_8 column of 4 μm particle size (Waters). To prolong the life of the analytical column, a precolumn (40 x 4 mm I.D.) containing 37-50 μm Corasil C_{18} (Waters) was incorporated into the system. Column elution was carried out at room temperature using a mixture of methanol with 75 mM acetate buffer pH 3.8 (45:55 v/v) as mobile phase at a fixed flow rate of 1 mL/min. Detection was performed using a glassy carbon working electrode maintained at +800 mV against Ag/AgCl and the resulting current was recorded.

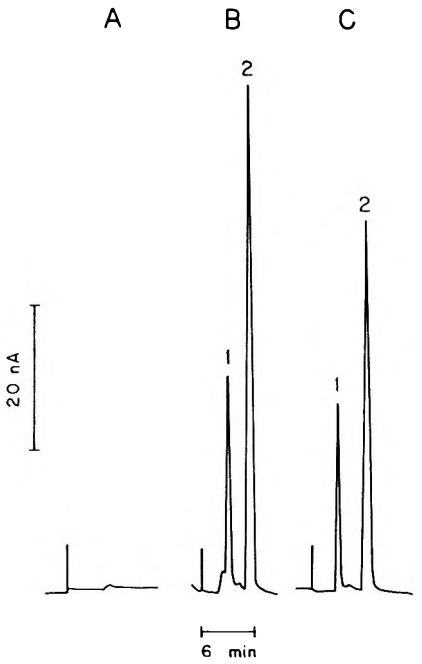

Figure 1. Chromatograms of human plasma extracts. A: Blank plasma. B: Plasma spiked with 2 μg/mL of etoposide (2) and 0.1 μg/mL of internal standard (1). C: Plasma sample drawn from a pacient 1 h after a 50 mg oral etoposide dose, spiked with 0.1 μg/mL of internal standard.

Calibration

The assay was calibrated by addition of known amounts of etoposide and of the internal standard to drug-free plasma samples. Samples used for calibration contained etoposide concentrations ranging from 0.02 to 4 μg/mL. The internal standard was used at a fixed concentration of 0.1 μg/mL. Calibration curves were constructed by plotting the peak-height ratio of etoposide to the internal standard (y-axis) as a function of the actual etoposide concentration in the sample (x-axis). The accuracy and precision of the method were evaluated by adding known drug amounts to replicate plasma samples over the concentration range used for calibration.

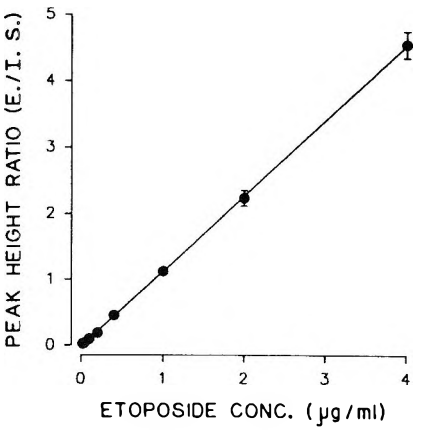

Figure 2. Calibration curves of etoposide in human plasma established in the range of 0.02 to 4 µg/mL. Data are expressed as mean ± S.E.M. of at least six determinations.

RESULTS

Typical chromatograms of extracted plasma samples are shown in Fig. 1. Retention times for the internal standard and etoposide were 3.0 and 5.6 min respectively. No interfering peaks occurred at these times. Any endogenous contaminants remaining in the extracts, which exhibited a response at the voltage level used in the assay, eluted before the internal standard. Therefore, samples could be injected immediately after elution of etoposide. The recoveries of etoposide and of the internal standard were assessed by comparison of peak heights from plasma extracts with those from standard solutions. Recoveries were similar for both compounds, being higher than 90%.

A linear relationship ($r = 0.9999$) was found when the peak height ratio of etoposide to that of the internal standard was plotted on the ordinate against etoposide plasma concentration in the abscissa (Fig. 2). The equation obtained by least-squares was $y = 1.1405x - 0.0160$. The precision and accuracy of the assay are shown in Table 1. It can be seen that a good accuracy was achieved, while the intra-assay coefficient of variation was always lower than 10%. Day-to-day precision of the assay was evaluated over a period of five weeks (n=6). Inter-assay coefficients of variation were 7.48, 5.07 and 9.71% for 0.04, 0.2 and 2 µg/mL respectively. The detection limit, defined as the etoposide plasma concentration producing a signal-to-noise ratio of 3, was 5 ng/mL

Table 1

Accuracy and Precision of the High Performance Liquid Chromatographic Assay for Determination of Etoposide Plasma Levels

Theoretical Concentration (μg/mL)	Measured Concentration (μg/mL; mean ± S.E.M.) (n=6)	Accuracy (%)	C.V. (%)
0.02	0.022 ± 0.002	106.40	5.21
0.04	0.043 ± 0.001	107.62	5.90
0.1	0.100 ± 0.003	100.40	8.12
0.2	0.194 ± 0.004	97.02	6.78
0.4	0.408 ± 0.016	101.98	9.59
1.0	1.009 ± 0.027	100.87	7.01
2.0	1.984 ± 0.078	99.20	9.61
4.0	4.064 ± 0.128	101.61	7.04

S.E.M. : Standard error of mean
C.V.: Intra-assay coefficient of variation

The application of the method for clinical pharmacokinetic studies was evaluated. Blood samples were obtained at selected times over a period of 24 h from a patient receiving a 50 mg oral etoposide as solution (Vepesid™, Bristol-Myers-Squibb de México, Mexico City). Plasma was obtained and the etoposide concentration was determined by the procedure described. The observed plasma concentration against time curve is shown in Fig. 3. The maximal etoposide plasma concentration was 3.1 μg/mL, being reached at 1.5 h. The terminal half-life was 6.1 h and the area under the curve amounted to 23.2 μg h/mL.

DISCUSSION

Several assays for the determination of etoposide plasma concentrations using HPLC coupled to electrochemical detection have been reported.[10-13] Although these procedures have shown to be reliable and sensitive, they are not suitable for routine monitoring in clinical practice. The procedure described by Duncan and coworkers[10] includes a back extraction step, which importantly prolongs the sample preparation time and does not use an internal standard.

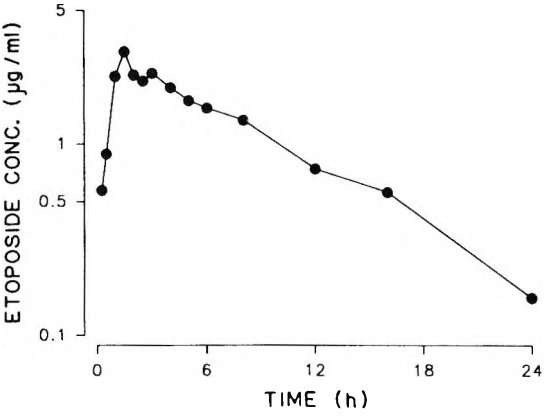

Figure 3. Time-course of etoposide plasma levels in a pacient after a single oral dose of 50 mg in solution.

Eisenberg and colleagues[11] reported a procedure requiring of a total run time of 19 min in order to elute all interfering compounds from the column before analysis of the following sample, and therefore it is not practical for pharmacokinetic purposes. The method of Littlewood and coworkers[12] appears to be superior, as the total run can be achieved in 9 min. This time, however, is 3 min longer than the one needed with our procedure which only requires of 6 min.

The cost of routine pharmacokinetic analysis is of great relevance, as it can significantly increase the price of treatments with drugs, such as etoposide, which require frequent monitoring in order to ensure an adequate therapeutic response. Van Opstal and colleagues[13] reported an assay in which etoposide analysis can be performed by direct injection into the chromatographic system without prior extraction. This procedure, although it is practical, requires an on-line sample preparation system which is expensive and not universally available. In the procedures described for etoposide determination in plasma using HPLC with electrochemical detection, in which an extraction step is included,[10-12] the solvent used is dichloroethane. This solvent is expensive, its cost being about three times higher than the ethyl ether-dichloromethane mixture employed in the present method. Furthermore, most procedures use teniposide, another antitumor agent, as internal standard.[11-13] The commercial price of teniposide is considerably higher than that of 2-acetamidophenol.

The procedure here presented fulfills the precision, accuracy and sensitivity requirements for the determination of etoposide clinical pharmacokinetics after administration of low oral doses. The procedure presents the advantage of a short run time, allowing the analysis of a considerable number of samples per working day. Furthermore, the procedure appears to be more economical than those previously reported. Hence, the present method appears to be a suitable alternative for the monitoring of etoposide pharmacokinetics in routine clinical practice.

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MAGNOLOL INHIBITS ARACHIDONIC ACID-INDUCED RABBIT PLATELET SEROTONIN RELEASE, MEASURE BY MICROBORE HPLC WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined simultaneously in rabbit platelet by a sensitive microbore high-performance liquid chromatographic method with electrochemical detection. The detection limit of 5-HT in rabbit platelet suspension was 0.1 ng/mL. To evaluate the 5 - HT release from platelet suspension, the aggregating agent arachidonic acid 30 and 100 μM were added and the 5-HT was elevated from

basal level 2.96±0.89 ng/mL to 154.72±28.03 and 246.77±24.38 ng/mL, respectively. The results demonstrated that magnolol inhibits arachidonic acid-induced 5-HT release from platelet suspension significantly.

INTRODUCTION

Magnolol is the major phenolic constituents of Magnolia bark.[1,2] The bark of Magnolia has been used as a folk medicine in China for the relief of fever, headache, anxiety, diarrhea and stroke.[3]

Recent studies indicate that magnolol inhibits intracellular calcium mobilization in platelets,[4] relaxes vascular smooth muscle,[5] antihemostatic, antithrombotic effects,[6] inhibits collagen-induced platelet 5-HT release[7] and modulatory effects of brain 5-HT release.[8]

In the present study, we have examined the inhibitory effects of magnolol on arachidonic acid-stimulated 5-HT release from platelet by a sensitive microbore HPLC/ECD system.[9]

MATERIALS AND METHOD

Materials

The methanol extract of magnolia bark was partitioned between water and chloroform. The chloroform layer was separated repeatedly by column chromatography on silica gel, magnolol (Fig. 1) was then extracted.[1] Identification and purity were compared with authentic compound by [^{13}C]NMR (Bruker, Germany) and HPLC, coupled with photodiode-array detection.[10] 5-HT and arachidonic acid, were purchased from Sigma Chemical (St. Louis, MO, USA). HPLC reagents and buffer reagents were obtained from E. Merck (Darmstadt, Germany). Thrice-deionized water (Millipore Corp., Bedford, MA, USA) was used for all preparations.

Platelet Suspension

Rabbit blood collected from the marginal ear vein, with one sixth volume of acid-citrate-dextrose was mixed. The blood was centrifuged by swinging centrifugation at 200 g for 15 min at room temperature. The upper platelet rich

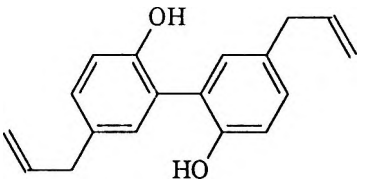

Figure. 1. Chemical structure of magnolol isolated from the bark of *Magnolia officinali.*

plasma was mixed with 2 mM EDTA and centrifuged at 1,000 g for 12 min. The supernatant was discarded and platelet pellet was suspended in Ca^{++}-free Tyrode's buffer (136.89 mM NaCl; 2.68 mM KCl; 2 mM $MgCl_2$ 0.33 mM NaH_2PO_4; 5 mM glucose, 10 mM HEPES; 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid) with 0.35 % bovine serum albumin, heparin (50 unit/mL) and apyrase (1 unit/mL). After 20 min incubation at 37°C, the washed platelet pellet was resuspended in Tyrode's buffer containing 1 mM calcium and the cell concentration was adjusted to around 1 x 10^8 platelets/mL. The reaction of drug treatment was terminated after 3 min by mixing the sample with one-fifth volume of 0.05 mM EDTA in ice. After centrifugation at 10,000 g for 3 min, the supernatant was filtered through a 0.2 μm membrane filter. Aliquot (10 μL) of the filtrate was directly injected onto the HPLC apparatus[11] for analysis.

Apparatus and Chromatography

The HPLC/ECD system consisted of a syringe pump (ISCO, Lincoln, NE, USA) at flow rate 0.06 mL/min for 5-HT analysis (Fig. 2). Samples were separated using a reverse phase C_{18} SepStik microbore column (150 x 1 mm; 5 μm; BAS, West Lafayette, IN, USA) fitted with a microbore guard column (14 x 1 mm; 5 μm; BAS). The injection volume was configured with a 10 μL sample loop. The mobile phase consisted of 110 mL acetonitrile, 2.08 mM sodium 1-octanesulfonate, 13.48 mM monosodium dihydrogen orthophosphate, 56.59 mM sodium citrate, 0.027 mM EDTA, and 1 mL diethylamine. The final volume of the mobile phase was added to 1 liter of triple-deionized water. The solution was adjusted to pH 3.0 by orthophosphoric acid. The mixture was filtered with a 0.22 μm Millipore membrane. 5-HT and 5-HIAA were detected using amperometric detector (BAS-4C) coupled to a glassy carbon working electrode and referenced to a Ag/AgCl electrode at +0.6 V. Output from the ECD was amplified and recorded using Waters Millennium 2010 software.[12]

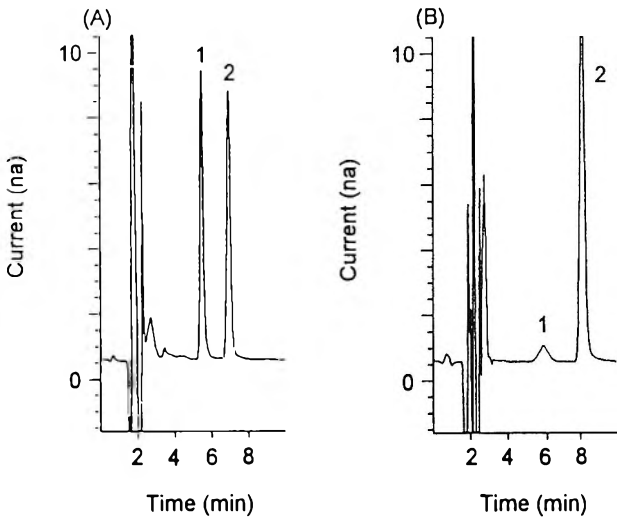

Figure 2. Typical chromatograms of (A) standard mixture of 5-HT (50 ng/mL) and 5-HIAA (50 ng/mL) (B) arachidonic acid (30 μM)-induced rabbit platelet 5-HT (155.86 ng/mL) release.

Statistical analysis

All results are expressed as the mean±S.E.M. from four experiments. Statistical analysis was performed by Student t test with the level of significance set at $p<0.01$.

RESULTS AND DISCUSSION

Peaks detection limit of 5-HT and 5-HIAA were about 0.1 ng/mL at a signal-to-noise ratio of 4. Fig. 2 (A) shows the typical chromatograms of a standard mixture containing 50 ng/mL of 5-HT and 5-HIAA. The retention times were 5.5 and 7.0 min, respectively. Fig. 2 (B) shows the arachidonic acid (30 μM) induced 5-HT (155.86 ng/mL) release.

The calibration curve was linear for 5-HT (range 1 to 200 ng/mL). The reproducibility of the method can be defined by examining both intra- and inter-assay variabilities. The intra-assay coefficients of variation (C.V.s) for 5-HT at concentrations of 10 and 50 ng/mL were 2.45 and 1.87%, respectively, the inter-assay C.V.s for 5-HT at the same concentrations were 5.09 and

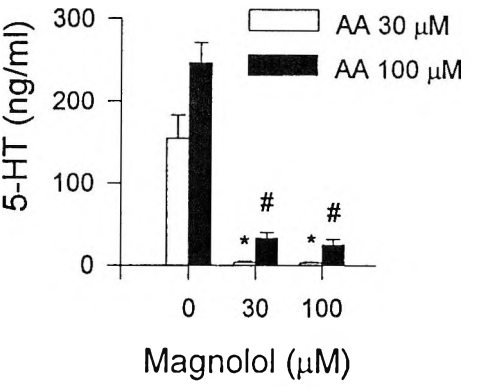

Figure 3. Arachidonic acid-induced 5-HT release from platelet suspension (n=4). * $p<0.01$ vs arachidonic acid (30 μM). # $p<0.01$ vs arachidonic acid (100 μM).

2.61%, respectively. Peaks detection limit of 5-HT and 5-HIAA were about 0.1 ng/mL at a signal-to-noise ratio of 4.

Using challenge doses of arachidonic acid 30 and 100 μM, the levels of platelet 5-HT increased from the basal value 2.96±0.89 ng/mL to 154.72±28.03 and 246.77±24.38 ng/mL, respectively. The antiplatelet agent magnolol dose-dependently inhibited arachidonic acid induced 5-HT release from rabbit platelet suspension. Fig. 3 shows that arachidonic acid (30 or 100 μM) induced 5-HT release from rabbit platelet suspension and magnolol inhibited arachidonic acid-induced 5-HT release.

Regardless, this HPLC/ECD system uses commercially available microbore column. The microbore column increase sensitivity through a decrease in band broadening that results in sharper peaks. Furthermore, the low flow rate (0.06 mL/min) is also a considerable advantage to the environment because of the organic solvent present in the mobile phase.[11]

The major activation of platelets induced arachidonic acid is due to the formation of thromboxane A_2.[13,14] Moreover, the thromboxane A_2 triggered secretion is mediated by increase intracellular calcium.[15] In addition, intracellular calcium increase has been regarded as the final common pathway for the platelet shape change, secretion and aggregation.[16]

Teng et al.[4] demonstrated that the mechanism of the antiplatelet agent, magnolol, was due to an inhibitory effect on thromboxane formation and

intracellular calcium mobilization. In the present study, we promote the above results that magnolol inhibits arachidonic acid-induced 5-HT release.

Serotonin displays complex properties on vascular tissues, producing constriction in venules and dilatation in arterioles. However, the relationship between serotonin content and platelet function in initiation and maintenance of vascular function are poorly understood.[17] The rapid measurement of platelet 5-HT by microbore HPLC/ECD system may be a useful tool in this area of investigation.

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HPLC METHOD FOR THE DETERMINATION OF KETOROLAC IN HUMAN PLASMA

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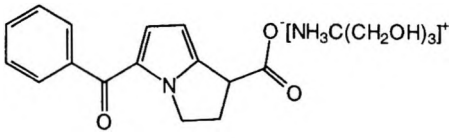
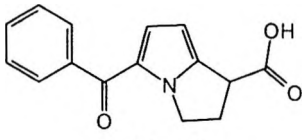
ABSTRACT

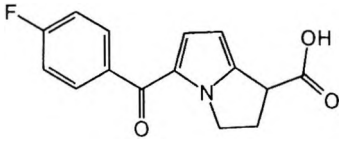
A precise and accurate HPLC method for the quantification of ketorolac in human plasma is described. Ketorolac and an added internal standard (a fluoro analog of ketorolac) are extracted from acidified plasma samples and the extract is further purified. The reconstituted sample is injected onto a reverse phase HPLC column. The method has a linear concentration range of 0.010-3.00 μg of ketorolac per aliquot of plasma, using 0.1 to 1.0 mL of plasma for analysis. The quantification limit of the method is 0.010 μg/mL. The intra- and inter-assay % CVs were less than 5.5%, and the mean recoveries ranged from 96% to 109%. The absolute recovery was 92%.



Ketorolac Tromethamine
Structure I



Ketorolac
Structure II



Internal Standard (IS)
Structure III

Figure 1. Molecular structures of ketorolac tromethamine (I), ketorolac (II), and internal standard (III).

INTRODUCTION

Ketorolac tromethamine (**I**, Figure 1) is a potent non-narcotic analgesic. The activities of the drug arise from its ability to inhibit prostaglandin synthesis.[1-4] Ketorolac tromethamine is marketed as a racemic mixture, although its biological activity is associated with the (-)*S* enantiomer.[5] It is available for intravenous (IV), intramuscular (IM), and oral administration.

To support the evaluation of the pharmacokinetics of ketorolac tromethamine in humans, we have developed a sensitive, specific, and accurate HPLC method for the determination of ketorolac in plasma. Previous unoptimized versions of this method have been summaried briefly,[6,7] but the fully optimized method with complete validation data is presented here. Two other methods for the determination of ketorolac in plasma or serum using HPLC have been reported.[8,9] These methods have quantification limits two to five times greater than those of the method reported here and are not always adequately sensitive for the study of ketorolac pharmacokinetics in humans.

EXPERIMENTAL

Chemicals and Supplies

Ketorolac tromethamine, (±)-5-benzoyl-1,2-dihydro-3H-pyrrolo[1,2a] pyrrole-1-carboxylic acid, tris(hydroxymethyl)aminomethane salt (**I**; Figure 1); ketorolac, (±)-5-benzoyl-1,2-dihydro-3H-pyrrolo[1,2a]pyrrole-1-carboxylic acid (**II**; Figure 1); and internal standard, RS-37414-000, (±)-5-p-fluorobenzoyl-1,2-dihydro-3H-pyrrolo[1,2a]-pyrrole-1-carboxylic acid (IS, **III**; Figure 1) were obtained from Syntex Research (Palo Alto, CA, USA). HPLC-grade methanol, acetonitrile, hexane, and ethyl acetate were purchased from Burdick and Jackson Laboratories (Muskegon, MI, USA), and water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Reagent grade phosphoric acid, 85%, analytical grade sodium acetate, and glacial acetic acid were purchased from Mallinckrodt (St. Louis, MO, USA). An aqueous solution of 0.5 M sodium acetate buffer, pH 3, was prepared in house. Heparinized human control plasma (blank plasma) was obtained from normal, healthy volunteers from the Clinical Studies Unit, Syntex Research (Palo Alto, CA, USA).

Instrumentation

The HPLC system consisted of a Hewlett-Packard Model 1090 L ternary solvent delivery system and autosampler (Santa Clara, CA, USA), a Kratos Spectroflow 785A variable wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ, USA), and a Nelson 6000 Laboratory Data System (PE-Nelson, Cupertino, CA, USA).

Chromatographic Conditions

For the determination of ketorolac, aliquots of sample extract (20 µl) were injected onto a Nova-Pak C_{18}, 4-µm, 4.6 mm x 150 mm column (Waters Associates, Milford, MA, USA) using a mobile phase of acetonitrile-0.05% aqueous phosphoric acid (34:66, v/v) and a flow rate of 1 mL/min. The UV detector was set at 317 nm. A 0.5-µm precolumn filter (Upchurch Scientific Inc., Oak Harbor, WA, USA) was connected to the analytical column and replaced every 1,000 injections.

Sample Preparation

1. Spiking procedure

A stock solution was prepared by dissolving ketorolac tromethamine in methanol. The stock solution was further diluted with methanol-water (9:1, v/v) to prepare spiking solutions at equivalent ketorolac free acid concentrations of 0.1, 0.2, 0.4, 1, 2, 10, 20, and 30 µg/mL. An internal standard spiking solution (IS) of 2 µg/mL was prepared by dissolving the internal standard in methanol and then further diluting the solution in methanol-water (9:1, v/v). For preparation of the calibration standards used for the construction of the calibration curve and for validation of the method, 0.1-mL aliquots of the ketorolac spiking solutions were added to 1.0-mL aliquots of blank human plasma to prepare a set of calibration standards at ketorolac concentrations of 0.01, 0.02, 0.04, 0.1, 0.2, 1.0, 2.0, and 3.0 µg/mL.

2. Extraction of calibration standards

To each calibration standard was added 0.1 mL of the internal standard spiking solution and 0.1 mL of the 0.5 M sodium acetate buffer. The combination was vortexed briefly, and 6 mL of ethyl acetate-hexane (3:7, v/v) was added to each tube. Tubes were vortexed vigorously for 5 minutes, then

centrifuged for 2 to 5 minutes at 2000 rpm, and then placed in a dry ice/isopropanol or dry ice/methanol bath to freeze the aqueous layer. The organic layer was then decanted, and the organic extract was evaporated to dryness at 38°C under a stream of nitrogen. Each tube then received 0.5 mL of methanol-water (9:1, v/v) and 3 mL of hexane. Tubes were sonicated for 15 seconds and then vortexed vigorously for 3 minutes. The contents were allowed to settle for at least 5 minutes before the upper hexane layer was aspirated and the remaining methanol-water layer was evaporated to dryness at 38°C under a stream of nitrogen. Then 0.1 mL of methanol was added, followed by 0.1 mL of the mobile phase, acetonitrile-aqueous 0.05% phosphoric acid (34:66, v/v). Tubes were sonicated for 30 seconds, then vortexed for 30 seconds. Tube contents were then transferred to HPLC vials.

3. Extraction of clinical samples

Samples of heparinized plasma obtained from healthy volunteers or patients treated with ketorolac were stored at -20°C before analysis. Samples were thawed at room temperature, vortexed briefly, and centrifuged for 2 minutes at approximately 2500 rpm; an aliquot of 0.1-1.0 mL was dispensed for analysis. When less than 1.0 mL was used for analysis, water was added to bring the total volume to 1.0 mL. The samples were extracted using the same procedure described for the calibration standards. The concentration of ketorolac in samples was calculated by reference to calibration curves generated from calibration standards analyzed along with each batch of clinical samples. Concentrations were reported in terms of μg of ketorolac free acid per mL of plasma.

Data Handling and Calculations

Linear least-squares regression was performed on the peak height ratio (analyte peak height:internal standard peak height) versus concentration data generated by the calibration standards to construct a linear standard curve of the form $peak\ height\ ratio = m\ (concentration) + b$. Calibration standards with ketorolac concentrations from 0.01-0.20 μg/mL ketorolac were used in the unweighted linear regression to construct a calibration curve, and calibration standards of higher concentrations were used to verify extrapolations of the curve up to 3.0 μg/mL. Concentrations in unknowns were then determined from their peak height ratios by the standard curve equation, with appropriate corrections for sample aliquot volumes that were less than 1.0 mL.

Preparation of Quality Control Samples

Quality control samples (QCs) prepared by spiking ketorolac into control human plasma were stored at -20°C in a manner similar to that used for the clinical samples. QCs were prepared at the following three ketorolac concentrations: QC #1 (0.020 μg/mL), QC #2 (0.415 μg/mL), and QC #3 (13.3 μg/mL). To prepare the bulk QCs, ketorolac was dissolved in methanol-water (9:1, v/v) and added to the appropriate amount of blank human plasma, which was then swirled briefly and stirred on a magnetic stirrer for 10 minutes before being apportioned into polypropylene tubes for storage at - 20°C. Two QCs of each of the three different concentrations were analyzed with each batch of clinical samples to monitor the performance of the method during routine use.

RESULTS AND DISCUSSION

Quantification Limit

The quantification limit of the method is 0.010 μg/mL using 1.0 mL of plasma for analysis. Concentrations below 0.010 μg/mL are reported as below the quantification limit of the method (BQL). At the quantification limit, the signal-to-noise ratio of the HPLC peak was approximately 8:1.

Precision and Accuracy

The precision of the assay was assessed by the intra-assay (within-day) and inter-assay (between-day) coefficients of variation (%CVs) of the method. The accuracy of the method was evaluated by the recovery, defined as the ratio of the concentration of ketorolac found in the sample to that added to the sample (found/added). Data for the intra- and inter-assay %CVs and the recoveries obtained using calibration standards are presented in Table 1. Corresponding data for the QC samples are also presented in Table 1. All intra-assay %CVs were less than 4% and all inter-assay %CVs were less than 6%. All recoveries were between 96% and 109%.

Specificity

The analysis of blank human plasma from six different sources showed no interfering peaks at the retention times of ketorolac and the internal standard.

Representative chromatograms from blank plasma, from a spiked calibration standard, and from a patient sample are shown in Figure 2.

Linearity

The linear range of the method, using 0.1 to 1.0 mL of plasma for analysis, was 0.010-3.0 μg per aliquot of plasma.

Table 1

Precision and Accuracy of Ketorolac HPLC in Plasma

	Nominal Concentration (μg/m L)	Replicates Intra-assay	Replicates Inter-assay	Mean Concentration Found (μg/mL) Intra-assay	Mean Concentration Found (μg/mL) Inter-assay	Precision (%CV) Intra-assay	Precision (%CV) Inter-assay	Accuracy (% Recovery) Intra-assay	Accuracy (% Recovery) Inter-assay
Calibration Standards	0.0100	4	4	0.0109	0.0103	3.85	3.84	109	103
	0.0200	4	4	0.0207	0.0201	1.86	2.45	103	101
	0.0400	4	4	0.0402	0.0402	0.610	3.81	101	101
	0.100	4	4	0.0983	0.0983	0.985	1.34	98.3	98.3
	0.200	4	4	0.200	0.201	0.289	0.642	100	101
	1.00	4	4	1.00	1.00	1.44	1.92	100	100
	2.00	4	4	2.07	1.99	0.623	1.71	103	99.5
	3.00	4	4	3.14	3.00	1.16	2.78	105	100
QC Samples									
QC #1	0.0200	4	48	0.0199	0.0192	3.99	5.31	99.5	96.0
QC#2	0.415	4	48	0.401	0.400	1.23	2.83	96.6	96.4
QC#3	13.3	4	48	14.1	13.5	0.816	2.40	106	102

Absolute Recovery

The absolute recovery of ketorolac from plasma, determined by the analysis of plasma spiked with [14C]-ketorolac, was 92%.

Effect of Volume

The effect of varying the volume of plasma used in the range of 0.1 to 1.0 mL was examined using QC samples. The results with aliquots of 0.1, 0.2, and 0.5 mL were equivalent to results with 1.0-mL aliquots. These data indicate that volumes of plasma from 0.1 to 1.0 mL may be used for the analysis.

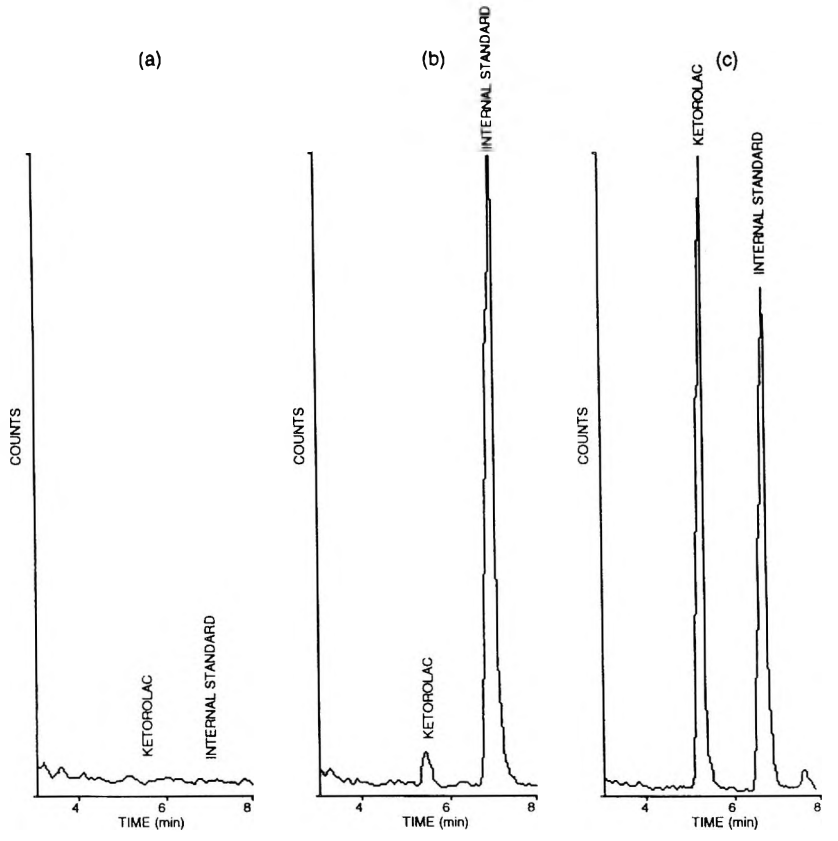


Figure 2. Chromatograms of (a) a blank human plasma from untreated subject; (b) a blank human plasma spiked with 0.010 μg/mL of ketorolac tromethamine and 0.2 μg/mL of internal standard; and (c) a patient plasma sample 40 minutes following oral administration of 10 mg of ketorolac tromethamine.

Stability

Ketorolac concentrations in QC plasma samples were found to be stable when stored in a -20°C freezer for at least 6 months. Ketorolac in QC plasma samples was found to be stable for at least 24 hours when stored on the laboratory bench at room temperature under normal laboratory conditions.

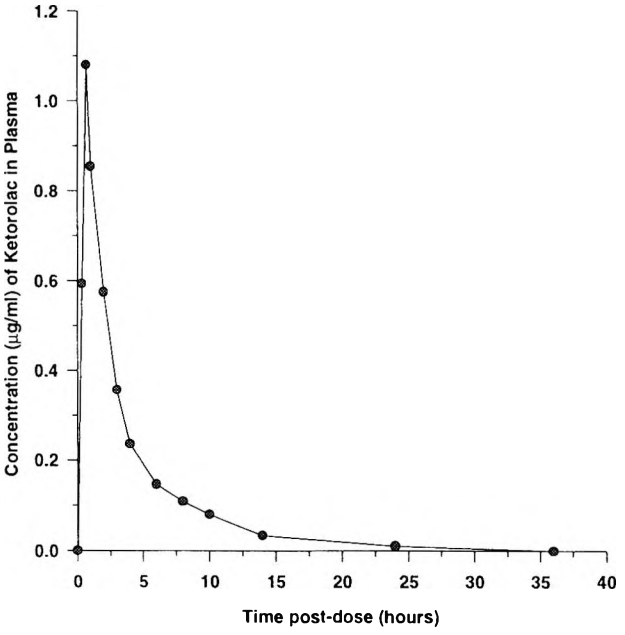


Figure 3. A representative plasma concentration vs time profile of a healthy subject following oral administration of 10 mg of ketorolac tromethamine. (Values reported as BQL are plotted as 0 µg/mL.)

Using refreezing in a -20°C freezer after thawing samples, ketorolac in both QC and clinical plasma samples was found to be stable for at least three freeze/thaw cycles.

Extracts prepared for HPLC analysis from calibration standards, from QC samples, and from clinical samples were analyzed by HPLC on the day samples were processed and several times after storage. They were stored refrigerated at 1-4°C or at room temperature (20-23°C) under normal laboratory conditions. Resulting data indicated that ketorolac in the extract is stable for at least 24 hours at room temperature and for at least 2 weeks when stored at 1-4°C.

Application

This method has been applied to the analysis of plasma from healthy subjects and patients treated with oral, IM, and IV ketorolac tromethamine. A representative profile for a healthy subject who received 10 mg of oral ketorolac is shown in Figure 3.

CONCLUSIONS

The method described here for the determination of ketorolac tromethamine in plasma is precise and accurate and can be used in samples obtained from subjects receiving ketorolac tromethamine by intramuscular, intravenous, or oral administration.

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A THIN LAYER CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION OF PROLINE AND HYDROXYPROLINE FROM BIOLOGICAL SAMPLES

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ABSTRACT

Studies of collagen metabolism often require the analysis of proline and hydroxyproline which necessitates their separation. A methodology was developed which: 1)separates proline and hydroxyproline using thin layer chromatography; 2) locates the imino acids by autoradiography and; 3) recovers them by dialysis in high yield with great precision.

INTRODUCTION

The separation of proline (Pro) and hydroxyproline (Hyp) is essential to studies of the incorporation of radioactive Pro into animal tissues or cells in tissue cultures.[1,2] Numerous separation techniques are currently available.[2-21]

These methods utilize various approaches (i.e. HPLC, supercritical fluid extraction, capillary zone electrophoresis, paper and thin layer chromatography). We report here a thin layer chromatographic procedure that separates Pro and Hyp, which is unique in that it demonstrates no interference from plasma hydrolysates and affords excellent recovery of Hyp. The procedure is simple, reliable and relatively inexpensive.

EXPERIMENTAL

Chromatography of Amino Acids Standards

Twenty-two amino acids (Sigma Chemical Co., St. Louis, MO) were dissolved in distilled water at a concentration of 5 mg/mL. Five microliter aliquots were spotted 2.5 cm from the lower edge of silicic acid gel impregnated glass fiber sheets (20 cm x 20 cm ITLC-SA, Gelman Sciences Inc., Ann Arbor, MI) at 3.5 cm intervals from the left edge. Warm air was blown under the sheet during the spotting procedure for rapid evaporation of the solvent, to minimize the spot size. The sheets were equilibrated for one hour, then lowered into the solvent (isopropanol:water, 7:3) and developed for eight hours. The dried chromatograms were sprayed with a 0.25% solution of ninhydrin (Pierce Chemical Co., Rockford, IL) in 95% ethanol and heated at 105^0C for five minutes for visualization of the amino acids.

Chromatography of ^{3}H-Pro and ^{3}H-Hyp in Plasma Hydrolysates

Reconstituted human plasma samples (Sigma) were made 6M with HCl and heated at 110^0C for 24 hours. The hydrolysates were desiccated under vacuum at 40^0C over sodium hydroxide and calcium chloride, then brought to the volume of the original plasma sample with distilled water. Thin layer chromatography sheets which had been spotted with 0.2 μCi of ^{3}H-Hyp (ICN, Irvine, CA) were overspotted with 0, 2, 4 or 6 microliters of plasma hydrolysate and chromatographed as described. The dry chromatograms were autoradiographed for two weeks at -20^0C using Kodak X-Omat film, which was subsequently developed in an automatic developer.

Sixteen gingival crevicular fluid and connective tissue hydrolysates were overspotted with 0.2 μCi 3H-Hyp standards (ICN), chromatographed and autoradiographed as described. The location of the tritiated imino acids in the chromatograms was determined by the image in the developed autoradiograms.

The portions containing the ^{3}H-Hyp were cut out and each chip was placed in a piece of dialysis tubing (10 mm flat width, 70 mm long). One end was sealed with a closure (Spectra/Por, Spectrum, Los Angeles, CA) and 0.5 mL of distilled water or 1M acetic acid was added to each sample. As much air as possible was excluded and the other end of the tubing was sealed with a second closure. Dialysis was carried out against two changes of 10 mL of the same solvent as had been added to the bag, i.e. water or 1M acetic acid, in one ounce capped polypropylene vials, which were agitated at approximately 30 reciprocations per minute. The combined dialysates and a 5 mL rinse (25 mL total) were lyophilized in 50 mL polypropylene tubes. Each sample was then dissolved in 5 mL of distilled water, transferred to a scintillation vial and 10 mL of scintillation cocktail (ACS, Amersham Co., Arlington Heights, IL) added. Counts per minute were determined for the chromatographed samples and 0.2 μCi ^{3}H-Hyp standards (ICN). Recovery of the test samples was calculated by the following formula:

CPM of test sample/ CPM of 0.2 μCi ^{3}H-Hyp x 100 = % recovery.

RESULTS

Chromatography of Amino Acid Standards

The chromatographic system described separated Hyp and Pro without interference from complex biological matrices such as plasma hydrolysates. While Hyp and Pro were completely separated other amino acids co-migrated with the imino acids. Aspartic acid, alanine, glutamic acid, glutamine, which is converted to glutamic acid on hydrolysis, and glycine co-migrated with Hyp. Asparginine, which is converted to aspartic acid on hydrolysis, co-migrated with Pro (Table I).

Chromatography of ^{3}H-Pro, ^{3}H-Hyp in Plasma Hydrolysates

Development of the chromatograms for eight hours gave a clear separation of Pro and Hyp. The addition of human plasma hydrolysate did not interfere with this separation in quantities as high as six microliters.

Table 1

Chromatographic Mobility of Assayed Amino Acids

Group	R_f Range	Amino Acids
A	.89-.81	leucine, tryptophan, tyrosine, isoleucine, methionine, phenylalanine, valine, cys-H, threonine, serine
B	.78-.75	hydroxyproline, aspartic acid, alanine, glutamic acid, glutamine*, glycine
C	.68-.69	proline, asparginine**
D	.55	histidine
E	.11-.10	arginine, lysine, 5-hydroxylysine

* Converted to glutamic acid during hydrolysis.
** Converted to aspartic acid during hydrolysis.

Elution of Hyp

Dialysis of the developed chromatograms for elution of Hyp gave a recovery of 84.4% +/- 4.3% (avg. +/- SD). Extractions using water as the solvent contained substantial quantities of a fine particulate material (presumably silica) whereas extractions using 1M acetic acid resulted in no detectable particulates on complete desiccation.

DISCUSSION

Resolution of Pro and Hyp was accomplished by thin layer chromatography using silicic acid gel impregnated glass fiber sheets and isopropanol:water (7:3) as the mobile phase. Chromatograms had to be developed for 8 hours to insure a clean separation of Pro and Hyp. The presence of human plasma hydrolysate had no effect on the separation of the imino acids. In addition, this system completely separated histidine from the other 21 amino acids that were examined.

When ^{3}H-Pro is used in animal or tissue culture experiments, about 10% is incorporated into glutamic acid, aspartic acid, alanine, arginine, valine and tyrosine.[1] In our system glutamic acid, aspartic acid and alanine co-migrate

with Hyp. Elimination of the co-migrating amino acids can be most easily accomplished by deamination of the sample prior to chromatography, a procedure that does not affect the imino acids.[22]

When elution of Hyp from the developed chromatograms was done by placing the chromatogram chips containing the imino acid in water or a weak acid the recovery was only 40 - 50%. In addition, the extracts contained significant amounts of particulate material. Elution of Hyp by dialysis of the chromatogram chips consistently gave an 85% recovery of the Hyp. When water was used as a solvent, the dialysates contained substantial quantities of a fine particulate material (presumably silica) that precipitated when the volume of the dialysates was reduced. The particulate material posed serious problems for spectrophotometric determination of Hyp using Ehrlich's reagent.[4] The particulates were eliminated by using 1M acetic acid for the dialysis procedure.

The presented chromatographic procedure effectively separates Pro and Hyp which can subsequently be localized by autoradiography and extracted by dialysis from the developed chromatograms for quantitation by standard methods such as scintillation counting or Ehrlich's reagent. This is a simple, accurate and inexpensive method for the separation of Pro and Hyp from complex biological samples.

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SIMULTANEOUS DETERMINATION OF THE 5-LIPOXYGENASE INHIBITOR "ZILEUTON" AND ITS N-DEHYDROXYLATED METABOLITE IN UNTREATED RAT URINE BY MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

A specific and sensitive HPLC procedure for the quantitative determination of Zileuton, (N- (1- (benzo-[b]-thien-2-yl)ethyl)-N-hydroxyurea), and its N-dehydroxylated metabolite in an untreated urine sample was developed. Separation of these compounds is achieved with micellar liquid chromatography, using sodium dodecyl sulfate (SDS) as the mobile phase and a CN-bonded silica column with UV detection at 262 nm. Because of the dissolving power of the micellar phase, urine samples were injected into the system without time-consuming protein precipitation and/or drug extraction steps.

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Changes in mobile phase variables such as SDS concentration, pH and/or organic modifier concentration profoundly affected both chromatographic selectivity and drug retention. Linear calibration curves of the relative peak heights versus concentrations were obtained from 0.10 - 5.0 ppm for Zileuton and its metabolite. The minimum quantifiable concentration using this column was 0.08 - 0.10 ppm.

INTRODUCTION

Zileuton (Abbott-64077, Figure 1) is a 5-lipoxygenase inhibitor with potential clinical applications in the treatment of inflammatory diseases.[1,2] When Zileuton is metabolized, its major conversion is the N-hydroxylated form (Abbott-66193, Figure 1). Although an analytical method exists for the determination of Zileuton and its metabolites in biological fluids,[3] this method requires a lengthy solid phase extraction pretreatment process. A C_{18} stationary phase was their column of choice with a complex mobile phase of acetonitrile-methanol-tetrahydrofuran-water with phosphate buffers. Disposal and use of these toxic solvents poses danger not only to the individual working with them but also to the environment.

Several analytical problems exist in the assay of drugs in physiological fluids.[4] Often the drugs are present in low concentration, are strongly bound to proteins, and are in a complex matrix. Frequently the technique of choice for these types of analyses is high performance liquid chromatography (HPLC). However, due to interference from numerous endogenous compounds in the sample as well as very low analyte concentrations, direct HPLC assay of physiological fluids is usually unfeasible. The high-molecular-mass proteins found in these samples are particularly troublesome since they tend to precipitate within the column leading to rapid loss of chromatographic efficiency.

Micellar liquid chromatography (MLC) with normal micelles is an alternative to conventional reverse phase liquid chromatography (RPLC), which employs aqueous solutions of surfactants above the micellar critical concentration as the mobile phases, instead of hydro-organic mixtures.[5] One of the main advantages of MLC is the possibility of determining drugs in physiological fluids without the need of a separation of the proteins present in the samples.[6] Micellar chromatography is suited for direct injection because

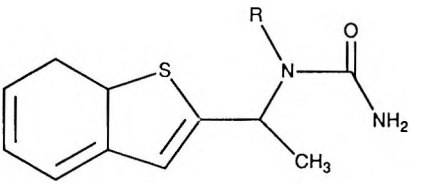

Figure 1: Structures of Zileuton, R = OH and Abbott-66193, R = H

the proteins are solubilized by the micellar aggregates in the mobile phase and are eluted with the void volume.[7]

The purpose of this study was to develop a specific and sensitive procedure for the simultaneous quantification of Zileuton and its N-dehydroxylated metabolite by direct injection of untreated urine sample into a micellar high performance liquid chromatographic system.

MATERIALS AND METHODS

Instrumentation

The chromatography mobile phase was delivered by an Applied Biosystems Model 400 isocratic pump. Samples were injected using a Rheodyne Model 7125 syringe-loading sample injector with a 200-µl loop. Peaks were detected with an Applied Biosystems Model 785A UV programmable detector set at 262 nm. The chromatograms were obtained using a Spectra-Physics chrom-jet integrator. A FlAtron CH-30 column heater controlled by a TC50 controller was used for the high temperature experiments. The column (25 cm long and 4.6 mm i.d.) was packed with 5 micron IB-sil CN-bonded silica (Phenomenex, Torrance, CA). The void volume of the system was calculated by using the peak of injected water. All pH measurements were performed with an Orion Model EA 920 Digital pH meter and an Orion combination glass electrode. The flow rate was set at 1.0 mL/min.

Chemicals and Reagents

2-Propanol, phosphoric acid (85%), sodium dodecylsulphate, SDS, (99%), and sodium phosphate dibasic heptahydrate were purchased from commercial vendors and used without purification. The water used was from a Millipore Milli-Q water purification system. Zileuton and its N-hydroxylated metabolite were gifts from Abbott Laboratories.

A stock solution of sodium dodecylsulphate was prepared in the Milli-Q water at a concentration of 0.2 M. Micellar mobile phases were prepared by adding the appropriate amounts of the stock SDS solution, sodium phosphate dibasic and 2-propanol. The mobile phases were diluted to the volume with the Milli-Q water to obtain different concentrations and adjusted to the appropriate pH with phosphoric acid. The mobile phases were vacuum filtered through a 0.45 μ nylon membrane.

Stock solutions of Zileuton and its metabolite were made by dissolving them in 2-propanol and then diluted to appropriate volume with 0.05 M SDS solution. These solutions were stored in a freezer and were stable for at least 2-weeks. Working solutions were prepared by dilution of the stock solutions.

Preparation of Standard in Rat Urine

Urine samples were obtained from female rats. Standard solutions were prepared by the addition of appropriate volumes of standard solutions to urine samples that had been diluted in half with water. Samples were then frozen and stored in the dark. Under such conditions spiked samples were stable for at least a week. The stability after one week was not investigated.

RESULTS AND DISCUSSION

Studies to determine the adequate composition of the mobile phase (pH, concentration of SDS, and concentration of modifier) for the separation and elution of Zileuton and its N-hydroxylated metabolite in both aqueous solutions and urine matrix were performed.

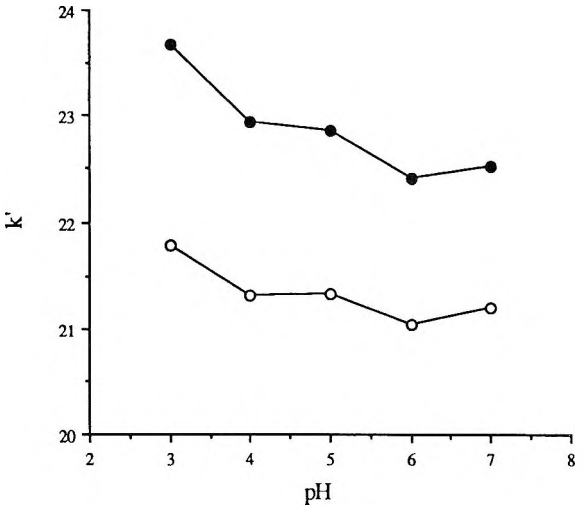

Figure 2: Dependence of capacity factor on pH of the mobile phase with 0.025 M SDS in 0.01 M phosphate buffer at room temperature. (o) = Zileuton and (•) = Abbott-66193.

Separation of Zileuton and Abbott-66193

The effect of pH on the retention of Zileuton and its metabolite was examined in the pH range of 3.00 - 7.00. For Zileuton there was no significant effect on the capacity factor in the region studied (Figure 2). This is expected since the drug is neutral in this region. For Abbott-66193, the capacity factor decreased when the pH of the mobile phase was increased from 3.00 to 5.00 and remained constant at higher pH (Figure 2). In this pH region, the metabolite Abbott-66193 is protonated and therefore will interact with the adsorbed anionic surfactant on the stationary phase, thus shifting the equilibrium from the mobile phase to the stationary phase. The resolution of the solutes decreased as the pH increased from 3.00 to 7.00 (Figure 3).

Figure 4 shows the dependence of the capacity factor on the surfactant concentration in the range of 0.02 - 0.10 M at pH 3.00. Increasing the SDS concentration results in a decrease of the retention times and the capacity factor of the solutes. This is most likely due to an increase in the number of micelles in the mobile phase as the concentration of surfactant increases.[8] When the results were replotted as 1/k' against [SDS], a linear relationship was

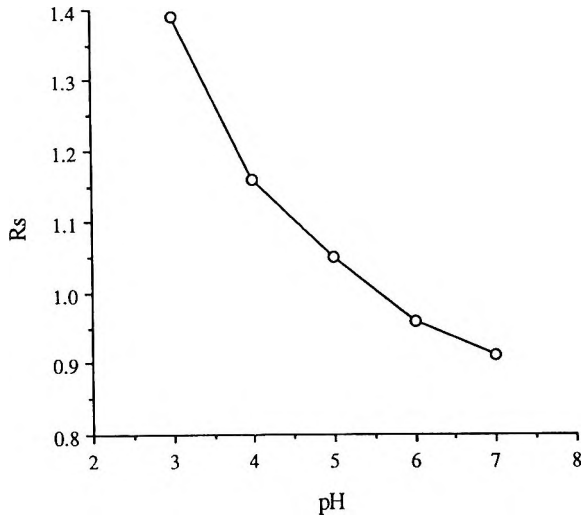

Figure 3: Effect of pH on the resolution of Zileuton and its metabolite. Conditions: 0.025 M SDS containing 0.01 M phosphate buffer.

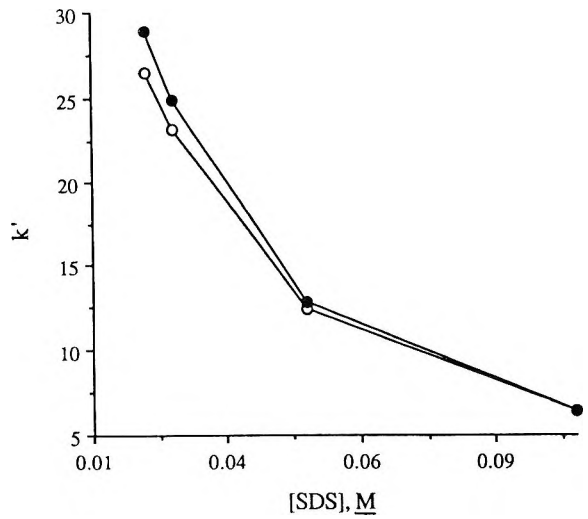

Figure 4: Dependence of capacity on [SDS] of the mobile phase pH 3.00 at room temperature. (o) = Zileuton and (•) = Abbott-66193

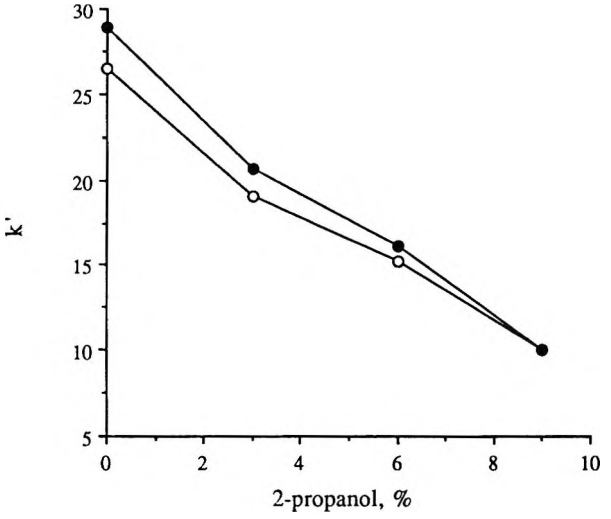

Figure 5: Dependence of capacity factor on the percentage of 2-propanol: 0.025 M SDS at pH 3.00. (o) = Zileuton and (•) = Abbott-66193

obtained. This behavior also is expected when hydrophobic interaction is the predominant factor contributing to the retention time.[9]

When 2-Propanol was added to the mobile phase in an effort to reduce the mass transfer of the solutes exiting out of the micelle and desorping from the column, both the capacity factor and resolution decreased (Figure 5). This may be related to a decrease in the polarity of the mobile phase and therefore a shift in the equilibrium of the lipophillic compounds from the stationary phase to the mobile phase.

In an attempt to improve the column efficiency as reported by other workers,[10] the effect of 2-propanol added to the mobile phase was studied. The number of theoretical plates (N) and the asymmetry ratios (B/A) from the observed peaks were compared. The values of N were calculated from the following equation which corrects for the asymmetry of skewed peaks:[10]

$$N = \frac{41.7\left(t_R / W_{0.1}\right)^2}{B/A + 1.25} \qquad (1)$$

where: t_R is the retention time,
$W_{0.1}$ is the peak width measured at 10% peak height,
B/A is the asymmetry ratio.

The results were shown in Table 1. The presence of 3% 2-propanol slightly increased the plate count and showed no significant effect on the asymmetry of both Zileuton and its metabolite, Abbott-66193.

The effect of column temperature on both the peak asymmetry and column efficiency was also studied. There were small improvements in the column efficiency as the temperature was increased from room temperature to 50°C (Table 2). However, we decided to carry out the remaining investigations at room temperature because of our experience that at higher temperatures, the column life, in particularly CN column, is shortened drastically.

Elution Studies on Urine Matrix

The chromatogram of the urine matrix shows a broad band due to the presence of the proteins at the solvent front and other smaller peaks due to the presence of endogenous compounds (Figure 6a and b). I. Pérez-Martinez et al.[11] indicated that in a purely micellar medium of SDS, the retention of the broad band was not large at pH 6.00 - 7.00 and was slightly affected by the SDS concentration. The retention of this band increased with decreasing pH.

Table 1

Variation of Efficiency and Asymmetry with 2-Propanol Concentration

	Zileuton, A64077		Metabolite, A66193	
2-Propanol, %	**N**	**B/A**	**N**	**B/A**
0	6960	1.02	6170	1.04
3	7167	1.00	6328	1.00
6	6061	1.07	6573	1.15
9		No Separation		

0.025 M SDS containing 0.01 M phosphate buffer and 3% 2-propanol pH 3.00 at 1.0 mL. min^{-1}; room temperature; 262 nm

Table 2

Variation of Efficiency and Assymmetry with Temperature

	Zileuton, A64077		Metabolite, A66193	
Temp,° C	**N**	**B/A**	**N**	**B/A**
Ambient	5095	1.12	5604	1.00
30	5993	1.14	5968	1.06
40	5877	1.25	6094	1.00
50	6269	1.18	5741	1.00

0.025 M SDS containing 0.01 M phosphate buffer and 3% 2-propanol pH 3.00 at 1.0 mL. min^{-1}; room temperature; 262 nm.

However, in order to obtain a satisfactory separation between Zileuton and its metabolite in the urine matrix (see Figure 3), the pH of the mobile phase was fixed at 3.00.

Direct Sample Injection

Based on these studies a mobile phase consisting of a solution containing 0.025M SDS and 3% 2-propanol at a pH of 3.00 were chosen as the optimum conditions for quantitation of Zileuton and its metabolite, Abbott-66193, in untreated urine.

Under these conditions, retention times for Zileuton and its metabolite were: (26.19 ± 0.20 minutes) and (27.71 ± 0.25 minutes) respectively, and all components in the urine sample eluted within 35 minutes (Figure 7). There were no significant changes in the retention times, pressure, or carry over peaks from one injection to another during this study.

The recovery of Zileuton and its metabolite from spiked urine was studied by adding different amount of these species to control (blank) urine samples. The results are shown in Table 3. The average percent recovery for Zileuton was 99.8 ± 3.6 over a 0.50 - 5.00 ppm concentration range and the recovery for Abbott-66193 was 101.1 ± 1.6 over the same concentration range as Zileuton. On the basis of on these results, both compounds can be quantitatively recovered.

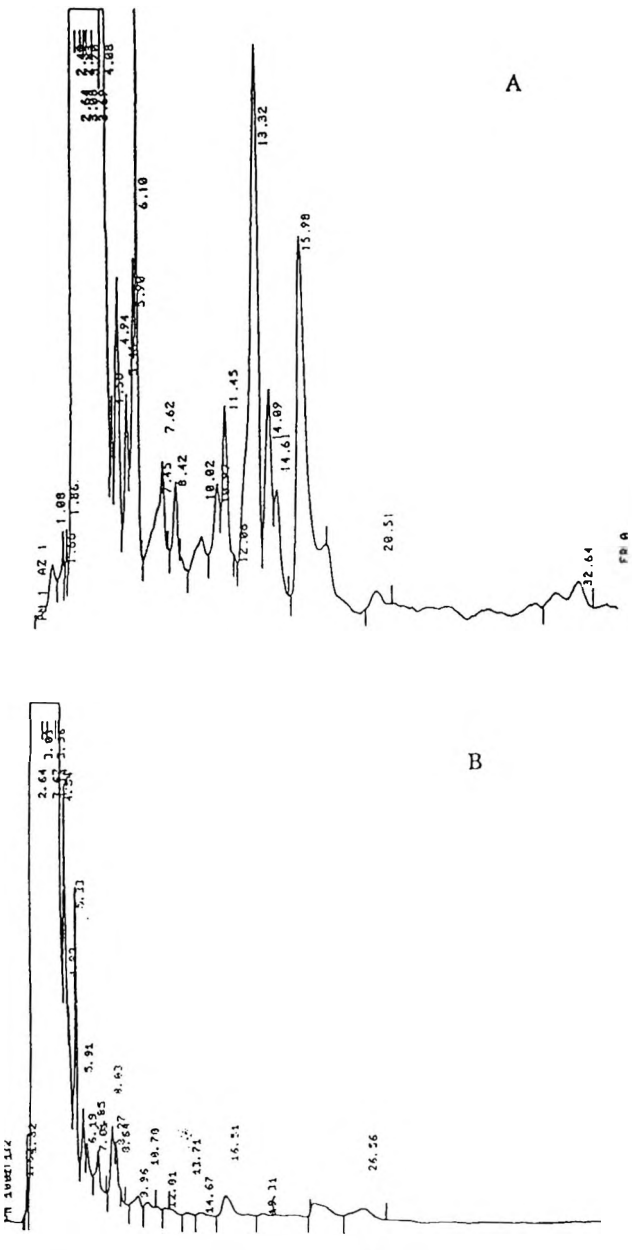

Figure 6: Experimental chromatograms: (a) urine sample (mobile phase 0.025 M SDS- 3% 2-propanol at pH 3.00); (b) urine sample (mobile phase 0.025 M SDS - 3% 2-propanol pH 7.00)

Table 3

Recovery of Zileuton and Abbott-66193 from Urine Samples

	Zileuton		Abbot-66193	
Concentration (ppm)	**% Recovery**	**Average Recovery**	**% Recovery**	**Average Recovery**
0.50	103 101 107	103.7 ± 3.0	104 106 95	101.7 ± 5.8
1.0	93 102 90	95.0 ± 6.2	96 105 95	98.7 ± 5.5
2.0	100 103 98	100 3 ± 2.5	103 102 102	102.3 ± 0.6
5.0	101 101 99	100.3 ± 1.2	100 102 103	101.7 ± 1.5

0.025 M SDS containing 0.01 M phcsphate buffer and 3% 2-propanol pH 3.00 at 1.0 mL. min^{-1}; room temperature; 262 nm.

The sensitivity of the method was investigated by preparing calibration plots of 50 µL each of Zileuton and Abbott-66193 in urine. Linear calibration curves of relative peak heights versus concentrations ($r > 0.9999$) were obtained from 0.25 to 5.00 ppm for Zileuton and Abbott-66193.

The typical linear relationship for the calibration curve can be expressed by the following regression equations:

$$\text{Peak Height} = 1850.7\,[\text{Zileuton}] + 234.12 \quad (2)$$

$$\text{Peak Height} = 1844.7\,[\text{Abbott-66193}] + 261.6 \quad (3)$$

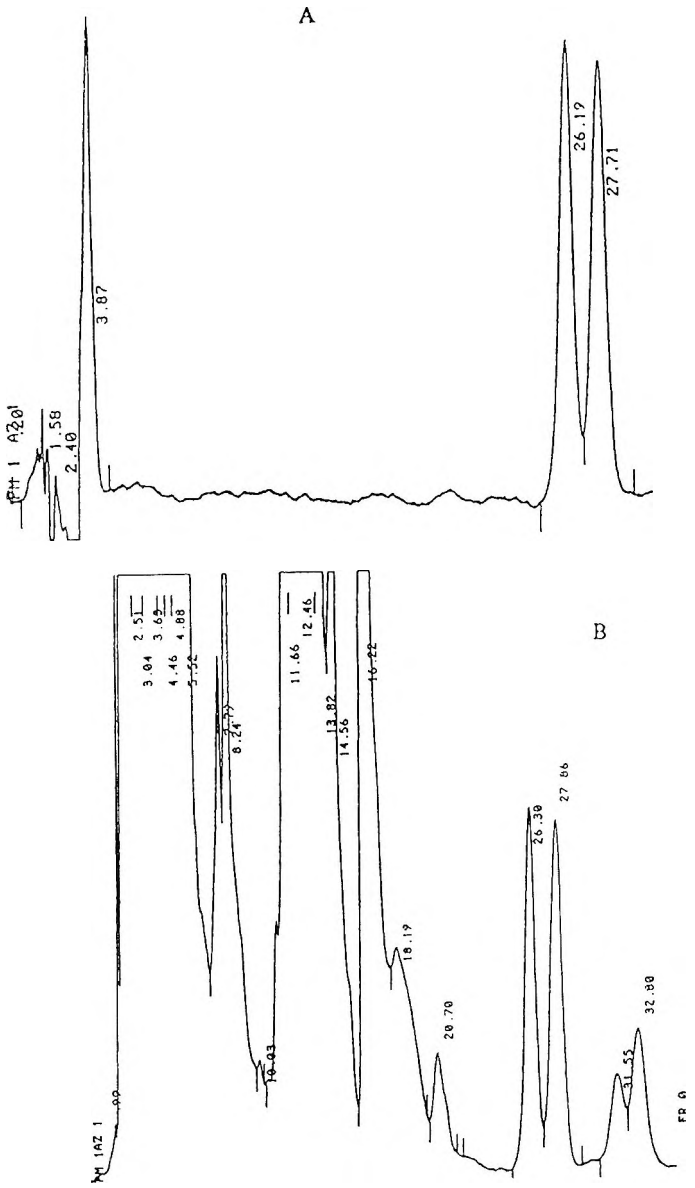

Figure 7: Typical chromatograms of (A) Zileuton and Abbott-66193 in surfactant (B) Zileuton and Abbott-66193 spiked into control (blank) rat urine. Conditions: 0.025 M SDS containing 0.01 M phosphate buffer pH 3.00, and 3% 2-propanol at room temperature.

Table 4

Precision and Accuracy of Concentration Measurements of Zileuton and Abbott-66193 in Rat Urine Samples

	Zileuton			Abbott-66193		
Conc. (ppm)	**Average ± SD**	**CV**	**Er, %**	**Average ± SD**	**CV**	**Er, %**
0.25	0.20 ± 0.02	10	-0.20	0.20 ± 0.01	5.0	-0.20
0.50	0.49 ± 0.03	6.1	-0.02	0.52 ± 0.05	9.6	0.04
1.00	1.11 ± 0.05	4.5	0.11	1.11 ± 0.03	2.7	0.11
2.00	2.48 ± 0.04	1.6	0.24	2.42 ± 0.03	1.2	0.21
5.00	5.20 ± 0.59	11	0.04	4.96 ± 0.07	1.4	-0.01

0.025 M SDS containing 0.01 M phosphate buffer and 3% 2-propanol pH 3.00 at 1.0 mL. min^{-1}; room temperature; 262 nm. Three replicates at each concentration.

The minimum detectable concentrations were 0.1 ppm for Zileuton and 0.08 ppm for Abbott-66193 based on a signal-to-noise ratio of 3 with an injection of 50 µL.

The precision and accuracy of this method was determined by making replicate measurements of five different standards within a concentration range of 0.25 to 5.0 ppm each of Zileuton and Abbott-66193. Very low relative standard deviations with low relative errors were obtained (Table 4).

Analytical Applications

The amount of Zileuton and Abbott-66193 in a pooled 24-hr female rat urine using the optimal conditions was determined. For a 50 µL sample, using the regression equation from the calibration curve and recovery values, the amount of Zileuton and Abbott-66193 was determined to be: 8.87 µg Zileuton / mL and 1.39 µg Abbott-66193/mL. These results are within the expected range from the dose amounts in the recovery studies by Abbott Laboratories.

CONCLUSIONS

The procedure reported allows the determination of Zileuton and its metabolite, Abbott-66193, in urine samples. Owing to the minimum handling time required, large series of samples can be processed without pretreatment. The results also show that the procedure could be a satisfactory choice to determine those compounds in other matrices. In addition, it utilizes a mobile phase which is nontoxic, nonflammable and relatively inexpensive . The sensitivity of the assay described is currently lower than the conventional assay employed by Abbott Laboratories.[3] Further investigations to increase the sensitivity of the assay are underway.

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DETERMINATION BY HPLC WITH ELECTROCHEMICAL DETECTION OF FORMOTEROL RR AND SS ENANTIOMERS IN URINE

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ABSTRACT

A method is described for the determination of the R,R- and S,S-enantiomer of the long-acting ß2-adrenoceptor agonist formoterol, which is marketed as a racemate for the treatment of asthma. The methodology is a modification of a previously published assay for formoterol. The sample clean-up from urine takes place by liquid liquid extraction followed by solid phase extraction. An AGP-column combined with electrochemical detection is used for the separation and detection of the enantiomers.

A diastereomer of formoterol (R,S- or S,R- configuration) is used as internal standard. The lower limits of detection of R,R-formoterol and S,S-formoterol were 60 and 75 pmol/L respectively. The method was validated by a cross-check of spiked urine samples with a GC/MS method for "racemic" formoterol (correlation of the sum of the enantiomers measured by HPLC with GC/MS results: r=0.999, n=17). The method appears to be well suited for pharmacokinetic studies of formoterol enantiomers in human subjects after inhalation of therapeutic doses of formoterol.

INTRODUCTION

Formoterol fumarate, 2-Hydroxy-5-[1RS)-1-hydroxy-2-{[(1RS)-(p-methoxyphenyl)-1-methylethyl]-amino}-ethyl]-formanilide fumarate dihydrate, is a new long-acting, $ß_2$-adrenoceptor agonist, with a high potency. Formoterol fumarate is a mixture of the two isomers with the configurations R,R and S,S; the RR-enantiomer being the active compound.[1,2] Pharmacokinetic data on the formoterol enantiomers are still missing due to the lack of a sensitive enantioselective assay.

Recently van den Berg et al. developed a high performance liquid chromatography (HPLC) assay with electrochemical detection to determine racemic formoterol in plasma.[3] This method was used to investigate the pharmacokinetics of racemic formoterol in plasma of human subjects after an oral dose of 168 μg of formoterol fumarate.[4] Despite the high sensitivity of this assay, it is still not sensitive enough to determine the plasma concentrations after inhaled therapeutic doses of formoterol fumarate and it is not suitable for the determination of the separate enantiomers. Formoterol is partly excreted into the urine, both after inhalation and oral administration.[5] Using a GC/MS method[7] it was shown that after inhalation of 12 μg formoterol, 24 ± 14 % of the dose was excreted as unchanged and conjugated formoterol into the urine within 12 hours, giving urinary concentrations which in pricipal could be determined using HPLC with electrochemical detection. Thus measuring the urinary excretion of formoterol is, at the moment, the only available option to get more information about the pharmacokinetics of formoterol and its enantiomers after inhalation of low therapeutic doses.

The four possible diastereomers of formoterol can be separated on a chiral column.[1] Based on this observation and our experience with electrochemical detection of formoterol, we have developed an enantioselective HPLC method

for the determination of R,R- and S,S-formoterol in human urine. Finding a good clean-up procedure for the urine samples, together with the selection of a suitable internal standard, was critical in the development of the assay.

MATERIALS

Apparatus

The HPLC system consisted of a Spectraflow 400 (Separations, Rotterdam, The Netherlands) with a Rheodyne 7125 injector with a PEEK 200 μL loop. An electrochemical detector Waters model EC 460 (Millipore-Waters Etten-Leur The Netherlands) was employed. The electrochemical detector was used with the original electrochemical cell or with an Antec electrochemical cell (Antec Leiden, The Netherlands), both with a glassy carbon working electrode, a reference Ag/AgCl and an auxiliary 316 stainlesss steel electrode. For appropiate functioning of the detector with the Antec electrochemical cell temperature fluctations must be minimized. For that reason the analytical column and the guard column were built into the cell-house of the detector, and the teflon tubing between column and cel was isolated with silicone tubing. The voltage of the working electrode was set on + 0.63 volt.

The enantioselective separation was achieved using a chiral α_1-acidglycoprotein (AGP) column 100x4.0 mm with an AGP guard column 10x4.0 mm (Baker, Deventer, The Netherlands) at ambient temperature (22°C).

Peak heights were recorded using a dual channel recorder (Kipp & Zonen, Delft, The Netherlands), one channel, connected with the 1.0 V output, recorded the low range concentrations the other, connected with the 10 mV output, the high range concentrations.

The mobile phase consisted of isopropanol: phosphate buffer 50 mM pH 7.0 (1.5 : 100) with 1mM KCL and a small quantity of complexing agent (EDTA). The mobile phase was filtered using 0,22 μm filter (Millipore-Waters Etten-Leur, The Netherlands). Electrochemical detector and pump (flow 0.9 mL/min) were continuously running 24 hours a day.

Chemicals

Chemicals, water (18 MΩ) and the organic solvents (Merck, Darmstadt, Germany) were of analytical grade and used without further pretreatment. Racemic formoterol fumarate, its enantiomers R,R- and S,S-formoterol fumarate as well as the racemic mixture of their diastereomers (configuration R,S and S,R) were kindly provided by Ciba-Geigy Ltd., Basle, Switzerland. Solid-phase SiOH 100mg 1mL extraction columns were obtained from Baker (Baker, Deventer, The Netherlands).

Preparation of theInternal Standard

The internal standard working solution was prepared as follows: 50 μL of a stock solution of the diastereomers of formoterol fumarate in methanol (concentration: 2.5 mg/mL) was injected into the HPLC apparatus equipped with the chiral AGP column. An UV detector (210 nm) was used instead of the electrochemical detector, the mobile phase being 10 mM phosphate buffer pH 7.0 (flow 0.9 mL/min). The last eluting peak (retention time 120 min) was collected to a total volume of about 50 mL. This solution containing either the R,S- or S,R- enantiomer (the absolute configuration was not determined) was used directly as internal standard working solution.

METHODS

Clean-up

A stock solution of racemic formoterol fumarate in methanol was prepared at a concentration of 100 μg/mL and was stored at - 20°C. To prepare calibration samples, working solutions were made by appropriate dilutions of the stock solution in double distilled water. 100 μL of the respective working solution were added to 900 μL of drug free urine, collected from healthy volunteers during the day. The calibration samples covered a concentration range of 0.15 to 48 nmol/L of R,R- and S,S-formoterol.

A two step extraction procedure was needed for a satisfactory clean-up of the urine samples. In glass tubes 100 μL of the IS solution and 100 μL of 250 mM phosphate buffer pH 8 were added to each calibration sample and to 1 mL aliquots of urine samples with unknown concentrations. The mixture was briefly vortexed and 3 mL of ethyl acetate was added. Extraction was

performed by mechanical tumbling during 30 minutes at 25 rpm followed by centrifugation for 10 minutes at 4000 rpm.

The organic layer was carefuly removed with a Pasteur pipette and brought upon a preconditioned (see below) solid-phase silica extraction column. The columns were washed by successive rinsing with 1 mL of ethyl acetate and 10 mL of 5% isopropanol in water, and were then centrifuged for 10 minutes at 4000 rpm.

Formoterol and the internal standard were eluted from the columns with 3 mL of methanol, and the eluate was evaporated to dryness at 30°C under a continuous stream of nitrogen. The residue was redissolved in 100 μL of the mobile phase and injected onto the HPLC column.

The solid-phase extraction columns were conditioned by prewashing with 3 mL methanol followed by 3 mL ethylacetate. Between the conditioning and the concentration step, care was taken to ensure that the sorbent bed did not dry.

AnalyticalCross-check

The following samples were prepared at Ciba-Geigy, Basle, using blank urine obtained from a healthy subject: Six samples spiked with racemic formoterol, six samples spiked with either R,R- or S,S- formoterol and six samples spiked with both enantiomers. The concentration range was 0.4 to 40 nmmol/L for each enantiomer. Two blank urine samples completed the set of 20 samples. Each sample was split into two aliquots. One set of samples was sent deep-frozen to the Academic Medical Center, Amsterdam, where the samples were analysed by the described enantiospecific HPLC method. The second set of samples was stored below -18°C until analysis by a non-stereospecific gas chromatographic/mass-spectrometric (GC/MS) method at Ciba-Geigy, Basle.

The GC/MS method used was based on the method described by Kamimura et al.[1] The main modifications were as follows: Deuterium labelled ($^2H_5$ to $^2H_7$) formoterol fumarate was used as internal standard. Gas chromatography was performed on a fused silica capillary column (12 m x 0.2 mm i.d.) coated with 5% diphenyl 95% dimethyl polysiloxane. The mass spectrometer was operated in the negative ion chemical ionisation mode (NCI) using methane as reagent gas and selected ion monitoring (SIM) was

performed on the fragment ions (M-HF) m/z 584 (derivative of formoterol) and m/z 589 (derivative of deuterated formoterol).

VALIDATION AND RESULTS

Calibration

To construct calibration curves, urine samples with known concentrations were prepared as described in a previous section.

After the samples were processed, the entire extract was injected into the injection valve and released onto the analytical column via the 200 μL loop. The peak-heights of the compounds of interest were measured and ratios of R,R- and S,S- enantiomers to the internal standard were calculated by hand. The calibration curves for both the enantiomers were calculated with linear regression analysis. The following terms for the calibration curve (Y=A+Bx) in the range of 0.1 to 50 nmol/L were obtained: RR-calibration curve; intercept 0.0108, slope 0.2575, correlation coefficient 0.9998, SS-calibration curve; intercept 0.0018, slope 0.1952 , correlation coefficient 0.9999.

Table 1

Comparison of HPLC Results (Sum of Both Enantiomers: = y) vs. GC/MS Results (=x); Linear Least-Squares Regression Line y = a + bx; R = Coefficient of Correlation; N = Number of Samples

	a	b	R	N
First HPLC assay vs. GC/MS	-0.3340	0.9885	0.9987	17*
Second HPLC assay vs. GC/MS	-1.2666	1.0549	0.9953	18

*One sample could not be evaluated due to distrubances in the HPLC chromatogram.

Table 2

Comparison of Found (= y) vs. Given (= x) Concentrations; Linear Least-Squares Regression Line y = a + bx; R = Coefficient of Correlation; N = Number of Samples

	a	b	R	N
First HPLC assay: R, R-formoterol	-0.0485	0.9529	0.9979	14*
Second HPLC assay: R, R-formoterol	-0.3330	0.9939	0.9878	15
First HPLC assay: S,S-formoterol	-0.0684	0.9642	0.9995	14*
Second HPLC assay: S, S-formoterol	-0.5168	1.0234	0.9966	15
GC/MS: sum enantiomers	0.0184	0.9797	0.9999	18

*One sample could not be evaluated due to disturbances in the HPLC chromatogram.

The recoveries of R,R-formoterol and S,S-formoterol were 93,2% (CV% 2.6) and 92.3 (CV% 4.1) respectively.

Analytical Cross-check

The concentrations obtained with the HPLC and the GC/MS assay and also the given and found concentrations were compared and evaluated by means of linear regression ('zero' concentrations were not included). The results are summarized in Tables 1 and 2.

Table 3

Between-Day Precision and Accuracy of R,R-Formoterol

Expected (nmol/L)	Found (n=3) (nmol/L)	Inter-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	29.12	2.8	-2.1
11.89	11.41	2.9	-4.0
2.97	2.84	2.2	-4.4
1.49	1.46	5.2	-2.0
0.48	0.51	12.4	+6.3

Table 4

Between-Day Precision and Accuracy of S,S-Formoterol

Expected (nmol/L)	Found (n=3) (nmol/L)	Inter-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	28.85	2.7	-3.0
11.89	12.05	7.5	+1.3
2.97	3.01	2.7	+1.3
1.49	1.52	7.0	+2.0
0.48	0.56	12.7	16.7

Between-day Precision and Accuracy

Five spiked human urine samples with concentrations in the range of 0.68 - 29.73 nmol/L R,R-formoterol and S,S-formoterol were analyzed on three different days (Table 3 and 4).

The inter-assay coefficient of variation ranged from 2.2 to 12.4 % for R,R-formoterol and 2.7 to 12.7 % for S,S-formoterol. The deviation from the mean values ranged from -4.4 to + 16.7 %.

Table 5

Within-Day Precision and Accuracy of R,R-Formoterol

Expected (nmol/L)	Found (n=5) (nmol/L)	Intra-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	28.68	2.6	-3.5
2.97	2.99	1.6	+0.7
1.49	1.48	4.8	+0.7

Table 6

Within-Day Precision and Accuracy of S,S-Formoterol

Expected (nmol/L)	Found (n=5) (nmol/L)	Intra-assay Precision (C.V. %)	Deviation From Theory (%)
29.73	29.02	2.1	-2.4
2.97	2.89	2.7	-2.7
1.49	1.43	4.0	-4.0

Within-Day Precision and Accuracy

Three spiked human urine samples with concentrations in the range of 1.49 to 29.73 nmol/L R,R-formoterol and S,S-formoterol were analyzed five times on the same day (Tables 5 and 6).

The coefficient of variation ranged from 1.6 to 4.8 % for R,R- enantiomer and 2.1 to 4.0 % for S,S-formoterol. The deviation from theory of the mean values ranged from -3.5 to +0.7 % for R,R-enantiomer and -4.0 to -2.4 % for S,S-formoterol.

DISCUSSION

Isolation of Formoterol from Urine

When developing the assay for urine samples with electrochemical detection and enantiomers separation with a AGP-column, we had to face problems concerning variable matrix composition of the urine samples, instability of the electrochemical detector and low resolution of the AGP column. The finding of an internal standard which has a close chemical resamblance to the components of interest, allowed a thorougly cleanup. We assume that both formoterol and the internal standard are trapped on silica by means of their formanilide and amine functional groups.

Chromatography and Detection

R,R-formoterol, S,S-formoterol and diastereomer R,S- or S,R-formoterol retention times were 8.7, 11.3 and 15.5 min., respectively (Figure 1). No endogenous substances with similar retention times were seen.

The use of an ANTEC electrochemical detector cell made it possible to detect the components of interest in the pmol/L range. According to a signal-to-noise ratio of 3:1, the lower limits of detection of R,R- formoterol and S,S-formoterol were 60 and 75 pmol/L respectively.

As was discussed before[3] it is especially the ortho position of the phenolic hydroxygroup of the molecule which makes electrochemical detection a viable choice for highly sensitive determination.

Tuning the mobile phase composition did not effect the enantioselectivity. Variable organic modifiers were used, such as acetonitril,methanol, aceton and isopropanol, and also the phosphate buffer molarity. These did not change the selectivity but only effect the capacity.

The method appeared to be well suited for pharmacokinetic studies of formoterol enantiomers in human subjects after inhalation of therapeutic doses of formoterol.

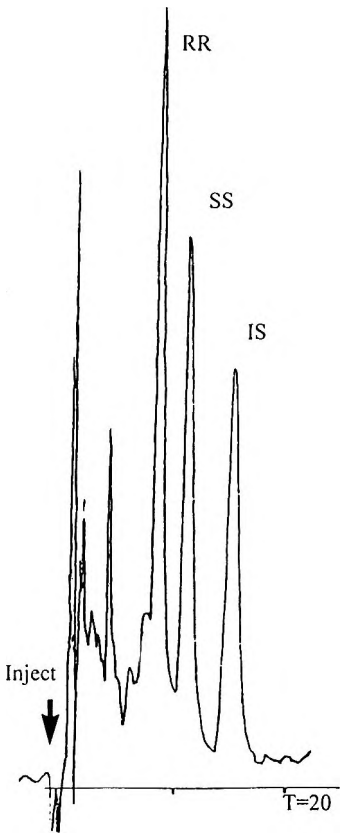

Figure 1. Separation of the enantiomers of formoterol (RR and SS) and its diastereomer used as internal standard (IS).

Application

The described method was applied to the determination of both enantiomers in urine of healthy volunteers after single inhaled doses of 12, 24, 48, 96 μg formoterol fumarate. The profiles of the urinary excretion rate of the enantiomers as obtained in a representative subject after inhalation of the 48 μg dose are shown in Figure 2.

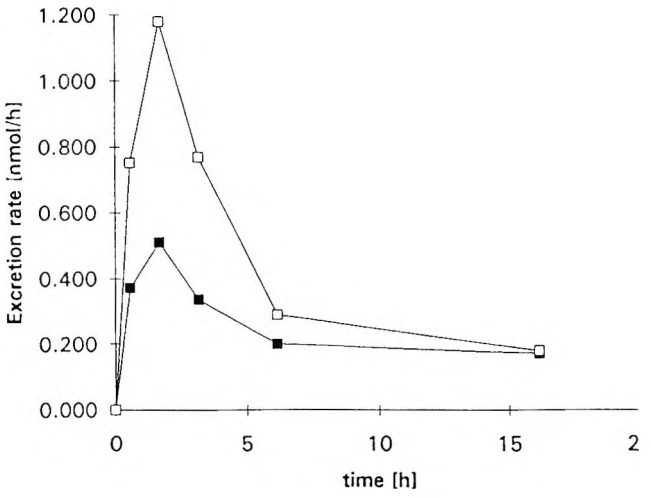

Figure 2. Urinary excretion rate of R,R- (9) and S,S-formoterol (;) in a healthy volunteer after a single inhaled dose of 48 μg of formoterol fumarate.

CONCLUSIONS

The two step clean-up procedure appears to be very well suited for determination of the enenatiomers of formoterol in urine using a AGP-column for the chiral separation with oxidative electrochemical detection. This method can be used in pharmacokinetic- pharmacodynamic studies of formoterol enantiomers in urine of healthy volunteers and patients.

ACKNOWLEDGEMENTS

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1996

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

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1997

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1998

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1999

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2000

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2001

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2002

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F. D. Pierce, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

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1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

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Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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