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**February 1997**

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# USE OF EXPERIMENTAL DESIGN IN THE OPTIMIZATION OF HPLC METHODOLOGY FOR THE SEPARATION OF STEREOISOMERS

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## ABSTRACT

Statistical experimental design was used to optimize chromatographic separations of two pharmaceutical compounds from their respective stereoisomeric impurities. The HPLC separations employ beta-cyclodextrin as a mobile phase additive to provide stereoselectivity. A fractional factorial design involving eleven separate experiments was used to generate the required data. The methods were optimized for percentage of organic in the mobile phase, concentration of cyclodextrin in the mobile phase, mobile phase buffer pH and stationary phase. Analysis of the data showed which of the factors needs to be critically controlled for robustness of the methods.

## INTRODUCTION

Lometrexol sodium and LY231514 are structurally similar compounds of pharmaceutical interest as anti-tumor agents. Lometrexol (Figure1), with two chiral centers has the potential of generating four stereoisomers. Because the stereochemistry at one of the chiral centers is determined early in the synthetic process using high-purity reagents, the potential for formation of two of the

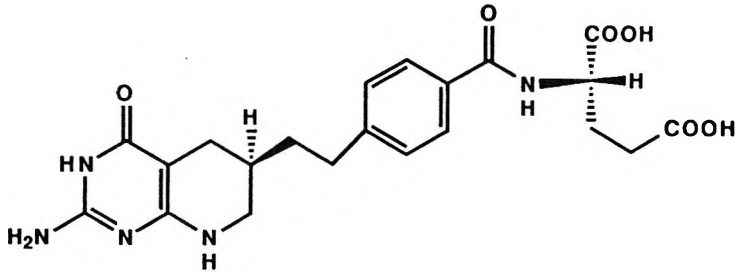


**Figure 1**. Structure of Lometrexol.

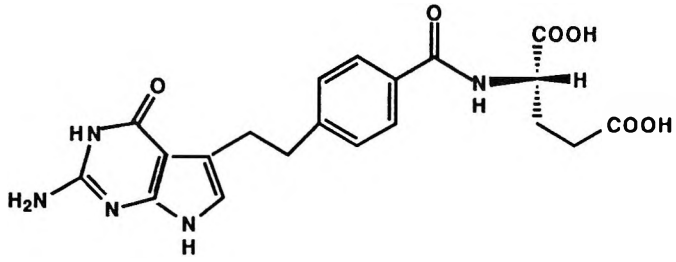


**Figure 2**. Structure of LY231514.

stereoisomers is negligible. The other pair of isomers, lometrexol and a diastereomeric impurity, must be analyzed by a suitable method. Although in theory these diastereomers should be separable by reversed phase HPLC in an achiral environment, repeated attempts to effect a separation using a variety of columns and conditions were unsuccessful. Thus, a chiral environment using beta-cyclodextrin as a mobile phase additive to a reversed phase HPLC system was invoked to effect a separation of the isomers.

In contrast to lometrexol, LY231514 (Figure 2) has a single chiral center. A chiral assay is required to assess the level of the undesired enantiomer. A similar assay to the one employed to separate lometrexol from its diastereomer using beta-cyclodextrin as a mobile phase additive to a reversed phase system was found to achieve a separation of LY231514 enantiomers.

Cyclodextrins have been utilized in liquid chromatographic chiral separations both as mobile phase additives as well as in bonded phases.[1-4] The approach of adding cyclodextrins to the mobile phase offers the advantages of lower cost and less concern about column ruggedness. In addition, the mobile phase additive approach offers more ways to optimize retention and selectivity.

Optimization of a method involving isomeric separation has the aim of achieving maximum resolution with minimum assay time. Robustness testing must be done to determine which operating conditions will significantly effect resolution and retention time in order to determine the degree of control required. Parameters may include: buffer pH and ionic strength, column type and temperature, type and amount of organic modifier, and flow rate. These methods were initially optimized to some extent by varying individual parameters independently, a traditional approach.[2,5,6] This practice is relatively laborious and makes it difficult to fully characterize interdependence of the parameters.

Another approach which can be used for method optimization and robustness testing is an experimental design.[7-8] Experimental designs are useful at all stages of method development and optimization. For compounds in early clinical studies a screening design with minimal experiments can be used to determine the important method parameters. For methodology included in a product registration, a full factorial design can determine the impact of method parameters and all interactions between those parameters. Described in this work is a fractional factorial design which allows for the determination of some interactions.

## EXPERIMENTAL

Chromatographic experiments were carried out using an Spectra-Physics P1500 HPLC pump equipped with a Kratos 757 UV detector, Micromeritics 728 autosampler and Reodyne 7010 injection valve. Fixed variables included flow rate (1.5 mL/min.), column temperature (30°C), detector wavelength (270 nm) and injection volume (20 µL). HPLC columns were 25 cm Phenomenex IB-SIL ODS and IB-SIL C1 with 5 micron particles.

HPLC grade acetonitrile (ACN) and water were used. Beta-cyclodextrin (ß-CD) was purchased from Ensuiko Sugar Refining Company, Ltd. Lometrexol, LY231514 and their associated isomers were synthesized in Lilly Research Laboratories. All experiments were conducted with a sample prepared at 50/50 ratio of desired/undesired isomer. JMP® statistical software was used to generate the experimental design.

## RESULTS AND DISCUSSION

### Initial Method Development and Optimization

Initial optimization of the isomeric purity assay for lometrexol involved testing one variable at a time. For each variable tested, resolution between lometrexol and the major diastereomer was measured and retention time noted. Parameters tested included flow rate, column temperature, ß-CD concentration, ACN/buffer ratio, buffer type and pH. The final mobile phase consisted of a 97% buffer (1% triethylamine-phosphate at a pH of 7.0) and 3% ACN with 8 g/L ß-CD. The flow rate was set at 1.5 mL/min. and column temperature at 30°C. The column selected was a 25 cm Phenomenex IB-SIL ODS with 5 micron particles. Assay time is 30 minutes.

The assay for LY231514 was adopted from the method conditions for lometrexol. Slight modifications were made to the mobile phase and flow rate to adjust retention time. The mobile phase was 96.5% buffer (1% triethylamine-phosphate at a pH of 7.0) and 3.5% ACN with 6 g/L ß-CD. The flow rate was set at 2.0 mL/min. and column temperature at 30°C. The column was a 25 cm Phenomenex IB-SIL ODS with 5 micron particles. Assay time is 42 minutes.

As these compounds progressed through clinical studies towards efficacy testing the sample load increased. In addition, the possibility of transferring methodology to other laboratories developed. The methods were reviewed and the decision made to use an experimental design to optimize assay conditions with the goal of shortening assay time and decreasing the ß-CD concentration. The high levels of ß-CD had a detrimental effect on the HPLC column and pump.

### Factorial Design

A full factorial design is used to determine the main effects and all interactions between the factors selected. The number of trials necessary is $2^k$, where k is the number of factors. For lometrexol and LY231514 methods the number of factors can include ß-CD concentration, stationary phase, buffer pH, buffer/organic modifier ratio, flow rate, and column temperature. Evaluating all of these parameters with a full factorial design would involve $2^6 = 64$ trials. This represents a significant amount of experimental time.

**Table 1**

**Experimental Design Factors and Values**

| Factors | Parameter | +1 | -1 |
|---|---|---|---|
| X1 | % Acetonitrile | 2 | 10 |
| X2 | g/L β-CD | 2 | 8 |
| X3 | Buffer pH | 3 | 7 |
| X4 | Stationary Phase | C1 | C18 |

**Table 2**

**Fractional Factorial Design**

| Trial | Factor | | | |
|---|---|---|---|---|
| | X1 | X2 | X3 | X4 |
| 1 | -1 | -1 | -1 | -1 |
| 2 | 0 | 0 | 0 | 0 |
| 3 | -1 | -1 | +1 | +1 |
| 4 | -1 | +1 | +1 | -1 |
| 5 | +1 | +1 | -1 | -1 |
| 6 | 0 | 0 | 0 | 0 |
| 7 | -1 | +1 | -1 | +1 |
| 8 | +1 | +1 | +1 | +1 |
| 9 | +1 | -1 | +1 | -1 |
| 10 | 0 | 0 | 0 | 0 |
| 11 | +1 | -1 | -1 | +1 |

In order to minimize experimental time, factors were carefully evaluated in light of what had been learned during the lometrexol method development. For example, limited testing of the impact of column temperature had found no significant change to retention time or resolution in a range of 30°C - 50°C.

**Table 3**

**Experimental Results**

| Trial | Lometrexol | | LY23154 | |
|---|---|---|---|---|
| | Rtention Time (min.) | Resolution | Retention Time (min.) | Resolution |
| 1 | 80 | 3.8 | 250 | 0 |
| 2 | 18 | 2.2 | 37 | 1.8 |
| 3 | 116 | 3.8 | 196 | 1.8 |
| 4 | 9 | 1.9 | 21 | 1.6 |
| 5 | 4 | 1.7 | 11 | 0 |
| 6 | 20 | 3.2 | 38 | 1.8 |
| 7 | 45 | 4.4 | 170 | 0 |
| 8 | 3 | 0.8 | 4 | 0.9 |
| 9 | 4 | 0.6 | 5 | 0 |
| 10 | 19 | 3.3 | 15 | 1.7 |
| 11 | 11 | 2.2 | 24 | 0 |

Therefore, column temperature was not considered a critical factor. Similar data existed regarding flow rate. Three key factors were selected, ß-CD concentration, buffer/ACN ratio, and buffer pH. A fourth factor, stationary phase was also selected.

The four factors in a full factorial would require 16 trials. This investment in experimental time is not extensive and would be more than appropriate for optimization of methodology intended for regulatory registrations. However, for these compounds, the goal was to improve existing methodology with a minimum amount of time. Therefore a fractional factorial design was selected. Fractional factorials measure main effects and some interactions with the number of trials is $2^{k-p}$ where p is an arbitrary number less than k. For these experiments p = 1, and the number of trials was $2^{4-1} = 8$.

Selection of maximum (+1) and minimum (-1) values for the factors is critical for generation of useful data. Practical considerations such as ß-CD solubility and stationary phase pH stability place limitations on value selection. However key information can be found from method development experiments. Factors selected and their +1 and -1 values are shown in Table 1.

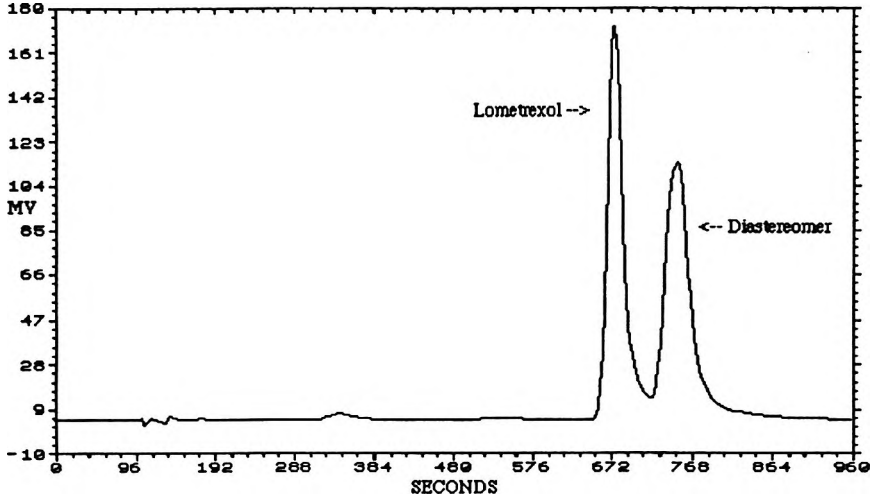


**Figure 3**. Chromatogram of Lometrexol separation.

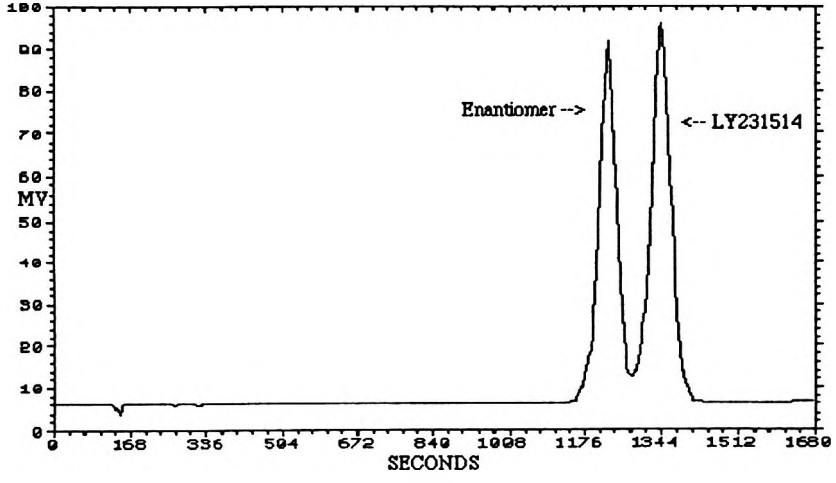


**Figure 4**. Chromatogram of LY231514 separation.

The fractional factorial design is shown in Table 2. In addition to the eight trials three center points were added. These serve as a control of system performance.

The same HPLC equipment, analyst and sample was used for all experiments. Because the design involved constant changing of column and mobile phase, a process for column and instrument rinsing was defined and used throughout the experiment.

The results of the trials for both compounds are shown in Table 3. Statistical analysis of the data using JMP software shows that the factor with the greatest significance on retention time is, not unexpectedly, the ACN concentration. For resolution, the most significant factor is buffer pH.

Additionally, the data showed that a decrease in ß-CD concentration combined with an increase in %ACN would maintain or improve the resolution obtained with the current method conditions. Column type had no statistical effect on either resolution or retention time.

## CONCLUSIONS

Use of a fractional factorial design with 11 experiments identified buffer pH as a significant factor in controlling resolution for both compounds. In addition, slight modifications in %ACN and ß-CD concentration will shorten assay time and maintain or improve resolution. This points the way to future method development.

For lometrexol, the data shows that changing the buffer pH from 7 to 3, decreasing the ß-CD concentration from 8 g/L to 2 g/L and increasing the %ACN from 3% to 10% will shorten assay time by 14 minutes and improve resolution (Figure 3). For LY231514, a decrease in %ACN (from 3.5% to 2%) combined with an increase in ß-CD (from 6 g/L to 8 g/L) will shorten assay time by 14 minutes and increase resolution (Figure 4).

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# A COMPARISON OF UV AND FLUORESCENCE DETECTORS IN THE LIQUID CHROMATOGRAPHIC ANALYSIS OF GLYPHOSATE DEPOSITS AFTER POST-COLUMN DERIVATIZATION

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## ABSTRACT

A sensitive and rapid liquid chromatographic method for the analysis of glyphosate using either post-column ninhydrin derivatization and UV detection (UV-D) or post-column reaction, fluorogenic labelling with *o*-phthalaldehyde in the presence of 2-mercaptoethanol and fluorescence detection (FD) is described. The sensitivity of the UV-D and two fluorescent detectors (HP-FD and Kratos-FD) were compared and evaluated to analyze glyphosate residues from glass fibre filter (GFF) discs used as deposit collectors in aerial application. The herbicide was extracted from the GFF discs using 30.0 mL of phosphate buffer adjusted to pH 2.1, derivatized and quantified. The method was tested initially, optimized and validated by using glyphosate standards. The average percent recoveries from fortified GFF samples were 93.7 (UV-D), 99.2 (HP-FD) and 94.9 (Kratos-FD). The corresponding standard deviation and coefficient of variation (percent) were 6.0 and 6.44; 1.6

and 1.67; and 4.4 and 4.59, respectively. The minimum quantifiable limits (ng/50 μL injection) for UV-D, HP-FD and Kratos-FD were 49, 16 and 28, respectively. The study showed that HP-FD was very sensitive compared to the other two detectors. The simplicity of the method allows high sample throughput for the routine analysis of glyphosate deposits.

## INTRODUCTION

Glyphosate [N-(phosphonomethyl) glycine, $HOOC\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}P(O)(OH)_2$] is a broad spectrum postemergence herbicide introduced by Monsanto Company, St. Louis, MO, USA in 1974 and is used extensively worldwide to control many annual and perennial weeds.[1] It translocates from the treated foliage to other plant parts inhibiting the biosynthesis of aromatic amino acids, thus causing the plant's death.[1,2] Vision®, a formulation concentrate containing 35.6% (w/v) of the acid equivalent of the isopropylamine salt $[(CH_3)_2HC\text{-}N^+H_3\ ^-O\text{-}P(O)(OH)\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}COOH]$ of glyphosate is registered in Canada for forestry use. The aqueous formulation is sprayed aerially for conifer release and site preparations.

Deposits found on the target foliage during aerial application of pesticides over forests are extremely variable and the overall efficiency in application is low.[3,4] Pesticide deposition during aerial spray applications is influenced by a series of complex, interdependent processes, such as meteorological conditions, aircraft parameters, physicochemical properties of the active ingredient, type of formulation, dosage, drop size, target shape and size, terrain, canopy characteristics, etc. In addition, pilot error and inappropriate swath positioning could contribute to the variability in deposition. Therefore, to determine the spray efficiency and resultant efficacy, it has become necessary to measure and quantify the amount of material deposited on the biological target, namely the foliage, during glyphosate sprays. For such deposit accountability studies, 2-dimensional artificial deposit collectors, such as glass fibre filter (GFF) discs, Mylar® discs, etc., are generally used[5] to simulate the leaves of broad-leaved weeds instead of natural foliage. This simplifies the cumbersome and tedious analytical procedures required to analyze glyphosate residues from foliage.[1]

In a recent glyphosate field trial, we used GFFs mounted onto sampling units, to collect spray deposits. The residues were solvent extracted, derivatized in a post-column reactor (PCR) using ninhydrin and analyzed by high performance liquid chromatography (HPLC) using ultraviolet detection (UV-D).[1,6] We also used the method of fluorogenic labelling with fluorescence detection (FD) by post-column oxidation of glyphosate to glycine using calcium hypochlorite $[Ca(OCl)_2]$ followed by derivatization with *o*-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol

(MERC) and detecting the fluorophore by Kratos and Hewlett-Packard fluorometers connected in series.[1,7-10] The influence of chromatographic conditions on performance and sensitivity between the two fluorometers and between the UV-D and FD methods were evaluated and compared. The methods and experimental conditions used in this study were validated initially and optimized for performance enhancement by analyzing the pure glyphosate standard under different experimental conditions by using the HPLC-PCR-ninhydrin and HPLC-PCR-fluorogenic labelling and the corresponding detection methods. Results are reported in this paper.

## MATERIALS AND METHODS

### Solvents

Methanol (BDH OmniSolv®, available from VWRCanlab, Mississauga, ON), water (deionized and purified by Milli-Q® Water System, Millipore Ltd., Mississauga, ON) and dimethyl sulfoxide (DMSO) (HPLC/spectrograde, available from Pierce Chemical Co., Rockford, IL) were used in the study after filtering using Nylaflo filter of 0.20 μm pore size (available from Gelman Sciences Inc., Rexdale, ON).

### Reagents

Potassium dihydrogen phosphate ($KH_2PO_4$), concentrated phosphoric acid (85%) ($H_3PO_4$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium acetate solution (4.0 M, $CH_3COONa$), 2-mercaptoethanol (MERC) and *o*-phthalaldehyde (OPA) were of primary standard grade obtained from Fisher Scientific Ltd. (Unionville, ON). Ninhydrin was obtained from Sigma Chemical Co. (St. Louis, MO). Hydrindantin (dihydrate), calcium hypochlorite [$Ca(OCl)_2$] (65% available chlorine) and disodium tetraborate ($Na_2B_4O_7.10H_2O$) were analytical grade supplied by VWRCanlab. Pure glyphosate, to prepare standard solutions for fortification and HPLC calibration, was supplied by Monsanto Company. Glass fibre filter (GFF) discs (9 cm diameter, 63.62 $cm^2$), used as deposit collectors, were purchased from Gelman Sciences.

### HPLC Instrumentation

The liquid chromatograph system consisted of a Hewlett-Packard (HP) 1090M HPLC, HP-9000/310 computer work station operated by HP-7995R software, HP-automatic sampler, variable volume auto-injector fitted with a 250-μL syringe, DR5

binary solvent delivery system with a helium-purge degassing system, two dual-syringe metering pumps which gave stable and reproducible flows and a diode-array detector (UV-D) with wavelength range of 190 to 600 nm. For the detection and quantification of the derivative formed between ninhydrin and glyphosate, the wavelength of UV-D was set at 570 nm.

A Kratos (Kratos Co., Ramsey, NJ) post-column derivatization system, URS 051, equipped with dual reagent pumps for delivering ninhydrin (single pump only) or $Ca(OCl)_2$ and OPA (both pumps) was used. The reagents were degassed with helium prior to use and kept under a helium atmosphere. The reaction between glyphosate and ninhydrin, at 100°C, and the oxidation of glyphosate to glycine, at 43°C, were carried out in a temperature controlled Pierce Reacti-Therm Heating Module (Pierce Chemical Company) consisting of 284 x 0.02 cm id coil. The OPA was allowed to react with glycine in the Kratos 1-mL reaction coil, to form the fluorophore.

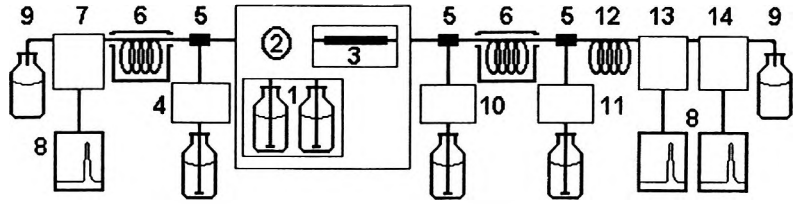
The FDs used were: a HP 1046A FD (HP-FD) equipped with a 5-µL flow-cell, variable excitation and emission wavelengths (range for both 190 to 800 nm and both can be scanned to optimize the maximum wavelength); and a Kratos FS970 HPLC fluorometer (Kratos-FD) equipped with a 10-µL flow-cell and automatic overload reset (FSA 986), with variable excitation wavelengths (190 to 700 nm; GM 970 monochromator) and fixed-wavelength emission filters.

The HPLC analytical column used was the Aminex A-9 cation exchange (10 x 0.46 cm id) preceded by Aminex A-9 cation exchange $K^+$ form cartridge guard column (Bio-Rad Laboratories, Richmond, CA), both thermostated at 50°C in the HPLC column compartment.

A schematic of the instrumental arrangement for the post-column derivatization using ninhydrin (UV-D) and OPA/MERC (FD with HP and Kratos fluorometers) is shown in Figure 1. The fluorescent derivatives formed in PCRs when glyphosate reacted with ninhydrin and with OPA/MERC after oxidation with $Ca(OCl)_2$ are given in Figures 2A and 2B, respectively.

**Procedure**

The phosphate buffer, used as the mobile phase in the HPLC and as the extractant for glyphosate deposits from GFFs, was prepared by dissolving 0.006 M $KH_2PO_4$ (0.8437 g/L) in water with 4% methanol and adjusting the pH to 2.1 with concentrated $H_3PO_4$. The solution was filtered (0.20-µm Nylaflo filter) and degassed prior to use. The mobile phase was delivered to the Aminex A-9 column at a rate of 0.5 mL/min.




1 Solvents and pump
2 Autosampler
3 Heated column
4 Pump - ninhydrin
5 Vortex T mixer
6 Heated reaction coil
7 Diode array detector
8 Integrator
9 Waste
10 Pump - hypochlorite
11 Pump - OPA
12 Reaction coil
13 Fluorometer - Hewlett Packard
14 Fluorometer - Kratos

**Figure 1**. Configuration of HPLC-PCR-ninhydrin and HPLC-PCR-fluorogenic labelling systems for the analysis of glyphosate in glass fibre filters.

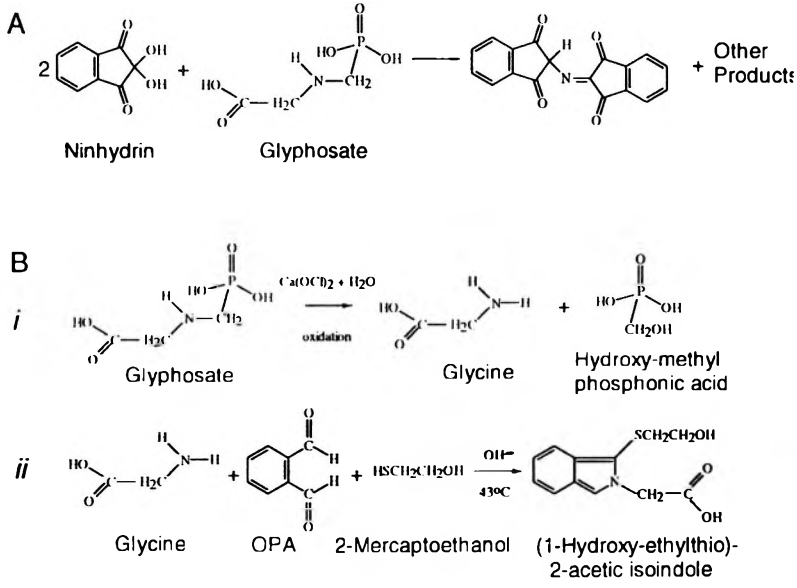



**Figure 2**. Reaction scheme of glyphosate with (A) ninhydrin, (B*i*) oxidation of glyphosate to glycine and (B*ii*) derivatization of glycine to isoindole derivative.

The ninhydrin solution was prepared as described by Thompson *et al.*[6] Briefly, 83.3 mL of 4 M sodium acetate and 13.33 g of ninhydrin were added successively with constant stirring under a nitrogen atmosphere to 833.4 mL of solution containing 1:1 ratio (volume) of deionized water and DMSO. To this, 0.461 g of hydrindantin dihydrate dissolved in 83.3 mL of DMSO was added. The solution was stored under nitrogen at 4°C in a tightly sealed bottle covered with aluminum foil. The ninhydrin solution was mixed with the column eluent at the rate of 0.5 mL/min.

The phosphate-hypochlorite reagent used in the oxidation of glyphosate was prepared by dissolving 1.36 g of $KH_2PO_4$, 11.6 g of NaCl and 0.4 g of NaOH in 0.5L water. A clear solution containing 0.060 g $Ca(OCl)_2$ in 50 mL water was then added to the above and the volume was brought to 1-L. The solution was shaken well, filtered (0.20-μm filter) and stored at 4°C in a tightly sealed bottle covered with aluminum foil. The oxidative solution was mixed with the column eluent at a rate of 0.1 mL/min.

The fluorogenic reagent was prepared daily by first dissolving 10.08 g of disodium tetraborate in 900 mL deionized water, under constant stirring. The pH was then brought up to 10.4 by adding concentrated NaOH dropwise. Subsequently 30 mL of a methanolic solution containing 1.75 g OPA and 4.0 mL MERC was added to the borate solution. The volume was adjusted to 1.0 L with water, mixed well, filtered and stored in a sealed bottle covered with aluminum foil. It was mixed with the oxidized effluent stream at a rate of 0.21 mL/min.

A stock standard solution of glyphosate was prepared by weighing exactly 10mg of pure glyphosate and dissolving in 100 mL of water. Analytical standards (0.01 to 10.0 μg/mL) for instrument calibration and fortification of GFF were prepared by serial dilution of the stock solution using the mobile phase. The concentrations of the analyte used for the calibration purposes were: 0.10, 0.50, 0.75, 1.0, 2.0, 5.0, 7.5 and 10.0 μg/mL. Fifty-μL volume of each standard was used during injections, which corresponded to the mass equivalent of glyphosate ranging from 5.0 to 500 ng.

GFF discs were fortified (n = 6 for each concentration) by placing droplets (*ca.* 300 μm in diameter) of known volume of glyphosate standards onto individual discs corresponding to 0.24 to 2.37 μg of glyphosate/$cm^2$. Samples were allowed to air-dry, cut into pieces, put in 50-mL polypropylene centrifuge tubes and allowed to equilibrate in darkness at 4°C for 24 h. Thirty mL of the mobile phase was then added to each tube and shaken in a mechanical shaker for 2 h. The extracts were filtered (0.20-μm), analyzed by HPLC using UV and FD and the percent recovery levels were computed.

During the field study, the GFF discs, fixed onto folding aluminum plates and mounted onto metallic stakes,[5] were placed at different locations in each spray block at 0.5 h prior to glyphosate spray application. They were collected at 1 h after application. Each GFF disc was placed in a polypropylene centrifuge tube containing 30 mL of the phosphate buffer and stored in a portable refrigerator at 4°C. The samples were brought to the residue laboratory, extracted and analyzed as discussed in the previous paragraph.

**Study Details**

The HPLC-PCR-ninhydrin configuration (Figure 1) was used first to quantify glyphosate from the fortified and field GFF samples. The instrument system was turned on for about 0.5 h for stabilization; the pump, mixing tee, detector, etc. were washed with deionized water and methanol and the column was conditioned by flushing with the mobile phase. The flow rates for the buffer (0.5 mL/min) and ninhydrin (0.5 mL/min) and the temperature (100°C) of the heating module where the derivatizing reagent reacted with glyphosate to form the derivative (Figure 2A), were the same as Thompson *et al.*[6] and found to produce good response. Fifty-μL aliquots of glyphosate standards were injected in quadruplicate onto the Aminex A-9 column and the absorbance after the PCR with ninhydrin was measured at 570 nm. The area counts of the four injections agreed within 1.4%, showing good repeatability.

A calibration curve [average area count ± SD (n = 4) *vs* amount glyphosate (ng)] was prepared (Figure 3) and it was validated daily. It was used in the subsequent determination of glyphosate deposits on GFFs used for the fortification and in the analysis of field samples.

Similarly for the HPLC-PCR-fluorogenic studies, the flow rates of the oxidative and OPA solutions were optimized at 0.10 and 0.21 mL/min, respectively, to produce maximum response for the analyte. The cleaving temperature for the glyphosate to form glycine (Figure 2B) in the presence of $Ca(OCl)_2$ was set at 43°C. The excitation and emission wavelength for the HP-FD was selected by scanning and optimizing at 230 and 445 nm, respectively. The excitation wavelength for the Kratos-FD was set at 230 nm and the filter for the emission wavelength was chosen as close as possible to 445 nm by using a 440 nm filter.

Calibration curves (Figure 3) for both the detectors were prepared from the fluorogenic responses recorded for the standard solutions of glyphosate. These curves were used subsequently in the quantification of the analyte from the GFFs.

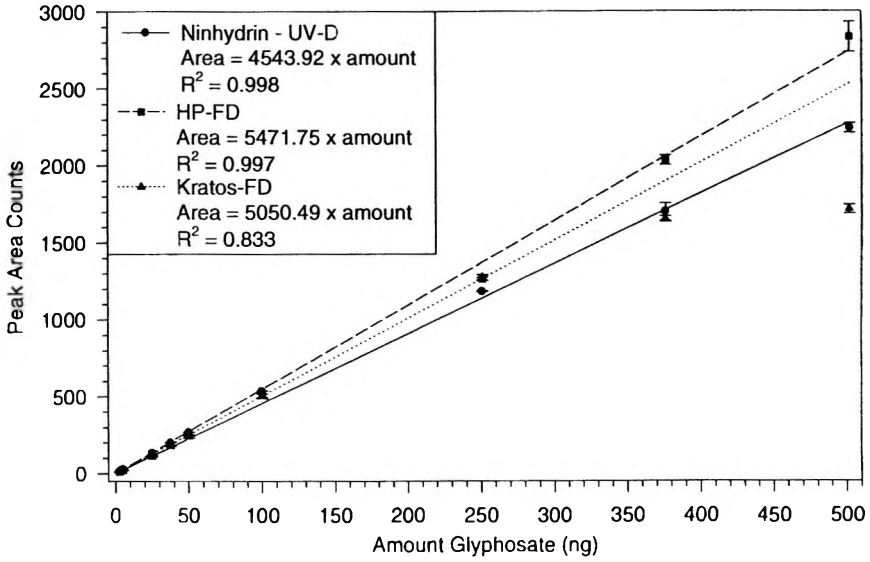



**Figure 3**. Calibration curves for glyphosate standards using UV and fluorescence detectors after post-column derivatization.

## Response Optimization

Typical chromatograms obtained by the UV and two fluorescent detectors for glyphosate standard, GFF extract blank, and GFF extract from the field (glyphosate peak overlaid on the blank chromatogram) are given in Figure 4.

Much experimentation was done, by adapting the methods used by Moye *et al.*,[11] to maximize the response of the detectors to glyphosate derivatives. The mobile phase pH (2.1), the flow rate (0.5 mL/min) and the temperature (100°C) chosen for the reaction to occur between ninhydrin and glyphosate were found to be optimum.

Similarly, hypochlorite concentration of 60 mg/L, its flow rate of 0.1 mL/min, and $Ca(OCl)_2$ effluent pH of about 9, conversion of glyphosate to glycine at 43°C, OPA/MERC flow rate of 0.21 mL/min and excitation (230 nm) and emission (445nm) wavelengths of the fluorometers were prime requirements to obtain maximum response (peak area counts) in fluorogenic labelling of glyphosate. Any deviation from these chosen parameters, yielded inconsistent results and lower sensitivity.

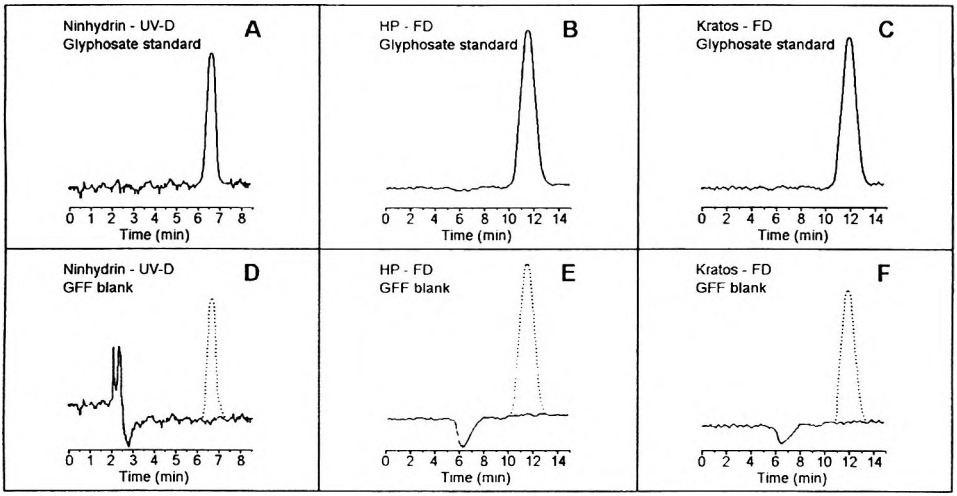



**Figure 4**. Representative chromatograms obtained from HPLC-PCR-ninhydrin (UV-D) and HPLC-PCR-fluorogenic (HP-FD and Kratos-FD) studies (glyphosate concentration injected 100 ng/50 µL) glyphosate peak in field extracts is shown by a dotted line over blank chromatograms in D to F.

## RESULTS AND DISCUSSION

Injection of different concentrations of calibration solutions of glyphosate in the aminex A-9 column, followed by post-column derivatization and detection using the three detectors (UV-D, HP-FD and Kratos-FD), gave consistent results, indicating good repeatability. Plots of peak area count against amount of glyphosate (ng) (on the basis of underivatized material) (Figure 3) were linear for the UV-D from 50 to 500 ng, HP-FD from 5 to 375 ng and Kratos-FD from 5 to 250 ng mass ranges for 50 µL sample size. Beyond 250 ng glyphosate, the Kratos-FD showed deviation. In contrast, the UV-D did not show noticeable deviation for a wide range of concentrations (50 to 500 ng/50 µL) studied.

Excluding the points which were far off from the straight line for Kratos-FD, the response of the detectors was found to obey the linear regression equation: Y (peak area count) = b (slope) x c (amount glyphosate in ng) and the coefficient of determination ($R^2$) values were 0.998, 0.997 and 0.833 for UV-D, HP-FD and Kratos-FD, respectively. The values for 'b' are given in Figure 3. The higher $R^2$ values for UV-D and HP-FD indicate that these detectors are rugged and reliable in responding to a wide range of concentrations of glyphosate compared to Kratos-FD.

**Table 1**

**Recoveries (%) of Glyphosate from Fortified Glass Fibre Filter Discs (Number of Replicates = 6)**

| Detector | Fortification Level ($\mu g/cm^2$)[a] | | | | Mean Recovery (%) | SD (±) | CV (%) |
|---|---|---|---|---|---|---|---|
| | 0.24[b] | 0.47[b] | 0.95[b] | 2.37[b] | | | |
| UV-D | NQ[c] | 86.7 | 97.2 | 97.1 | 93.7 | 6.0 | 6.44 |
| HP-FD | 101.2 | 99.8 | 98.4 | 97.4 | 99.2 | 1.6 | 1.67 |
| Kratos-FD | 88.4 | 97.7 | 96.7 | 96.8 | 94.9 | 4.4 | 4.59 |

[a] Volume of buffer used in extraction = 30 mL.
[b] Amount injected in 50 µL at 0.24, 0.47, 0.95 and 2.37 $\mu g/cm^2$ were 25, 50, 100 and 250 ng, respectively. [c] NQ = not quantifiable below 0.46 $\mu g/cm^2$.

Detector sensitivity indicated by the slopes of straight lines in Figure 3 show that HP-FD is very sensitive among the three, and sensitivity decreased in the order HP-FD > Kratos-FD > UV-D. The corresponding sensitivity ratio computed from the slopes were 1.0 : 0.92 : 0.83. Together with slopes and accompanied sensitivity ratio, it is apparent that for the determination of glyphosate residues from GFF, HP-FD following PCR, would be preferable in comparison to the other two detectors if the deposit concentrations were low and higher analytical sensitivity was required.

The recovery, on the basis of underivatized glyphosate obtained from the GFF discs (area 63.64 $cm^2$) fortified at 4 concentrations (range: 0.24 to 2.37 $\mu g/cm^2$ or 15.27 to 150.83 µg/disc) followed by extraction with 30 mL of buffer (mobile phase) and injection of 50 µL volumes (25 to 250 ng/injection, for the four fortification levels), is given in Table 1. The mean percentage recovery for each fortification level obtained from each detector with its standard deviation (± SD) and average coefficient of variation (CV), recorded in Table 1, were derived from multiple injections of the quadruplicate samples at each fortification level.

The mean percent recoveries observed in UV-D, HP-FD and Kratos-FD were 93.7, 99.2 and 94.9, respectively. However at the lowest fortification level (0.24 $\mu g/cm^2$), the UV-D was not sensitive enough to quantify the amount of glyphosate present in the extract [minimum quantifiable limit (MQL) = 0.46 $\mu g/cm^2$]. The SD (±) for UV-D, HP-FD and Kratos-FD were 6.0, 1.6 and

4.4, respectively. This indicates that the degree of reproducibility, as given by the SD values, decreased in the order HP-FD > Kratos-FD > UV-D. A similar trend among the detectors was also observed in the precision obtained for glyphosate measurements, as represented by the respective CV values. The precision was high (low % CV) in HP-FD (CV = 1.67 %), moderate in Kratos-FD (CV = 4.59 %) and low in UV-D (CV = 6.44 %). The minimum detection limit (MDL) was defined as three times the SD value[12] obtained for the four GFF discs fortified with glyphosate at 0.47 μg/cm$^2$ (29.91 μg/disc) and analyzed using the three detectors, after extraction with 30 mL of buffer and injecting 50 μL volume. The SD values found for UV-D, HP-FD and Kratos-FD were 4.9, 1.6 and 2.8, respectively, and the corresponding MDL values were 14.7, 4.8 and 8.4 ng per 50 μL injection. The MQL was defined as 10 times the SD[12] from the same four GFF samples and were 49, 16 and 28 ng per 50 μL injection for UV-D, HP-FD and Kratos-FD, respectively. These values are translatable to the MQL of 462, 151 and 264 ng/cm$^2$. The MQL values obtained in this study indicate that HP-FD is more sensitive, hence preferable, than the other two detectors to quantify glyphosate residues from GFF discs.

Figures 4A, 4B and 4C illustrate the chromatographic response of glyphosate standard (100 ng/50 μL injection) obtained in the UV-D, HP-FD and Kratos-FD, respectively. The mean retention times (RT) (min) for the analyte in the three detectors were 6.7 (UV-D), 11.5 (HP-FD) and 11.8 (Kratos-FD). The percent CV in RT for UV-D, HP-FD and Kratos-FD were 2.9, 1.3 and 2.1, respectively. The analyte peak obtained from each detector, depending on its sensitivity, was sharp and well defined; however, the baseline stability in UV-D was poor and consequently the chromatographic efficiency was rather low, compared to the two FDs. The chromatograms of the blank GFF extracts (Figs. 4D, 4E and 4F) did not show any interfering peak corresponding to the RT of glyphosate.

**Analysis of GFF Discs from Field after Glyphosate Spray**

The PCR-ninhydrin-UV-D and PCR-fluorogenic labelling with FD methods were extended and validated to quantify the glyphosate deposits from GFF discs used in field efficiency studies. The results are presented in Table 2. The data show that the inter-assay variations were not much and that all the three methods were quite adequate and suitable to quantify the analyte from GFF discs as long as the concentration of glyphosate in the 30-mL extract was above the MQL (980 ng/mL or 462 ng/cm$^2$ on the disc, so that a 50 μL injection contained above 49 ng). Because of the lower sensitivity of UV-D compared to FD, if the deposit levels were below 462 ng/cm$^2$, then the use of FDs was preferred in glyphosate quantification. This is amply evident from the residue data recorded in Table 2.

**Table 2**

**Analysis of GFF Deposit Collectors from Field for Glyphosate Residues[a] using UV-D, HP-FD and Kratos-FD**

| | Glyphosate Concentration ($\mu g/cm^2$) | | |
|---|---|---|---|
| **Sample No.** | **UV-D** | **HP-FD** | **Kratos-FD** |
| 1 | 3.53 ± 0.19 | 3.89 ± 0.11 | 3.66 ± 0.22 |
| 2 | NQ[b] | 0.17 ± 0.06 | NQ |
| 3 | 1.93 ± 0.16 | 2.05 ± 0.13 | 2.11 ± 0.31 |
| 4 | NQ | 0.36 ± 0.09 | 0.29 ± 0.13 |
| 5 | 5.66 ± 0.21 | 5.79 ± 0.17 | 5.60 ±0.27 |
| 6 | NQ | 0.45 ± 0.16 | 0.38 ± 0.19 |

[a] See footnotes in Table 1.

[b] NQ - not quantifiable. MQL for UV-D 49 ng/50 µL, or 980 ng/mL or 462 ng/cm$^2$; for HP-FD 16 ng/50 µL, or 320 ng/mL or 151 ng/cm$^2$; for Kratos-FD 28 ng/50 µL, or 560 ng/mL or 264 ng/cm$^2$.

The data also reaffirmed the relatively higher sensitivity (*i.e.* lower MQL) of HP-FD over the Kratos-FD and it was a choice detector and eminently suitable to monitor and quantify glyphosate residues from GFF discs. if the deposit levels were around or just above the 151 ng/cm$^2$ level, which would normally be the case. if off-target drift studies are planned for glyphosate.

None of the chromatograms of the extracts obtained from either the GFF discs fortified with glyphosate or the GFF discs collected from the field after spray application (glyphosate peak shown as dotted line, Figure 4D to 4F) exhibited any peak that would interfere with the analysis of glyphosate.

## CONCLUSIONS

The method for the determination of glyphosate residue from GFF deposit collectors reported in this paper, was simple and shorter than the method reported in literature[6] because no pre-concentration by anion-exchange chromatography was

necessary. The use of extremely acidic mobile phase, instead of aqueous ammonia,[6] to extract the glyphosate residues from GFF discs readily facilitated the removal of the analyte in its zwitterionic and monoanionic forms.[2] Although the use of UV-D and Kratos-FD are acceptable for the quantification of glyphosate, the use of HP-FD offers advantages such as its relatively high detection sensitivity and reproducibility, especially if the analyte levels are low in the deposit collectors.

The use of post-column fluorogenic labelling and detection by HP-FD would allow for relatively high sample throughput if the HPLC instrument is equipped with an autosampler and a computerized data acquisition system, and in such cases a large number of samples can be injected without much pretreatment.

## ACKNOWLEDGEMENTS

The authors are thankful to Dr. R. Campbell for conducting the aerial spray application of glyphosate and inviting them to participate in deposit measurements. Sincere appreciation is extended to Reg Nott for collecting the GFF discs from the field.

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# FORMATION OF A VINYL ALCOHOL DERIVATIVE DURING SAMPLE PREPARATION OF 7-CHLORO-6,4'-DIFLUOROQUINOLONE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

During analysis of 7-chloro-6,4'-difluoroquinolone (CDFQ) by high performance liquid chromatography (HPLC), a reaction product was formed during sample preparation. This compound was identified as a vinyl alcohol derivative of CDFQ, formed by loss of carbon monoxide from the carboxylate group of CDFQ during sonication in tetrahydrofuran (THF). The reaction to form the vinyl alcohol was shown to depend upon the presence of trace amounts of potassium acetate in the CDFQ, the peroxide content of the THF, and sonication variables of time and bath temperature. The sample preparation procedure was modified so that this reaction would not occur and meaningful HPLC analyses of CDFQ could be achieved. This study demonstrates that caution is required when quinolone compounds are prepared for HPLC analysis by heating or sonicating in THF solution.

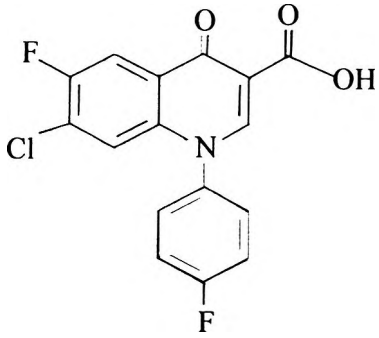



**Figure 1**. Chemical Structure of CDFQ.

## INTRODUCTION

7-Chloro-6-fluoro-1-( 4-fluorophenyl)-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid or, more commonly, 7-chloro-6,4'-difluoroquinolone (CDFQ) is an intermediate in the chemical synthesis of aryl-fluoroquinolone antibacterial agents difloxacin and sarafloxacin, intended for use in animal health products. Fluoroquinolones have been shown to be orally active antibacterial agents with activity against Gram-negative bacteria.[1] They act to inhibit DNA gyrase, a key enzyme in DNA replication.[2] The chemical structure for CDFQ is shown in Figure 1. Synthetic routes for CDFQ[3-5] and HPLC analyses of difloxacin,[6-7] sarafloxacin,[8-9] and other quinolones[10-11] have been reported.

Impurity analysis of CDFQ is carried out in our laboratories by reverse phase gradient HPLC on a $C_{18}$ column with mobile phase consisting of tetrahydrofuran (THF), methanol, and potassium phosphate buffer at pH 5.3. During HPLC testing on three CDFQ lots, lots A and B showed the presence of a new peak which was not present in lot C. The magnitude of this peak was generally consistent within a set of samples, but varied greatly from one sample preparation set to the next. This observation led us to consider that this peak was being formed during the sample preparation process.

It was shown that this peak was indeed formed from CDFQ during sample preparation. The new component was identified as a vinyl alcohol analog of CDFQ, hereafter referred to as the vinyl alcohol. Critical parameters were evaluated and the sample preparation process altered to prevent this reaction.

## MATERIALS AND METHODS

The HPLC instrumentation used most frequently in this study was from Spectra-Physics (Thermo Separation Products, Division of Thermo Instrument Systems, Inc., Riviera Beach, FL, USA), which included a P2000 HPLC pump, an AS3000 autosampler, and UV1000 detector. Data aquisition and processing were accomplished by a PE Nelson ACCESS*CHROM GC/LC Data System (Perkin-Elmer Systems, Inc., Cupertino, CA, USA). The HPLC column was either a YMC-Pack ODS-A, A-302-5 (5 μm, 150 x 4.6 mm) (YMC, Inc., Morris Plains, NJ, USA) or Zorbax RX-C18 column of the same dimensions (Mac-Mod Analytical, Inc., Chadds Ford, PA). The injection volume was 20 μL and the flow rate of the mobile phase was 1.0 mL/min. The wavelength employed for UV detection was 325 nm.

A gradient system was employed for HPLC impurity analysis. Mobile phase A consisted of 68% 0.02 M aqueous potassium phosphate (pH 5.3-5.4), 27% tetrahydrofuran (THF), and 5% methanol. Mobile phase B was 35% buffer, 60% THF, and 5% methanol. The gradient program consisted of 100% mobile phase A for 30 min, linear change to 100% mobile phase B from 30 to 60 min, linear return to mobile phase A from 60-62 min, and mobile phase A continued from 62-80 minutes. HPLC grade THF, acetonitrile, and methanol were obtained from EM Science (Gibbstown, NJ), reagent grade monobasic potassium phosphate was from J. T. Baker, (Phillipsburg, NJ) and water was from a Millipore Milli-Q system (Millipore, Milford, MA).

Sample dissolutions for HPLC were performed with a Branson 2200 Ultrasonic Cleaner (Branson Ultrasonic Corp., Danbury, CT, USA). Quantofix peroxide test sticks obtained from Aldrich Chemical Company (Milwaukee, WI) were used to measure peroxide concentration in THF.

Inductively Coupled Plasma (ICP) analyses were performed with a Thermo Jarrell Ash Atomscan 25 instrument (Division of Thermo Instrument Systems, Inc., Franklin, MA, USA). Samples were prepared for analysis by dissolution in 20% ammonium hydroxide.

Gas chromatography was performed with a Hewlett-Packard Model 5890 instrument (Hewlett-Packard Company, Palo Alto, CA, USA). Acetate was determined as acetic acid using a AT-1000 megabore column and flame ionization detector.

Proton nmr, C13 nmr, and mass spectral data for identification of the vinyl alcohol were provided by the Structural Chemistry Group, Pharmaceutical Products Division, Abbott Laboratories. The vinyl alcohol sample for spectral

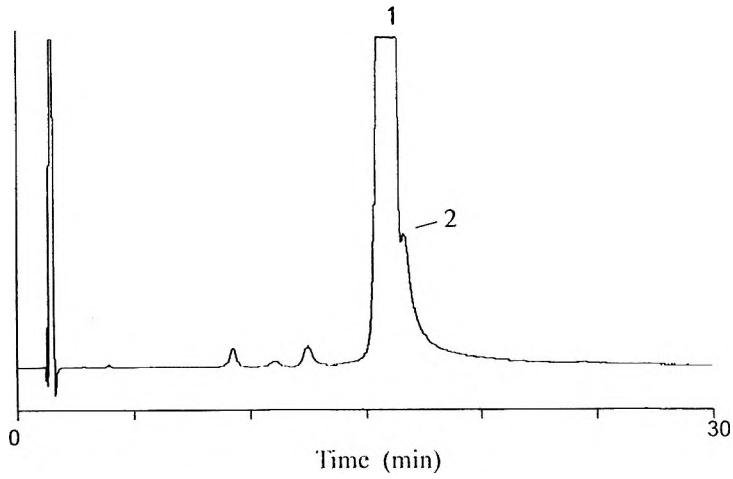



**Figure 2.** Chromatogram for CDFQ, lot A on Zorbax C18. Principal peaks: 1: CDFQ; 2: vinyl alcohol..

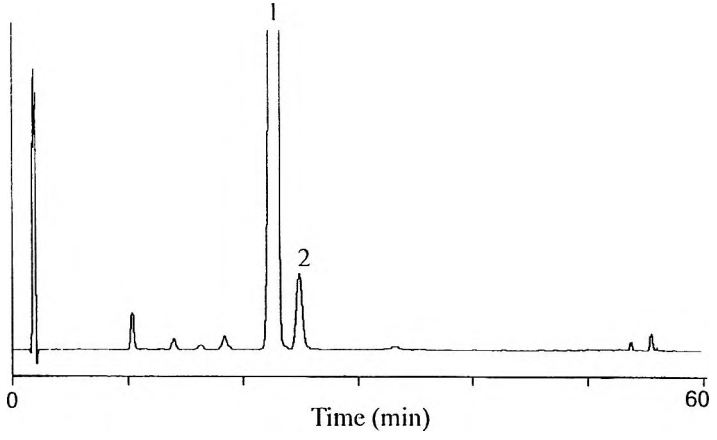



**Figure 3.** Chromatogram for CDFQ, lot A on YMC C18. Principal peaks: 1:CDFQ; 2:vinyl alcohol.

**Table 1**

**Variation in Vinyl Alcohol PA (%) for Independent Sample Preparation of CDFQ, Lot A**

| Sample Prep No. | Peak Area Percent |
|---|---|
| 1 | 1.3 |
| 2 | 3.6 |
| 3 | 1.6 |
| 4 | 7.4 |
| 5 | 0.6 |

analysis was obtained by sonication of CDFQ lot A (30 mg in 25 mL THF) for 85 minutes. HPLC analysis indicated near quantitative conversion of CDFQ to the vinyl alcohol.

### Sample Preparation

The sample preparation procedure for CDFQ employed during most of this work consisted of dissolving ~30 mg sample in 25 mL THF in an ultrasonic bath followed by dilution to 100 mL with 1:1 (v/v) acetonitrile/water. This procedure was later modified to prevent formation of the vinyl alcohol as described in the Results and Discussion section.

## RESULTS AND DISCUSSION

Release testing of CDFQ includes HPLC assay and HPLC impurity analyses. During HPLC assay analysis of three lots of CDFQ, designated A, B, and C, lots A and B showed the presence of a new peak. Inspection of a typical chromatogram of lot A (Figure 2) shows a shoulder on the main component when a Zorbax RX-C18 column was used. With a YMC ODS-A column and other conditions unchanged, a large peak was completely resolved from the main component (Figure 3). This peak was later shown to result from the vinyl alcohol.

Following the initial HPLC analyses of these CDFQ lots, many more sample preparations and assays were performed. The peak area percentage (PA%) of the new peak varied substantially from preparation to preparation (Table 1).

It was considered that the variations observed were due to sample non-uniformity. However, after the lots (A, B) were well blended the variability in PA% for this peak persisted.

After further study of sample preparation and HPLC analyses, the following experimental facts became clear: (1) the amount of vinyl alcohol formed from a given CDFQ sample often varied substantially from one sample preparation to the next and (2) the amount of vinyl alcohol present did not change after the sample preparation step. These observations led to the hypothesis that the vinyl alcohol was being formed *during sample preparation* and that variations in the amount formed for a given sample were mainly due to differences in duration and water temperature in the ultrasonic bath.

In order to test this, a sample of CDFQ lot A was dissolved in THF by vortexing at room temperature. The sample was then divided and half of the sample solution was further treated by sonication for 20 minutes. The sonicated sample preparation gave 2.0% of the vinyl alcohol while the sample solution which was only vortexed at room temperature yielded only a trace. These results provided strong evidence that the vinyl alcohol was being formed during sonication of CDFQ in THF.

In order to probe the effect of sonication time on the amount of vinyl alcohol formed, CDFQ lot A was sonicated in THF for 0.5 and 1.0, hour (Figure 4). In a related experiment, another sample from lot A was heated in THF at 60°C for 1, 2, and 3 hours (Figure 5). In both cases, a steady conversion of CDFQ into the vinyl alcohol was observed. As shown in Figure 5, the conversion of CDFQ was virtually complete for the sample heated at 60°C for 3 hours. Spectral analysis of the converted sample by proton nmr, C13 nmr, and mass spectrometry provided identification of this component as 7-chloro-6-fluoro-1-(4-fluorophenyl)-1,4-dihydro-4-oxoquinoline-3-carbinol, a vinyl alcohol analog of CDFQ, resulting from loss of carbon monoxide (CO) from the carboxylate group. The chemical structures involved in the conversion of CDFQ to the vinyl alcohol are shown in Figure 6.

With the vinyl alcohol structure and mode of formation identified (ie. heating CDFQ in THF), the question remaining was why these two particular lots of CDFQ (A, B) were most susceptible to this reaction. Additional testing was performed on CDFQ lots A, B, and C in order to probe for differences (Table 2). The problem lots (A, B) had residues on ignition (0.1-0.2%), whereas lot C did not. Metals analyses by ICP and acetate determinations by gas chromatography revealed significantly higher potassium and acetate levels, respectively, in lots A and B (Table 2).

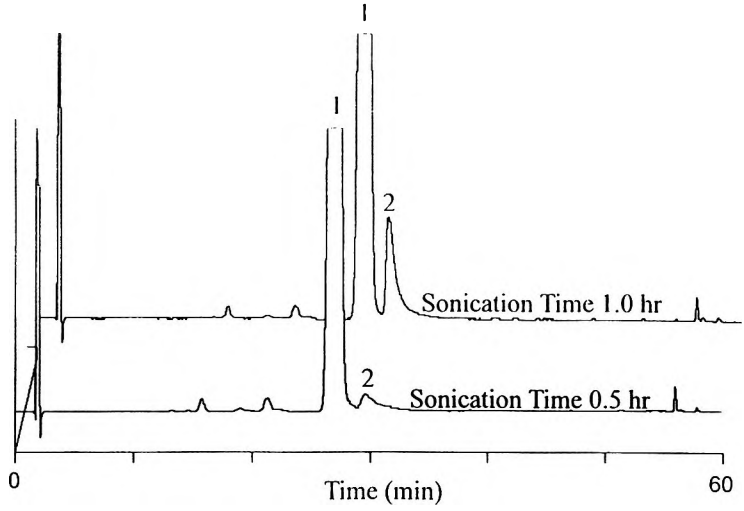



**Figure 4**. Chromatograms for CDFQ, lot A, sonicated for times indicated. Principal peaks: 1:CDFQ; 2:vinyl alcohol.

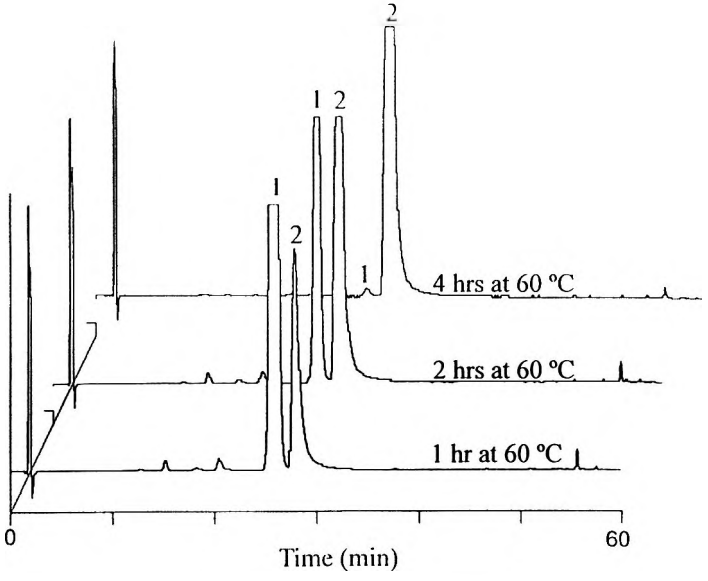
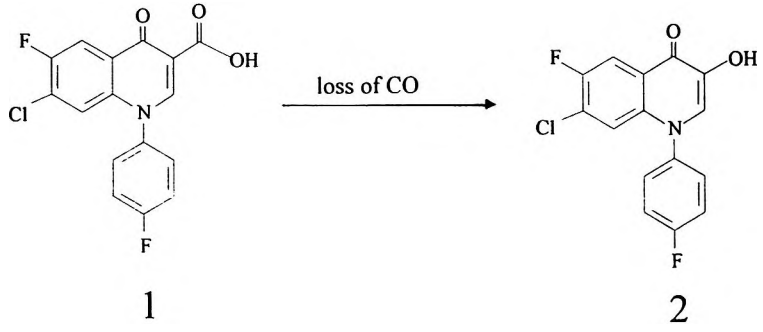



**Figure 5**. Chromatograms for CDFQ, lot A, heated at 60 °C for times indicated. Principal peaks: 1:CDFQ; 2:vinyl alcohol.




1 2

**Figure 6**. Chemical structures for the conversion of CDFQ (1) to the corresponding vinyl alcohol (2).

**Table 2**

**Potassium and Acetate Determinations on Blended CDFQ Lots**

| Lot ID | Potassium (%) | Acetate (%) |
|---|---|---|
| A | 0.07 | 0.05 |
| B | 0.14 | 0.07 |
| C | 0.007 | 0.005 |

The extent to which a residual amount of potassium acetate in CDFQ promotes the formation of the vinyl alcohol was explored. For this experiment, CDFQ lot C, unspiked and spiked with 0.1%, 0.5%, and 1.0% (w/w) potassium acetate was sonicated for 60 minutes in THF. Unspiked CDFQ formed only 0.5% of the vinyl alcohol under these stringent conditions, whereas the preparations spiked with potassium acetate produced greatly increased amounts of this reaction product. A 50-fold increase in vinyl alcohol formation resulted from the addition of only 0.1% potassium acetate. These results (Table 3) clearly demonstrate that low levels of potassium acetate promote vinyl alcohol formation from CDFQ sonicated in THF.

The peroxide content of the THF used for CDFQ sample preparations was also suspected to impact vinyl alcohol formation. To examine the effect of peroxides, the peroxide concentration in four THF lots was determined using peroxide test strips.

**Table 3**

**Dependence of Vinyl Alcohol Formation on % Potassium Acetate for CDFQ, Lot C**

| Sample Number | % KOAc | Vinyl Alcohol PA% |
|---|---|---|
| 1 | 0.0 | 0.5 |
| 2 | 0.1 | 2.5 |
| 3 | 0.5 | 96 |
| 4 | 1.0 | 88 |

**Table 4**

**Dependence of Vinyl Alcohol Formation on Peroxide Content in THF for CDFQ, Lot A**

| Sample Number | Peroxides ppm | Vinyl Alcohol PA% |
|---|---|---|
| 1 | 25 | 32 |
| 2 | 50 | 39 |
| 3 | 70 | 95 |
| 4 | 125 | 95 |

CDFQ (lot A) was sonicated for 60 minutes in each of these four THF lots. Results summarized in Table 4 show that CDFQ solutions in THF containing higher peroxide concentrations produced significantly more of the vinyl alcohol.

It was desired to change the sample preparation procedure to prevent formation of the vinyl alcohol. This was accomplished by omitting THF from the sonication step: 60 mg CDFQ was dissolved by sonication in 100 mL acetonitrile then diluted to 200 mL with 1:3 THF/water. With this procedure, CDFQ dissolved readily and did not recrystallize upon cooling. HPLC analysis showed the vinyl alcohol was not present in either lot A (Figure 7) or lot B.

When CDFQ samples were prepared in this fashion, both of the problem lots (A, B) passed HPLC testing requirements for assay and impurities. To further ensure that vinyl alcohol formation would not be a problem for the

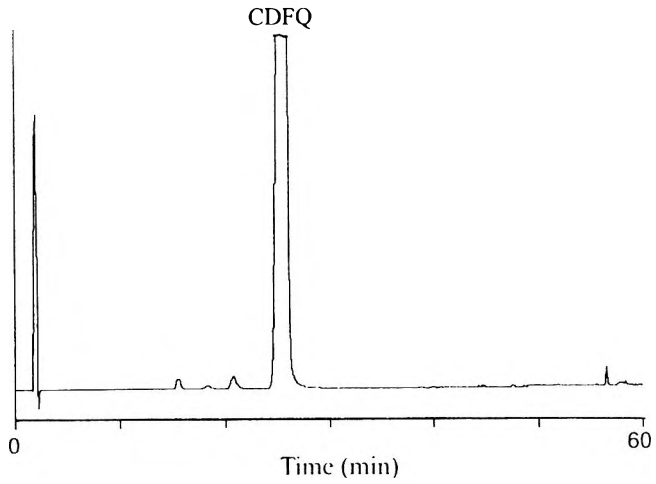



**Figure 7**. Chromatogram for CDFQ, lot A, prepared by modified procedure.

future HPLC analyses of CDFQ, a maximum limit of 50 ppm was set for total peroxides in the THF. We urge caution when THF is used for HPLC sample preparations, particularly when the solutions are heated or sonicated. Although no attempt was made here to generalize this reaction, in principle it could occur for quinolones other than CDFQ.

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# SUPERCRITICAL FLUID EXTRACTION OF MYCOTOXINS FROM FEEDS WITH ANALYSIS BY LC/UV AND LC/MS

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## ABSTRACT

The use of supercritical fluid extraction with supercritical carbon dioxide and a matrix modifier is described for the isolation of three trichothecene mycotoxins from yellow corn meal and rolled oats. The quantity of supercritical carbon dioxide used in each extraction was 30 mL which contained 5% methanol used as a modifier. The pressure was maintained at 550 atm., and the temperature of the extraction chamber was set at 60 degrees. Recovery of the target analytes from cereal and corn feed by supercritical fluid extraction (SFE) varied from 85 to 95 % depending on the target compound. The mycotoxins, deoxynivalenol (DON), deacetoxyscirpenol (DAS) and T-2 toxin (T-2) were monitored in the SFE extracts by high performance liquid chromatography with UV-detection (HPLC-UV) or ion spray mass spectrometry under full-scan, selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes. There are significant restrictions in the HPLC-UV detection limits for T-2 and DAS, in particular, due to their low UV absorbance. With

HPLC-UV and also HPLC-MS in the full-scan mode of acquisition the considerable interference from co-eluting fat-soluble compounds precluded detection of these compounds in feed extracts. In the SIM and SRM LC/MS modes the target analytes could be readily detected at the 250 ppb level in the cereal SFE matrix extracts.

## INTRODUCTION

Trichothecenes are sesquiterpenoid mycotoxins produced by a variety of species of *fusarium* fungi. These fungi have been established as plant-pathogenic fungi which invade various agricultural crops and plants. These fungi thus have the potential for producing tricothecene mycotoxins in grains and cereals. Trichothecenes show a wide range of toxicity, which is dependent on the structure of the molecule. Over 150 trichothecenes have been isolated and characterized, but it is still a challenging analytical task to isolate and characterize these compounds from foods and feeds.[1,2]

Conventional methods for isolation of trichothecenes involve extensive and time-consuming sample preparation steps. According to a recent survey, two thirds of the analysis time is devoted to sample preparation and this step accounts for at least one-third of the errors generated during the performance of an analytical method.[3] Supercritical fluid extraction (SFE) has shown great potential in offering shorter extraction times with good recoveries and low consumption of organic solvents. Supercritical carbon dioxide ($SC\text{-}CO_2$) is the most widely used fluid for supercritical techniques because it is non toxic, nonflammable and inexpensive. Supercritical carbon dioxide provides extraction capability at relatively low temperatures and pressures because its critical pressure (7.38 MPa) and temperature (31.1°C) are relatively low.[4] This enables the use of low extraction temperatures thereby reducing the risk of analyte degradation during extraction. Due to favorable physical properties the supercritical fluids are also amenable with other chromatographic techniques.[5]

Supercritical fluid extraction has been used for industrial-scale separation and isolation of a variety of compounds.[6] An increasing number of quantitative applications of SFE especially for the extraction of environmental pollutants has been reported in recent years.[7-10] SFE is also utilized in the field of food science to isolate not only natural food components including fat,[11] cholesterol,[12] and volatiles,[13] but also unnatural components like organic contaminants including pesticides and PAHs .[14,15]

Mycotoxins are often separated, identified and quantitated using thin layer chromatography,[16] thin layer chromatography mass spectrometry,[17] gas chromatography-mass spectrometry,[18-22] high performance liquid chromatography (HPLC) methods with fluorescence[23,24] or light scattering[25] detection. Conventional liquid and gas chromatography methods, however, suffer serious drawbacks. The sensitivity of HPLC is limited since most trichothecenes have minimal fluorescent or ultraviolet absorbing properties. In the case of GC-methods, derivatization is often required, which may cause problems with quantitative analysis procedures. HPLC combined with mass spectrometry via thermospray, plasmaspray or fast atom bombardment[26-29] have also been reported.

Only limited studies have been published with the use of supercritical fluid extraction for the isolation of mycotoxins or with a comparison of different identification methods. Supercritical fluid chromatography (SFC) combined with UV-detection or mass spectrometry via the moving belt interface has been used for the determination of some *Fusarium*-toxins.[30] Kalinoski *et al.*[31] have used SFE combined with direct fluid injection mass spectrometry (DFI/MS) for the determination of trichothecenes from wheat samples. SFE is also used in the isolation of aflatoxin $B_1$ from field-inoculated corn[32] and peanut meal.[33] In both cases analytes were monitored using HPLC equipped with a fluorescence detector.

The aim of this study was to evaluate the feasibility of supercritical fluid extraction in the isolation of some trichothecene toxins from cereals and feed. A comparison of HPLC-UV, HPLC/MS and HPLC/MS/MS was made to determine the preferred method for monitoring the analytes isolated by SFE. Extracts were analyzed without further purification. One of the topics of this report is to describe how and why HPLC/APIMS/MS may be a preferred means of developing routine, high-sensitive methods for analyzing biological samples using limited sample preparation procedures.

## MATERIALS AND METHODS

### Samples and Standards

Samples, rolled oats and yellow cornmeal, were purchased from a local supplier (Ithaca NY, USA). The feed samples were available in the laboratory from previous veterinary toxicology case studies. All samples were ground to a fine powder with a homogenizer (Waring commercial blender, Model 7011S, Fisher Scientific, Rochester, NY). These samples were stored in the capped

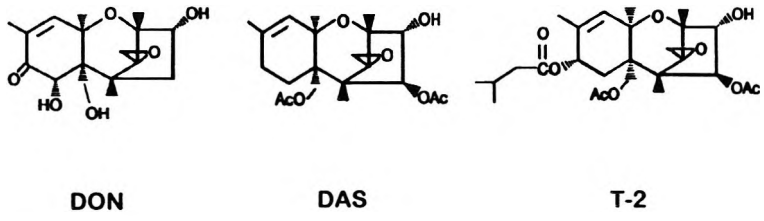


**Figure 1**. Structures of three trichothecenes investigated.

Corning 250 mL, 8oz polypropylene containers, (Fisher Scientific, Rochester, NY) at the room temperature. A standard stock solution of the three mycotoxins (500 ng/μL methanol) was prepared from pure standards purchased from Sigma Chemical Co (St. Louis, MO, USA). The stock solution was diluted 1:100 with methanol. All reagent-grade solvents, buffers and common reagents were purchased from Fisher Scientific (Rochester, NY, USA). The mycotoxins used in this study were deoxynivalenol (DON), deacetoxyscirpenol (DAS) and T-2 toxin (T-2). The structures of these compounds are shown in Figure 1. Fortification of the samples was done by injecting an appropriate amount of the diluted standard stock solution of mycotoxins into the sample in the extraction vessel. The concentrations prepared were 250, 500 and 1500-ppb. Repeatability of SFE was based on the data obtained from the tests, where samples were spiked before and after supercritical fluid extraction. The LC/MS ion current profiles for the extract of the fortified sample and the post-extract spike of a control sample were compared to calculate the repeatability.

**Supercritical Fluid Extraction**

Four grams of sample were extracted with an ISCO model 100 DX dual syringe pump system coupled with an ISCO SFX 2-10 extractor and a two-channel adjustable restrictor device (ISCO Inc., Lincoln, NE, USA). A layer of anhydrous sodium sulfate was placed in a 10mL extraction vessel followed by four grams of sample, and the remaining void filled with additional anhydrous sodium sulfate. SFC-grade $CO_2$ was used from an aluminum cylinder charged with a helium head pressure and equipped with a dip tube (Scott Specialty Gases, Plumbsteadville, PA, USA). The ISCO SFE pump head was maintained at +5°C with an external cryostat (Neslab, Model RTE-110). The other pump of the ISCO system was used for adding methanol as a modifier. Extraction conditions used were as follows: pressure 550 atm (1 atm = 0.10132 MPa), extraction temperature was 60°C, restrictor temperature was 65°C, fluid volume used was 30 mL with 5 % of HPLC-grade methanol. The flow rate of

supercritical fluid was maintained at 1.2 mL/min. The syringe pump that delivered the $CO_2$ was refilled after each extraction. The analytes were collected by bubbling the SFE-extracted material into 10 mL of methanol. Extracts were defatted with 5 mL of hexane, which was removed by aspiration with Pasteur pipette. Samples were evaporated to dryness under nitrogen, diluted with 500 μL of mobile phase and stored at + 5°C prior HPLC-UV and HPLC/MS analysis.

## High Performance Liquid Chromatography

Reconstituted SFE extracts as well as standard mixtures were injected into a Waters HPLC system, Model # 600-MS (Waters Inc., Milford, MA, USA) using a Rheodyne 7125 injector (Rheodyne Inc., Cotati, CA, USA) equipped with 5 μL sample loop. The mobile phase was methanol: acetonitrile:ammonium acetate (3 mM) 45:5:50 (v/v/v). The HPLC flow rate was 200 μL/min. and the column utilized was 2 mm ID x 10 cm, 5 μm, 100 Å, BETASIL C18 (Keystone Scientific Inc., Bellefonte, PA, USA). A variable wavelength detector, Spectroflow (ABI Analytical Kratos Division, Ramsey, NJ, USA) was used for monitoring the three mycotoxins, deoxynivalenol (DON), diacetoxyscirpenol (DAS) and T-2 toxin (T-2) at 220 nm.

## Mass Spectrometry

A PE Sciex API 300 LC/MS/MS System (Perkin Elmer Sciex Instruments, Thornhill, Ontario, Canada) equipped with an ion spray LC/MS interface was used for all mass-spectrometry studies. The HPLC flow was maintained at 200 μL/min. without post-column splitting of the HPLC effluent. The ammonium adduct ions of the three mycotoxins were detected by HPLC/MS under full-scan and selected ion monitoring (SIM) modes. When HPLC/MS/MS experiments were undertaken, the mass spectrometer was operated in the product ion mode under selected reaction monitoring (SRM) conditions using the ammoniated molecules as the precursor ions. The mass range in full-scan experiments was 50-600 Da using a step size of 0.2 Da. Under scanning conditions a scan rate of 4 s per scan was used, while under SIM conditions a dwell time of 200.0 msec was used. Two product ions from each compound were monitored under SRM conditions. For all MS/MS experiments nitrogen was used as the collision gas at a setting of 3 on the PE-SCIEX API 300. Quantitation was performed using reference to an external standard analyzed under identical experiment conditions.

## RESULTS AND DISCUSSION

Supercritical fluid extraction with pure $CO_2$ is a useful approach for extraction of relatively nonpolar compounds. However, when polar compounds *e.g.* trichothecenes are present in a sample, either ultrahigh pressures (> 10 000 psi) or organic modifiers must be used. In this work very high pressures were not available, so the highest pressure available from the ISCO system was used combined with MeOH as a modifier for the supercritical carbon dioxide. Under these conditions trichothecenes are readily extracted from grain samples by supercritical fluid extraction. However, the aggressive conditions used also recovers many other interfering compounds, which leads to considerable chemical interference when UV detection is used following HPLC separation. Furthermore, the detection of trichothecenes by HPLC-UV is relatively nonspecific due to the lack of strong chromophores. These problems could potentially be resolved by using further purification and derivatization procedures or by employing more specific detection such as mass spectrometry.

The SFE conditions used in this work were as follows: pressure 550 atm (=8085 psi), extraction temperature 60°C and restrictor temperature 65°C. Thirty mL of supercritical $CO_2$ doped with 5 % methanol were used for each sample extraction. The optimum conditions used were based on our systematic studies designed to determine the optimum pressure and temperature to afford the best recovery of the target compounds with a minimum of chemical interference. Higher pressures and temperatures as well as varying quantities of the MeOH modifier were used, but coeluting lipophilic compounds interfered with UV-detection. As can be seen from Figure 1, DON is perhaps the most polar mycotoxin while DAS and T-2 are less polar. T-2 and DAS do not, however, have functional groups which absorb or fluoresce at 220 nm, whereas DON has somewhat better UV absorbance. Due to the relatively weak absorbance of these three compounds, interfering chemical constituents in the extract can easily interfere with the detection of the target analytes in the supercritical extracts. Recoveries of SFE were calculated by spiking samples before and after SFE. Spiking levels in all cases were 250, 500 and 1500-ppb.

Recovery studies with references to external standards showed that DON gave the highest recovery (95 %) while DAS and T-2 each were recovered at the 85 % level under the SFE conditions used. The results were a little surprising given that less polar compounds are usually isolated more easily with SFE than polar compounds. When multiple samples were extracted by SFE, the variable restrictor was wiped clean with MeOH to ensure minimal carryover between samples. Systematic studies with blank samples showed no evidence for carryover between samples using either HPLC/UV of HPLC/MS.

**Table 1**

**Mass Spectral Data of Three Trichothecenes Investigated**

| Compound | $[M + H]^{\pm}$ | $[M + NH_4]^{\pm}$ | Product Ions |
|---|---|---|---|
| | m/z | m/z | m/z |
| DON | 297 | 314.2 | 249.0 and 297.0 |
| DAS | 367 | 384.1 | 247.0 and 307.0 |
| T-2 | 467 | 484.1 | 245.0 and 305.0 |

HPLC conditions were based on our earlier studies[34] with the exception that ammonium acetate was used in this work to afford ammonium adduct ions from the ion spray LC/MS experiments. In this study isocratic elution of the test compounds was used. The HPLC conditions were chosen so that the elution of three toxins investigated could be accomplished in a minimum time period. The organic solvent content in the mobile phase was also maintained as high as possible to afford maximum sensitivity for electrospray ionization. The total HPLC analysis time was 6 min. per sample. The elution order of the analytes was as follows: DON, DAS and T-2. First, UV-detection was used at 220 nm, but with this wavelength DAS and T-2 could not be detected in a 500 ppb sample extract. The chemical background in the HPLC chromatogram was sufficiently high that even DON cold not be detected when the SFE extracts from real samples were analyzed. A preferred approach is to use a more specific detection system such as mass spectrometry.

Ion spray mass spectrometric analysis was performed in three different acquisition modes. First, full-scan acquisition was carried over the mass range of 50-600 amu Da. To gain higher specificity as well as sensitivity the instrument was then operated in the selected ion monitoring mode (SIM). The ions selected were the ammonium adduct ions of three trichothecenes. These ions as well as the corresponding protonated molecules are listed in Table 1. The ammoniated adduct ions were selected because of their high abundance in the mass spectra of these compounds under the experimental conditions used. Finally, to obtain a combination of high sensitivity as well as high specificity the selected reaction monitoring (SRM) mode of operation was used. In these experiments nitrogen was used as a collision gas in the central collision cell positioned between the two quadrupole mass analyzers. When the precursor ions derived from electrospray ionization are focused into the collision cell, fragmentation occurs that affords a high level of specificity for the target compounds. In addition, the pre selection of the ammoniated adduct ions from

each target compound affords a significant reduction of chemical noise thereby providing a higher signal-to-noise in the final ion current profile of the chromatogram. A total of six ions were monitored in these experiments since two unique product ions for each of the three target compounds were monitored. The relevant product ions are listed in Table 1.

Figure 2A-D shows the results obtained from the four HPLC analyses described above. In each case, supercritical fluid extraction was applied for isolation of the three trichotecenes DON, DAS, T-2 from cereal samples containing 500-ppb levels of each compound. Figure 2A shows the HPLC chromatogram when UV-detection was used. The sample used was rolled oats spiked with 500-ppb of each mycotoxin. The total run time was 20 min. DON could be observed at the 500 ppb level only as a shoulder on the first peak. DAS and T-2 were not detected in the HPLC/UV chromatogram under these experimental conditions. Neither quantitative nor qualitative determination of these compounds could be possible with this experimental approach.

At the 250-ppb level DON was not detected at all by HPLC/UV (data not shown). A more specific detector such as mass spectrometry can provide a more reliable way to detect compounds present in complex mixtures. Figure 2B-D shows an example of this point. These data were obtained by the LC/MS analysis of an SFE extract of rolled oats spiked with 250-ppb of each compound. Figure 2B shows the chromatogram obtained from the full-scan LC/MS analysis of the supercritical fluid extract of ground oats. Although each of the target analytes may be detected in this LC/MS TIC chromatogram, there is still considerable chemical interference. In spite of this, one may obtain background-subtracted mass spectra from each of the target compounds in this chromatogram (data not shown) which readily verify the molecular weight for each compound.

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**Figure 2 (right)**. HPLC/UV and HPLC/MS chromatograms obtained from the determination of the target trichothecenes isolated from rolled oats fortified at the 500 ppb level (HPLC/UV) and 250 ppb levels (HPLC/MS) of the three test compounds.
A) HPLC/UV chromatogram obtained from the analysis of a supercritical fluid extract of rolled oats spiked with 500 ppb levels of the three test compounds. B) Full-scan LC/MS TIC chromatogram obtained from the analysis of a supercritical fluid extract of rolled oats spiked with 250 ppb levels of the three test compounds. C) SRM LC/MS chromatogram obtained from the analysis of a supercritical fluid extract of rolled oats spiked with 250 ppb levels of the three test compounds. See text for experimental details.

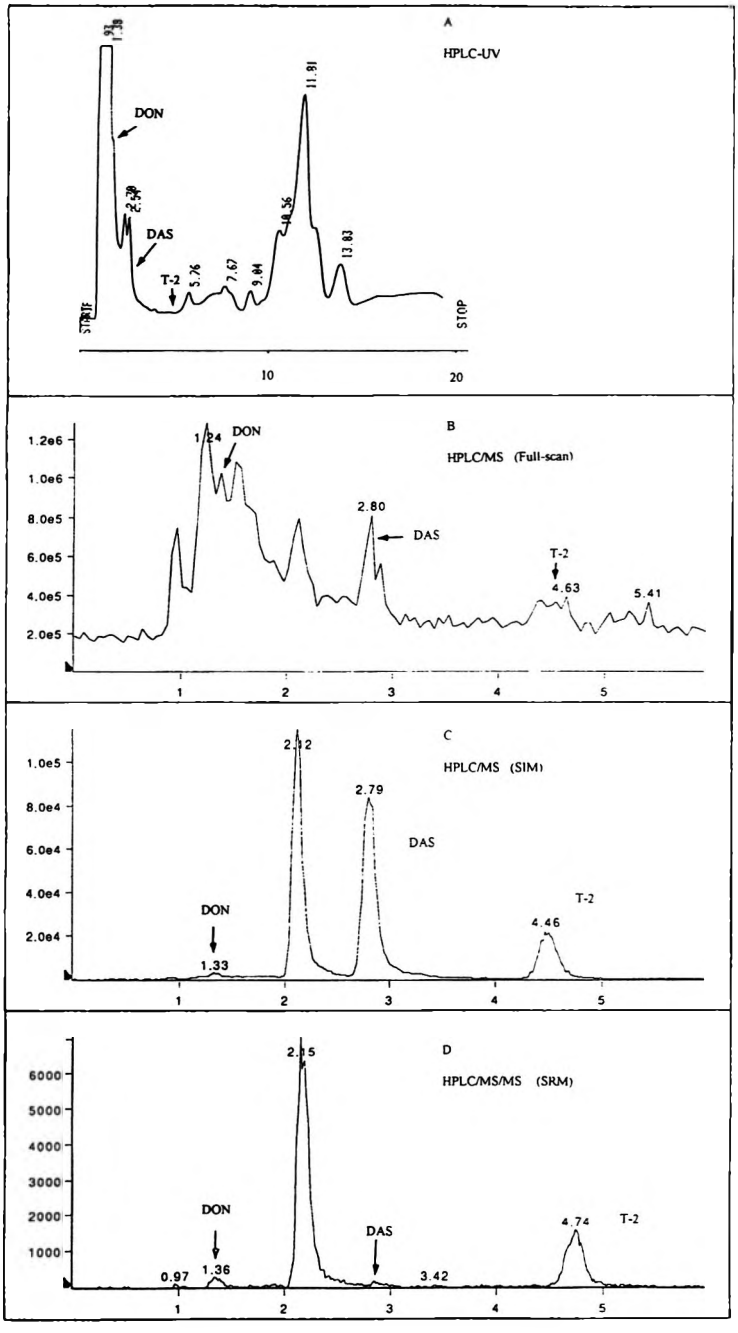
A
HPLC-UV
DON
DAS
T-2
11.91
13.83
STOP
10
20
B
HPLC/MS (Full-scan)
1.24
DON
2.80
DAS
T-2
4.63
5.41
1.2e6
1.0e6
8.0e5
6.0e5
4.0e5
2.0e5
C
HPLC/MS (SIM)
2.79
DAS
DON
1.33
T-2
4.46
1.0e5
8.0e4
6.0e4
4.0e4
2.0e4
D
HPLC/MS/MS (SRM)
DON
1.36
DAS
3.42
T-2
4.74
6000
5000
4000
3000
2000
1000

Figure 2C shows a much improved ion current chromatogram signal-to-noise ratio when SIM LC/MS analysis of the same sample is performed. The large peak eluting at 2.12 min. is an endogenous compound that is detected under these conditions since it appears to have ions common to the target compounds. Finally, the results from the SRM LC/MS analysis of the same sample are shown in Figure 2D. The signal-to-noise ratio improves further in this experiment, although the gains from tandem mass spectrometry are not as large as often observed. However, since two precursor-product ion transitions are monitored for each compound (Table 1), one has considerably more specificity from these data than from the SIM results.

Unfortunately, the response from DON under these positive ion spray LC/MS conditions is not as good as for the other compounds. This is sometimes observed when compounds have significantly different proton or ammonium ion affinities. In addition, the high specificity of MS/MS has not removed the large component eluting at 2.12 min. However, the ion current profiles shown in Figure 2C,D display a significantly improved sensitivity and specificity over the LC/UV chromatogram shown in Figure 2A. Similar results were obtained when the sample extracted was yellow cornmeal spiked with 250-, 500- and 1500-ppb of each mycotoxin using these same experimental conditions. In each case only the SIM- or SRM LC/MS analyses reliably detected the target analytes.

It is clear from the above data that mass spectrometry provides a more selective and sensitive method for analyzing samples containing these trichothecenes. The total ion current chromatogram (Figure 2B), however, shows only slightly more information than the HPLC/UV chromatogram shown in Figure 2A. However, when either SIM or SRM LC/MS are used, all three mycotoxins could be easily detected and identified at the 250 ppb level (Figure 2C and D). Good sensitivity and specificity were achieved in the SIM LC/MS determination of trichothecenes in the SF-extracts (Figure 2C), but the lack of fragmentation decreases the reliability of the identification and does not provide any fragmentation information that can facilitate qualitative confirmation of these compounds. It is for these reasons that more selectivity is needed. Figure 2D shows the most powerful feature of MS if tandem mass spectrometry (MS/MS) is available. The ion spray LC/MS/MS experiment was set to monitor the precursor-product ion transitions characteristic of the three target compounds. This experiment, therefore, provides very high specificity for the qualitative determination for each of the three compounds even when they are present in complex matrix. It should also be true that improved detection limits could be achieved for quantitative analyses using this approach, provided an appropriate internal standard was used.

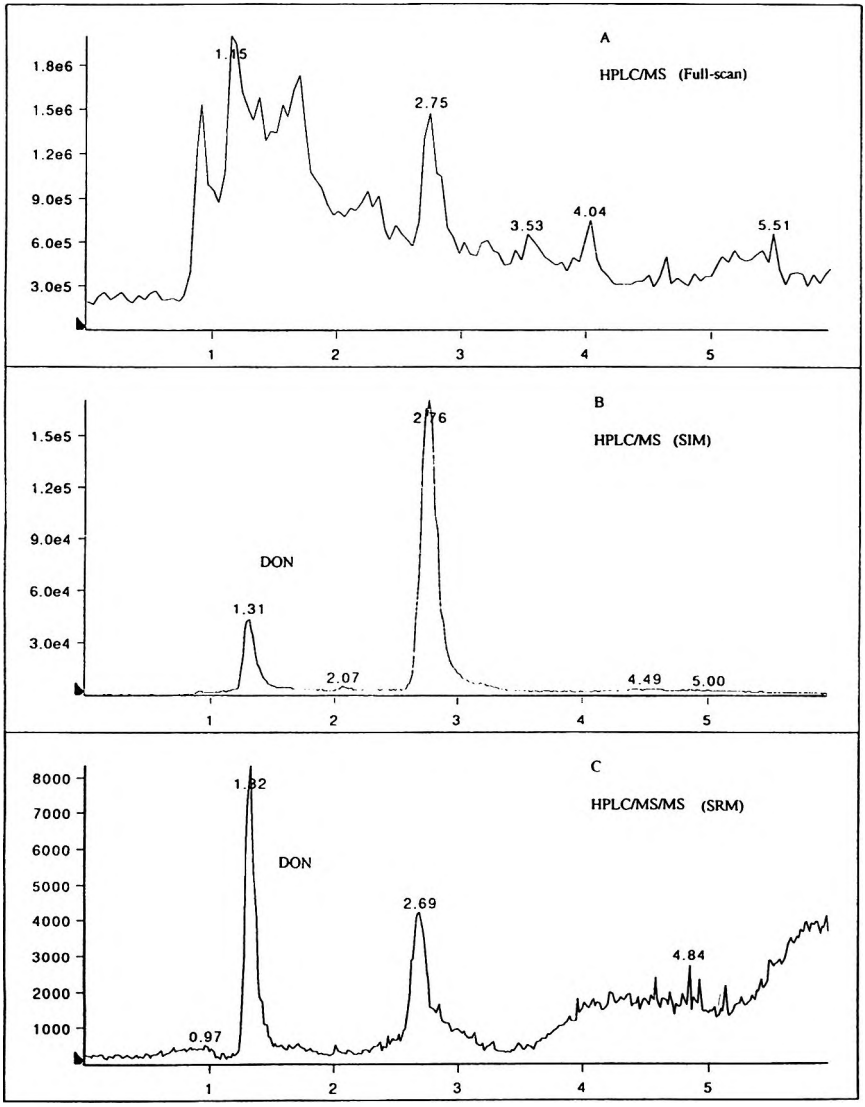


**Figure 3**. HPLC/MS analysis of corn feed spiked with 1 ppm levels of the three test compounds. A) Full-scan LC/MS TIC from the analysis of the supercritical fluid extract of the sample. B) SIM LC/MS analysis of the supercritical fluid extract of the sample. C) SRM LC/MS analysis of the supercritical fluid extract of the sample. See text for experimental details

Figure 3 shows the LC/MS and LC/MS/MS chromatograms obtained from a supercritical fluid extract of a feed sample fortified at the 1 ppm level. The SFE extract was yellow in color and a few droplets of fat were observed in the collection tube. This material was removed by extraction with hexane before LC/MS/MS analysis of the SFE extract. The DON in this corn feed is easily determined using this approach. Figure 3A shows the corresponding full-scan TIC chromatogram and Figure 3 B and C show the SIM and SRM LC/MS chromatograms, respectively. HPLC/UV analysis was not attempted due to the high levels of interfering endogenous chemical background present in the supercritical fluid extract.

## CONCLUSIONS

The results described show that trichothecenes may be extracted from cereal and grain samples by supercritical fluid extraction with high recoveries. If the analytes are monitored by high performance liquid chromatography-tandem quadrupole mass spectrometry via atmospheric pressure ionization interface, the target compounds may then be quantitatively and qualitatively determined with an LC/MS sample throughput of approximately 10 samples per hour. The limit of detection with HPLC-UV was 500-ppb for DON and not detectable for T-2 and DAX. The detection limit using HPLC/MS/MS techniques are estimated to be in the low ppb range for each of the three analytes. HPLC-UV analyses of the extracts were hampered by co-eluting fat-soluble compounds that produced high chemical interference with the target compounds. LC/MS and LC/MS/MS techniques circumvent this problem by providing much higher specificity for the test compounds in the presence of high chemical interference.

SFE-API/HPLC/MS/MS analyses are an efficient and rapid means of analyzing biological samples for the determination of unknown and target compounds. The API source operates at ambient temperature and easily handles HPLC column flow rates of 200 μL/min. without post-column splitting of the HPLC effluent. Results presented in this work suggest that the sensitivity and ruggedness of LC/MS/MS is suitable if not preferred when combined with the merits of supercritical fluid extraction of feed and cereal matrices containing the target compounds studied in this work.

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# HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ERYTHROMYCIN AND RELATED IMPURITIES IN PHARMACEUTICAL FORMULATIONS

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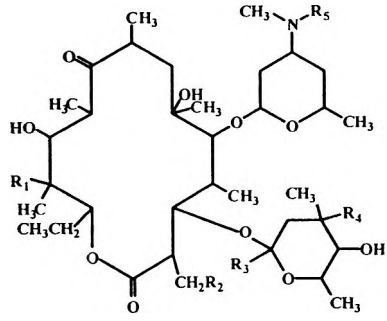
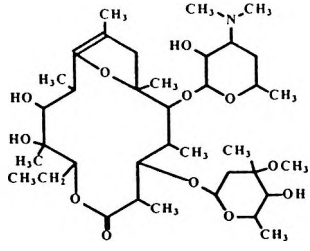
## ABSTRACT

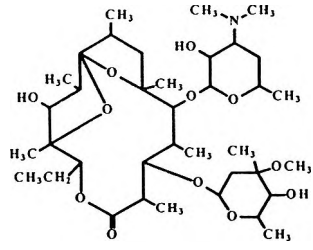
In the last few years, several liquid chromatographic methods for the analysis of erythromycin have been published. Until recently, most of the published methods were either complex or lacked the selectivity needed for the assay of erythromycin in the presence of erythromycin derivatives and impurities. Recently, we developed a significantly improved $C_{18}$ liquid chromatographic method for the assay of erythromycin. In comparison, our method is simpler, more rugged, faster, and more sensitive and selective than other published methods. In this investigation, the developed method was successfully used for the assay of erythromycin in several pharmaceutical formulations.



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Erythromycin A (EA) | OH | H | H | $OCH_3$ | $CH_3$ |
| Erythromycin B (EB) | H | H | H | $OCH_3$ | $CH_3$ |
| Erythromycin C (EC) | OH | H | H | OH | $CH_3$ |
| Erythromycin E (EE) | OH | —O— | | $OCH_3$ | $CH_3$ |
| N-demethylerythromycin A (NDEA) | OH | H | H | $OCH_3$ | H |



Erythromycin A enol ether (EEEA)



Anhydroerythromycin A (AE)

**Figure 1**. Chemical Structures of Erythromycin A and related substances.

## INTRODUCTION

Erythromycin (free base) is a widely used broad spectrum antibiotic marketed in several pharmaceutical formulations. Erythromycin base can be found in a variety of liquid and solid erythromycin formulations. Solid formulations include delayed-release tablets and capsules, where erythromycin is enteric-coated to protect against degradation by gastric acidity.

Erythromycin topical solutions (2%) are alcoholic solutions of erythromycin base and are indicated for the topical control of *acne vulgaris*.[1] The antimicrobial activity of erythromycin is due mainly to the erythromycin A (EA), erythromycin B (EB), and erythromycin C (EC) forms. Erythromycin E (EE), N-demethyl erythromycin A (NDEA), erythromycin A enol ether (EEEA), anhydroerythromycin A (AE), and other erythromycin derivatives can be present as impurities. Chemical structures of erythromycin A and related substances are illustrated in Figure 1.

There is a considerable interest in developing chemical assay methods of antibiotics. Several methods for the assay of erythromycin and related substances in bulk, biological fluids, and pharmaceutical formulations have been published.[2-9] These methods lack the resolution needed for the separation of several related substances, such as EE, and other common erythromycin impurities. In order to improve chromatographic selectivity, a method utilizing poly(styrene-divinylbenzene) stationary phase, capable of the separation of EE and EA, was developed[10] and adopted by the European Pharmacopoeia.[11] However, the polymer column method lacks the simplicity and ruggedness needed for the routine assay of erythromycin.

Recently, we developed a $C_{18}$ based gradient LC method[12] that is simple, sensitive, rugged, and able to separate and assay erythromycin and most related substances commonly found in commercial erythromycin products. In this manuscript, we are illustrating the application of this method in the assay of erythromycin in different pharmaceutical formulations.

## EXPERIMENTAL

### Chemicals and Reagents

USP erythromycin reference standard (RS) was used throughout the study. Other erythromycin standards (EB, EC, EE, NDEA, AE, and EEEA) were kindly provided by Abbott Laboratories, North Chicago, Illinois. Commercial samples of erythromycin powder, erythromycin topical solutions, delayed-release capsules, and delayed-release tablets were purchased from available sources. Ammonium hydrogen phosphate, ammonium hydroxide, and tetrabutylammonium hydrogen sulfate (of the highest available purity) were purchased from different sources and used without additional purification. Acetonitrile used was HPLC grade and water was deionized and filtered through a Milli-Q™ water purification system (Millipore, New Bedford, MA).

**Solutions**

Stock 0.20 M ammonium phosphate buffer was made by dissolving the calculated amount of $(NH_4)H_2PO_4$ in Milli-Q water, the pH adjusted to 6.5 using ammonium hydroxide, and the solution filtered through a 0.45 μm nylon membrane filter. Stock 0.20 M tetrabutylammonium sulfate (mobile phase additive) was made by dissolving the calculated amount of $(C_4H_9)_4NHSO_4$ in Milli-Q water, the pH adjusted to 6.5 using ammonium hydroxide, and the solution filtered through a 0.45 μm nylon membrane filter.

**Chromatographic Conditions**

The HPLC system used in this investigation consisted of Spectra-Physics SP 8800 pump, Spectra-Physics SP 8880 autosampler, Spectra FOCUS Forward Optical Scanning detector set at 205 nm, COMPAQ DESKPRO XL 5100 computer, and PC1000 System Software™ (Ver. 3.0). A Prodigy 5μ ODS-2 (150 Å) 250 x 4.6 mm I.D. column (Phenomenex, Torrance, CA) was used in this study. In addition, a Prodigy 5μ ODS-2 30 x 4.6 mm I.D. guard column was used. The gradient profile and mobile phase compositions are described in Table I. The concentration of both the buffer and tetrabutylammonium sulfate were maintained constant in both mobile phases and the only difference between mobile phase A and B is the percentage of acetonitrile (10 vs 50%, respectively). The gradient delay volume was determined to be 5.5 mL and no equilibration time was needed between injections. The mobile phase flow rate was set at 1.3 mL/min. The column temperature was controlled at 50°C with a block column heater (Jones Chromatography, Lakewood, CO) and sample injection volume was 50 μL.

**Sample Preparation**

The sample solvent was prepared by mixing equal volumes of the two mobile phases (50%A-50%B) and used as the sample solvent throughout this study.

**Powders**

Samples were prepared by dissolving the weighed amount in the sample preparation solvent to give a final concentration of 8-10 mg/mL. Samples were then placed in an ultrasonic bath for approximately 5 minutes to enhance dissolution.

**Table 1**

**Gradient System for the Erythromycin Assay**

| Time (min.) | %A[a] | %B[b] |
|---|---|---|
| 0.0 | 78.0 | 22.0 |
| 39.0 | 26.0 | 74.0 |
| 47.0 | 26.0 | 74.0 |

[a]Mobile phase A (10% $CH_3CN$) was prepared by mixing 60 mL stock ammonium phosphate buffer (0.20 M, pH=6.5), 60 mL stock tetrabutylammonium sulfate (0.20 M, pH=6.5), and about 250 mL Milli-Q water. This was followed by the addition of 100 mL acetonitrile, diluting to 1L with Milli-Q water, mixing well and filtering through a 0.45μm nylon membrane filter.

[b]Mobile phase B (50% $CH_3CN$) was prepared as in "A" with the only exception being the use of 500 mL acetonitrile in the mobile phase preparation.

**Topical Solutions**

Topical solutions were used as such without any additional treatment. In other words, samples of topical solutions were transferred directly into autosampler vials.

**Delayed-Release Capsules**

The contents of four capsules were transferred into a 100 mL volumetric flask, sample preparation solvent was added to the volume, and the flask sonicated for 15 minutes to ensure complete dissolution of erythromycin. The resulting solution was filtered through a 0.45 μm type HVLP filter. The first few milliliters of the filtrate were discarded.

**Delayed-Release Tablets**

Ten tablets were weighed in order to determine the average tablet weight, transferred into a mortar, and pulverized to fine powder. A 50 to 70 mg portion of the powdered tablets was placed into a 10-mL volumetric flask,

**Table 2**

**HPLC Assay of Commercial Erythromycin Powders**

| Commercial Erythromycin Powder[a] | % Erythromycin Found[b,c] ± SD |
|---|---|
| Product I | 101.1 ± 1.0 |
| Product II | 98.2 ± 0.7 |

[a] Samples were dissolved in a solvent made of 50%A and 50%B (as described in the experimental section) at a concentration of 8-10 mg/mL.
[b]Average of 6 runs.
[c]Calculated as the sum of erythromycins A, B, and C. Erythromycins B and C calculations were based on the response factor of erythromycin A.

**Table 3**

**HPLC Assay of Ten Commercial Erythromycin 2% Topical Solutions**

| Topical Solutions[a] | Erythromycin Found[b,c] ± SD | % of Label Claim[d] |
|---|---|---|
| Product I | 1.60 ± 0.01 | 80.0 |
| Product II | 1.63 ± 0.01 | 81.5 |
| Product III | 1.70 ± 0.01 | 85.0 |
| Product IV | 1.83 ± 0.02 | 91.5 |
| Product V | 1.69 ± 0.02 | 84.5 |
| Product VI | 1.69 ± 0.00 | 84.5 |
| Product VII | 1.96 ± 0.01 | 98.0 |
| Product VIII | 1.68 ± 0.03 | 84.0 |
| Product IX | 1.84 ± 0.02 | 92.0 |
| Product X | 1.87 ± 0.02 | 93.5 |

[a] Solutions were used as such without any prior treatment.
[b]% erythromycin (weight/volume), average of three runs.
[c]Calculated as the sum of erythromycins A, B, and C. Erythromycins B and C calculations were based on the response factor of erythromycin A.
[d]USP limits: 90.0 - 125.0 % of labeled amount.[13]

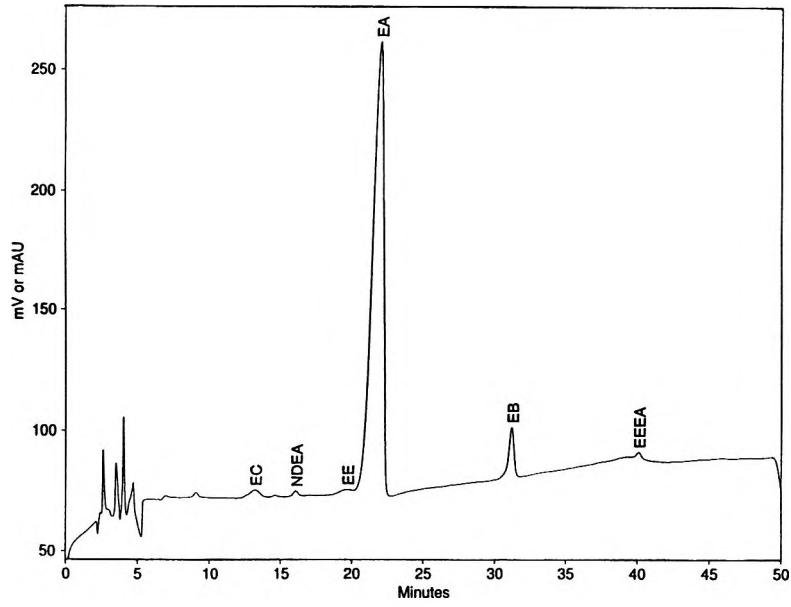



**Figure 2**. Typical chromatogram (monitored at 205 nm) obtained from the analysis of a commercial sample of erythromycin powder. Details of chromatographic conditions are described in the experimental section and Table 1.

sample preparation solvent was added to the volume, the suspension was sonicated for about 10 minutes, and the resulting solutions were filtered twice through a 0.45μm type HVLP filter. The first few milliliters of the filtrate were discarded.

The effect of changing the sample preparation solvent, its volume, sonication time, and the use of different shakers and blenders was investigated. It was found that the sample preparation method described above was the simplest to provide complete recovery of erythromycin.

## RESULTS AND DISCUSSION

A system suitability test mixture was prepared and used to test method performance and ruggedness as described before.[12] Increasing the temperature

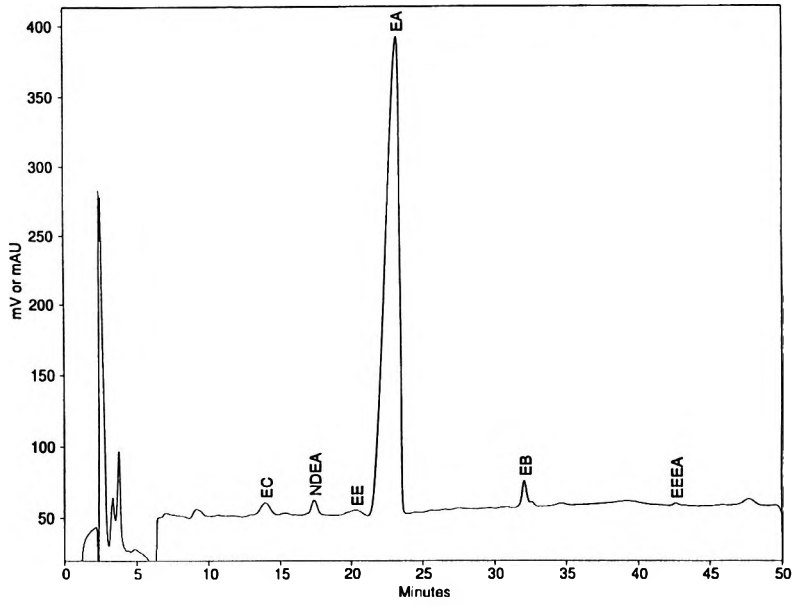



**Figure 3**. Typical chromatogram (monitored at 205 nm) obtained from the analysis of a commercial sample of erythromycin 2% topical solution. Details of chromatographic conditions are described in the experimental section and Table 1.

to 50°C was the only modification made to the previously described method.[12] The slight increase in temperature enhances the chromatographic selectivity and eliminates interference from additives present in tested pharmaceutical formulations.

## Assay of Erythromycin Powders

Two commercial samples of erythromycin powder, suitable for prescription compounding, were obtained and assayed using the developed method. The assay results are summarized in Table 2. The percentage of erythromycin in these samples was calculated as the sum of EA, EB, and EC. The absorptivities of erythromycin isomers and related substances in the sample preparation solution were determined before[12] and in spite of differences in absorptivity, the concentrations of both EB and EC were estimated using the same response factor as for EA. Figure 2 provides an illustration of a typical chromatogram obtained from the analysis of a commercial erythromycin powder.

**Table 4**

**HPLC Assay of Six Commercial Erythromycin 250 mg Delayed-Release Capsules**

| Delayed-Release Capsules[a] | mg of Erythromycin Found[b,c] | % of Label Claim[d] |
|---|---|---|
| Product I | 241 | 96 |
| Product II | 246 | 98 |
| Product III | 260 | 104 |
| Product IV | 253 | 101 |
| Product V | 236 | 94 |
| Product VI | 252 | 101 |

[a] Samples were obtained and prepared for the assay as described in the experimental section

[b] Average of 6 runs.

[c] Calculated as the sum of erythromycins A, B, and C. Erythromycins B and C calculations were based on the response factor of erythromycin A.

[d] USP limits: 90.0 - 115.0 % of labeled amount.[13]

**Assay of Erythromycin Topical Solutions**

Ten commercially available samples of erythromycin (2%) topical solutions were obtained and assayed using the developed method (Table 3). The assay was performed directly on the topical solutions without any prior treatment. This is clearly an added advantage of using the developed method for the assay of erythromycin topical solutions. Other solution ingredients present (alcohol, propylene glycol,..etc) had no effect on chromatographic quality or separation (Figure 3).

**Assay of Erythromycin Delayed-Release Capsules**

Six commercially available erythromycin delayed-release capsules were analyzed as described in the experimental section. The assay worked well and can be utilized as a measure of content uniformity. The percentage of erythromycin in the six tested formulations varied from a lower value of 94 percent of declared to a higher value of 101 percent of declared (Table 4). The chromatographic method used was able to separate inert material interferences from the different erythromycin forms and related substances (Figure 4).

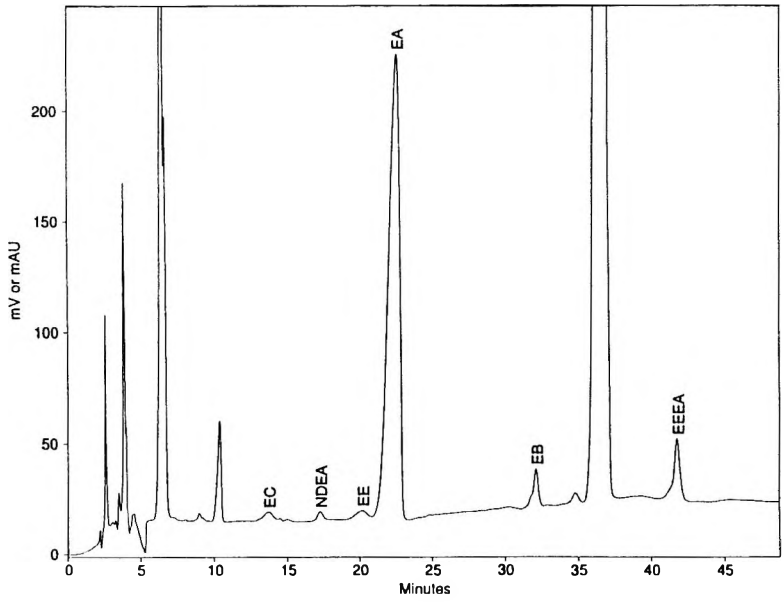



**Figure 4**. Typical chromatogram (monitored at 205 nm) obtained from the analysis of a commercial sample of erythromycin delayed-release capsule. Details of chromatographic conditions are described in the experimental section and Table I.

**Table 5**

**HPLC Assay of Commercial Erythromycin Delayed-Release Tablets**

| Delayed-Release Tablet[a] | mg of Erythromycin Found[b,c] | % of Label Claim[d] |
|---|---|---|
| Product I, 250 mg | 277 | 111 |
| Product II, 333 mg | 359 | 108 |

[a] Samples were obtained and prepared for the assay as described in the experimental section

[b] Average of 6 runs.

[c] Calculated as the sum of erythromycins A, B, and C. Erythromycins B and C calculations were based on the response factor of erythromycin A.

[d] USP limits: 90.0 - 120.0 % of labeled amount.[13]

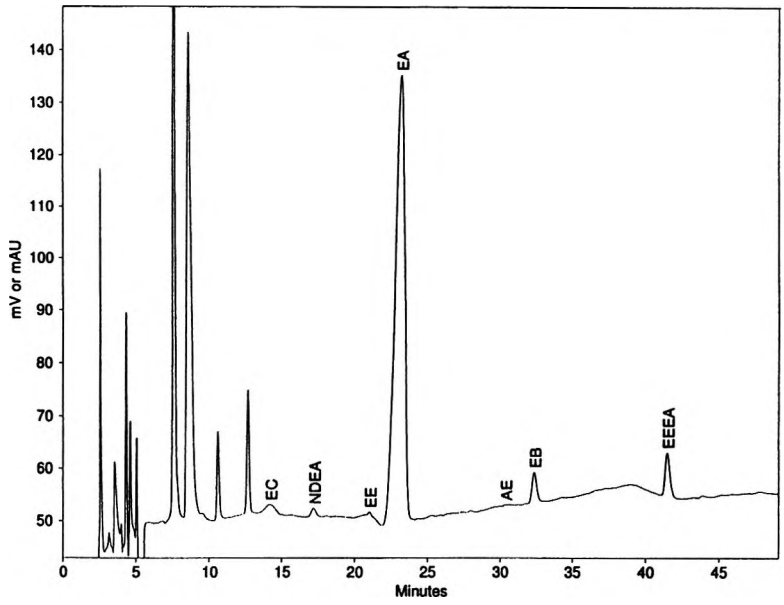



**Figure 5**. Typical chromatogram (monitored at 205 nm) obtained from the analysis of a commercial sample of erythromycin delayed-release tablet. Details of chromatographic conditions are described in the experimental section and Table 1.

### Assay of Erythromycin Delayed-Release Tablets

Two commercially available erythromycin delayed-release tablets were analyzed as described in the experimental section (Table 5, Figure 5).

## CONCLUSION

The developed method has been applied successfully for the assay of erythromycin and related substances in different pharmaceutical formulations. The method proves to be simple, versatile, and easy to use. In most cases, the assay results of commercially available products are within the USP[13] specifications for the tested products. However, the assay results of topical solutions (Table 3) shows that most topical solutions tested are not within the USP limit of 90 - 125 % of the label amount or 1.8 - 2.5% erythromycin.[13]

## ACKNOWLEDGMENT

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# A STABILITY-INDICATING HPLC METHOD FOR THE DETERMINATION OF OXYTOCIN ACETATE IN OXYTOCIN INJECTION, USP, SYNTHETIC†

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## ABSTRACT

A gradient high performance liquid chromatographic (HPLC) procedure employing ultraviolet (UV) detection for the analysis of oxytocin acetate in Oxytocin Injection, USP, Synthetic is reported. The method requires no sample pretreatment and is accurate, reproducible and selective. The peak area versus oxytocin acetate concentration is linear over the range of 50-150% of its label claim of 10 Oxytocin USP Units/mL. The mean absolute recovery of oxytocin acetate using the described method is 98.3 ± 0.6%, (mean ±SD, n = 9). The precision, relative standard deviation (RSD), of label claim, amongst five sample preparations is not more than 0.4%. Intermediate precision, as determined from fifteen sample preparations, generated by two Analysts on different HPLC systems over three days, exhibits an RSD of 1.9%. The Standard and Assay Preparations are stable for up to 48 hours at room temperature.

The selectivity was evaluated by subjecting the finished product (Oxytocin Injection, USP, Synthetic) to thermal, acidic, basic, oxidative and fluorescent radiation stress conditions. No interference in the analysis of oxytocin acetate was observed from degradation products or from the preservative, chlorobutanol, showing the method is stability-indicating.

## INTRODUCTION

Oxytocin is an endogenous nonapeptide hormone produced in the supraoptic and paraventricular nuclei of the hypothalamus and stored in the posterior pituitary.[1] The release of oxytocin from the posterior pituitary causes contractions in uterine, vascular, and other smooth muscle.[1] Synthetic oxytocin is the active component of the finished product, Oxytocin Injection, USP, Synthetic, which is administered to aid in the progression of labor and delivery in modern obstetrics.

The analysis of oxytocin is primarily determined immunologically[2-4] or chromatographically.[5-11] Although several methods exist for the determination of oxytocin, a computer search (Medline and Chemical Abstracts) indicated only two methods applicable to parenteral solutions.[9,10] The first method by Krummen and Frei[9] does not provide selectivity data for various stress conditions of the finished product, while the second method by Brown and Jenke[10] requires postcolumn derivatization with fluorescamine, which is time consuming and costly. The method described herein is a modification of the United States Pharmacopeia (USP) method,[11] whereby the effect of Mobile Phase A pH on resolution, column length and selectivity were examined.

This manuscript describes a sensitive, accurate, and reproducible, gradient reversed phase HPLC method for the determination of oxytocin acetate in Oxytocin Injection, USP, Synthetic. Moreover, this method was determined to be stability-indicating.

According to the USP 23 <1225> guidelines, analytical methods for the quantitation of major components of bulk drug substance or preservatives in finished pharmaceutical products fall under Assay Category I.[12] Data elements required for Assay Category I include precision, accuracy, specificity, range, linearity, and ruggedness. The method for oxytocin acetate in Oxytocin Injection, USP, Synthetic satisfies all of these requirements.

## EXPERIMENTAL

### Chemicals and Reagents

Oxytocin Injection, USP, Synthetic was formulated at Fujisawa USA, Inc. (Melrose Park, IL, USA). Oxytocin acetate was an in-house reference standard, while chlorobutanol was obtained from Akzo Nobel (Edison, NJ, USA). ACS reagent grade glacial acetic acid, sodium acetate trihydrate, sodium phosphate monobasic, sodium hydroxide, hydrochloric acid, and hydrogen peroxide were purchased from Mallinckrodt (Paris, KY, USA), while phosphoric acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade acetonitrile was purchased from Baxter (Deerfield, IL, USA) and ethyl alcohol, absolute, was purchased from McCormick, Distilling Co. (Perkin, IL, USA). The water was deionized and distilled. All reagents were used without further purification.

### Apparatus

The chromatographic system consisted of a Waters 680 Automated Gradient Controller, two 510 pumps, a WISP 717 autosampler, and a 486 variable-wavelength UV detector set at 220 nm (Waters Associates, Milford, MA, USA).

Two columns were evaluated; an Alltech Hypersil ODS (12 cm x 4.6 mm, 5 μm, Alltech, Deerfield, IL, USA) and a Beckman Ultrasphere ODS column (15 cm x 4.6 mm, 5 μm, Beckman, Fullerton, CA, USA). Both were maintained at ambient temperature.

### Mobile Phases

Mobile Phase A consisted of 100 mM sodium phosphate monobasic. The pH of Mobile Phase a was varied from 3.1 to 4.5 in 0.1 pH intervals by adjustment with phosphoric acid. Mobile Phase B consisted of acetonitrile-water (1:1, v/v). Each mobile phase was filtered through a 0.45 μm filter and degassed. The gradient conditions are provided in Table 1. The flow rate was 1.5 mL/minute with a typical operating pressure of *ca.* 850 psi. Under these conditions, the retention times of oxytocin acetate and chlorobutanol were 10.2 and 21.1 minutes, respectively.

**Table 1**

**Gradient Parameters**

| Interval ( min) | Mobile Phase A (%) | Mobile Phase B (%) |
|---|---|---|
| 0 | 70 | 30 |
| 30 | 50 | 50 |
| 35 | 50 | 50 |
| 45 | 70 | 30 |
| 60 | 70 | 30 |

**Preparation of Solutions**

**A. Diluent**

Dissolve 5 g/L chlorobutanol, 5 mL/L glacial acetic acid, 5 g/L ethyl alcohol, and 1.82 g/L sodium acetate trihydrate in water, and mix.

**B. Oxytocin standard stock solution**

Dissolve enough Oxytocin Standard in Diluent to yield a concentration of 200 Oxytocin USP Units/mL.

**C. Standard preparation**

Dilute the Oxytocin Standard Stock Solution with Diluent to yield a concentration of 10 Oxytocin USP Units/mL.

**D. Assay preparation**

The concentration of Oxytocin Injection, USP, Synthetic is 10 Oxytocin USP Units/mL so, consequently, no dilution is required.

**E. Resolution solution**

Transfer 15.0 mL of Oxytocin Standard Stock Solution into a 100 mL volumetric flask, dilute to volume with Diluent, mix, and adjust the pH to 2.0 ± 0.1 with 0.1N hydrochloric acid. Heat the solution at 1000C for 40 minutes, then adjust the pH to 4.0 ± 0.1 with 0.1N sodium hydroxide.

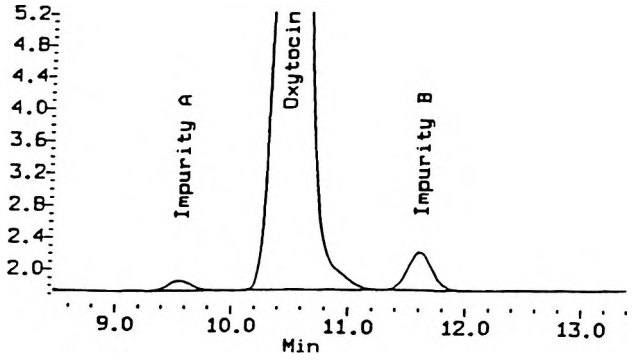


**Figure 1**. Typical chromatogram of the Resolution Solution.

## System Suitability

The system suitability results were calculated according to the USP 23 <621> from typical chromatograms.[13] The instrument precision as determined by five successive injections of the Standard Preparation should provide a relative standard deviation (RSD) not more than (NMT) 2.0%. The column efficiency should be greater than 6000 theoretical plates. The tailing factor should not exceed 1.5 at 5% peak height. Finally, inject 100 μL of the Resolution Solution. The resolution between oxytocin acetate and the nearest adjacent peak (Impurity A or Impurity B) is not less than (NLT) 1.5 (Figure 1).

## Specificity

The specificity of the method was studied through the analysis of stressed Oxytocin Injection, USP, Synthetic (finished product, 10 Oxytocin USP Units/mL) and stressed Placebo Solutions (finished product without oxytocin acetate). The finished product was subjected to thermal, acidic, basic, oxidative and fluorescent light environments to cause oxytocin degradation of 10-30%, as determined by peak area percent.

Five mL aliquots of the finished product and Placebo solution were sealed in transparent glass containers with equal head space and exposed to various stress conditions. Thermal stressed samples were stored at 105°C. Acid stressed samples were adjusted to pH 2 with concentrated HCl.

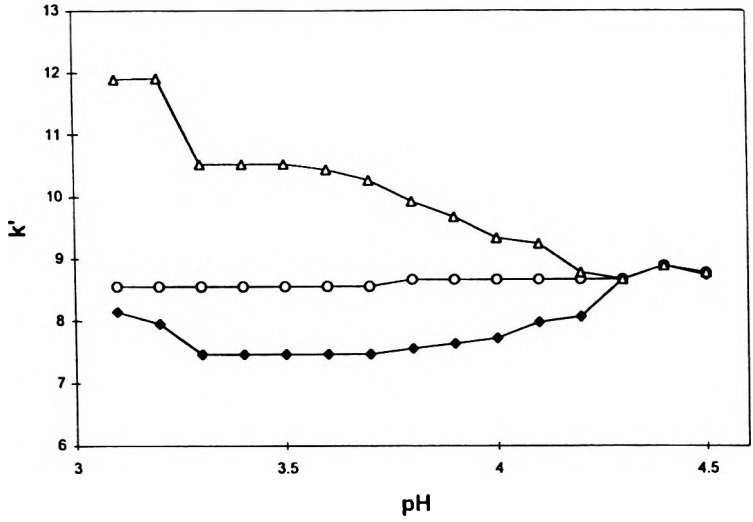


**Figure 2**. Effect of pH on k*, where o = oxytocin, ● = impurity A and ■ = impurity B.

Base stressed samples were adjusted to pH 12 with 50% NaOH. Oxidative stressed samples were subjected to 30% $H_2O_2$. Fluorescent stressed samples were subjected to 500-700 foot-candles of radiation.

### Data Acquisition

The peak areas of oxytocin acetate were measured using PE Nelson 6000 (Perkin-Elmer Corporation, Cupertino, CA, USA). The chromatographic data was automatically processed for peak area followed by an unweighted linear regression analysis.

## RESULTS AND DISCUSSION

### Effect of Mobile Phase A pH on Resolution

The unadjusted pH of 100 mM sodium phosphate monobasic is about 4.5, which results in an resolution of less than 1.5 between oxytocin acetate and the nearest adjacent peak (Figure 2). Consequently, the Resolution Solution of oxytocin acetate at different pH values (pH 3.1 - 4.5 in 0.1 intervals) of Mobile Phase A were analyzed.

**Table 2**

**Effect of Column on Resolution and Sensitivity**

| Column | Rs Between Impurity A & Oxytocin | Rs Between Impurity B & Oxytocin | S/N Ratio of Sensitivity Solution |
|---|---|---|---|
| Alltech 12 cm x 4.6 mm | 1.9 | 1.9 | 15.0 |
| Beckman 15 cm 4.6 mm | 2.4 | 2.7 | 14.8 |

From Figure 2, it is apparent that the resolution is significantly affected by the pH of Mobile Phase A. Based on these results, a pH range of 4.0 ± 0.1 was selected.

## Column Choice

The USP method[11] for Oxytocin Injection utilizes a 12 cm column, and due to its odd length needs to be specially ordered. This could result in delay times since the column is not a standard length and, furthermore, costs more.

Hence, a standard 15 cm x 4.6 mm column (Beckman ODS, 5 μm) was evaluated. The 15 cm column was selected since the resolution of oxytocin acetate from the nearest adjacent peak was higher, while maintaining sensitivity (Table 2).

## Chromatography

Typical chromatograms obtained from a 100 μL injection of a Standard Preparation, Assay Preparation and Placebo using Mobile Phase A (pH 4.0 ± 0.1) and a 15 cm column are illustrated in Figures 3 (a-c), respectively. The retention times of oxytocin acetate and chlorobutanol were 10.2 and 21.1 minutes, respectively. The overall chromatographic run time was 60 minutes.

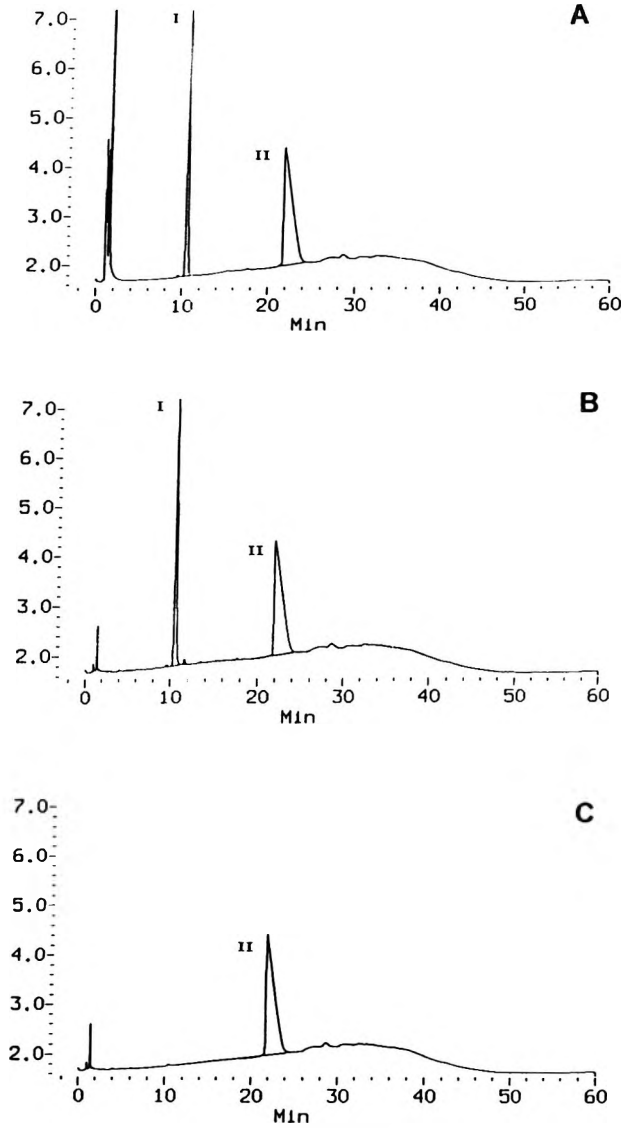


**Figure 3**. Typical chromatograms of (a) a Standard Solution of Oxytocin, (b) Oxytocin Injection, USP, Synthetic and (c) Placebo (Oxytocin Injection, USP, Synthetic not containing oxytocin acetate). I = oxytocin acetate and II = chlorobutanol.

**Table 3**

**System Suitability***

| Run | %RSD (n=5) | Tailing Factor Limit: NMT 2.0 | Theoretical Plates Limit: NLT 2000 | Resolution Limit:NLT 1.5 | |
|---|---|---|---|---|---|
| | | | | Impurity A | Impurity B |
| 1 | 0.3 | 1.1 | 7861 | 2.0 | 2.7 |
| 2 | 1.8 | 1.1 | 8341 | 2.4 | 3.1 |
| 3 | 0.5 | 1.1 | 9801 | 1.9 | 2.2 |
| 4 | 0.2 | 1.3 | 10625 | 1.6 | 1.8 |
| 5 | 1.2 | 1.3 | 12407 | 1.8 | 2.4 |
| 6 | 0.1 | 1.1 | 9604 | 2.0 | 2.7 |
| 7 | 0.4 | 1.2 | 9523 | 2.0 | 2.4 |
| 8 | 0.2 | 1.2 | 8281 | 2.1 | 2.4 |
| 9 | 0.1 | 1.2 | 7798 | 2.0 | 2.4 |
| 10 | 0.2 | 1.0 | 8649 | 2.1 | 3.1 |

**System Suitability**

In all cases, the column efficiency for oxytocin acetate was greater than 7700 theoretical plates. The tailing factors of oxytocin acetate were not more than 1.3. The resolution between Impurity A or Impurity B and oxytocin acetate was not less than 1.6 and 1.8, respectively. The instrument precision, determined by 5 replicate injections of the Standard Preparation, exhibited a maximum RSD of 1.8%. Table 3 illustrates the system suitability results obtained over 10 independent runs spanning 3 months.

**Precision**

The precision (repeatability and intermediate precision) of the method was determined from one lot of finished product.

**A. Repeatability**

Five Assay Preparations were analyzed in a single session by Chemist I with HPLC System I. The RSD of the five results was 0.4%, which was within the 2% limit (Table 4).

**Table 4**

**Assay Precision***

| Run | Assay Value (USP Units/mL) | Average Assay (n=5) (USP Units/mL) | RSD (%) |
|---|---|---|---|
| 1 Chemist I HPLC System I | 10.18<br>10.26<br>10.27<br>10.20<br>10.25 | 10.23 | 0.4 |
| 2 Chemist I HPLC System I | 9.89<br>9.85<br>9.90<br>9.89<br>9.90 | 9.89 | 0.2 |
| 3 Chemist II HPLC System II | 10.32<br>10.32<br>10.27<br>10.31<br>10.30 | 10.30 | 0.2 |
| Immediate Precision (n=15) | | 10.14 | 1.9 |

* Repeatability Acceptance Criteria: NMT 2%.

## B. Intermediate Precision

Intermediate precision was evaluated using Chemist I/HPLC System I to independently analyze another five Assay Preparations from the same lot of finished product, and to have another analyst using a different chromatographic system (Chemist II/HPLC System II) analyze another five Assay Preparations from the same lot. The RSD of each individual precision run was not more than 2% (Table 4).

**Table 5**

**Assay Accuracy***

| Approximate % Claim of Sample | Amount Determined (% Claim) | Theoretical Amount (% Claim) | Amount Recovered (%) | Average Recovered (n = 3) | RSD (%) |
|---|---|---|---|---|---|
| | 50.86 | 51.97 | 97.86 | | |
| 50 | 50.45 | 51.07 | 98.78 | 98.15 | 0.6 |
| | 47.33 | 48.38 | 97.83 | | |
| | 101.80 | 103.94 | 97.94 | | |
| 100 | 101.20 | 102.14 | 99.08 | 98.21 | 0.8 |
| | 94.46 | 96.77 | 97.61 | | |
| | 153.20 | 155.90 | 98.27 | | |
| 150 | 152.00 | 153.22 | 99.20 | 98.55 | 0.6 |
| | 152.50 | 145.15 | 98.17 | | |
| Overall Recovery (n = 9) | | | | 98.30 | 0.6 |

*Accuracy Acceptance Criteria: 97.0 to 103.0%. Precision Acceptance Criteria: 3% within each level.

Furthermore, the average assay values obtained were 10.23, 9.89, and 10.30 Oxytocin USP Units/mL for runs 1, 2, and 3, respectively (Table 4). This yields an intermediate precision RSD value of 1.9% ($\bar{X}$=10.14 Oxytocin USP Units/mL, n=15) amongst the three runs. The low scatter in the data supports the high degree of robustness of the analytical method.

**Accuracy**

The accuracy of the method was shown by analyzing spiked finished product samples of a known concentration, in Placebo, and comparing the analytical result to the known added value. The average percent recovery was calculated at each concentration level. The average amounts recovered were 98.15, 98.21, and 98.55% for concentrations of about 50, 100, and 150% of label claim, respectively. This yields an overall average recovery of 98.30% (n=9) for the analytical method in general (Table 5). Since the results obtained are within the acceptable range of 97.0 to 103.0%, the method is deemed to be accurate.

**Table 6**

**Linearity of Oxytocin Response***

| % Label Claim | Average Peak Area Response |
|---|---|
| 51.97 | 236953 |
| 83.15 | 378900 |
| 103.94 | 476167 |
| 124.72 | 565728 |
| 155.90 | 713080 |

slope, m = 4569.2
y - intercept, b = -742.9
correlation, r = 1.000
Bias = 0.2%

*Coefficient of correlation acceptance criteria: NLT 0.999. Bias acceptance criteria: ± 3.0%.

**Table 7**

**Stability of Analytical Solutions**

| Time (Hours) | Potency (Oxytocin USP Units/mL) Standard Preparation | % Change | Assay Preparation | % Change |
|---|---|---|---|---|
| Zero Time | 10.09 | NA | 10.69 | NA |
| 25 | 10.15 | 0.6 | 10.79 | 0.9 |
| 49 | 9.95 | -1.4 | 10.51 | -1.7 |

**Linearity**

A linear response in peak area for oxytocin acetate over the range of 50-150% of its label claim in Oxytocin Injection, USP, Synthetic was observed. The correlation coefficient was 1.000 and the bias was -0.2% (Table 6).

**Table 8**

**Specificity Results**

| Stress Condition of Finished Product | % Degradation | Peak Homogeneity Limit: NLT 990 |
|---|---|---|
| Thermal (105°C, 5 hours) | 22.2 | 998.4 |
| Acid (20 hours) | 29.8 | 999.9 |
| Base (20 hours) | 13.3 | 998.0 |
| Oxidation (16 hours) | 20.0 | 999.9 |
| Fluorescence (500-700 ft-candles, 48 hours) | 0.0 | 1000 |

**Range**

The range of the assay method has been set at 50 to 150% of the finished product label claim (10 Oxytocin USP Units/mL), since the method has been shown to be precise, accurate, and linear within this region.

**Stability of Analytical Solutions**

The stability of the analytical solutions was determined from the Standard Preparation (prepared from In-house Reference Standard) and Assay Preparation (prepared from finished product) at room temperature. These solutions were analyzed at 0, 24, and 48 hours. Solutions were analyzed against a freshly prepared standard at each time interval. The oxytocin acetate concentrations were examined as a function of time (Table 7). These data were evaluated for percent change of potency from time zero. The Standard Preparation and Assay Preparation were found to be stable for 48 hours, respectively. Since the percent change in potency is within ± 2%, the solutions are considered stable at room temperature.

**Specificity**

Oxytocin Injection, USP, Synthetic was stressed by thermal, acidic, basic, oxidative and fluorescent radiation for up to 48 hours or until approximately 10-30% degradation of oxytocin acetate was achieved, as determined by peak

area percent. The results of the stress study are presented in Table 8. No interfering peaks at the retention time of oxytocin acetate were observed in any of the stressed sample.

**Peak Homogeneity**

The control sample, degradation samples, test samples, and placebo samples were analyzed using an HPLC equipped with a photodiode array detector. The oxytocin acetate peak was determined to be homogeneous since a purity value ≥ 990 was obtained in all cases (Table 8).

## CONCLUSION

The described gradient HPLC method for the analysis of oxytocin acetate in Oxytocin Injection, USP, Synthetic has been evaluated for system suitability, linearity, precision, accuracy, stability of solutions, and specificity. The oxytocin acetate peak response has been shown to be linear, precise and accurate in the range of 50 to 150% label claim. Precision between two chemists on two different chromatographic systems was demonstrated to be within 1.9%. The Standard and Assay Preparations were found to be stable for 48 hours, respectively, at room temperature. Finally, the method has proven to be specific under a variety of stress conditions, while maintaining peak homogeneity. Consequently, the validated method for the determination of oxytocin acetate in Oxytocin Injection, USP, Synthetic is regarded as stability-indicating.

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# HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE CARDIOPROTECTIVE AGENT DEXRAZOXANE IN HUMAN PLASMA AND URINE

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## ABSTRACT

For the purpose of a pharmacokinetic study in the comparison of two intravenous pharmaceutical formulations of the cardioprotective agent dexrazoxane, we have developed an High Performance Liquid Chromatographic (HPLC) assay to quantify the drug in human plasma and urine. The plasma sample pretreatment involved a protein precipitation step with acetonitrile followed by an extraction with 10% 2-methyl-2-propanol in chloroform (v/v). Urine samples were diluted in distilled water and subsequently extracted with 10% 2-methyl-2-propanol in chloroform (v/v). After evaporation of the organic solvents, the residues were dissolved and analysed on a μBondapak Phenyl column with a mobile phase consisting of 0.01 M potassium phosphate pH 4.7 and methanol (8:2, v/v). Detection was performed at 208 nm. The lower limit of

quantitation was 0.1 μg/mL and 10 μg/mL for plasma and urine respectively, using 1.0 mL sample volumes. The usefulness of the method has been demonstrated in clinical samples originating from patients treated with dexrazoxane.

Dexrazoxane is not stable in plasma at ambient temperature: after 6.5 hours the initial concentration was 42.6 ± 1.0% (n=3) of the original concentration of 100 μg/mL. After sampling in the clinic, plasma samples should be stored immediately at -30°C. Under these conditions dexrazoxane is stable for at least 5 months. In urine the drug is stable for 24 hours when stored at 4-8°C. An aliquot of the voided urine sample can be stored at -30°C for at least 4 months without drug decomposition.

## INTRODUCTION

A major impediment in the clinical use of anthracycline antitumour drugs *e.g.* doxorubicin, is a cumulative dose-related cardiac toxicity that manifests itself as congestive heart failure. Many strategies have been pursued in an attempt to prevent this serious complication. In particular, the development of a treatment with the protective agent dexrazoxane (ICRF-187, ADR-529, NCS-169780, (+)-1,2-bis(3,5 dioxopiperazinyl-1-yl)propane) seems an attractive approach. Dexrazoxane is the S(+) enantiomer of the racemic compound razoxane (ICRF-159). The (+) enantiomer was developed further because it appeared about five times more water soluble than the racemic mixture.[1]

It is hypothesized that dexrazoxane hydrolyses intracellularly into a metabolite (Figure 1) which complexes iron ions by which the generation of hydroxyl radicals through the Fenton reaction is prevented.[2] Generation of hydroxyl radicals by anthracyclines in the heart muscle may cause the cardiomyopathy.

The cardioprotective effects of dexrazoxane have been demonstrated in animals[3-11] and in patients.[12-19] The timing of drug administration appears to be very important whereby simultaneous administration of doxorubicin and dexrazoxane resulted in a better protection than when dexrazoxane is administered 2 hours after doxorubicin.[20] More pharmacokinetic data of this combination therapy are necessary.

In our hospital patients with breast cancer were treated according to the FDC-regimen: 500 mg/m$^2$ fluorouracil, 50 mg/m$^2$ doxorubicin and 500 mg/m$^2$ cyclophosphamide given as bolus injections. The patients also received in a

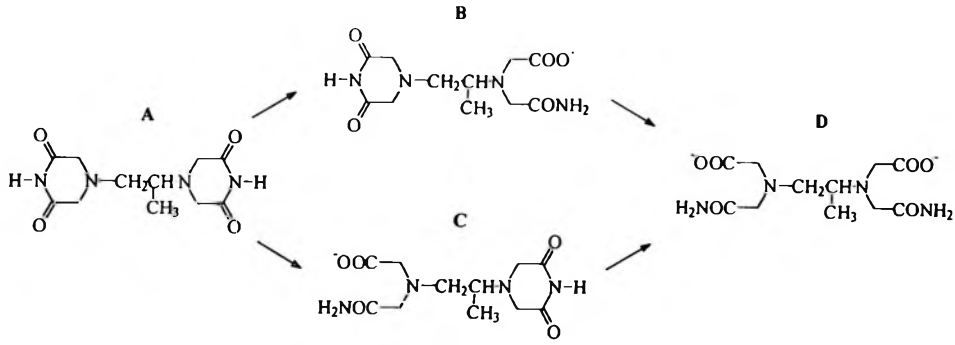



**Figure 1**. Chemical structure and intracellular activation of dexrazoxane (A). B, C and D are hydrolysis products.

randomized fashion two formulations of dexrazoxane (1,000 mg/m$^2$) as a 12 minutes intravenous infusion in normal saline in which the drug is stable for 6 hours when stored at ambient temperature.[21] To investigate the pharmacokinetic bioequivalence of the tested formulations we developed an HPLC assay for the determination of dexrazoxane in human plasma and urine.

Several analytical methods for the analysis of dexrazoxane in biological matrices have been published.[22-28] Sadee and colleagues described a method based on gas chromatography with flame ionization detection or mass fragmentography.[22,23] The mass spectrometrical equipment was not available in our laboratory at that moment, therefore, this technique could not be utilized. Flame ionization detection provides a detection limit of 5 μg/mL which was found not sufficient for our studies. Other methods all made use of HPLC, though with different sample pretreatment procedures/detection methods, including: ultrafiltration/UV-detection,[24,25] liquid-liquid extraction/UV-detection,[26] solid phase extraction/amperometric detection[27] and pre-column derivatization/fluorescence detection.[28] In our hands, the Collins method[24] was not selective: chromatograms of blank plasma samples showed a major endogenous interference after the ultrafiltration. Lewis et al.[27] developed a very sensitive (limit of quantitation: 5 ng/mL plasma) and selective method for the quantitation of dexrazoxane in biological fluids using two solid phase extraction procedures, followed by straight phase HPLC with column switching and amperometric detection. This method is, however, very laborious and a lower limit of quantitation of 0.1 μg/mL was considered sufficient for our purposes. The HPLC method with pre-column derivatization to form a

fluorescence product[28] was not supported by validation data. The method published by Earhart et al.[26] was used by us as a reference but, the assay appeared to be afflicted with several problems: interferences by endogenous components, degradation of the analyte in the eluent and the use of an internal standard which was not commercially available. In the presented assay the 'Earhart'-method has been adapted substantially in order to overcome these problems.

## EXPERIMENTAL

### Equipment

The chromatographic system consisted of a Model 510 solvent delivery system (Waters Assoc. Inc., Milford, MA), a Spectra 200 programmable wavelength detector, an automatic sample injection device model SP8875, and a SP4600 integrator coupled to the WINner® data system (all from Thermo Separations Products, Fremont, CA). Chromatographic separation was achieved on a μBondapak Phenyl column (internal diameter: 4.6 mm; length: 300 mm; particle size: 10μm,) (Waters Assoc. Inc., Milford, MA) protected by a guard column (3 x 10 mm) packed with reversed phase material (Chrompack, Middelburg, The Netherlands).

### Chemicals

Dexrazoxane, (lot. 3354) originated from Chiron (Amsterdam, The Netherlands). Chloroform, acetonitrile and methanol were obtained from Promochem (Wesel, Germany). 2-Methyl-2-propanol, hexane, diethyl ether, ethyl acetate, potassium dihydrogen phosphate and hydrochloric acid were of analytical grade and purchased from Merck (Darmstadt, Germany). Home-made double-distilled water was used throughout. Drug-free edathamil (EDTA) human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). Drug-free urine was provided from healthy volunteers.

### Stock solutions

A stock solution of dexrazoxane (1 mg/mL) was prepared by dissolving the appropriate amount of drug, accurately weighed, in distilled water. Typical

amounts of the stock solution were diluted with distilled water to give standard solutions with concentrations of 500 μg/mL, 100 μg/mL and 10 μg/mL. The solutions were stored at -30°C and were found to be stable for at least 2 months under these conditions.

**Calibration Samples**

**Plasma**

Blank human plasma samples (1,000 μL), in 10 mL brown glass containers, were spiked with 10 to 100 μL of a dexrazoxane standard solution with appropriate concentration to obtain calibration samples in the range of 0.1 - 1 μg/mL, 1 - 10 μg/mL and 10 - 100 μg/mL. Distilled water was then added to the plasma samples to achieve a total volume of 1.1 mL. To precipitate the plasma proteins 2.0 mL of acetonitrile was added. The containers were shaken for 10 minutes and centrifugated at 2,500 g for 10 minutes. The clear supernatant was transferred to a clean glass container of 30 mL. Next, 20.0 mL of extraction fluid (chloroform - 2-methyl-2-propanol; 9:1, v/v) was added and the flasks were shaken for 30 minutes and subsequently centrifugated for 10 minutes at 2,500 g. The water phase was discarded and 15.0 mL of the organic extract was transferred into clean glass container and evaporated to dryness under a stream of nitrogen at 40°C. The residues were redissolved in 500 μL 0.1 M hydrochloric acid - methanol (8:2, v/v) and subjected to HPLC analysis.

**Urine**

Blank human urine samples (1,000 μL) were transferred to 100.0 mL volumetric flasks and appropriate volumes (100 - 1,000 μL) of dexrazoxane standard solutions were added to obtain calibration samples in the range of 10 - 100 μg/mL and 100 - 1,000 μg/mL. Distilled water was then added to achieve a total volume of 100.0 mL. From each diluted urine solution 1.00 mL was pipetted into a clean glass container of 30 mL. Next, 20.0 mL of extraction fluid (chloroform - 2-methyl-2-propanol; 9:1, v/v) was added and the sample pretreatment was followed as described for the plasma samples.

**Clinical samples**

Blood samples (5 mL) of patients were collected in EDTA-tubes. Plasma was immediately isolated by centrifugation (10 minutes at 2,500 g) and subsequently stored at -30°C prior to analysis.

Before treatment, an aliquot of the patient's urine sample was stored at -30°C and was used as a blank. During treatment, urine voided over 24-hours was pooled and stored in a refrigerator at 4-8°C.

The total volume was recorded and approximately 20 mL of the homogenized sample was taken and stored at -30°C prior to analysis.

**Chromatography**

Chromatography was performed at ambient temperature with the mobile phase composed of 0.01 M potassium phosphate pH 4.7 and methanol (8:2, v/v). Prior to use, the mobile phase was filtered under reduced pressure through a 0.2 μm cellulose acetate filter (Sartorius, Spruyt-Hillen, Utrecht, The Netherlands).

The absorbance of the effluent was monitored at 208 nm. The flow rate was maintained at 1.0 mL/min and a volume of 50 μL was injected into the chromatograph.

**Validation Parameters**

A validation programme was completed for the determination of dexrazoxane in plasma and urine. The following parameters were determined: linearity, precision, accuracy, specificity, selectivity, absolute recovery and stability.

**Linearity**

For each calibration range five standards were prepared and analysed in duplicate, producing calibration curves in plasma ranging from 0.1 - 1 μg/mL, 1 - 10 μg/mL and 10 - 100 μg/mL and in urine ranging from 10 - 100 μg/mL and 100 - 1,000 μg/mL. Unweighted linear regression analysis of the peak area of dexrazoxane versus the nominal concentration was applied.

A split-curve approach was used to improve accuracy at the low concentration levels. The lack of fit test ($\alpha$=0.05) was used to evaluate the linearity of the calibration curves.

**Accuracy and precision**

Quality control samples were prepared from a second, fresh stock solution with analyte concentrations of 0.5, 5 and 50 μg/mL in plasma and 50 and 500 μg/mL in urine. Six replicates of each quality control sample were processed and analysed with calibration standards to determine the accuracy and precision of the method.

**Specificity and selectivity**

Six batches control human plasma and urine were tested whether endogenous constituents co-eluted with dexrazoxane.

The medication used by patients treated according to the FDC-regimen were investigated for interference with the analytical method: fluorouracil, doxorubicin, cyclophosphamide, codeine, paracetamol and indometacin.

**Absolute recovery**

The extraction efficiencies were determined at 5 and 50 μg/mL in plasma and at 50 and 500 μg/mL in urine by comparing the response of three processed quality control samples to the mean response of two unprocessed samples of dexrazoxane in 0.1 M hydrochloric acid in methanol (8:2, v/v).

**Stability**

The stability of dexrazoxane at ambient temperature, has been studied at a concentration of 5 μg/mL in the following solutions: 0.01 M phosphate buffer pH 6.1 - methanol (8:2, v/v), 0.01 M potassium phosphate pH=4.7 - methanol (8:2, v/v) and 0.1 M hydrochloric acid - methanol (8:2, v/v).

The stability of dexrazoxane in human plasma at a concentration of 100 μg/mL has been studied during a period of 6.5 hours at ambient temperature. Triplicates were analysed at every time point.

Five plasma samples from patients treated with dexrazoxane containing about 70 μg/mL were re-analysed in duplicate after 5 months storage at -30°C. The stability of dexrazoxane has been investigated at a concentration of 500 μg/mL in human urine during a period of 29 hours at 4°C. Three replicates were analysed at every time point.

Four urine samples from patients treated with dexrazoxane containing about 900 μg/mL were re-analysed in duplicate after 4 months storage at -30°C. The stability of the drug in the organic extract originating from plasma spiked with dexrazoxane at a concentration of 100 μg/mL was evaluated after 24 hours at -30°C while the stability of the dry extract was investigated after storage at -30°C for one week.

The stability of the drug extracted from plasma and reconstituted in 0.1 M hydrochloric acid - methanol (8:2, v/v) was evaluated during storage at ambient temperature for 16 hours in the autosampler. Triplicates were analysed at every time point.

### Statistical Quality Control

Quality control samples were prepared from a second, fresh stock solution at concentrations of 0.5, 5 and 50 μg/mL in plasma (see 'Accuracy and precision') and were stored together with the clinical samples. Three replicates of each quality control sample were processed and analysed with each calibration curve for statistical process control during the study. Exponentially weighted moving-average (EWMA) control charts were constructed using Statgraphics® version 7.0 (Manugistics Inc., MA).

### Pharmacokinetics

To demonstrate the applicability of the assay for pharmacokinetic studies an example of a treated patient is presented. Serial blood samples and urine were taken from a patient suffering from breast cancer treated with dexrazoxane (1,000 mg/m$^2$) given as a 12 minute intravenous infusion prior to the FDC-regimen: 500 mg/m$^2$ fluorouracil, 50 mg/m$^2$ doxorubicin and 500 mg/m$^2$ cyclophosphamide given as bolus injections. The plasma concentration time curve was analysed using MW\PHARM$^{29}$ (MEDI\WARE, Groningen, Netherlands).

## RESULTS AND DISCUSSION

The extraction properties of dexrazoxane with different organic solvents were investigated because the bio-analysis of the drug is hampered by poor extractability. Chloroform, 2-methyl-2-propanol in chloroform (10%, v/v), hexane, diethyl ether and ethyl acetate were tested and blank plasma samples were injected to examine the sample clean-up. All extractions were performed

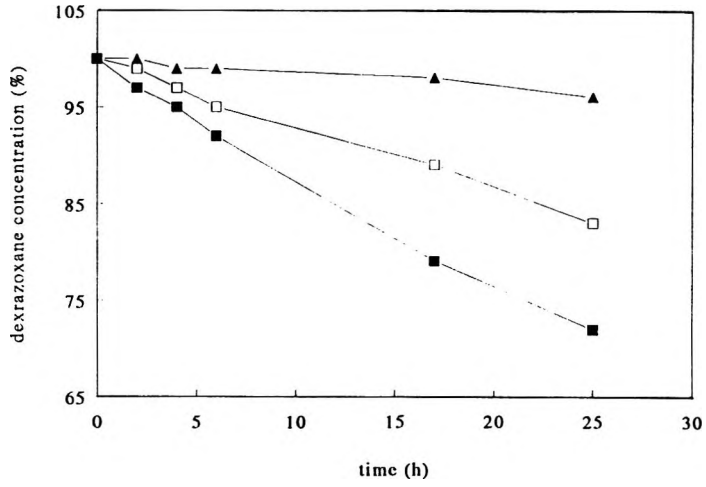



**Figure 2**. The stability of dexrazoxane during storage at ambient temperature at a concentration of 5 μg/mL in 0.01 M phosphate buffer pH 6.1 - methanol (8:2, v/v, -■-), 0.01 M potassium phosphate pH 4.7 - methanol (8:2, v/v, -□-) and 0.1 M hydrochloric acid - methanol (8:2, v/v, -▲-).

in glass containers. Polypropylene could not be used in consequence of additional peaks in the chromatograms originating from the container material used. 2-Methyl-2-propanol in chloroform (10%, v/v) gave the best results (recovery >60%), however the sample pretreatment method in combination with the chromatographic system was not very selective: there was still an interfering endogenous component present. Using a μBondapak Phenyl column, dexrazoxane had a significantly longer retention time compared with the μBondapak C-18 column and the drug was well separated from all endogenous compounds.

Earhart et al.[26] used the analog ICRF-192 as the internal standard of the assay and residues were reconstituted in the eluent: 20% methanol (v/v) in 0.01 M sodium-potassium phosphate buffer pH 6.1. In this solvent dexrazoxane is not stable (Figure 2) but the peak ratio with ICRF-192 was stable over at least 24-hours.[26] Unfortunately this compound was not available to us and we decided to reconstitute the residues in 0.1 M hydrochloric acid - methanol (8:2, v/v) in which dexrazoxane is stable for at least 24 hours at ambient temperature (Figure 2). This allows the use of an autosampler injection device with samples stored for 24 hours. In addition we have tested a range of compounds as potential internal standards for the bio-analytical assay (Table 1). These

**Table 1**

**Compounds Tested as Potential Internal Standards for the Bio-analytical Assay and the Medication used by Patients Treated according to the FDC-Regimen**

| Compound | Concentration (μg/mL) | Retention Time (min) |
|---|---|---|
| Dexrazoxane | 50 | 6.2 |
| 5-Bromouracil | 10 | 4.5 |
| 5-Chlorouracil | 10 | 4.5 |
| 8-Chlorotheophylline | 5 | 16.0 |
| Caffeine | 5 | 19.8 |
| 5-Fluorocytosine | 6 | 3.4 |
| Methylparaben | 5 | N.D. |
| Phenazone | 5 | N.D. |
| Phenacetine | 5 | N.D. |
| Phenylethyleneglycol | 10 | 7.3 |
| Prednisone | 25 | N.D. |
| Theophylline | 5 | 10.7 |
| Tiamcinolonacetate | 0.5 | N.D. |
| Triamcinolone | 0.2 | N.D. |
| Codeine | 10 | N.D. |
| Cyclophosphamide | 200 | N.D. |
| Doxorubicin | 200 | N.D. |
| 5-Fluorouracil | 500 | 4.0 |
| Indomethacin | 10 | N.D. |
| Paracetamol | 5 | front |

N.D.: not detected; 50μL injections.

compounds were eluted with a mobile phase composed of methanol/0.01 M phosphate buffer pH 4.7 to minimize on-column degradation of dexrazoxane. 8-Chlorotheophylline was added as an internal standard during initial studies at a concentration of 25 μg/mL in plasma and 250 μg/mL in urine.

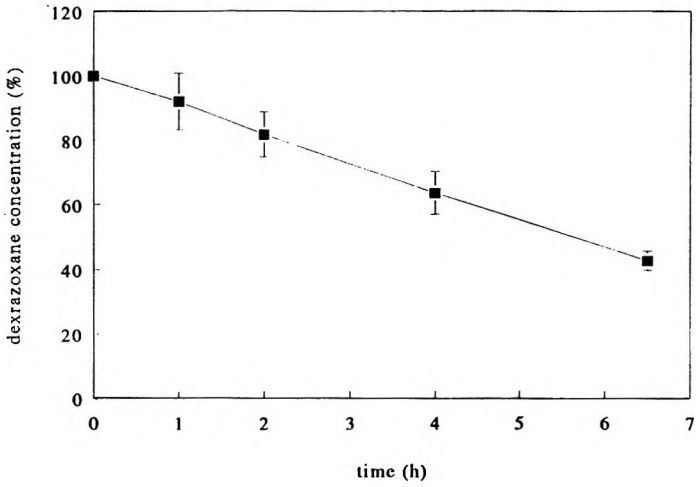



**Figure 3**. The stability of dexrazoxane in human plasma at a concentration of 100 μg/mL, measured during a period of 6.5 hours at ambient temperature. Percentages of the initial dexrazoxane concentrations are plotted (± the standard deviation).

**Table 2**

**Validation Parameters for the Determination of Dexrazoxane in Human Plasma and Urine**

| Matrix | Nominal Concentration (μg/mL) | Concentration Found (μg/mL) | Accuracy (%) | Precision (%) | Number of of Replicates |
|---|---|---|---|---|---|
| Plasma | 0.500 | 0.498 | 99.6 | 4.1 | 6 |
| Plasma | 5.00 | 5.00 | 100.0 | 3.4 | 6 |
| Plasma | 50.0 | 50.3 | 100.6 | 2.9 | 6 |
| Urine | 50.0 | 47.3 | 96.4 | 5.4 | 6 |
| Urine | 500 | 493 | 98.6 | 8.2 | 6 |

Comparable accuracies and precisions were obtained when the internal standard was rejected. The recovery of 8-chlorotheophylline from plasma was very poor (< 20%) unlike the recovery from urine (79% ± 3.4%, n=6). Caffeine,

**Table 3**

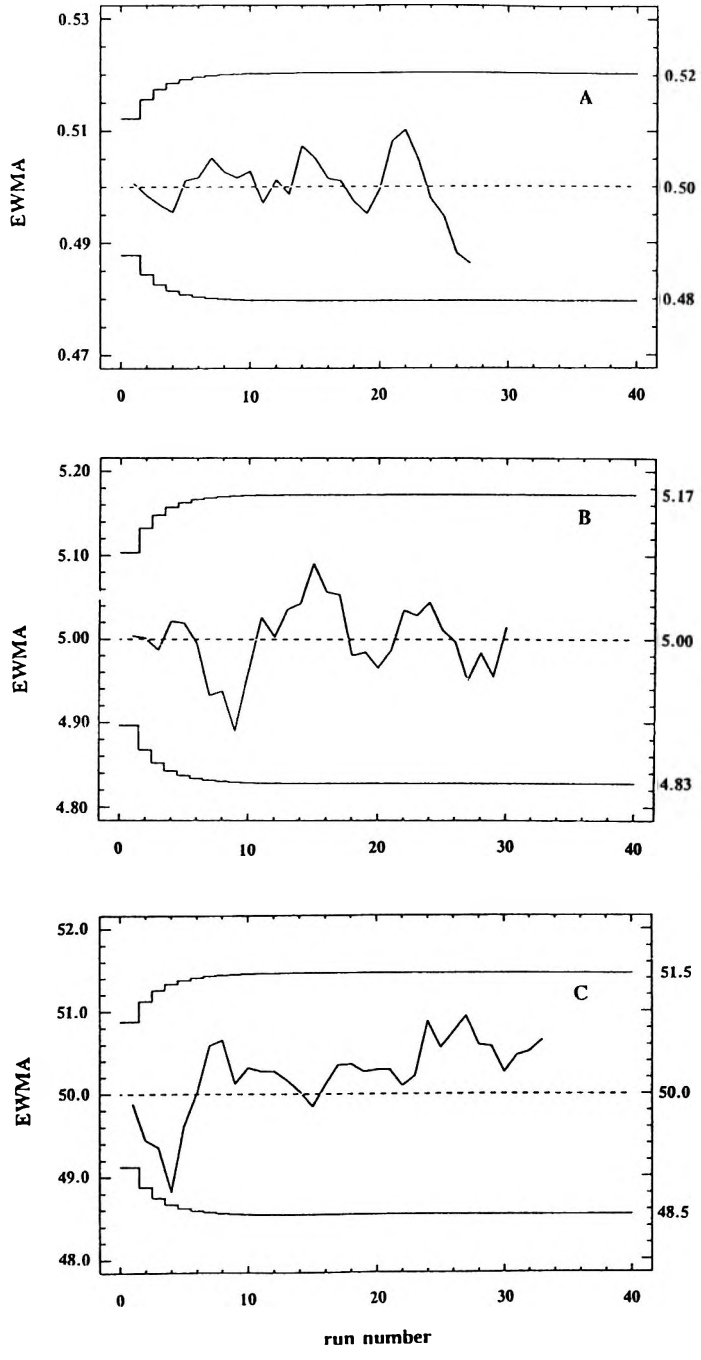
**Stability Data of Dexrazoxane at Several Storage Conditions**

| Matrix | Storage Conditions | Initial Conc. (µg/mL) | Recovery (%) | C.V. (%) | N |
|---|---|---|---|---|---|
| Spiked human plasma | 6.5 hours at 21°C | 100 | 42.6 | 1.0 | 3 |
| Plasma originating from patients | 5 months at -30°C | 70 | 99.6 | 3.1 | 5 |
| Spiked urine (pH = 6.92) | 29 hours at 4-8°C | 500 | 91.7 | 2.5 | 3 |
| Urine originating from patients | 4 months at -30°C | 900 | 88.8 | 6.1 | 4 |
| Organic layer after extraction from spiked plasma | 24 hours at -30°C | 100 | 100.2 | 6.2 | 4 |
| Dry residue after extraction from spiked plasma | 1 week at -30°C | 100 | 100.5 | 2.1 | 4 |
| Plasma residue after reconst. in 20% (v/v) methanol in 0.1M HCL | 16 hours at 21°C | 10 | 93.0 | 2.0 | 3 |

C.V. = Coefficient of Variation.
N = Number of determinations.

phenylethyleneglycol and theophylline were not tested as potential internal standards as their chromatographic characteristics were not ideal in terms of symmetry. It was then decided to execute the validation programme without internal standard.

**Figure 4 (right)**. EWMA charts of the quality control samples in human plasma at nominal concentrations of 0.5 (A), 5 (B) and 50 µg/mL (C) measured during the pharmacokinetic study.


A
B
C
EWMA
EWMA
EWMA
0.53
0.51
0.49
0.47
0.52
0.50
0.48
5.20
5.10
5.00
4.90
4.80
5.17
5.00
4.83
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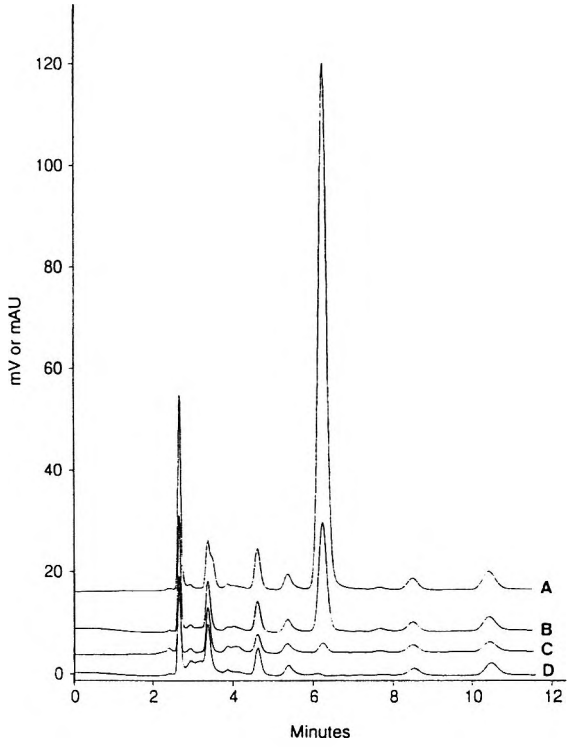


**Figure 5**. HPLC chromatograms of a patient treated with dexrazoxane (1,000 mg/m$^2$) as a 12 minutes intravenous infusion prior to adminitration of the FDC-regimen: samples were taken at 2 (A, 13.75 μg/mL), 6 (B, 2.75 μg/mL) and 12 hours (C, 0.22 μg/mL) after the end of the infusion and a blank plasma sample before treatment (D). Dexrazoxane elutes after 6.2 minutes.

The calibration curves were linear over a concentration range of 0.1 μg/mL to 100 μg/mL in human plasma and over a range of 10 to 1,000 μg/mL in urine. Assay validation parameters are summarized in Table 2.

The precision was less than 10% for all quality control samples. The average accuracies were within 95 and 105%. The chromatograms of six batches of control human plasma and urine contained no endogenous constituents co-eluting with dexrazoxane.

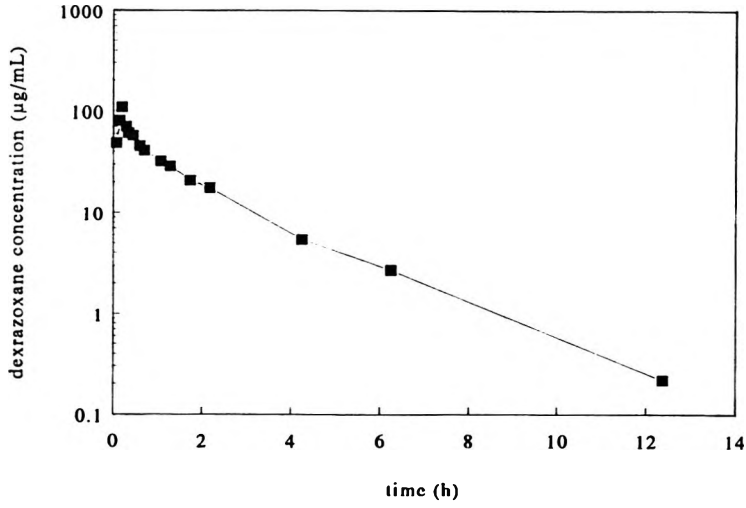


**Figure 6**. The plasma concentration versus time curve of a patient treated with dexrazoxane (1,000 mg/m$^2$) as a 12 minutes intravenous infusion prior to the FDC-regimen.

The medication used by patients treated according to the FDC-regimen (Table 1) showed no interference with the analytical method.

The extraction efficiencies in plasma were 76% ± 1.0% at 5 µg/mL and 71% ± 2.3% at 50 µg/mL (n=3). In urine comparable extraction efficiencies were found: 66% ± 1.0% at 50 µg/mL and 70% ± 0.6% at 500 µg/mL (n=3).

Stability data of dexrazoxane at several storage conditions are presented in Table 3. Dexrazoxane is not stable in plasma at ambient temperature (Figure 3): after 6.5 hours the dexrazoxane concentration was only about 40% of the original value. Consequently for the determination of dexrazoxane, plasma should be isolated immediately after sampling in the clinic and can then be stored at -30°C for at least 5 months.

A basic method for statistical process control is the Shewhart control chart.[30] A major disadvantage of a these charts, however, is that it only uses the information about the process obtained during the last analytical run, and it ignores any information gathered before. This feature makes the Shewart control chart relatively insensitive to small shifts in the process. A very effective alternative is the exponentially weighted moving-average (EWMA) control chart.[30]

**Table 4**

**Pharmacokinetic Parameters of Dexrazoxane in a Patient Suffering from Breast Cancer Treated with Dexrazoxane (1,000 mg/m$^2$; Total Dose 2000 mg) as a 12 Minutes Intravenous Infusion using a Two Compartment Model**

| | |
|---|---|
| AUC (Area Under the Curve, $0 \rightarrow \infty$) | 118 mg.h/L |
| $Cl_{tot}$ (Total Body Clearance) | 16.9 L/h |
| Distribution volume | 38.7 L |
| $t_{½}$ ($\alpha$) | 0.16 h |
| $t_{½}$ ($\beta$) | 1.59 h |
| MRT (Mean Residence Time) | 1.93 h |
| $Cl_{ren}$ (Renal Clearance) | 6.8 L/h |
| Urinary excretion | 40% |

These charts were constructed for the quality control samples in human plasma at nominal concentrations of 0.5, 5 and 50 μg/mL with a weighting factor ($\lambda$) of 0.2 and estimations of $\sigma$ determined at the validation phase (Figure 4). No 'out-of-control points' were detected indicating that the bio-analytical method was in control during the analysis of dexrazoxane for the pharmacokinetic study.

The applicability of the assay has been demonstrated in a patient with breast cancer treated with dexrazoxane (1,000 mg/m$^2$) as a 12 minutes intravenous infusion prior to the FDC-regimen. Typical chromatograms of extracted patient samples are presented in Figure 5 and the plasma concentration-time curve is depicted in Figure 6. An open two compartment model was used to calculate the pharmacokinetic parameters (Table 4).

In summary, a simple, validated HPLC assay for the quantitative determination of dexrazoxane in human plasma and urine is described. The assay quantifies dexrazoxane concentrations in plasma in a range of 0.1 to 100 μg/mL and in urine of 10 to 1,000 μg/mL using a 1,000 μL sample volumes.

The sensitivity of the assay is sufficient to monitor patients during 24 hours after the end of the infusion when treated with a dexrazoxane dose of 1,000 mg/m$^2$. The method has been succesfully used in our hospital for pharmacokinetic studies (>1000 samples).

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# LIQUID CHROMATOGRAPHIC DETERMINATION OF ASULAM AND AMITROLE WITH PRE-COLUMN DERIVATIZATION

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## ABSTRACT

A liquid chromatographic pre-column derivatization method with fluorimetric detection for the simultaneous determination of asulam and amitrole was developed. The separation was accomplished in less than 15 min. R.S.D.'s (n = 10) of 1.13% and 1.6% (concentration) and 1.4% and 0.97% (retention time), were obtained for asulam and amitrole respectively. Recoveries from spiked tap water ranged from 90% to 118%, and detection limits of 0.04 ng for asulam and 7.5 ng for amitrole, were obtained.

## INTRODUCTION

The pesticide asulam is a translocation herbicide, absorbed by leaves and roots causing slow chlorosis in susceptible plants. It interferes with cell division and expansion and is used to control the growth of grasses.[1]

The pesticide amitrole is a non - selective herbicide absorbed by roots and leaves, and translocated. It inhibits chlorophyll formation and regrowth from buds. It is used around established apple and pear trees between fall harvest and the following summer. It is also used as a non selective herbicide before planting kale, maize, oilseed rape, potatoes, wheat; on fallow land and in other non crop situations. Its activity is enhanced by the addition of ammonium thiocyanate.[2]

Methods for determination of asulam include liquid chromatography,[3-7] gas chromatography[8] and fluorescence synchronous derivative technique.[9] Amitrole was determined by liquid chromatography[10-15] and gas chromatography.[16] The detection limits of the methods previously reported was ranged from 0.5 mg to 0.1 ng and from 0.5 mg to 0.05 μg for asulam and amitrole, respectively. The proposed method in this work is more sensitive than the methods previously described for asulam and amitrole. The liquid chromatographic method with amperometric detection[15] presents a detection limit of 200 pg for amitrole. This method is more sensitive than the method proposed in this work but its relative standard deviation is 11% while 1.6% is obtained in this work.

Amitrole does not show native fluorescence, thus the technique of spectrofluorimetry has not been previously applied to its determination. Asulam presents a low native fluorescence, thus the derivatization of fluorescamine reagent increases the sensitivity for the spectrofluorimetric determination of asulam.[9]

Udenfriend et al.,[17] introduced fluorescamine as a labeling reagent to determine primary amines; this is superior to dansyl chloride because both the reagent and its hydrolysis products are non-fluorescent and permit homogeneous fluorogenic labelling. Such approach has proved its usefulness in numerous analytical applications for some 30 years.[18-19]

Considerable efforts have been made to develop highly selective and sensitive derivatization reagents for use in liquid chromatography with fluorescence detection. Several excellent reagents are currently available for most functional groups, e.g., hydroxy, amino, thiol, carbonyl and carboxyl groups. The most useful application of fluorescamine in high performance liquid chromatography derivatization is its ability to hydrolyze the excess of reagent after the derivatization process, releasing non-fluorescent products. Thus, the fluorimetric detection is blind to the fluorescamine excess. This is specially useful in pre-column derivatization HPLC methods.

In this work the optimum experimental conditions for the spectrofluorimetric determination of asulam and amitrole based on fluorophore generation by derivatization with fluorescamine (FC) were investigated. Reverse phase LC determination of asulam and amitrole with pre-column derivatization was also carried out and applied to spiked tap water.

## EXPERIMENTAL

### Instrumentation

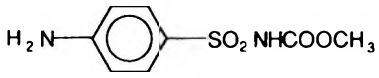
A Merck - Hitachi (Darmstadt, Germany) liquid chromatograph was used consisting of a L-6200 pump, an AS-4000 autosampler, a F-8000 fluorescence detector and a D-6000 interface. Integration was carried out with a PC/AT computer and the instrumental parameters were controlled by Hitachi - Merck HM software. All the derivatization steps are performed automatically by the AS-4000 autosampler.

Fluorescence measurements were made with a Perkin Elmer LS50 Spectrofluorimeter (Beaconsfield U.K) and equipped with a Xenon discharge lamp and two monochromators. Fluorescence Data Manager (FLDM) Software and a RS232C interface were used to send information to an external computer.

### Reagents

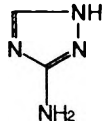
Potassium hydrogen phthalate and borax were obtained from Panreac (Barcelona, Spain), potassium chloride from J. T. Baker Chemicals (B.V. - Deventer - Holland), potassium phosphate dibasic from Codex (Milano, Italy), potassium phosphate monobasic from Probus (Barcelona, Spain), HCl from Merck (Darmstadt, Germany) and Fluorescamine (FC) (98%) from Aldrich (Milwaukee -USA). Asulam (purity 99.9%) and amitrole (98%) were purchased from Dr. S. Ehrenstorfer (Augsburg, Germany). Methanol was of LiChrosolv gradient grade (Merck) and acetone of analytical - reagent grade (Merck). The solvents were previously sonicated for 30 min and filtered through 0.2 μm Nylon membrane filters.

Stock standard solutions of asulam ($4.34 \times 10^{-3}$ M) and amitrole ($1.19 \times 10^{-2}$ M) were prepared by dissolving the compounds in water and stored at 4°C. Working standard solutions were prepared by dilution with water. Fluorescamine ($3.59 \times 10^{-3}$ M) was dissolved in acetone.




ASULAM

Methyl sulphanylcarbamate




AMITROLE

3 - amino - 1 H - 1,2,4 - triazole

**Figure 1**. Structures of the pesticides.

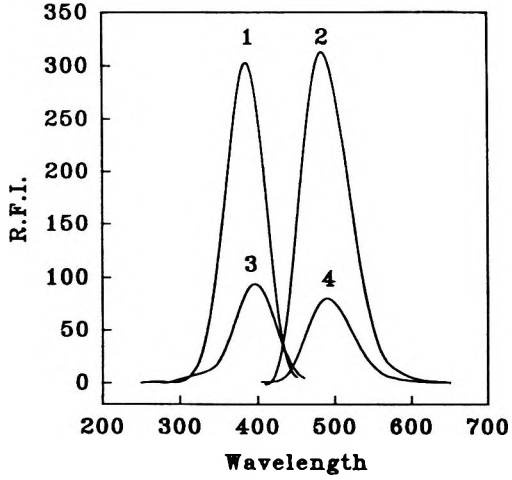



**Figure 2**. Excitation and emission spectra of FC derivatives of (1,2) amitrole and (3,4) asulam. Asulam $8.7 \times 10^{-7}$ M and amitrole $2.4 \times 10^{-4}$ M pH = 3; [FC] = $3.6 \times 10^{-4}$ M.

Solutions of potassium chloride (0.1 M), potassium hydrogen phthalate (0.05 M), borax (0.1 M) and phosphate buffer (0.1 M) were prepared in doubly deionized water. The solutions were filtered through 0.2 μm Nylon membrane filters.

**Derivatization**

Aliquots of 100 μL of aqueous standard solutions of asulam (0.04 - 4 μg/mL) and 100 μL amitrole (0.8 - 20 μg/mL) were introduced in a 1.5 mL flask, and then 300 μL of a $3{,}6 \times 10^{-3}$ M acetone solution of fluorescamine and 300 μL of pH buffer solution were added. The mixture was diluted in water up to 1.5 mL. After each reagent addition the mixture was agitated. A volume of 20μL of this solution was injected into the chromatograph and analyzed. All these operations were automatically performed by the autosampler.

**LC Operating Conditions**

The pesticide sample was analyzed using a LiChrospher 100 RP-18 reverse phase column (25 cm x 4 mm I.D.; 10 μm particle size) from Merck. The injection volume was 20 μL for the standard aqueous solutions and samples. The mobile phase composition was 25% methanol aqueous at a 1 $mL.min^{-1}$ flow rate. The peak - area response was measured at the retention times of asulam (5.96 min) and amitrole (10.14 min). A calibration graph was constructed using the responses.

**Recovery test**

Tap water samples from Antequera (Spain) were used to prepare two samples with known levels of added asulam and amitrole (0.05 ng of asulam + 20 ng of amitrole and 2 ng of asulam + 100 ng of amitrole). The mixture was filtered through a Sep-Pak silica 3 cc cartridges. The solutions were diluted with water to a final volume of 5 mL. These solutions were used for analysis.

## RESULTS AND DISCUSSION

Asulam and amitrole (Fig 1) react with FC to form two fluorophores whose spectra are very similar. Figure 2 shows the excitation and emission spectra of the FC derivatives of asulam and amitrole under the final experimental conditions. As expected, the spectral parameters for both compounds are similar. Each compound is characterized by its well resolved excitation maximum (398 nm) and its single emission peak (490 nm) for asulam and amitrole.

The operating parameters for the individual compounds can be optimized to give an analytical method for each. Consequently, after fixing the individual optimum conditions in order to determine isolates asulam and amitrole, a new set of conditions was selected to obtain good emission signals for each compound before carrying out the analysis of mixtures of asulam and amitrole by high performance liquid chromatography.

As a fluorigenic reagent for amino compounds, FC lacks selectivity, which emphasizes the need for more detailed information about the effect of the main reaction conditions so that the fluorescence yield might be improved to permit the selective analysis of mixtures of fluorophores with FC.

**Influence of Reaction Variables**

The effect of pH on fluorescence intensity was explored by carrying out several assays of solutions in 5 mL volumetric flasks containing 0.2 μg/mL of asulam (or 20 μg/mL of amitrole) and 1 mL of different buffer solutions that covered the pH range 1 - 10, together with 0.5 mL of FC standard solution (1 mL in the case of amitrole), the solution was then diluted with water.[18]

Figure 3 shows that the maximum fluorescence of the asulam fluorophore occurred at pH 2 and that of amitrole at pH 4. In both instances, the narrow range in which the fluorescence intensity was maximum suggests that careful control of the pH solution is required.

On the other hand, to obtain good yields in the labelling reactions of mixtures of both compounds, the pH setting must be a compromise and in this work, pH 3 appeared to be the optimum.

FC reacts very quickly with primary amines ($t_{1/2}$ = 100 - 500 ms), but frequently a great excess of FC is needed to produce good thermodynamic equilibrium conditions, as described previously.[19]

The effect of FC concentration on fluorophore formation was observed by measuring the fluorescence intensity for each compound at different FC concentrations, while all other experimental conditions were kept constant at the optimum values. Figure 4 shows that the maximum response was obtained when the FC concentration was $5.03 \times 10^{-4}$ M for asulam and $2.16 \times 10^{-4}$ M for amitrole. For the simultaneous determination of the two compounds a [FC]= $7.20 \times 10^{-4}$ M was selected.

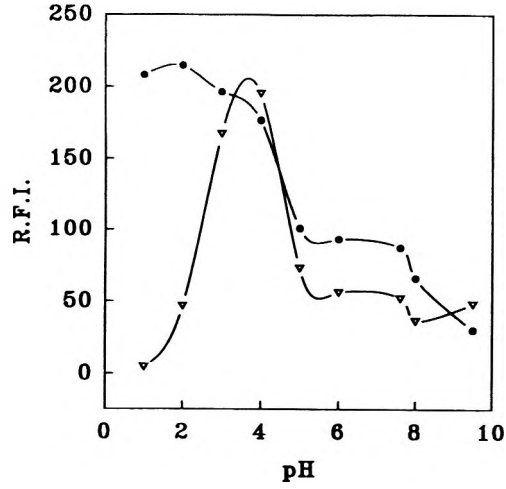



**Figure 3**. Influence of pH on the relative fluorescence intensity of (●) asulam and (▼) amitrole. Asulam $8.7 \times 10^{-7}$ M and amitrole $2.4 \times 10^{-4}$ M [FC] = $3.6 \times 10^{-4}$ M.

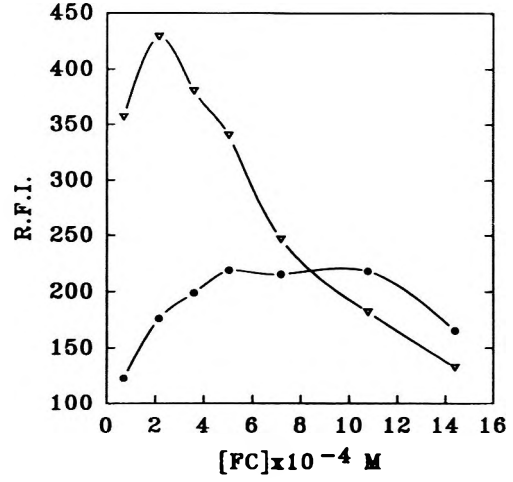



**Figure 4**. Influence of the [FC] on the relative fluorescence intensity of (●) asulam (pH = 2) and (▼) amitrole (pH = 4).

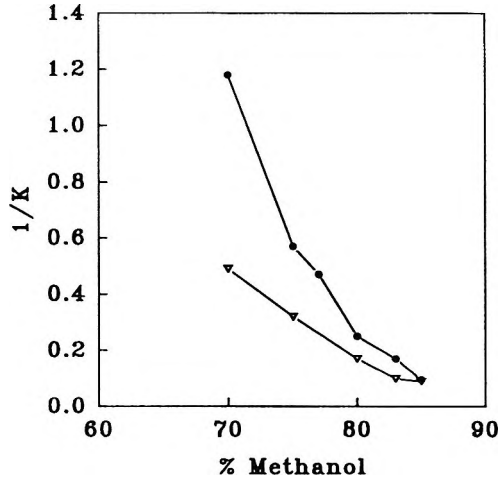



**Figure 5**. Capacity factor of the asulam (●) and amitrole (▼) vs methanol percentage.

## Optimization of the Chromatographic Conditions

The detection was accomplished to the wavelength obtained by the emission and excitation spectra ($\lambda_{exc}$ = 398 nm, $\lambda_{ems}$ = 490 nm). The column used was a LiChrospher 100 RP - 18 and the solvents for the mobile phase water - methanol. A form of choosing the better composition of the mobile phase for the resolution of the mixture of pesticides is plotting capacity factor, K, against the percentage of methanol. Taking into account results shown in Fig. 5, while the methanol proportion in the mobile phase increases the capacity factor also increases, therefore the pesticides are more retained by the stationary phase and the retention times are greater.

It can be concluded that a 25% methanol percentage gives the best separation of the pesticides and the overall chromatographic time is not very high (15 min).

## Calibration Graphs

The calibration graphs are linear between 0.04 ng - 4 ng for asulam and between 16 ng - 400 ng for amitrole. The lower limit of the linear dynamic range is determined by the quantification ($C_Q$) limit. Typical relative standard deviations (R.D.S.s) are between 1.13% - 1.6%. Linear regression analysis gave the following fit.

**Table 1**

**Spectrofluorimetric Interference Study**

| Interfer. | Asulam:Interfer. | Recovery(%) | Amitrole:Interfer. | Recovery(%) |
|---|---|---|---|---|
| Carbaryl | 1:500 | 109 | 1:5 | 103.8 |
| Warfarin | 1:500 | 96.4 | 1:5 | 91.4 |
| | | | 1:2.5 | 101.17 |
| Fuberidazol | 1:500 | 100 | 1:5 | 88.9 |
| | | | 1:2.5 | 94.74 |
| MCPA | 1:500 | 103 | 1:5 | 98.3 |
| Bentazone | 1:500 | 69.17 | 1:5 | 60.13 |
| | 1:250 | 95.8 | 1:2.500 | 70.80 |
| | | | 1:1.875 | 73.46 |
| | | | 1:1.250 | 90.06 |
| | | | 1:1 | 96.79 |

Asulam: $Y = 2436813.6\ X - 17230.5$ $r = 0.999$ ($n = 8$)
Amitrole: $Y = 22514.1\ X - 117219.7$ $r = 0.999$ ($n = 7$)

where Y is area under peak, in arbitrary units, and X is injected quantities in ng.

## Application

Prior to the application in real samples, the method was evaluated with synthetic mixtures of the most commonly used pesticides in pre- or post-harvest treatment. Five potential interferents were selected among insecticides, fungicides, and herbicides usually found in cereals, fruits, vegetables and other types of crops. The synthetic mixtures were prepared using a fixed concentration of the pesticide to be recovered and adding the potential interferents at several levels. The pesticides carbaryl, warfarin, fuberidazol, MCPA and bentazone, were added separately. Recoveries from these synthetic mixtures ranged from 94 to 104% for the non-interferent pesticides and from 60 to 94% for those pesticides causing interference. The tolerance criterion was a deviation of ± 5% in the signal, referred to the blank (pesticide alone). Table 1 shows the results obtained.

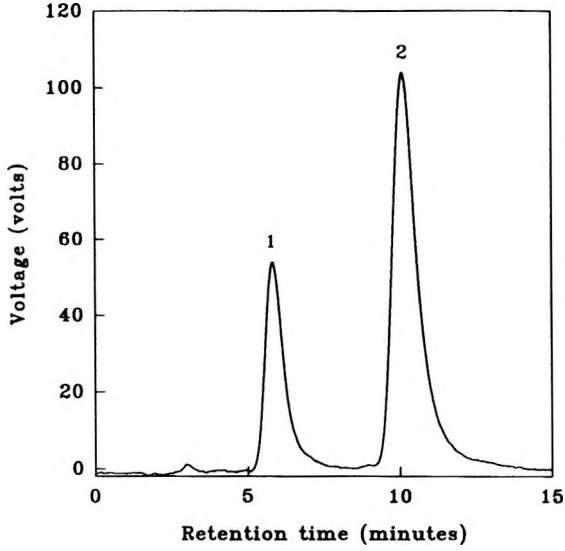



**Figure 6**. Chromatogram of spiked tap water. (1) 1 ng asulam and (2) 200 ng amitrole.

**Table 2**

**Recovery of Pesticides from Spiked Tap Water**

| Compound | Taken (ng) | $D_l$[a] (ng) | $C_q$[b] (ng) | Recovery % | R.S.D.[c] % |
|---|---|---|---|---|---|
| Asulam | | 0.04 | 0.13 | | |
| | 00.050 | | | 118 | 1.7 |
| | 2 | | | 90 | 2.2 |
| Amitrole | | 7.5 | 25 | | |
| | 20 | | | 108.5 | 6.9 |
| | 104.8 | | | 104.8 | 6.2 |

[a] detection limit for a signal-to-noise ratio = 3
[b] quantification limit for signal -to-noise ratio = 10
[c] n = 3

The application of herbicides in agriculture may cause the contamination of ground water and subsequently drinking water. The 1989 European Community Water Act states that the maximum admissible concentration of all pesticides in drinking water should be below 0.5 μg/L and the maximum individual pesticides concentration is 0.1 $\mu g.L^{-1}$.[20] The high water solubility of the pesticides studied (30 mg/L for asulam and 280 g/L for amitrole) facilitates their mobilization in water streams and localization at high levels in water.

Two different water samples were spiked, prior to the extraction, with a mixture of the pesticides prepared in doubly deionized water, after checking for the absence of the pesticides under study. After extraction, the samples were subjected to the LC procedure. The chromatogram of water extracts are reported in Fig. 6.

Table 2 presents the results obtained in the determination of asulam and amitrole in water by applying the LC method. As can be seen, recoveries are within 90 - 118%. The results obtained demonstrate the effectiveness of the proposed methods in determining the analytes assayed in these types of samples.

## ACKNOWLEDGEMENT

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# QUANTIFICATION OF BASAL AMOUNTS OF ISOMETALLOTHIONEINS IN CULTURED CELLS BY REVERSED-PHASE HPLC

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## ABSTRACT

A reversed phase HPLC method was developed to separate and quantify basal amounts of the major isometallothionein, i. e. MT-2, in cell extracts of human liver cells. To separate MT-2 from interfering proteins an ultrafiltration step was included in the preparation of the sample. Calibration was performed with varying amounts of pure human MT-2 quantified by amino acid analysis. Using MT-2 diluted with 25 mM Tris/HCl a non-linear calibration curve with a detection limit of 70 pmol was obtained. Linear correlation between the amount applied and the integrated area resulted when MT-2 was diluted with cytosol equivalent to 5.5 x $10^6$ cells. The detection limit under these conditions was 5 pmol, sufficient to monitor basal metallothionein concentrations and to follow changes in the accretion of these proteins under physiological conditions.

## INTRODUCTION

Metallothioneins (MT's) are cysteine- and metal-rich polypeptides identified in all animal phyla, in some plants and some eukaryotic and prokaryotic microorganisms (for review see,[1,2] and references therein). In mammals the MTs form a polymorphous family of proteins subdividable in several structurally distinct and in part tissue specifically expressed subclasses, i.e. MT-1, MT-2, MT-3 and MT-4.[3,4] MT-1 and MT-2 are the most abundant forms and subsume in some mammalian genera a number of genetically distinct isoforms. All have a molecular weight of approximately 6500 and contain seven equivalents of bivalent $d^{10}$ metal ions, usually $Zn^{2+}$, bound through thiolate coordination to 20 cysteine residues of the polypeptide chain. The best supported biological functions of the mammalian MTs are in attenuating the harmful influences of toxic metal ions and of various stress conditions.[5]

The abundance of MTs in tissues and cultured cells varies widely as its biosynthesis can be greatly stimulated by exposure to heavy metal ions, certain hormones, cytokines, tumor promoters and toxic electrophiles as well as by a number of stress conditions.[2] Thus, addition of $Zn^{2+}$ or of dexamethasone has resulted in massive cell- and inducer-specific isoMT accumulation.[6] Naturally enhanced expression of some of the MT-genes has also been found in fetal and perinatal development.[7,8]

In the absence of such biosynthesis promoting influences the cellular iso-MT concentrations range at much lower basal levels which are at the limit of detection by the commonly used chromatographic methods of analysis.[9,10] Thus, for exploring potential physiological variations of iso-MT's under basal conditions sensitive and specific analytical methods are needed.

In previous studies, we have successfully developed and employed a method which allowed the separation and accurate quantification of partially purified samples of induced human and rabbit MT's.[6,11]

In this report we describe an adaptation of this reversed phase HPLC method for a more direct quantification of human MT isoforms in the cytosol fraction of cultured Chang liver cells. The new method allows the detection of as little as 5 pmol of MT isoforms and provides thus an adequate and highly convenient means to assess variations in basal iso-MT concentrations under a variety of experimental conditions.

## MATERIALS

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, gentamycine, amphotericin B (Fungizone), trypsin-EDTA solution (10x) and cell culture dishes were purchased from Gibco and fetal-calf serum (FCS) from Amimed. Ultrafree-CL ultrafiltration tubes (exclusion size 30kDa) were obtained from Millipore and Tris (Trizma base) from Sigma. All other chemicals were of analytical grade and obtained from either Merck or Fluka. All water used was obtained from an ultra pure water system (Milli-Q Plus, Millipore).

## METHODS

### Cell Culture

Chang liver cells were obtained from Flow Laboratories. DMEM cell culture media was supplemented with 2 mM glutamine, 50 mg/L gentamycine, 2.5 mg/L fungizone and 10% (v/v) FCS. Cells were grown at 37°C, 100% humidity and 8% $CO_2$. Confluent cultures were harvested by trypsinisation and cell numbers were determined with a haemocytometer.

### Sample Preparation

Cells were harvested in DMEM at 4°C, suspensions of known cell count were centrifuged at 250 x g for 10 min and resuspended in 2.5 mL 25 mM Tris/HCl, pH 7.5. The cells were disrupted by ultrasonication (Sonifier cell disruptor B15, Branson) with 25 bursts of 1 sec duration at 10-20 W. The resulting homogenate was centrifuged at 100000 x g for 60 min. The supernatant was incubated for 4 min at 80°C, cooled on ice and centrifuged at 10000 x g for 10 min. The supernatant was diluted to a constant protein concentration equivalent to that obtainable from 5.5 x $10^6$ cells/mL and passed through a Ultrafree-CL ultrafiltration tube. 1 mL of filtrate was loaded onto the HPLC column.

### HPLC

An HPLC system Model 215 (LKB/Pharmacia) was used. Samples were injected with an autosampler Model 231 (Gilson) equipped with a 1 mL sample loop. The column (Spheri 5 RP-18, 2.1 mm (i.d.) x 220 mm, 5 μm particle size, Brownlee) was thermostated to 28°C. Chromatography was performed at a flow rate of 0.4 mL/min using a linear gradient formed between buffer A (25 mM

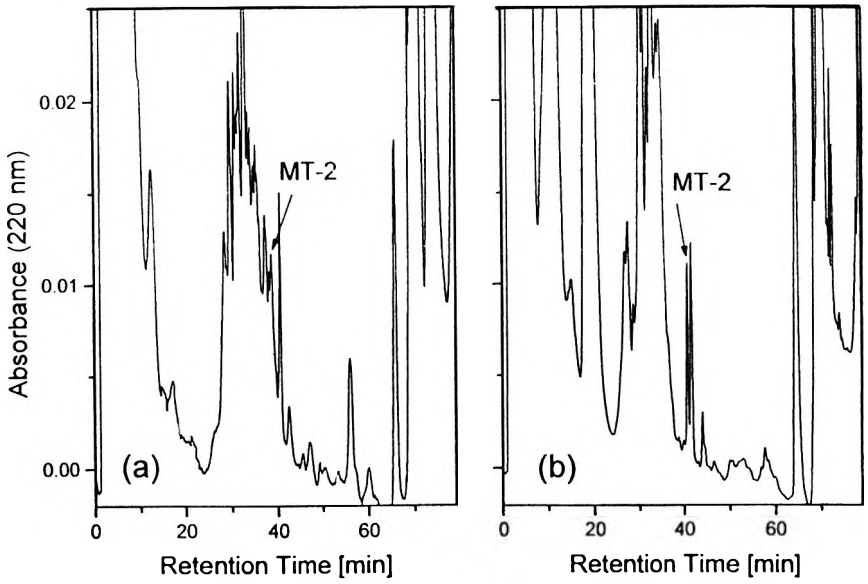


**Figure 1**. HPLC of cytosols obtained from proliferating Chang liver cells by (a) ultracentrifugation and heat denaturation and (b) additional ultrafiltration (30 kDa exclusion molecular weight).
Samples of 1 mL were injected on a Spheri-5 RP-18 column (2.1 (i.d.) x 220 mm) and eluted with a gradient formed between buffer A (25 mM Tris/HCl, pH 7.5) and buffer B (same as A, containing 60% (v/v) acetonitrile) at a flow-rate of 0.4 mL/min. The gradient used was as follows: 0% buffer B for 15 min.; from 0 to 23% buffer B during 45 min. The effluent was monitored at 220 nm

Tris/HCl, pH 7.5) and buffer B (same as buffer A containing 60% (v/v) acetonitrile). The gradient used was as follows: 0% buffer B for 15 min; from 0 to 23% buffer B during 45 min. The effluent was monitored at 220 nm and peak integration was performed on a Data System 450 (Kontron Instruments).

## Calibration

The concentration of a stock solution of chromatographically pure human MT-2 (27.2 nmol/mL) was determined by quantitative amino acid analysis (Model 420A/H, Applied Biosystems). The concentrations (pmol/mL) used for calibration were prepared by diluting the stock solution either with 25 mM Tris/HCl, pH 7.5 or with cytosol prepared from Chang liver cells. For each calibration point, 1mL of the

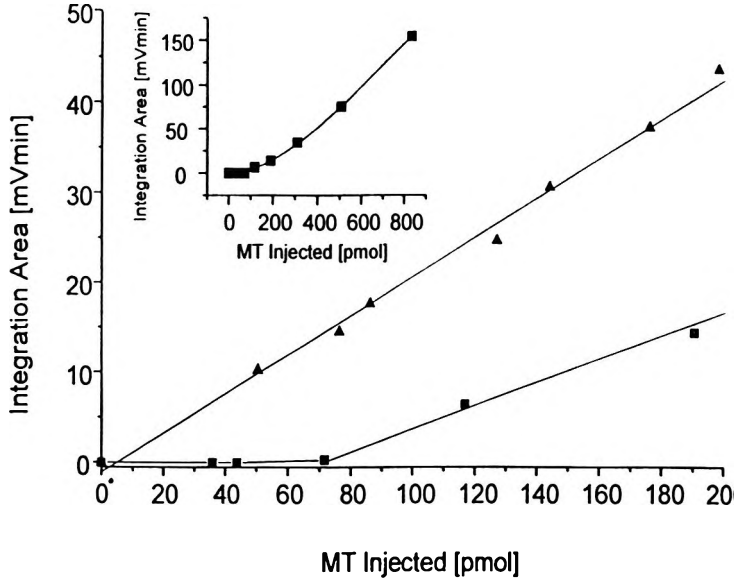


**Figure 2.** Calibration plots for MT-2 solutions in protein-free 25 mM Tris/HCl, pH 7.5 (■) and in 25 mM Tris/HCl, pH 7.5, containing cytosolic supernatant of Chang liver cells (▲_). Standard quality MT-2 was quantified by amino acid analysis before preparing the calibration solution. ■: 35 to 190 pmol of MT-2 were diluted in buffer A (25 mM Tris/HCl pH 7.5) and 1 mL was injected onto the HPLC column. The complete range examined under these conditions (35 to 800 pmol of MT-2) is shown in the insert. ▲: 50 to 200 pmol of MT-2 were diluted in 1 mL of cytosol prepared from a suspension of 5.5 x $10^6$ cells/mL and injected onto the HPLC column. Cytosol was prepared by ultracentrifugation, heat denaturation and ultrafiltration. Chromatography was performed as described in Fig. 1. The effluent was monitored at 220 nm and the peak area of MT-2 was integrated using a Data System 450 (Kontron). In presence of cytosol the area of MT-2 was corrected for the contribution of endogenous MT-2 (0.49 ± 0.01 mVmin). Correlation coefficients ($r^2$) for linear and second order polynomial regression were 0.980 and 0.999, respectively, in the absence of cytosol (■). In the presence of cytosol (▲) $r^2 = 0.994$ for linear regression.

appropriate calibration solution was loaded onto the HPLC column. The influence of the concentration of cytosolic proteins on the recovery of MT-2 was monitored by diluting increasing amounts of cytosol containing approximately 7 pmol endogenous MT-2/$10^6$ cells with 25 mM Tris/HCl, pH 7.5 to a total volume of 1 mL. Samples corresponding to 0.25 x $10^6$ to 14 x $10^6$ cells were analyzed by HPLC and MT-2 was quantified by peak integration.

## RESULTS

Figure 1 compares the profiles of cytosols of Chang liver cells obtained by two different preparation protocols which differ by an ultrafiltration step designed to eliminate interfering proteins from the cytosolic fraction. While MT-2 was hardly recoverable from cytosol prepared by ultracentrifugation of a cell homogenate and a heat denaturation step (Fig. 1(a)) this protein was easily resolved and quantified when an ultrafiltration step was included in the preparation of the sample (Fig. 1(b)). With the extended purification scheme a clear separation and a reproducible retention time of MT-2 with a relative standard deviation of ± 0.5% were achieved.

The HPLC system was calibrated using purified human MT-2 as external standard protein. Two different calibration curves which correlate the amount injected onto the HPLC column and the peak integration area of the effluent are shown in Fig. 2. The relative recovery of the aqueous standard samples of MT-2 from HPLC decreased progressively with decreasing load. The plot is not linear (Fig. 2, insert) and at sample quantities below 70 pmol no MT-2 was recovered from the column. This effect was avoided when ultrafiltrated cytosol from $5.5 \times 10^6$ cells was included in the MT-2 calibration solution. After subtracting the endogenous amount of MT-2 present in the cytosol preparation, i.e. 0.49 ± 0.01 mVmin, from the integration area the correlation between the injected amount of MT-2 and the integrated area of the peak recovered between 45 and 200 pmol was linear. The extrapolated detection limit under these conditions was approximately 5 pmol.

The dependence on the amount of cell cytosol needed for the full recovery of MT-2 is shown in Fig. 3. The detection of MT-2 failed when the amount of cytosolic protein of less than $3.2 \times 10^6$ cells was present in the analyzed sample. Using cytosol derived from $3.7 \times 10^6$ cells the yield increased sharply to 86% and a quantitative recovery is reproducibly obtained in the presence of cytosolic proteins of more than $5.1 \times 10^6$ cells.

## DISCUSSION

A method to quantify the basal accretion of MT in culture human cells by reversed phase HPLC was developed. Because MT-2 is the most abundant isoform in human cells,[6] all data reported here relate to this isoprotein although other isoMTs have also been reliably quantified by the same protocol.

For better separation from protein background in chromatography and accurate quantification of MT in cytosol an additional purification step was necessary (Fig. 1). Calibration of the system using buffered solutions of human

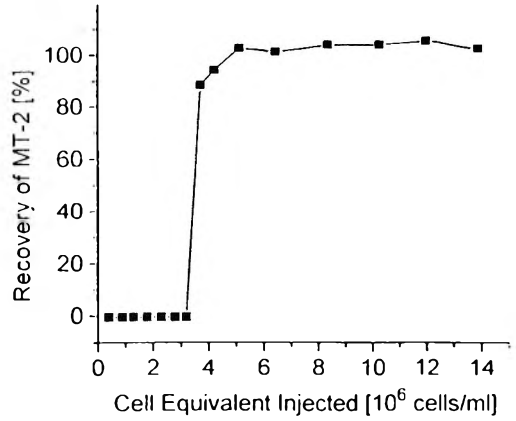


**Figure 3**. Influence of the Concentration of Cytosolic Proteins on the Recovery of MT-2. Cytosol of proliferating Chang liver cells was prepared by ultracentrifugation, heat denaturation and ultrafiltration and diluted with 25 mM Tris, pH 7.5, (HPLC buffer A). Samples of 1 mL corresponding to the different cell equivalents indicated were chromatographed as described in Fig. 1. and the recovery of endogenous MT-2 (7.2 ± 0.2 pmol/$10^6$ cells) was determined by using the linear calibration curve of Fig. 2.

MT-2 standard revealed a non-linear relation between the amount injected and the integrated area of the peak in the effluent (Fig. 2, insert). The skewed shape of the calibration curve and the overall lower recovery suggest that, under the conditions used, a constant amount of the MT standard is lost by unspecific binding to the column matrix. These losses are significantly reduced when the MT standard is injected together with prepurified cytosol. The cytosolic proteins apparently compete for the unspecific binding sites within the column matrix and thus allow for a good recovery of MT-2. The calibration curve becomes linear within the range examined and the detection limit is lowered approximately 14-fold (Fig. 2). However, this effect depends critically on the amount of protein in the cytosol, i. e. on the number of cells providing the cytosol. Below a minimal amount of cytosolic protein (1 mg/mL) in the sample the MTs are lost completely on the column (Fig. 3). Above a critical concentration the recovery of MT increases and reaches with a protein concentration of 1.3 mg/mL, derivable from 5.1 x $10^6$ cells/mL, a value of 100%. At protein concentrations derived from more than 14 x $10^6$ cells/mL the yield of MT decreases again and MT emerges, in part, with the void volume of the column indicating that the capacity factor of the column is reduced by saturating the stationary phase with cytosolic proteins (data not shown). This change in the capacity factor is irreversible necessitating replacement of the HPLC column.

In preliminary experiments conducted, the present method proved its usefulness as a reliable means to quantify changes in basal concentrations of iso-MT's during cell growth. As the protein content of non-synchronized growing Chang liver cells (260 μg ± 50 μg/$10^6$ cells) remains constant and is only slightly lower in density-arrested cells (220 μg ± 42 μg/$10^6$ cells), a standardisation of all samples to the same number of cells assures an adequate cytosol protein admixture for a proper analysis of MT. The optimal number of cells allowing for accurate measurement of basal MT concentrations lies in the range between 5 to 6 x $10^6$.

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# HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY : A PROMISING METHOD FOR THE SEPARATION OF THE ANNONACEOUS ACETOGENINS[1]

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## ABSTRACT

High-speed countercurrent chromatography (HSCCC) was applied, for the first time, to the separation of Annonaceous acetogenins. The two major acetogenins of the seeds of *Annona atemoya*, squamocin and rolliniastatin-2, were isolated in one

step using a biphasic mixture of heptane/ethyl acetate/methanol/water. In addition, this method yielded four pairs of highly purified acetogenins: squamocin/molvizarin, rolliniastatin-2/asimicin, neoannonin/atemoyin and isodesacetyl-uvaricin/desacetyluvaricin. These pairs were further resolved by HPLC, leading to a total of eight pure acetogenins in two steps. Isolated acetogenins, which are homologous, position and configurational isomers, have been identified by comparison with standards and by spectroscopic techniques.

## INTRODUCTION

Annonaceous acetogenins are recently discovered polyketides which were isolated, until now, only from the tropical and subtropical plants of the Annonaceae family.[2-4] They are characterized by common features: a long chain of 35-37 carbon atoms, including one or two tetrahydrofuran rings, several oxygenated functions (hydroxyl, ketone), and a γ-methyl-γ-lactone terminal group. In addition to these natural biologically active products, some natural precursors have been isolated, which bear only a γ-lactone and either hydroxyl groups and/or 1,2-epoxides with double bonds on the alkyl chain. Most of these compounds exhibit interesting cytotoxic activity with promising antitumor potential, and some have parasiticide, insecticide and immunomodulating properties.[2-4] They exist in the plant as mixtures of numerous isomers or homologs of close polarities making their isolation difficult. Various successive and repeated chromatographic steps on silica gel, Sephadex LH 20, preparative TLC, and normal and reversed phase HPLC, were needed to purify acetogenins, with generally poor overall yields.

We were interested in applying, for the first time, high-speed countercurrent chromatography (HSCCC)[5,6] for purification of acetogenins and we report, in this paper, the isolation of two major acetogenins, squamocin[2] and rolliniastatin-2,[3] from the seeds of *Annona atemoya*.[7,8]

## MATERIALS AND METHODS

### Extraction

Seeds of *Annona atemoya* were collected in September 1993 in Australia and authenticated by Dr. D. J. Batten (Tropical Fruit Research Station, Alstonville, New South Wales, Australia). The dried and pulverized seeds

(930g) were macerated with MeOH. The MeOH extract (79.7 g) was partitioned between $H_2O$ and hexane to yield 6 g of hexane extract. The aqueous alcohol fraction was partially evaporated and extracted with dichloromethane to afford 29.6 g of $CH_2Cl_2$-soluble extract.

**Apparatus**

CCC runs were performed using a HSCCC[5] (P.C. Inc., Potomac, MD, USA); the large coil of the triple-coil set was used (i.d. = 1.68 mm, total volume $V_c$ = 240 mL). The rotational speed was 800 rpm. The pump was a model LKB P-500 (Pharmacia, Uppsala, Sweden), and the injector a medium pressure Rheodyne model 50110 injector with a 5 mL sample loop. Fractions (5 mL) were collected with a fraction collector model 202 (Gilson, Villiers-le-Bel, France).

**Fractionation by HSCCC**

The HSCCC experiment was performed with the quaternary system heptane/ethylacetate/methanol/water (HEP/EtOAc/MeOH/$H_2O$) [3:10:10:7] v/v/v/v. The coil was first entirely filled with the upper phase and rotation was set up at the desired speed (800 rpm). The lower phase was then pumped into the column at a flow-rate of 4 mL/min, in the head to tail mode. After the mobile phase front emerged and the equilibrium between the two phases was established (mobile phase volume = 86 mL), the sample solution (500 mg of the dichloromethane extract in 5 mL of mobile phase) was injected through the injection valve at a flow-rate of 1 mL/min. The flow-rate was then gradually increased to 4 mL/min and the effluent was collected from the outlet of the column. After the fraction 190 had been collected, the rotation was stopped and the content of the column was pushed out by the mobile phase and collected.

**Analysis of the Collected Fractions**

The composition of each fraction was determined by TLC on silicagel $60F_{254}$ plates (Merck, Darmstadt, Germany) using toluene/EtOAc/EtOH [30:70:5] v/v/v as mobile phase. Solutes were visualized by vanillin-$H_2SO_4$ spray and heat (100°C for 10 min.) while acetogenins were characterized by the more specific Kedde reagent (typical of an unsaturated $\gamma$-lactone). Fractions

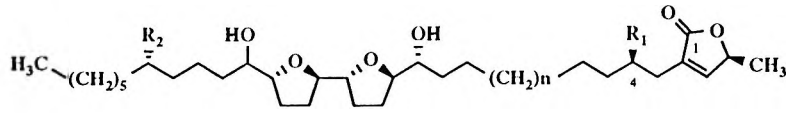
containing only one spot were analyzed by HPLC, MS and NMR and identified by comparison with authentic samples of molvizarin **[1]**, squamocin **[2]**, rolliniastatin-2 **[3]** (=bullatacin), asimicin **[4]**, neoannonin **[5]**, atemoyin **[6]**, isodesacetyluvaricin **[7]** and desacetyluvaricin **[8]**.[2-4] Analyses were performed with an HPLC system equipped with a Beckman pump 112 (Beckman Instruments, Berkeley, CA, USA), a Varian 9050 spectrophotometer (Varian, San Fernando, CA, USA) and a U6K Waters injector (Millipore, Waters Chromatography Div., Milford, MA, USA). A Spherisorb S5-ODS2 $C_{18}$ column, 25 x 0.46 cm (Prolabo, Paris, France) and mobile phases MeOH/$H_2O$ [85:15] v/v or [90:10] v/v were used. The flow-rate was 0.8 mL/min. and the effluent was monitored at 214 nm. Similar fractions were pooled, evaporated to dryness, and pairs of acetogenins were separated with a preparative HPLC Millipore-Waters system equipped with a model 590 pump, an SSV injector, and a 484 UV detector (214 nm). A μBondapak $C_{18}$ prepacked cartridge (10 mm, 25 x 100 mm), eluted with MeOH/$H_2O$ [82:18] v/v for molvizarin **[1]** / squamocin **[2]** and rolliniastatin-2 **[3]** / asimicin **[4]** or [90:10] v/v for neoannonin **[5]** / atemoyin **[6]** and isodesacetyluvaricin **[7]** / desacetyluvaricin **[8]** was used. Pure isolated products were finally analyzed by NMR and MS. NMR spectra ($CDCl_3$) were obtained with a Bruker AC-200 instrument (Bruker, Wissembourg, France) while EIMS and CIMS (methane) were performed on a Nermag R10-10C spectrometer (Nermag, Rueil-Malmaison, France).

### Solvents

All organic solvents were of analytical grade and came from Prolabo (Paris, France). Authentic samples of acetogenins have been previously isolated from several Annonaceae species and were supplied by Prof. A. Cavé and collaborators.

## RESULTS AND DISCUSSION

The large number of acetogenins present in the same annonaceous species, and their very close chemical structures (homologous, positional and configurational isomers), account for the difficulty in separating these kinds of compounds.[2-4] Many successive and repeated conventional column chromatographic operations are needed to isolate pure acetogenins. They are often obtained as mixtures and must be purified by HPLC. In recent years, HSCCC has gained increasing popularity for isolation of bioactive natural products. HSCCC, based on the fundamental principle of liquid-liquid



*X/trans/threo/trans/threo*

| | $R_1$ | $R_2$ | n | X |
|---|---|---|---|---|
| Molvizarin [1] : | OH | H | 4 | *erythro* |
| Squamocin [2] : | H | OH | 6 | *erythro* |
| Rolliniastatin-2 [3] : | OH | H | 6 | *erythro* |
| Asimicin [4] : | OH | H | 6 | *threo* |
| Neoannonin [5] : | H | H | 4 | *erythro* |
| Atemoyin [6] : | H | H | 4 | *threo* |
| Desacetyluvaricin [7] : | H | H | 6 | *erythro* |
| Isodesacetyluvaricin [8] : | H | H | 6 | *threo* |

**Figure 1**. Acetogenins isolated from *Annona atemoya* seeds.

partition, utilizes a particular combination of coil orientation and planetary motion to produce a hydrodynamic phenomenon in the distribution of two immiscible solvents in a coiled column.[5,6] We found it very attractive to use this method, for the first time, to purify acetogenins.

The dichloromethane extract of *Annona atemoya* seeds is a very complex mixture, having its components spread over a wide range of polarity. So a compromise between resolution and run time was necessary. Considering the solubility of the compounds present in our extract, classical biphasic solvent systems like $CH_2Cl_2$/MeOH/$H_2O$ and EtOAc/MeOH/$H_2O$ were first examined. The adjustment of the partition coefficients to desired levels by changing the ratio of MeOH was impossible, acetogenins partitioning always entirely into the organic phases. Addition of heptane as a modifying solvent was required to decrease the solubility in the organic phase by increasing its hydrophobicity and expelling more polar components into the aqueous phase. Finally, the quaternary system HEP/EtOAc/MeOH/$H_2O$ [3:10:10:7] v/v/v/v, combining a large capacity for dissolving extract with different partition coefficients for the acetogenins (checked by TLC[9]) was selected. Two pure compounds have been obtained in one step, using this solvent system after injection of 500 mg of the dichloromethane extract; the major acetogenins of the *Annona atemoya* seeds, squamocin **[1]** (fractions 58 to 59, 12 mg, 2.4% of the dichloromethane extract, 94% pure by HPLC) and rolliniastatin-2 **[3]** (fractions 67 to 69, 5 mg, 1% of the extract, 98% pure by HPLC). These two acetogenins differ only in the position of one hydroxyl group, *i.e.*, C-28 for squamocin and C-4 for rolliniastatin-2 (Figure 1). Four pairs of acetogenins, molvizarin **[1]** /

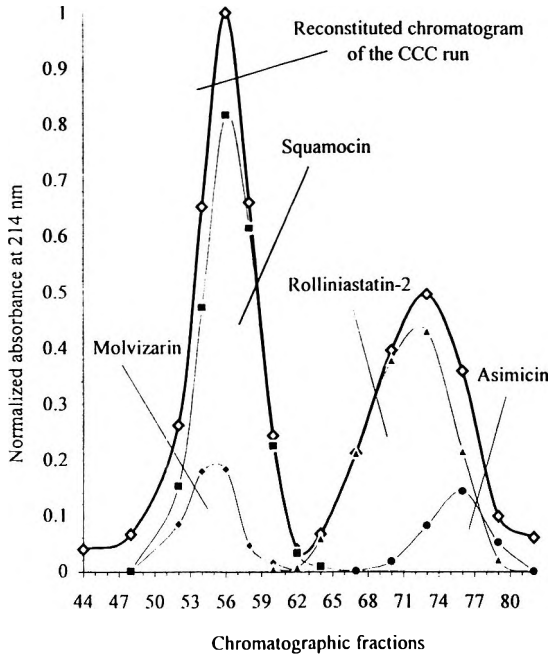



**Figure 2**. Chromatogram drawn from HPLC analyses of HSCCC fractions 44 to 82, showing the separation of molvizarin **[1]**, squamocin **[2]**, rolliniastatin-2 **[3]** and asimicin **[4]**.

squamocin **[2]** (fractions 52 to 57, 87 mg, 17.4% of the extract), rolliniastatin-2 **[3]** / asimicin **[4]** (fractions 70 to 78, 34 mg, 6.8% of the extract), neoannonin **[5]** /atemoyin **[6]** (fractions 130 to 155, 4 mg, 0.8% of the extract) and isodesacetyluvaricin **[7]** / desacetyluvaricin **[8]** (fractions 170 to 181, 6 mg, 1.2% of the extract) were also obtained with these chromatographic conditions. These mixtures were further purified by HPLC and pure compounds were identified by comparison (HPLC, MS and NMR) with standards previously isolated from the same crude extract by combination of numerous successive chromatographies on silica gel and tedious repeated reversed phase HPLC.[7]

The HSCCC chromatogram drawn from the HPLC analysis of fractions 44 to 82 (Figure 2) showed an elution in conformity with the reversed phase mode of chromatography which was used. Differences in polarities of acetogenins are mainly due to their oxygenated groups, carbon atom number, and relative configurations of their THF rings. Although squamocin **[2]** and rolliniastatin-2

**[3]** are both bis-THF acetogenins with 37 carbon atoms, three hydroxyl groups and the same relative configuration (*threo/trans/threo/trans/erythro*), squamocin is eluted first. This is probably due to the 4-OH in **3** which takes part in a hydrogen bond with the carbonyl group of the lactone. The 28-OH of **2** should be more easily submitted to interactions of polar solvents, explaining the differences in partition of the squamocin and rolliniastatin-2.

Elution of acetogenins in HSCCC follows an order similar to that found in $C_{18}$ reversed phase HPLC: molvizarin **[1]** first with squamocin **[2]**, then rolliniastatin-2 **[3]**, then its configuration isomer asimicin **[4]** (HSCCC chromatogram shows a small but significant difference in their elution times) then neoannonin **[5]** / atemoyin **[6]** which are configuration isomers with 35 carbon atoms and two hydroxyl groups, and finally a mixture of two configuration isomers, isodesacetyluvaricin **[7]** / desacetyluvaricin **[8]**, which contain 37 carbon atoms and two hydroxyl groups (Figure 1).

Efficient separations of acetogenins are possible by optimization of the chromatographic conditions. For instance, HEP/EtOAc/MeOH/$H_2O$ [3:10:10:7] v/v/v/v is an appropriate solvent system to separate **[1]** or **[2]** and **[3-8]**, **[3]** or **[4]** and **[5-8]**, **[5]** or **[6]** and **[7-8]** but inappropriate for **[1]** and **[2]**, **[5]** and **[6]**, **[7]** and **[8]**. Although a combination of HSCCC in initial fractionation work, with HPLC in the final step of purification seems to be a good approach (large amounts of mixtures can be treated and crude extracts pose no problem), pure compounds can be also obtained with this method in one step. Therefore, a judicious choice of the biphasic solvent system, determining the relative proportions of solutes passing into each of the two phases, is crucial to the success of the purification.

## CONCLUSION

The two main acetogenins of the seeds of *Annona atemoya*, squamocin and rolliniastatin-2 were obtained in one step and with a high purity by using high-speed countercurrent chromatography. In addition, this method yielded four pairs of acetogenins, the separation of which, by HPLC, led to six additional compounds in a second step (molvizarin, asimicin, neoannonin, atemoyin, isodesacetyluvaricin and desacetyluvaricin). HSCCC allowed us to separate acetogenins with very closely related chemical structures: neoannonin/atemoyin and isodesacetyluvaricin/desacetyluvaricin, which differ by two methylene, position isomers (squamocin and rolliniastatin-2), as well as relative configurational isomers (rolliniastatin-2 and asimicin). HSCCC seems to be a very effective and promising tool for the purification of biologically

active annonaceous acetogenins, and would be a good alternative to column chromatography by using appropriate biphasic solvent systems and well adjusted partition coefficients.

## ACKNOWLEDGMENTS

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# HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROCHEMICAL DETECTION APPLIED TO MONITORING CLOPAMIDE IN HUMAN URINE AND PHARMACEUTICAL FORMULATIONS

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## ABSTRACT

A simple and sensitive HPLC-EC method has been developed for the determination of the benzothiazidic diuretic 4-Chloro-N-(2,6-dimethylpiperidino)-3-sulphamoylbenzamide (Clopamide), using a μBondapack C18 column with a mobile phase of acetonitrile-water (35:65) containing 5mM sodium acetate/acetic acid at a flow rate of 1mL/min. After a simple alkaline liquid-liquid extraction procedure with ethyl acetate, clopamide can be extracted from urine with percentages of recovery greater than 95%. The compound can be detected and measured with good reproducibility and an experimental quantitation limit of 20 ng/mL at a positive potential of +1400 mV. Clopamide has been determined in the pharmaceutical formulation Brinerdine (clopamide 10 mg, reserpine 0.1 mg, dihydroergotoxine mesilate

0.58 mg and tartrazine 0.064 mg). The method developed has been also applied to the determination of clopamide in human urine samples obtained at different time intervals after the ingestion of a single dose of Brinerdine.

## INTRODUCTION

4-Chloro-N-(2,6-dimethylpiperidino)-3-sulphamoylbenzamide (Clopamide) is an oral mild diuretic agent of the benzothiazide family that shares the same aromatic sulphonamide base as the thiazide diuretics but without the double-ring structure characteristic of these compounds.[1] Clopamide acts on the proximal convoluted tubule to inhibit the reabsorption of sodium, chloride and hence water, with a mechanism of action similar to that of the thiazide diuretics. Clopamide also causes an increase in potassium excretion in the urine and hypokalemia may occur.[2] The drug is often given with a supplementary potassium to minimize this effect.[3] The diuretic effect is less intense than that of the loop diuretics.

The main therapeutic use is the control of mild to moderate hypertension but it is also used for the management of congestive heart failure and hepatic disorders.[4] For these purposes a daily dose of 5 to 20 mg/day is recommended.[5]

Clopamide causes a diuresis that starts within 2 hours from drug administration. The maximum diuretic effect is usually seen within 3 to 6 hours, corresponding to the peak plasma drug concentrations, and the effect will last for 12-24 hours.[6] Metabolism is complex; the main products are hydroxiderivatives on the piperidinic ring, which then can be conjugated with sulphate or glucuronic acid. Different authors have reported that clopamide is excreted 25% or 30-40% unchanged in urine.[7]

Due to its mild diuretic effect, clopamide is available in oral forms alone or in combination with a b-blocker: pindolol. The combined oral administration of pindolol and clopamide has been found to be more effective than either drug alone on treatment of hypertension.[8] Also the triple combination of clopamide, reserpine and dihidroergocristine was shown to lower blood pressure both at rest and during exercise.

Some methods have been reported for the analysis of clopamide. Most of them involve the determination in plasma using high performance liquid chromatography with photometric detection.[9,10] The determination of clopamide in tablets using spectrophotometric methods has also been reported.[11,12] For the assay of the clopamide-pindolol combination in tablets, a

thin layer chromatography method[13] has been published, which allows the simultaneous determination of the two drugs in pharmaceutical formulations.

The aim of this paper is the development of a HPLC-EC method for the determination of clopamide in pharmaceutical formulations and human urine obtained from healthy volunteers.

## MATERIALS AND METHODS

### Apparatus and Column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump, and a Rheodyne (Pharmacia) Model 7125 injector with a loop of 20μL.

An amperometric detector PAR Model 400 equipped with a glassy carbon cell (EG&G Princeton Applied Research, Madrid, Spain) was used to carry out the electrochemical detection. The detector potential was set at +1400 mV vs a Ag/AgCl electrode, in the DC mode with a 5-s low-pass filter, and a current range between 0.2 and 100 nA. The chromatographic response was recorded by a LKB Model 2221 integrator setting with an attenuation of 8 mV full scale and a chart speed of 0.5 cm/min.

The column used was a 125 Å μBondapak C18, 30 cm x 3.9 mm I.D., 10-μm, (Waters Assoc.). A μBondapak C18 precolumn module (Waters Assoc.) was used to protect column from degradation. To study the influence of the temperature, a Waters TMC temperature control system was used.

The extracted urine samples were evaporated to dryness with a Zymark TurboVap LV evaporator (Barcelona, Spain).

### Reagents and Stock Solutions

Clopamide was kindly supplied by Sandoz Pharma (AG Basel/Schweiz), and was used without any further purification. HPLC grade acetonitrile and methanol were purchased from Lab-Scan (Dublin, Ireland), and water used was obtained by the Milli-RO and Milli-Q Waters systems. All the reagents used were Merck Suprapur (Bilbao, Spain).

A stock solution containing 1000 μg/mL of clopamide was prepared in pure methanol and stored in the dark under refrigeration. Standard solutions were made from this one and prepared fresh every week.

Urine samples were collected, frozen and kept at -18°C until required for the analysis.

**Chromatographic Conditions**

A mixture acetonitrile-water (35:65) containing 5 mM sodium acetate/acetic acid was used as mobile phase. pH was adjusted to 5.5 and the buffer served as the supporting electrolyte. This phase was filtered through Millipore membrane filters of 0.45-μm porosity, and the filtrate was degassed by bubbing helium through. The μBondapak C18 column head-pressure was 69 bar at a flow rate of 1.0 mL/min. The injection volume was 20 μL. Chromatographic separations were performed at room temperature.

**Electrode Maintenance**

The electrode was cleaned electrochemically by keeping it at -800mV for 2 min and after that at +1600 mV for 5 min. This operation was carried out at the end of each working day, using a mobile phase of pure methanol at a flow rate of 1.5 mL/min. When the base line was poorly defined, the glassy carbon electrode was cleaned with a tissue wet with methanol to remove posible adsorbed compounds, and rinsed with deionized water.

**Assay of Commercial Tablets**

Tablets were reduced to a homogeneous fine powder in a mortar. A suitable amount of this powder was weighed and methanol was added. After shaking for 20 min, the mixture was ultrasonicated for 5 minutes and filtered, and the precipitate washed with the solvent. Solutions obtained after this procedure were made up to 100 mL with methanol. Aliquots of these concentrated solutions were diluted with the mobile phase, and injected into the chromatographic system. Different amounts of initial solid sample were assayed and this procedure was repeated for different tablets in order to obtain a mean value.

### Clean-up Procedure for Urine Samples

Urine samples were treated following a liquid-liquid extraction procedure based on the method proposed by Ventura et al.[14] with some modifications. 2 mL urine were alkalinized with 0.5 mL NaOH 5 M, 1.5 mg NaCl(s) were added and 4 mL ethyl acetate. The mixture was shaken for 10 min and centrifuged for 5 min at 734 g. After that, the organic layer was separated and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The dried residue was dissolved in 2 mL of the mobile phase and 20 μL were injected into the HPLC column.

## RESULTS AND DISCUSSION

In static conditions, the oxidation of clopamide on a glassy carbon electrode is unable, due to the high potential neccessary for the oxidative process which makes the peak appear behind the cut-off of the electrolyte. However, in the chromatographic system, because of the displacement that takes place in peak potential, it is possible to reach a value which allows the determination of clopamide.

Hydrodynamic voltammetry of the compound was carried out in order to choose the optimum potential value for the amperometric detection of clopamide (Figure 1). An oxidative potential of +1400 mV was chosen as the working potential, since it was the potential which provided the maximum sensitivity for clopamide without increasing the background to high levels.

The chromatographic behaviour of clopamide was not very affected by the pH of the mobile phase in the pH range 3.5-6.5 since the two pKa values of clopamide (2.9 and 9.2 )[15] are out of this interval. The study of the influence of pH gave an optimum value of 5.5 which allowed the separation of clopamide from the electrooxidable interferences found in urine, keeping a low retention time (6 min).

The supporting electrolyte used, which is necessary for the amperometric detection, was the buffer sodium acetate/acetic acid. The best signal to noise ratio was provided by an electrolyte concentration of 5mM.

Different ratios of methanol-water and acetonitrile-water containing 5 mM sodium acetate/acetic acid were assayed as the mobile phase. Acetonitrile proved to be better than methanol for the separation of this diuretic since the chromatographic peak was better defined.

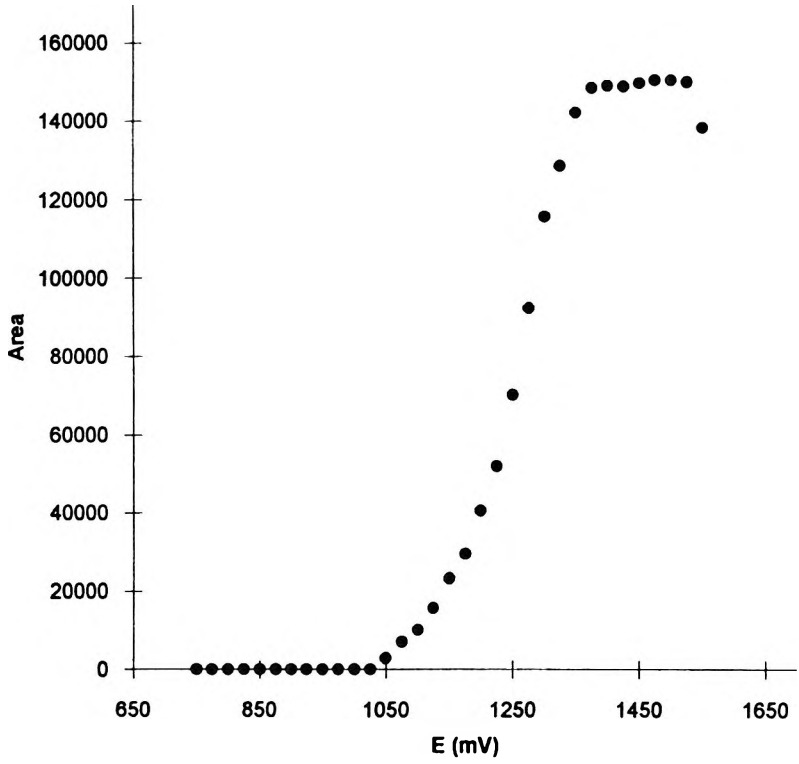


**Figure 1**. Hydrodynamic voltammogram of clopamide(•). Amount of drug injected: 100 ng in acetonitrile-water (35:65) containing 5mM $NaCH_3COO/CH_3COOH$, pH 5.5.

A study of the influence of the flow rate on the chromatographic behaviour was carried out. As was expected, the peak area decreased with the increase of flow rate, while the effect on the capacity factor k′ (k′=Retention time/injection peak time) was practically negiglible. A value of 1mL/min was chosen as optimum.

A variation in temperature from 26 to 55°C produced small variations on the peak area of chromatograms. A linear relationship between k′-log 1/T was obtained. Since the influence of the temperature was not very relevant, the work was carried out at room temperature.

**Table 1**

**Analytical Parameters for the Determination of Clopamide**

| Diuretic | Clopamide |
|---|---|
| Linear concentration range | 20 ng/mL- 10μg/mL |
| Slope of calibration graph (area/[μg/mL]) | 11501.87 |
| Correlation Coeficient ($r^2$) | 0.9996 |
| Experimental limit of quantitation | 20 ng/mL |
| Reproducibility (%RSD) within-day | 1.58 % |
| Reproducibility (%RSD) day-to-day | 8.6 % |

The stability of the system was evaluated checking the retention time corresponding to different injections of clopamide. A standard deviation of ± 0.07 min was observed. This deviation is caused by the different integration of the peak area makes by the integrator and not by a real change in the retention time of the compound.

When optimum chromatographic conditions had been established, a quantitative method for the determination of clopamide was developed. In Table 1 are collected the linear regresion of the calibration curve, reproducibility studies (intra-day and inter-day) made on n=10 solutions, in terms of relative standard deviations (% RSD), and the experimental quantitation limit, defined as the minimum concentration of clopamide which gives rise to a signal able to be quantified for the integrator.

The calibration curve for clopamide was linear over the concentration range 20 ng/mL-10 mg/L. The method showed good accuracy since spiked urine samples with different clopamide concentrations gave values very close to the nominal concentrations (Table 2).

## Analytical Applications

The method developed was applied to the determination of clopamide in tablets and urine samples obtained from human volunteers.

**Table 2**

**Evaluation of the Precision and Accuracy of the Assay of Clopamide**

| Nominal Concentration (μg/mL) | Number of Replicates | Observed Concentration (ng/mL) | %Accuracy=(Observ. Conc./Nom. Conc.) x 100 |
|---|---|---|---|
| 0.250 | 3 | 0.253 ± 0.042 | 101.2 % |
| 0.500 | 3 | 0.499± 0.005 | 99.8 % |
| 1.000 | 3 | 0.991 ± 0.027 | 99.1 % |

**Table 3**

**Recoveries for Clopamide Liquid-Liquid Extraction using Different Organic Solvents and NaCl(s)**

| Organic Solvent | % Recovery |
|---|---|
| Ethyl acetate | 95.96 ± 3.49 |
| Diethyl ether | 40.41 ± 2.78 |
| Chloroform | 81.12 ± 3.05 |

Results obtained for pharmaceutical formulations containing clopamide (9.96±0.02 mg/tablet) are in accordance with the values certified by the Pharmaceutical Company (10 mg/tablet). The liquid-liquid extraction procedure of clopamide from urine was evaluated at different concentration levels and with different organic solvents: ethyl acetate, chloroform and diethyl ether. Recovery of clopamide from urine was estimated by comparing the peak area of clopamide in spiked urine samples with those obtained by direct injection of the pure standard solutions of clopamide.

Ethyl acetate provided the best results with high recoveries as can be seen in Table 3 as well as less interfering peaks from endogenous compounds. The extraction procedure was performed with and without NaCl(s), and an increase in recovery was observed with the use of the salt ( from 86.19±4.88% to 95.96± 3.49%).

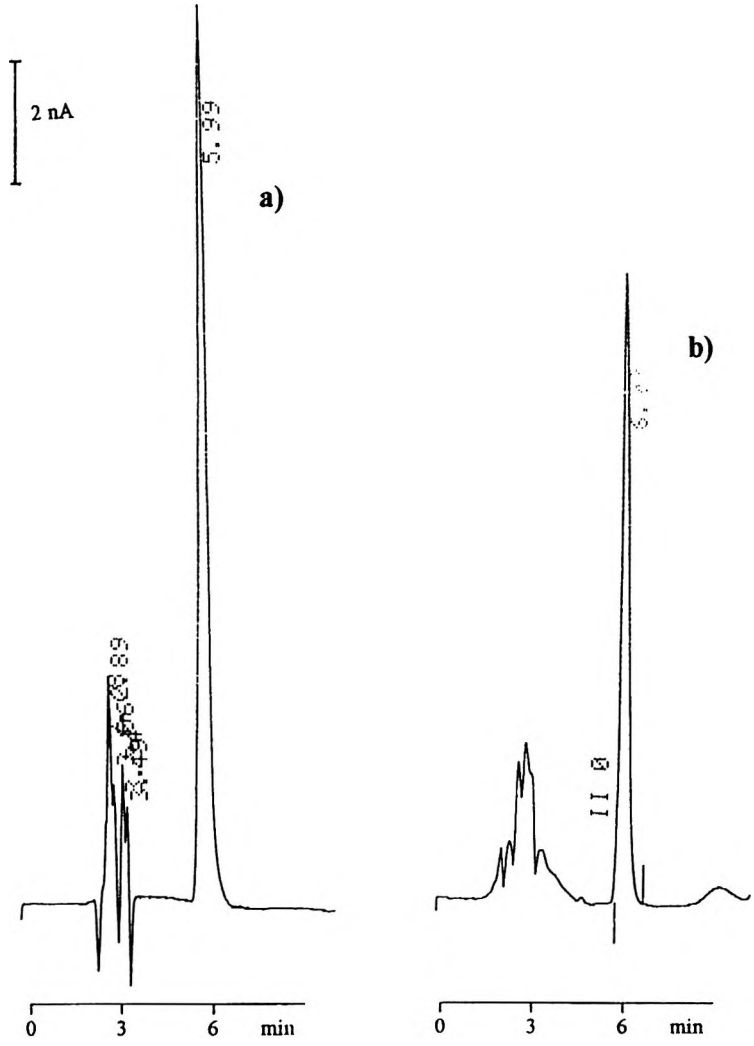


**Figure 2**. Comparative chromatograms of a) a standard solution of clopamide 5 µg/mL and b) a diluted solution of a pharmaceutical formulation containing clopamide: Brinerdine (-clopamide 10 mg, dyhydroergotoxine mesilate 0.58 mg, reserpine 0.1 mg and tartrazine 0.064 mg-). E= +1400 mV; Flow rate: 1mL/min; Full current scale: 20 nA.

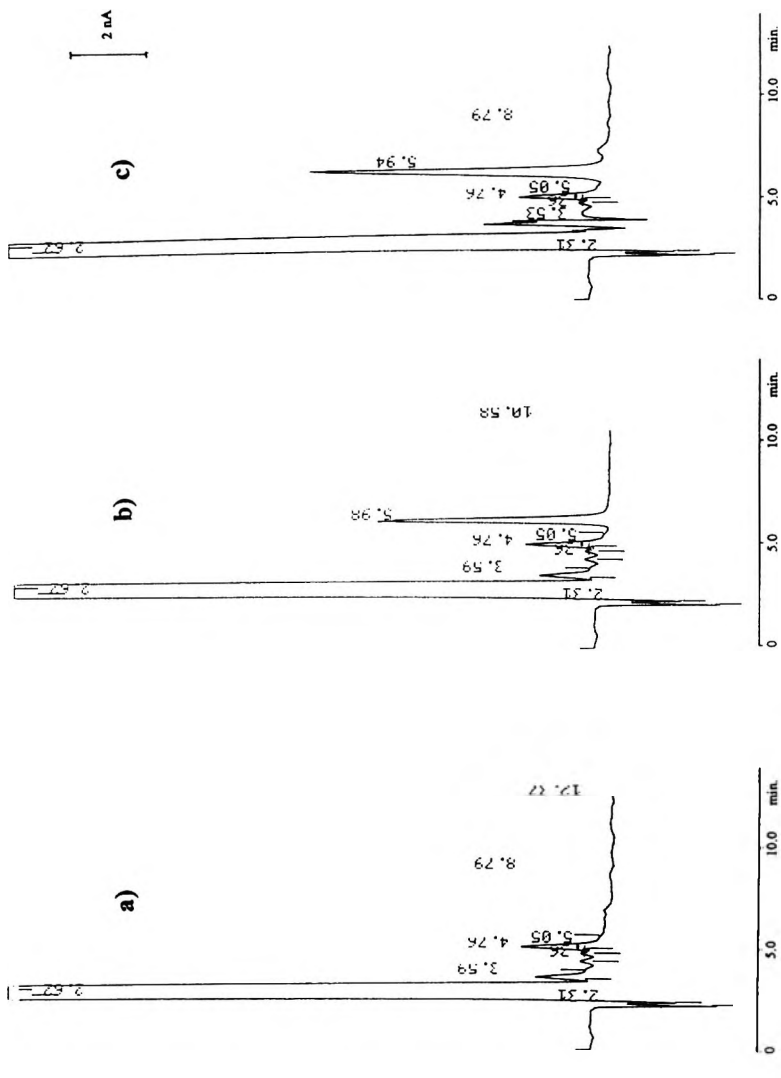


**Figure 3**. Typical chromatograms of extracts of human urine: a) blank urine sample, b) urine spiked with 1 mg/mL clopamide and c) urine with clopamide from a healthy subject 2-8 hours after an oral dose of Brinerdina (clopamide, dihidroergotoxine mesilate, reserpine and tartrazine). Full current scale: 20 nA. Chromatographic conditions as in Figure 2.

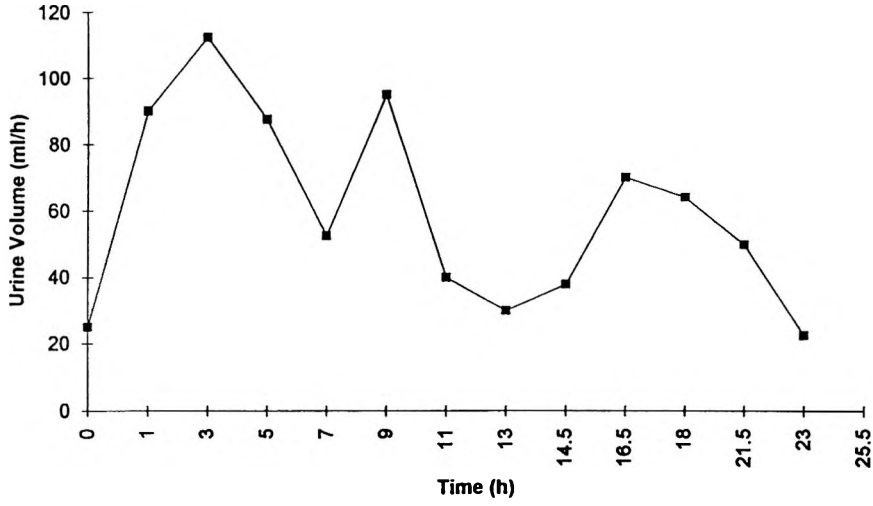


**Figure 4**. Excretion rate of urine after oral administration of a single dose of Brinerdine (clopamide 10 mg) to a healthy female volunteer.

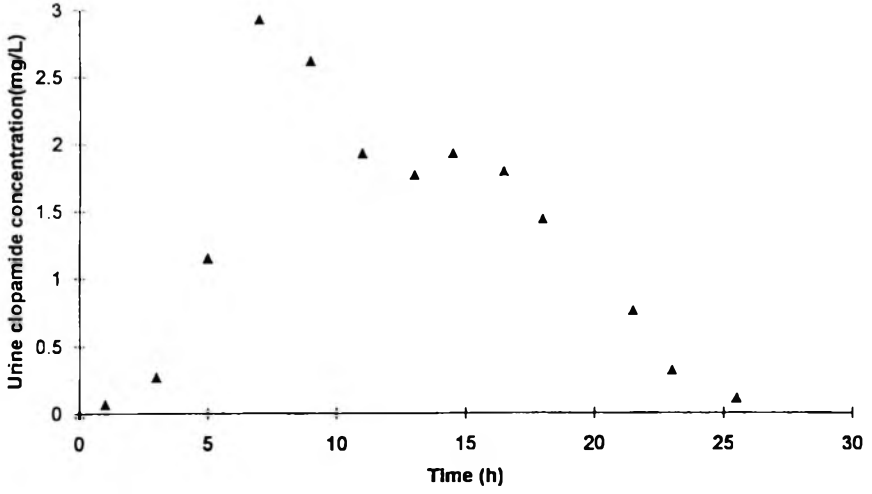


**Figure 5**. Mean urine concentrations of clopamide versus time in a healthy subject after an oral administration of a single dose of Brinerdine (clopamide 10 mg).

Figure 2 shows the comparative chromatograms of standard clopamide and a dilute solution of the pharmaceutical formulation containing this diuretic.

In Fig. 3, the chromatograms corresponding to blank urine and clopamide in spiked and real urine samples can be observed. In Figs. 4 and 5, the excretion rates of urine and urinary clopamide are plotted.

Both excretion rates represent the average during the collection interval and have been plotted at the midpoint of the interval and are in accordance with the values found in literature.[7] The peak excretion rate of clopamide is about 6 hours which is in agreement with its mild diuretic potency.

## DISCUSSION

Clopamide is a usual oral diuretic agent for the treatment of hypertension, alone or in combination with other drugs as beta-blockers. Due to this fact, its determination in urine samples is of great interest.

Different attempts were made to develop a static voltammmetric method for the determination of clopamide. It was impossible, since due to the high potential value neccessary for its oxidation, the peak appears after the cutt-off and only a shoulder was observed in acidic media.

But, with the chromatographic system used, the peak of clopamide can be observed without too many interferences even at high positive potential values.

The method developed allows the determination of clopamide in human urine samples. This paper is not intended to be a study of the pharmacodynamic properties of clopamide, since only one volunteer was used for the sample collection and results may be of no significance. It only shows that the posibility of monitoring this diuretic makes the method useful for pharmacokinetic and pharmacodynamic purposes.

The system was stable despite the high potential neccessary for the oxidation of clopamide. The base line was free from drifts and noises, allowing one to set the scale range at the low nA level necessary for the detection of the small peaks corresponding to very low concentrations of clopamide.

The main interest of this paper is its contribution to a better knowledge of clopamide behaviour since studies about this diuretic are scarce and some of its effects have not been evaluated yet.

## ACKNOWLEDGEMENTS

The authors thank the Interministerial Commision of Science & Technology (project SAF 93-0464) for financial support and Sandoz Pharma for kindly supplying the clopamide. M. B. Barroso thanks the Spanish Ministery of Education & Science for a FPI grant.

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J. LIQ. CHROM. & REL. TECHNOL., 20(4), 651–659 (1997)

# HPLC MEASUREMENT OF THE NOVEL NON-NUCLEOSIDE ANTI-HIV AGENT, (+)DIHYDROCALANOLIDE A

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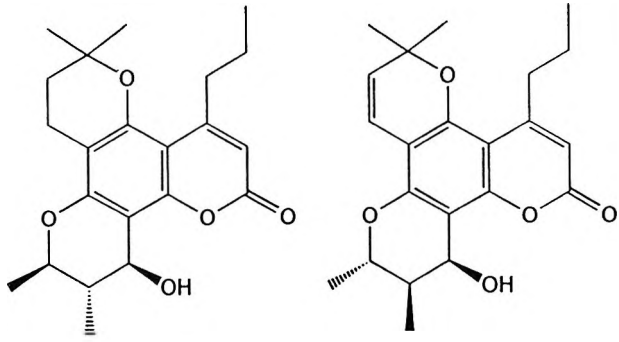
1515 Holcombe Boulevard
Houston, TX 77030

## ABSTRACT

An HPLC assay was established to determine levels of the promising anti-HIV agent (+)dihydrocalanolide A (DHCal-A; NSC 678323) in murine plasma. The structurally related compound costatolide (NSC 661122) was found to be a suitable internal standard. Drugs were extracted from plasma using a solid phase $C_{18}$ cartridge. After injection onto an ODS analytical HPLC column, compounds were eluted using an acetonitrile: water mobile phase. Drugs were quantified over the assay range of 19.5 to 625 ng/mL with excellent within- and between-day reproducibility. Results are obtained from the application of the described method to determination of dihydrocalanolide A pharmacokinetics in mice. This is the first report of a validated HPLC assay for determining dihydrocalanolide A levels in biological fluids such as mouse plasma.




Dihydrocalanolide A Costatolide

**Figure 1**. Structures of (+)dihydrocalanolide A (NSC 678323) and the internal standard, costatolide (NSC 661122).

## INTRODUCTION

(+)Dihydrocalanolide A was produced by chemical modification of extracts of latex from the Malaysian plant *Calophyllum teysmannii var. ionphylloide* as part of a National Cancer Institute sponsored program to identify novel, natural product compounds with anti-HIV activity. While the structures of calanolide and costatolide related compounds have been known since 1964,[1] discovery of the anti-HIV activity of this class of agents is a relatively recent event.[2] In anticipation of potential clinical tests of these compounds, analytical assays were developed which would be suitable for evaluation of bioavailability as well as pharmacokinetic parameters of lead candidate compounds from this series of natural products.[3] Here we present a validated HPLC method for the determination of (+)dihydrocalanolide A in mouse plasma. Also presented is the application of this method towards determination of murine derived pharmacokinetic data.

## MATERIALS AND METHODS

### Reagents and Chemicals

DHCal-A as well as the internal standard, costatolide, were provided as dry powders by the National Cancer Institute (for structures, see Figure 1). Acetonitrile used in the mobile phase was of analytical grade and was obtained

from EM Science (Gibbstown, NJ, USA). Water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA). Mouse plasma was purchased from Pel-Freeze (Rogers, AR, USA).

Stock solutions of DHCal-A and costatolide (1 mg/mL), were prepared in 100% acetonitrile and stored at -20°C. Under these conditions the solutions were stable for at least 1 month.

**Extraction Procedure**

A 25-μL volume of a stock solution of internal standard (25 μg/mL) was added to 1 mL of each plasma sample to be analyzed. After vortex mixing, the sample was loaded onto a conditioned $C_{18}$ Sep-Pak solid-phase extraction cartridge (Millipore, Bedford, MA, USA). The cartridges were then washed with 5 mL of water and the eluate discarded. Drug was eluted with 2 mL of ethyl acetate and the eluants dried under nitrogen. Samples were reconstituted in 250 μL of 100% acetonitrile and transferred to an automatic injector vial for HPLC analyses.

**Calibration Curve and Linearity**

Calibration curves were prepared by spiking control mouse plasma with increasing amounts of DHCal-A and a constant amount of internal standard. Linearity in this matrix was assessed with drug concentrations ranging from 19.5 to 625 ng/mL (19.5, 39, 78, 156, 312 and 625 ng/mL). The ratios of the peak areas for DHCal-A were plotted against those of the DHCal-A concentration to check for linearity, and the correlation coefficient was calculated.

**Chromatography**

The isocratic liquid chromatography system (Waters Assoc., Div. of Millipore, Milford, MA, USA) consisted of a Model 510 solvent delivery pump, a Model 717 autosampler and a Model 990 photodiode array detector. A wavelength of 318 nm was used.

Chromatographic separations were performed on a 250 x 4.6 mm I.D. Spherisorb ODS analytical column, 5 μm particle size (Phenomenex, Torrence, CA, USA). DHCal-A and costatolide were eluted using a mobile phase of 80% acetonitrile in water.

No addition of buffer or adjustment of pH was necessary. The flow rate was 1.2 mL/min. The limit of detection was defined as the lowest concentration of extracted DHCal-A which resulted in a signal-to-noise ratio of 4.

### Reproducibility and Accuracy

Both within- and between-day reproducibilities were determined. Two concentrations of drug (125 and 500 ng/mL) were included in these studies. For within-day reproducibility, six replicates of each sample were tested on the same day and the resulting percent relative deviation (reproducibility) and percent relative error (accuracy) determined. To measure between-day reproducibility, two concentrations of drug (125 and 500 ng/mL) were run in triplicate on each of three separate days. Percent relative deviation and percent errors were determined.

### Calanolide Pharmacokinetics

Preliminary pharmacokinetics of DHCal-A were determined in male $CD_2F_1$ mice obtained from the National Cancer Institute (Bethesda, MD). Drug was prepared for injection by dissolving it in analytical grade dimethylsulfoxide. Mice were administered DHCal-A at a dose of 20 mg/kg by either oral gavage or intravenous routes. Samples of blood were obtained from groups of 3 mice each at 2, 5, 15, 30 min, 1 h, 2 h and 4 h after injection of drug. Plasma was obtained from the blood samples by centrifugation and then frozen at -20°C until analysis. Pharmacokinetic parameters were obtained from the data through the use of the computer program WinNonlin (Scientific Consulting, Inc., Apex, NC).

## RESULTS AND DISCUSSION

This is the first report of an analytical method for determination of DHCal-A concentrations in biological matrices such as plasma. The assay has been successfully applied to determination of pharmacokinetic parameters of DHCal-A in mice following either oral or intravenous administration. Shown in Figure 2 are typical chromatograms obtained from analyses of DHCal-A in mouse plasma. Chromatograms of extracted blank plasma and of extracted plasma containing DHCal-A and costatolide as the internal standard are presented. As can be seen, both DHCal-A and costatolide are resolved from endogenous peaks. Retention times in extracts from mouse plasma were 7.3 min for costatolide and 8.5 min for DHCal-A.

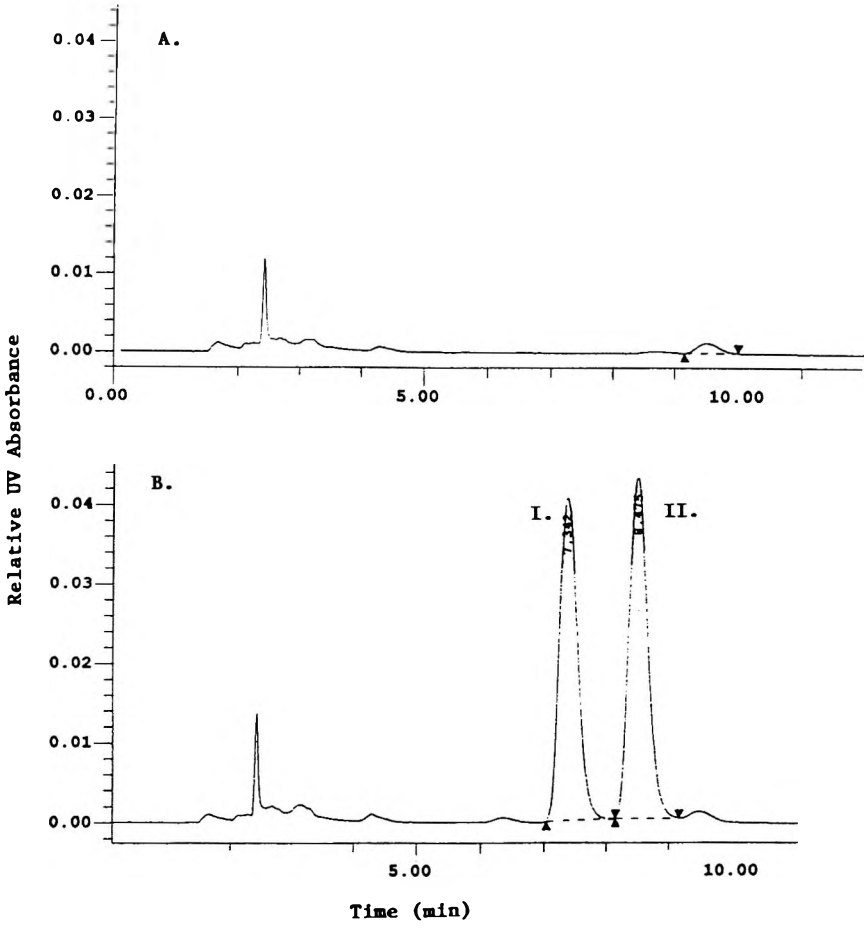



**Figure 2**. HPLC chromatograms of (A) blank murine plasma, (B) murine plasma containing the internal standard, costatolide (peak I: 335 ng) and (+)dihydrocalanolide A (peak II: 300 ng). Detector sensitivity, 0.001 AUFS.

Within-day and between-day reproducibilities for extracted plasma DHCal-A concentrations are shown in Tables 1 and 2, respectively. In Table 1, the relative standard deviations were less than 2%. Percent relative errors ranged from -1.2 to -1.4% in murine plasma. With between-day studies (Table 2), the relative standard deviations were low, ranging from 0.9 to 3.5. Accuracies (as percent relative errors) were also excellent, ranging from -3.5 to 2.4%.

**Table 1**

**Within-Day Reproducibility and Accuracy: (+)Dihydrocalanolide A Assay**

| Nominal Conc. (ng/mL) | Calculated Conc. Mean ± S.D.* (ng/mL) | Percent Relative Deviation[a] | Percent Relative Error[b] |
|---|---|---|---|
| Mouse Plasma | | | |
| 125 | 123.5 ± 1.1 | 0.9 | -1.2 |
| 500 | 492.8 ± 8.3 | 1.7 | -1.4 |

* n = 6

[a] % Relative deviation: [standard deviation (calculated mean)/calculated mean] • 100.

[b] % Relative error: [(calcualted mean - nominal value)/nominal value] • 100.

**Table 2**

**Between-Day Reproducibility and Accuracy: (+)Dihydrocalanolide A Assay**

| Day | Nominal Conc. (ng/mL) | Calculated Con. Conc. Mean ± S.D.* (ng/mL) | Percent Relative Deviation[a] | Perecent Relative Error[b] |
|---|---|---|---|---|
| 1 | 500 | 482.3 ± 9.5 | 2.0 | -3.5 |
| 2 | 500 | 491.2 ± 7.8 | 1.6 | -1.8 |
| 3 | 500 | 500.7 ± 4.4 | 0.9 | 0.1 |
| 1 | 125 | 128.0 ± 1.2 | 0.9 | 2.4 |
| 2 | 125 | 126.0 ± 4.4 | 3.5 | 0.8 |
| 3 | 125 | 125.9 ± 2.2 | 1.7 | 0.7 |

* n = 6

[a] % Relative deviation: [standard deviation (calculated mean)/calculated mean] • 100.

[b] % Relative error: [(calcualted mean - nominal value)/nominal value] • 100.

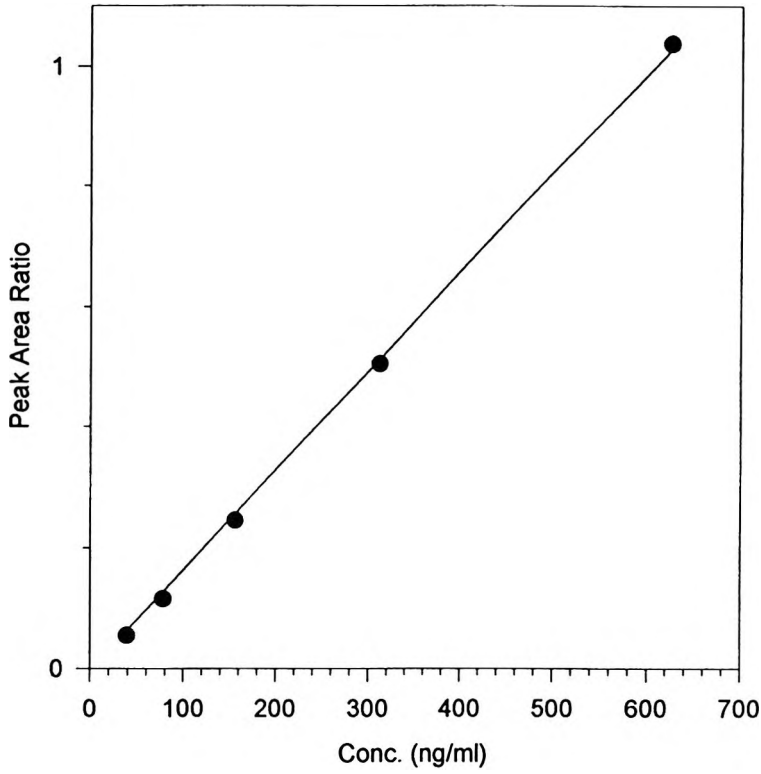



**Figure 3**. Standard curve (working range) for dihydrocostatolide. Data are presented as mean values of triplicate determinations per point; $r^2 = 0.999$.

Using mouse plasma as a matrix, the limit of detection of DHCal-A was 1.5 ng/mL while the useful limit of quantitation was 19.5 ng/mL. The linearity study was carried out over 3 days with concentrations ranging from 19.5 to 625 ng/mL. The coefficient of correlation between DHCal-A/costatolide peak-area ratios and DHCal-A concentrations in plasma was 0.999 (see Fig. 3). The equation resulting from the analysis of the regression plot for DHCal-A in mouse plasma was $y$ *(conc.)* = 5.89 +546.4 $x$ *(peak area ratio)*.

The extraction recoveries (n= 5) from plasma samples spiked with DHCal-A (125 and 500 ng/mL) were 58 ± 7% and 84 ± 9% , respectively. The variances in recoveries observed for different concentrations of DHCal-A were controlled for by use of costatolide as an internal standard.

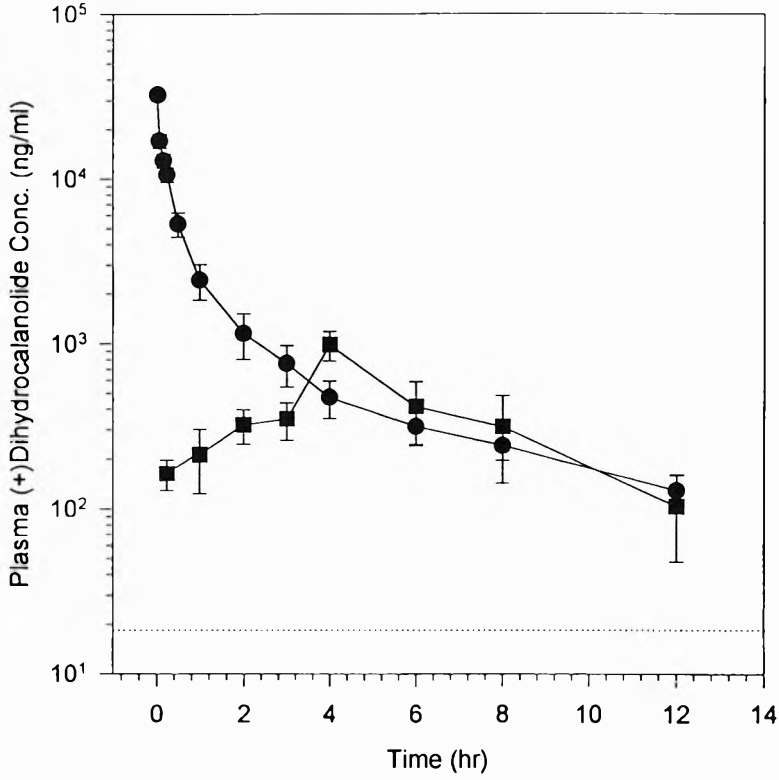



**Figure 4.** Plot of DC-A plasma concentrations versus time obtained with mice administered drug (20 mg/kg) by either oral (-●-) or intrav enous routes (-■-). Data are presented as the mean ± S.D. of determinations from 3 separate mice per time point. The dashed line indicates the lower limit of quantititation.

Drug concentration-time curves for DHCal-A after both i.v. and oral administration are presented in Fig. 4. Pharmacokinetic parameters of dihydrocostatolide after intravenous and oral administration of drug to mice were determined using the pharmacokinetic computer program WinNonlin. The initial estimates of plasma DHCal-A $t_{1/2}\alpha$ and $t_{1/2}\beta$ half-lives (derived from the obtained pharmacokinetic data are 0.013 hr and 2.26 hr, respectively, following i.v. injection and 1.25 hr and 6.10 hr, respectively, after oral administration. The data obtained permitted calculation of F, the fraction of drug absorbed. as a measure of oral bioavailability. This was a reasonable 36%.

## ACKNOWLEDGEMENTS

This investigation was supported in part by N01-CM-57203 and CA-16672 from the National Cancer Institute.

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J. LIQ. CHROM. & REL. TECHNOL., 20(4), 661–662 (1997)

**ERRATUM**

# HPLC SEPARATION OF NADOLOL AND ENANTIOMERS ON CHIRALCEL OD COLUMN

Hassan Y. Aboul-Enein and Laila I. Abou-Basha
J. Liq. Chrom. & Rel. Technol., **19(3)**, 383-392 (1996).

Errors were discovered in Figure 1 and Table 2 of the above article for SQ12150 nadolol enantiomers optical rotation sign. The corrected Figure 1 and Table 2 are given below. The authors apologize for any confusion.

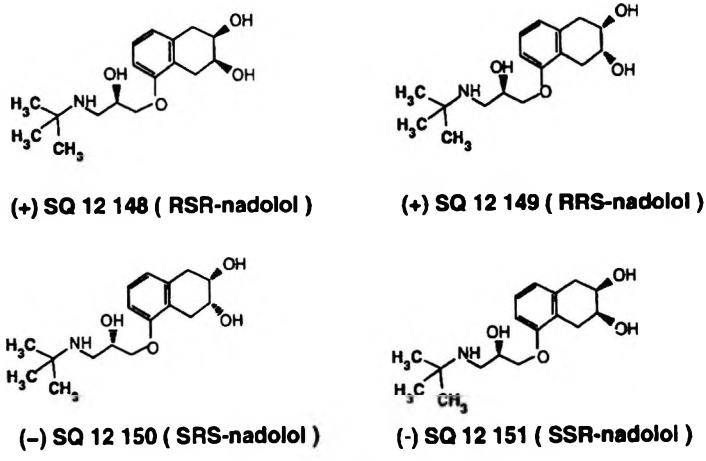


**Figure 1**. Chemical structures of nadolol enantiomers.

**Table 2**

**The Chromatographic Parameters; Capacity (k'), Separation Factors (α), and Optical Rotation of Nadolol Enantiomers**

| Nadolol Enantiomers | k' | α | Optical Rotation |
|---|---|---|---|
| SQ 12148 | 4.24 | 1.13 | (+) |
| SQ 12149 | 3.73 | 0.87 | (+) |
| SQ 12150 | 6.5 | 1.55 | (–) |
| SQ 12151 | 8.25 | 1.25 | (–) |

Mobile phase composition: Hexane:ethanol:diethylamine (85:15:0.4% v/v); see Figure 2 for other chromatographic conditions.

J. LIQ. CHROM. & REL. TECHNOL., 20(4), 663 (1997)

**EDUCATION ANNOUNCEMENT**

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The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Serics Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on Gel Permeation Chromatography for many years.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097, USA. (954) 973-8516; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(4), 665–670 (1997)

# LIQUID CHROMATOGRAPHY CALENDAR

**1997**

**MARCH 16 - 21: PittCon '97, Atlanta, Georgia.** Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

**APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California.** Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

**APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes**, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

**MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California.** Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

**MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California.** Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

**MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium.** Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

**MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan.** Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

**MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY.** Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

**MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois.** Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

**JUNE 1 - 4: 1997 International Symposium, Exhibit & Workshops on Preparative Chromatography: Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington, DC.** Contact: J. Cunningham, Barr Enterprises, 10120 Kelly Road, Box 279, Walkersville, MD 21793, USA. (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

**JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York.** Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

**JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas.** Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

**SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (703) 231-8222.

**SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio.** Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

**OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia.** Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

**OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California.** Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

**OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain.** Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

**OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri.** Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

**NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico.** Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

**NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey.** Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

**1998**

**MARCH 1 - 6: PittCon '98, New Orleans, Louisiana.** Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

**MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington.** Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

**JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia.** Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

**AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

**SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland.** Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

**SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania.** Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

**NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina.** Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

**1999**

**MARCH 7 - 12: PittCon '99, Orlando, Florida.** Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

**MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee.** Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

**2000**

**MARCH 5 - 10: PittCon 2000, Chicago, Illinois.** Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

**MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

**AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**2001**

**APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**2002**

**APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida**. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

**SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

**2003**

**MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**2004**

**MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

**AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

**2005**

**MARCH 13 - 17: 229th ACS National Meeting, San Diego, California.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

**AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC.** Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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