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formerly Journal of Liquid Chromatography

OLUME 19 NUMBER 5 1996

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

March 1996

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CODEN: JLCTFC 19(5) i–iv, 687–852 (1996)
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Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 687–698 (1996)

SIMPLE THIN LAYER CHROMATOGRAPHY METHOD WITH FIBRE OPTIC REMOTE SENSOR FOR FLUORIMETRIC QUANTIFICATION OF TRYPTOPHAN AND RELATED METABOLITES

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ABSTRACT

Tryptophan (TP), 5-hydroxytryptophan (5-HTP), 3-indoleacetic acid (IAA) and serotonin (5-HT) were separated by TLC, with chloroform-methanol-ammonia (12-7-1) (v-v-v) as eluent and cellulose as stationary phase. A fibre optic-based fluorescence instrument for in situ scanning was used for quantitative measurements. The compounds were determined over the range 10-100 ng, with relative standard deviations between 1.70-6.52% and detection limits over the range 16.39-22.50 ng.

INTRODUCTION

Thin layer chromatography (TLC) offers significant advantages for the separation and identification of compounds of analytical interest.[1] In the quantification of analytes separated by TLC, densitometry has proved most useful.[2-4] Recently, as an alternative, the use of fibre optic sensors has been suggested since it

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allows the measurement of fluorescence emitted by fluorophors at some distance from the source of excitation and the detector.[5-8]

The possibility of transporting light from one place to another, by means of optical fibres, facilitates taking readings of TLC plates. Thus it is possible to transmit useful spectral information for qualitative and quantitative analysis with minimal loss of precision and resolution.

Tryptophan (TP), 5-hydroxytryptophan (5-HTP), 3-indoleacetic acid (IAA) and serotonin (5-hydroxytryptamine) (5-HT) are highly important biological compounds that actively participate in biosynthetic routes in both the animal and vegetable worlds.[9-11] Among other aspects of interest, the analysis of some of these derivatives in brain tissue or cerebrospinal liquid is used in the diagnosis of mental disease and nervous disorders.[12]

Owing to their natural fluorescence, 5-HT,[13-15] TP[16] and IAA[17] have been determined spectrofluorimetricaly. Other analytical techniques, such as voltametry[18] and phosphorescence[19] have also been proposed for the quantification of these substances. However, in the analysis of real samples, these determinations lack selectivity, which makes previous separation imperative. Thus, the most important techniques of determination are HPLC, in the normal or inverse phase modes, and electrochemical,[20] UV[21] or fluorescence detection, with chemical derivatization,[22] or without it.[23]

In this study we set out to quantify TP, 5-HTP, IAA and 5-HT with conventional spectrofluorimetry, after separation by TLC, by means of in situ reading of the analytes. Excitation and emission radiations were transmitted by optical fibres.

EXPERIMENTAL

Apparatus

Fluorescence measurements and spectra were made with a Perkin-Elmer LS-50 luminescence spectrometer equipped with a Perkin-Elmer fluorescence plate-reader accessory. A bifurcated fibre optic was used to transfer excitation and emission energy between the plate and the spectrometer. The spectrometer was connected via an RS232C interface to an Epson PCAX2e, containing Fluorescence Data Manager Software (FLDM) that controls the instrument.

Reagents

3-Indoleacetic acid (IAA), 5-hydroxytryptophan (5-HTP) and serotonin-creatinine sulphate (5-HT) were obtained from Aldrich, D-tryptophan (TP) was from Sigma and solvents were purchased from Merck.

Solutions of the indolic compounds were prepared in methanol-water (60-40) (v-v) at a concentration of 1 mg mL^{-1} and diluted as required.

Whatman-41 filter paper, ion exchange chromatography paper (P81 and DE81, Whatman), and TLC plates of silica gel (Merck), cellulose (Merck) and KC18 reverse phase (Whatman) were used as solid surfaces.

All chemicals used were of analytical reagent grade and used without further purification.

Thin Layer Chromatography and Analytical Method

Sample application was by the spray-on technique using a microprocessor-controlled Camag Linomat IV device. Sample volumes of 1-10 μL (containing from 10 to 100 ng of each one of the analytes) were applied to the plates at a rate of 15 $sec \cdot \mu L^{-1}$.

Indolic compounds were chromatographied on 10x10 cellulose TLC plastic sheets, layer thickness 0.1 mm (Merck). The TLC plates were without fluorescence indicator and activated before use. The thin layer plate was developed in chloroform-methanol-ammonia (12-7-1) (v-v-v), light protected, until the solvent migrated a distance of 8 cm up to the plate.

Once the spot corresponding to the analyte had been located, in situ quantitative scans were done at λ_{exc}= 280 nm and λ_{em}= 347 nm, using slits of 5 nm for excitation and emission. As a blank to correct fluorescence intensity measurements, we used the signal corresponding to the dry stationary phase, after elution with the above-mentioned mobile phase.

Procedure for the Determination of 3-Indoleacetic Acid, Tryptophan, 5-Hydroxytryptophan and Serotonin in Serum

A 1 mL aliquot of the serum was deproteinized with 100 μL of perchloric acid

Table 1

Spectrofluorimetric Characteristics of the Indolic Derivatives on Different Solid Surfaces. C_a = 40 ng/μL, Slits 5 nm.

Surfaces	5-HT		TP		IAA		5-HTP	
	$\lambda_{ex}/\lambda_{em}$	I^*_f	$\lambda_{ex}/\lambda_{em}$	I^*_f	$\lambda_{ex}/\lambda_{em}$	I^*_f	$\lambda_{ex}/\lambda_{em}$	I^*_f
Whatman-41	277/337	392	278/346	334	278/361	20	278/337	464
Cellulose	276/347	10	278/350	21	280/358	15	278/337	41
Silica gel	278/336	46	278/338	15	275/348[b]	10	278/337	30
P-81	278/336	210	279/344	210	278/364	8	277/338	264
DE-81	278/336	212	279/344	252	279/335	23	277/337	172
C-18	277/334	118	279/334	212	277/335	8	276/335	127

[b] C_a = 400 ng/μL, Slits 10 nm.

(70%) and centrifuged (speed: 3000 rpm, 5 min.). An aliquot of 400 μL was taken from the supernatant, spiked with 32 μg of each indole and dilute 1:2 with methanol-water (60-40) (v-v). The sample was analyzed in accordance with the analytical method previously described.

RESULTS AND DISCUSSION

The fluorescence emitted by a fluorophor largely depends on the nature of the medium in which it is found. In TLC, the solid constituting the stationary phase is, at the same time, the medium in which solutes are retained after separation occurs. Consequently, selection of the stationary phase must be made bearing in mind its chromatographic properties and its possible influence on the spectrofluorimetric characteristics of the analytes.

The combination of a fibre optic sensor and conventional spectrofluorimeter enables one to obtain spectra of excitation and emission of solutes trapped on a solid surface. Figure 1 shows representative examples of emission spectra of the indolic derivatives considered in this study deposited on a cellulose plate. The main characteristics of 5-HT, TP, IAA and 5-HTP, adsorbed on different solid surfaces, are summarized in Table 1. In general, the excitation wavelengths are similar for all the derivatives. The greatest differences appear in the wavelengths of maximum emission, especially in the case of IAA, although with respect to the spectral band widths these differences are not too significant from an analytical point of view.

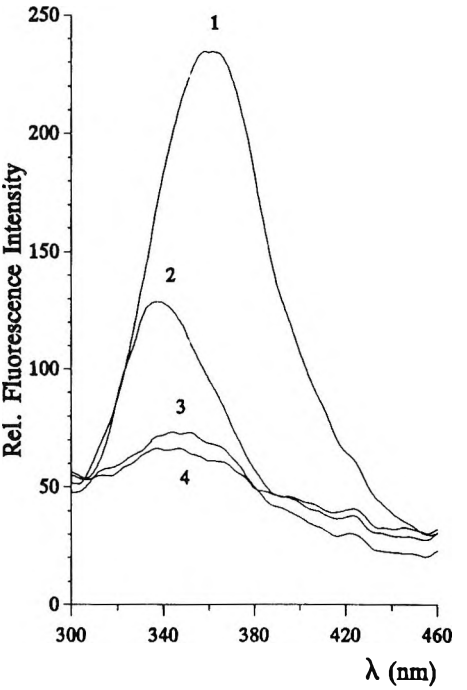

Figure 1. In situ emission spectra of 500 ng IAA (1), 60 ng 5-HTP (2), 60 ng TP (3) and 60 ng 5-HT (4) adsorbed onto cellulose obtained with the fibre optic sensor. Spectrofluorimetric conditions as described in Table 1.

The intensities of fluorescence emitted by TP, 5-HT, IAA and 5-HTP largely depend on the nature of the solid surface used. The corrected values(I^*_f) for this parameter, taking into account the signal emitted by the respective surfaces, are grouped in Table 1. In general terms, the low values of I^*_f, corresponding to IAA, stand out. With regard to the nature of the surfaces, the different types of paper and silica gel provide the greatest intensities of fluorescence.

When different solid surfaces were used as stationary phases for the chromatographic separation of indolic derivatives, the best resolutions were obtained using cellulose plates and silica gel. With the latter, the analytic sensitivity of IAA is far less than that of the other analytes. For this reason cellulose was selected as the stationary phase for the separation and quantification of TP, 5-HTP, 5-HT and IAA.

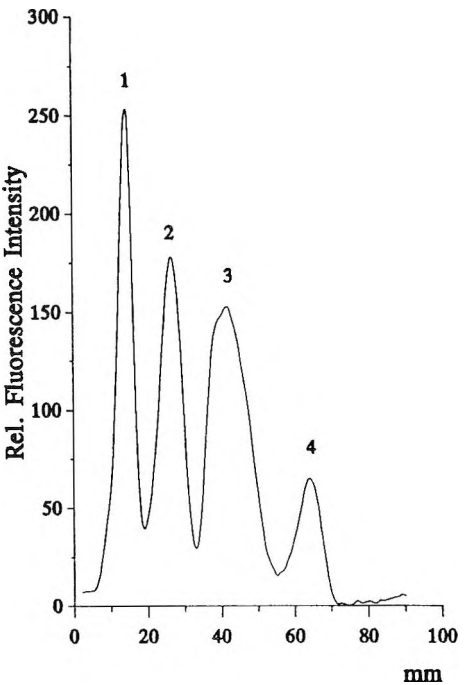

Figure 2. TLC chromatogram of a mixture of 5-HTP (1), TP (2), IAA (3) and 5-HT (4) obtained with the fibre optic sensor. Chromatographic conditions as described in the text. C_a= 100 ng, Slits 5 nm, λ_{ex}=280 nm, λ_{em}=347 nm.

Chromatographic Separation

Once the stationary phase had been chosen, we tested the behavior of different mobile phases used in the separation of similar substances.[24] One of them was the mixture of chloroform-methanol-ammonia (12-7-1) (v-v-v), which provided the best results.

Table 2 shows the most representative chromatographic properties of the indolic derivatives. For the statistical evaluation of the R_f values, we performed nine elutions of each solute in the stationary phase of cellulose, with the selected mobile phase. For a probability of 95%, uncertainty in R_f values ranged between ±0.001 and ±0.028. Coefficients of Variation (CV) showed that the dispersion of results corresponding to serotonin was considerably lower than those of the other analytes.

Table 2

Chromatographic Characteristics of the Indolic Derivatives
C_a= 40 ng/μL, Slits 5 nm, λ_{ex}=280 nm, λ_{em}=347 nm.

Compound	Rf ± I	CV(%)	R
5-HTP	0.015 ± $7 \cdot 10^{-4}$	5.52	
			1.85
TP	0.193 ± 0.015	9.53	
			1.5
IAA	0.370 ± 0.028	8.16	
			2.3
5-HT	0.846 ± 0.013	1.90	

Figure 2 shows a representative chromatogram of the separation of the four indolic derivatives. For each pair of analytes eluted consecutively, the spatial resolving power of the chromatographic system was calculated by means of the following expression:

$$R = \frac{P_2 - P_1}{(W_2 + W_1)/2} \qquad (1)$$

in which P_2 and P_1 represent the position of the adjacent peaks in mm, while W_2 and W_1 are the peak width at half-height. R values as ranged from 1.5 to 2.3.

Analytical Characteristics

Analytical determinations on solid surfaces are strongly conditioned by background signal and reproducibility of the measurements.

Radiation reaching the detector consists of background signal superimposed on fluorescence emitted by the components of the sample. Thus the ability of the system to detect and measure the sample emission is limited by the magnitude of the background signal and noise.

The background signal is generally elevated by the high amount of diffusely

Table 3

S/N Ratios for the Detection of 40 ng of Each Compound Spotted on Silica Gel After Elution

Compound	E_{bk}	E_S	σ_s	σ_{bk}	S/N
5-HTP	50.33	30.76	2.43	1.78	10.2
TP	50.99	15.61	2.89	0.72	5.2
IAA	47.82	23.19	2.08	1.55	8.9
5-HT	48.37	18.06	1.19	2.37	6.8

Table 4

Representative Statistical Parameters of the Analytical Methods

Compound	LDR(ng)	LOD(ng)	S_M	CV(%)	E(%)
5-HT	10 - 100	19.56	0.40	1.70	2.90
TP	10 - 100	19.32	0.95	4.00	3.27
IAA	10 - 100	22.50	0.76	2.84	2.50
5-HTP	10 - 100	16.39	0.86	6.52	5.80

scattered radiation. Moreover, the use of a fibre optic to transport the radiation allows a certain amount of light from the surroundings to be added to the fluorescence emitted by the analyte, thus increasing the background signal. Table 3 shows the values obtained for the signal/noise ratios for each of the compounds studied, using the expression:[25]

$$S/N = E_s / (\sigma_s^2 + \sigma_{bk}^2)^{1/2} \qquad (2)$$

in which E_s is the analytical signal, obtained by subtracting the blank signal from the total, while σ_s and σ_{bk} express the standard deviations of the analytical and blank signals respectively. A minimum of nine determinations were performed for each of the parameters included in the expression (2).

The quantification of the solutes separated by TLC was seen to be notably

Table 5

Recoveries of Tryptophan, Serotonin, 3-Indoleacetic Acid and 5-Hydroxytryptophan in Enriched Samples of Deproteinized Rat Serum.

Compounds	Added (ng)	Found (ng)	R(%)
5-HT	50	48.50	97.20
	100	107.2	107.21
TP	40	39.60	99.00
	80	78.79	98.49
IAA	40	42.31	105.78
	80	75.44	94.30
5-HTP	40	38.44	96.10
	80	78.44	98.05

attained in the sample application and in the measurements and efficacy provided by the chromatographic system.

For the four indolic derivatives, fluorescence intensities, registered as peak heights, showed a lineal function for concentrations below 100 ng. In all cases the correlation coefficients were higher than 0.996. Table 4 shows the most representative statistical parameters of the analytical methods established, as well as the detection limits.

Coefficients of Variation were relatively small, with the greatest dispersion of results corresponding to TP and 5-HTP determinations. Detection limits[26] oscillated between 16.39 and 22.50 ng. being higher than those found in the literature where HPLC was used.[11]

However, the detection limit obtained in this study for IAA was approximately half of that described by others after derivatization with o-phthalaldehyde (OPA) by TLC.[27]

By means of the elution of synthetic samples containing variable quantities of the four analytes studied, from 40 to 80 ng, we obtained mean recoveries ranging from 87.2% to 119.67%.

Determinations in Rat Serum

In order to test the applicability of the methods established for the determination of TP, 5-HT, IAA and 5-HTP in complex matrices, we used samples of deproteinized rat serum,[28] enriched by the addition of dissolutions containing different amounts of each of the four analytes. The results obtained, Table 5, show recoveries whose values range from 94.3% to 107.2%.

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Received May 20, 1995
Accepted June 8, 1995
Manuscript 3882

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 699–709 (1996)

SIMULTANEOUS DETERMINATION OF ZOPICLONE AND ITS TWO MAJOR METABOLITES (N-OXIDE AND N-DESMETHYL) IN HUMAN BIOLOGICAL FLUIDS BY COLUMN LIQUID CHROMATOGRAPHY AFTER SOLID-PHASE EXTRACTION

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ABSTRACT

A reverse phase liquid chromatographic procedure with fluorescence detection for the simultaneous determination of zopiclone and its main metabolites, N-desmethylzopiclone and zopiclone-N-oxide, in serum, blood and urine is described. An aliquot (0.5 - 1 mL) of the sample after the addition of 0.25 mL of 250 ng/mL solution of harmane in 0.2 M NaH_2PO_4 as the internal standard is passed through a 1-mL BondElut C_{18} silica extraction column. The column is selectively washed with water and acetonitrile to remove polar, neutral and acidic compounds. The desired compounds are eluted with a 0.25 mL aliquot of a mixture of methanol + 35% perchloric acid (100:1 v/v). A 10 - 25 μL aliquot of the eluate is injected onto a 150 X 4.6 mm I.D. column packed with 5-μm C_{18} silica particles which is eluted at ambient temperature with a mobile phase of acetonitrile - 0.1% tetramethylammonium perchlorate (17:83 v/v) adjusted to pH 3.8

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with 10% perchloric acid at a flow rate of 1.8 mL/min. The peaks are detected with a fluorescence detector (ex = 320 nm, em = 520 nm). The extraction recovery of all the compounds is in the range of 90 - 95%. The chromatogram is clean and the desired peaks are well separated from each other and from extraneous peaks.

INTRODUCTION

Zopiclone (Imovane®) belongs to a novel chemical class (cyclopyrrolone) of hypnotics-sedatives which is structurally unrelated to benzodiazepines or barbiturates. However, the pharmacological profile of zopiclone is similar to that of benzodiazepines.[1] Like other sedatives, zopiclone also has a considerable potential of being ingested in overdose. Severe overdose with zopiclone produces somnolence, confusion and coma with reduced or absent reflexes. Though the treatment of zopiclone overdose like that of benzodiazepines overdose is supportive in response to clinical signs and symptoms, it is desirable to include zopiclone in hypnotic-sedative screen in clinical and forensic laboratories.

Mannaert and Daenens[2] have recently described a radioimmunoassay for the determination of N-desmethylzopiclone, the most persistant metabolite of zopiclone. However, immunoassay reagents for the detection or determination of zopiclone or its metabolites are not yet commercially available. Antibodies for barbiturates or banzodiazepines do not show any cross reactivity with this drug. Relatively low serum peak drug concentration of 20 - 60 ng/mL observed after a therapeutic dose of 7.5 mg of zopiclone does not allow the use of spectrophotometric or spectrofluorometric procedures for the determination of zopiclone. Gas chromatgraphy is not particularly suitable as zopiclone is thermally labile and produces multiple peaks.[3,4]

At present, column liquid chromatgraphy (LC) appears to be the most suitable technique for the identification and quantification of zopiclone in biological fluids. A number of LC procedures using fluorescence[5-8] or UV absorbance detection[3,9,10] have been described for the determination of zopiclone in serum/blood. However, only Liboux et al.[7] describe the simultaneous determination of zopiclone and its metabolites in serum and urine. Analysis of urine is required for forensic detection of zopiclone use because of the short half-life (3.5 h) of zopiclone. A high performance thin layer chromatographic procedure for the determination of zopiclone has also been described.[12]

In all the LC procedures described so far, the sample has been extracted by liquid/liquid extraction and the extract evaporated prior to chromatography. Gaillard et al.[4] have described a solid-phase extraction (SPE) procedure requiring evaporation of the extract. In this procedure, zopiclone is isolated as its decomposition product for its determination by gas chromatography. Now I describe a rapid SPE procedure which allows the direct injection of the extract without its prior evaporation for the simultaneous determination of intact zopiclone and its main metabolites.

EXPERIMENTAL

Materials

Stock solutions of zopiclone, N-desmethylzopiclone and zopiclone-N-oxide (all from Rhone-Poulenc Rorer, Canada) of 1 mg/mL each were prepared in acetonitrile. The solutions were stored at -20°C.

A serum standard of only zopiclone of 2 μg/mL and a urine standard of zopiclone and its two metabolites of 2 μg/mL each were prepared in blood bank outdated plasma and drug free pooled urine. Serum standard was serially diluted to prepare 8 standards and the urine standard was serially diluted to prepare 6 standards.

Stock internal standard (IS) solution of 1 mg/mL of harmane hydrochloride (Sigma Chemical Co. St.Louis, MO) was prepared in methanol and stored at -20°C. Working IS solution was prepared by diluting 5 μL of the stock solution with 20 mL of 0.2 M NaH_2PO_4 This solution was stored at 4°C for one week.

Procedures:

Extraction

The required number of 1-mL BondElut C_{18} extraction columns (Varian, Harbor City, CA, USA) was placed on a VacElut system. The columns were washed once (one column volume) with 1 M HCl , twice with methanol and once with water, each time aspirating the liquid completely with suction. A

0.25 mL aliquot of the working IS solution was placed in each column, then 1 mL of serum sample or supernatant of blood hemolysate (prepared by diluting 1.0 mL of blood with 0.5 mL of water and centrifugation) or 0.5 mL of urine sample was applied. The liquid was allowed to pass through the columns at a slow rate of about 1 mL/min.. using mild suction. The columns were washed twice with water and twice with 0.5 mL aliquots of acetonitrile making sure that each column was drained completely after every wash. The tips of each column was wiped with tissue and placed on 16 X 100 mm glass tubes containing correspondingly labelled 1.5 mL plastic sample cups. An aliquot of 0.25 mL of methanol containing 1 mL/100 mL of 35% perchloric acid was applied to each column. The liquid was allowed to pass through the column bed by gravity and finally drained completely by centrifugation for 20 s. The cups were covered with aluminium foil and loaded in the autosampler. A 25-µl aliquot of serum extract or a 10-µL aliquot of urine extract was injected onto the chromatographic system.

Chromatography

A modular chromatographic system comprising of a Model LC-6A pump, a Model SPD-10A absorbance detector, a Model RF-535 fluorescence detector and a Model CR-501 integrator plotter (all from Shimadzu Scientific Co., Columbia MD, USA) was used. A 150 X 4.6 mm I.D. Ultrasphere ODS reverse phase column packed with 5-µm C_{18} bonded silica particles (Beckman Instruments, San Ramon, CA, USA) protected by a 15 X 3.2 mm I.D. RP-18 guard cartridge packed with 7-µm silica particles (Applied Biosystems, San Jose, CA, USA) was used as the analytical column. A mobile phase consisting of acetonitrile - 0.1% tetramethylammonium perchlorate (17:83 v/v) adjusted to pH 3.8 with 10% perchloric acid was pumped at a flow rate of 1.8 mL/min. resulting in an operating pressure of 12 Mpa. Chromatography was performed at ambient temperature. The fluorescence was monitored at 520 nm (excitation at 320 nm).

RESULTS AND DISCUSSION

Detection

Zopiclone has been determined by both UV absorbance[3,9,10] and fluorescence[5-8] detection. The two modes of detection were compared by connecting the exit of the absorbance detector to the inlet of the fluorescence

detector. There was virtually no distortion in the fluorescence peaks. Both detectors working optimally were set for maximum possible sensitivity. The fluorescence response of zopiclone and its two metabolites (ex = 310 nm; em = 500 nm) was about 4 times that of absorbance detection (305 nm) with a stable base line for both the detectors. Extracts of serum samples showed clean chromatograms by both detection modes. However, extracts of some urine samples showed additional extraneous peaks than observed by fluorescence detection. Therefore, fluorescence detection was selected for quantification of zopiclone and its metabolites. Simultaneous UV/fluorescence detection is used to confirm the identification of zopiclone and its metabolites by comparing the ratios of peak areas of absorbance/fluorescence of the unknown peak to that of the zopiclone standard.

Internal Standard

Tracqui et al.[10] did not use any internal standard as this publication describes only a screening procedure for the detection of zopiclone and other analogous sedatives. Royer-Morrot et al.[9] used dihydroquinidine as the internal standard for the determination of zopiclone in plasma. This compound is present in the blood of patients receiving quinidine therapy and elutes close to quinidine. Foster et al.[11] have used chlordiazepoxide as the IS for stereospecific assay of zopiclone. However, this compound does not appear to be a suitable compound as an IS for toxicological and forensic determinatioon of zopiclone as chlordiazepoxide is a commonly prescribed sedative drug.

In all other procedures,[3,5-8] a quinolyl analogue of zopiclone (RP 29481) has been used as the internal standard. It is an appropriate compound to be used as an IS for the determination of zopiclone. However, the chromatographic run time in the procedure of Liboux et al.[7] was more than 35 min. and there is an interval of about 20 min. between the last peak of the IS and the preceeding peak of zopiclone. In an attempt to reduce the chromatographic run time, a number of commercially available fluorescent non-drug compounds were screened for use as an IS for the simultaneous determination of zopiclone and its metabolites. Harmane proved to be the most suitable compound. It elutes as the first peak in the selected chromatographic system. The chromatographic run time of 20 min. in the present system is still quite long. However, this long time is due to the requirement of baseline separation of zopiclone from its metabolite N-desmethylzopiclone. Chromatographic time can be reduced to about 12 min. by increasing the acetonitrile content to about 25%, when the presence of only zopiclone is expected in a given sample. Harmane is highly fluorescent at the optimal

conditions of fluorescence of zopiclone (ex = 310 nm; em = 500 nm). Fluorescence response of harmane is reduced to one-half at ex = 320 nm and em = 520 nm with only a 10% reduction in the fluorescence of zopiclone. These settings were selected as a compromise so that harmane could be monitored by both fluorescence and UV absorbance detection. It is not possible to do so if harmane fluorescence peak is reduced by decreasing the concentration of the IS solution. These settings also reduce the fluorescence of procainamide, quinidine and quinine and other indole compounds.

Extraction

Liboux et al.[7] have used a mixture of dichloromethane and 2-propanol to extract biological samples for the isolation of zopiclone and metabolites. It seems that the extraction recovery of the various compounds has not been described in this publication. The SPE procedure described in this report is environmentally friendly as water immiscible solvents, particularly halogenated hydrocarbons, are not used. The extraction recovery determined by comparing the peak areas of extracts of serum and urine of 0.1 and 1 μg/mL of each compound with those of unextracted standards of corresponding concentrations showed extraction recovery of each compound in the range of 90 - 95%. The internal standard, harmane, behaves similarly to zopiclone during extraction as there is no change in the ratios of peak areas of analyte/IS after extraction by the described SPE.

Gaillard et al.[4] have used a SPE for the isolation of zopiclone only and give the impression that zopiclone is completely converted to a decomposition product during extraction. Some compounds do undergo decomposition during SPE because compounds are exposed to large volumes of air during wash and elution steps. However, there is no indication of any change in the structure of zopiclone or that of zopiclone metabolites as a result of SPE. The retention times of these compounds in the methanolic extract are identical to those observed when an unextracted acetonitrile solution of these compounds is chromatographed.

It is now well established that zopiclone is unstable in nucleophilic solvents such as methanol or ethanol[3] even when stored at 4°C or even at -20C. However, this decomposition is quite slow. Zopiclone concentration decreased by about 20% over a period of 4 weeks when 1 mg/mL methanolic solution of zopiclone was stored at 4°C. The methanolic eluate did not show any decrease in the peak area of any of the three compounds or show any change in the ratio of peak area of analyte/IS when the extract was stored at room temperature for

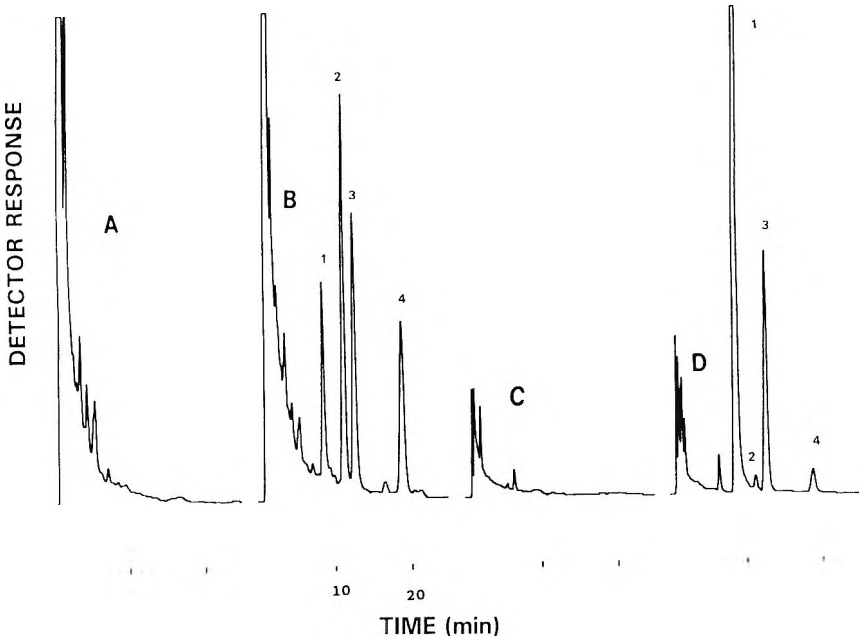

Figure 1. Chromatograms of extracts obtained from (A) drug free urine; urine of a volunteer collected 15 h after the ingestion of a 15-mg dose of zopiclone; (C) drug free serum; (D) serum 1.5 h after zopiclone dose. Peaks: 1 = Harmane (8.3 min.), 2 = N-desmethylzopiclone (10.8 min.), 3 = zopiclone (12.2 min.), 4 = zopiclone-N-oxide (18.1 min.). Detector: signal output 1 V, sensitivity = high, response time = slow. Integrator: attenuation = 2, chart speed 2 mm/min.

5 h or at 4°C for 24 h. There was a 20% decrease in the peak areas of each of three compounds when the extract was kept at 60°C for 1h in a stoppered tube to avoid evaporation. The extraction recovery of zopiclone is reduced to about 70% when the extraction column is eluted with a 0.25 mL aliquot of acetonitrile containing 1% of 35% perchloric acid.

Chromatograms of extracts of drug free urine (Fig. 1A) and of drug free serum (Fig. 1C) show the absence of extraneous peaks from the biological matrices or materials of extraction columns after the solvent peaks.

Method Validation

The relationship between the ratios of peak areas of zopiclone/IS (**y**) and

Table 1

Precision and Accuracy of the Method

	Zopiclone			N-Desmethylzopiclone			Zopiclpone-N-oxide		
	Mean ng/mL	%CV	%Dev[a]	Mean ng/mL	%CV	%DEV	Mean ng/mL	%CV	%DEV
Within Batch (n=8)									
u-low	120	2.6	-4.0	238	2.9	-4.9	251	2.8	+0.4
u-high	1263	2.2	+1.0	2410	2.2	-3.6	2510	1.2	+0.4
s-low	53	3.3	+6.0		---			---	
s-high	1000	1.4	0.0		---			---	
Between Batch (n=8)									
u-low	117	10.4	-6.4	242	5	-3.2	248	5.3	-0.8
u-high	1168	3.8	-6.6	2285	2.2	-9.4	2439	3.4	-2.4
s-low	51	11.1	+2.0		---			---	
s-high	1016	5.3	+1.6		---			---	

[a]Bias from the spiked value

the serum zopiclone concentration (x) is linear and the curve passes through the origin ($y = -0.01 + 0.883x$, $r^2 = 1.000$). The relationship between the peak area ratios of analyte/IS and urine zopiclone and its metabolite concentrations are also linear and the curves pass through the origin ($y = -0.03 + 0.048x$, $r^2 = 0.998$ for zopiclone; $y = 0.04 + 0.03x$, $r^2 = 0.995$ for N-desmethylzopiclone and $y = -0.031 + 0.054x$, $r^2 = 0.997$ for zopiclone-N-oxide).

Sensitive fluorescence detection and high extraction recovery allow quite low limits of quantitation. Zopiclone and its metabolites can be quantitated down to 2 ng/mL in serum and 10 ng/mL in urine. The sensitivity of detection can be further improved by injecting a larger volume of the extract. There is no distortion of peaks when up to 40 µl of the extract are injected. Analysis of serum spiked with zopiclone and of urine spiked with zopiclone and its metabolites showed acceptable precision (Table 1).

Fig. 1B shows a chromatogram of an extract of a random urine sample

obtained from a volunteer who had ingested a 15-mg dose of zopiclone, 15 hours prior to sample collection. This 64 yr old male volunteer has been on chronic therapy of 20 mg/day of glyburide, 1 g/day of metformin and 325 mg/day of aspirin. The concentration of zopiclone corresponds to 1.2 μg/mL; of N-desmethylzopiclone to 1.3 μg/mL and of zopiclone-N-oxide to 0.84 μg/mL. Fig. 1D shows a chromatogram of an extract of serum obtained from blood collected 1.5 h after the ingestion of zopiclone dose. The concentration of zopiclone corresponds to 57 ng/mL. It is generally believed that zopiclone metabolites are non-detectable in serum after therapeutic or mild overdoses (6). It is interesting that in this case both N-desmethylzopiclone (4 ng/mL) and zopiclone-N-oxide (8 ng/mL) can be observed in serum after only a high therapeutic dose of zopiclone. Renal and liver function tests are normal in this individual.

Specificity

The described procedure has a high specificity due to selective extraction and fluorescence detection in the visible range. Acidic and neutral compounds including barbiturates, salicylates and acetaminophen are removed during wash steps with acetonitrile. However, basic drugs including benzodiazepines, if present are co-extracted. Only a few drugs show fluorescence at the selected excitation and emission wavelengths.

In a number of LC procedures, some basic drugs e.g. morphine, codeine, beta blockers (atenolol, metoprolol, nadolol etc.) and antidepressants (paroxetine, fluoxetine, impramine etc.) have been determined by monitoring their native fluorescence under optimal conditions for the detection of these drugs. However, under the present conditions these drugs show poor response. Foster et al.[11] have used chlordiaepoxide as the IS for the determination of zopiclone enantiomers by LC with fluorescence detection. However, in the present procedure, chlordiazepoxide and other benzodiazepines show very poor fluorescence response. Further, benzodiazepines and antidepressants which are commonly ingested in overdose, elute after zopiclone-N-oxide and do not interfere with the assay of zopiclone and its metabolites.

CONCLUSION

This report describes a simple procedure which is quite suitable for use in routine clinical laboratories for sensitive screening and quantification of

zopiclone and its major metabolites, N-desmethylzopiclone and zopiclone-N-oxide.

ACKNOWLEDGEMENTS

Thanks are due to Ms Barbara O'Donoghue of Rhone-Poulenc Rorer, Canada, for arranging to provide us a gift of pure compounds of zopiclone, quinolyl analogue of zopiclone (RP29481), N-desmethylzopiclone (RP 32773) and zopiclone-N-oxide (RP29753).

Miss Abha Gupta helped with the library search and in the preparation of the manuscript.

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Received September 14, 1995
Accepted October 12, 1995
Manuscript 5131

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 711–718 (1996)

HPLC DETERMINATION OF AN ONDANSETRON AND DIPHENHYDRAMINE MIXTURE IN 0.9% SODIUM CHLORIDE INJECTION

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ABSTRACT

A high performance liquid chromatography procedure has been developed for the assay of an ondansetron hydrochloride and diphenhydramine hydrochloride mixture in 0.9% sodium chloride injection. The separation and quantitation were achieved on a 5-μm Spherisorb ODS-1 column at ambient temperature using a mobile phase of 60:40 v/v 0.1 M phosphate buffer pH 4.5-acetonitrile at flow rate of 1.2 mL/min. with detection of both analytes at 210 nm. The separation was achieved within 22 min. with sensitivity in the ng/mL range for each analyte. The method showed linearity for ondansetron and diphenhydramine in the 0.40 - 6.40 and 5.0 - 80.0 μg/mL ranges, respectively. Intra- and inter-day RSD values were 1.8% and 2.8 - 3.8% for ondansetron, and 1.4 - 1.7% and 2.0 - 2.7% for diphenhydramine, respectively. Accuracy of intra and inter-day were in the 1.0 - 1.6% and 1.2% for ondansetron and 0.7 - 2.0% and 0.3 - 3.8% for diphenhydramine, respectively. The limits of detection for ondansetron and diphenhydramine were 70 and 105 ng/mL, respectively, based on a signal to noise ratio of 3 and a 20 μL injection.

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INTRODUCTION

A mixture of ondansetron hydrochloride and diphenhydramine hydrochloride can be administered as a perioperative injection in a hospital operating room. Interest in our laboratories in the stability and compatibility of the drug mixture over time in 0.9% sodium chloride injection required the development of an HPLC method. A search of the literature indicated that an HPLC method was not available to assay for both compounds concurrently with a single injection.

Ondansetron has been assayed by high performance thin layer chromatography (HPTLC) and HPLC methods.[1-3] The HPTLC method was developed especially for plasma samples, but the sample throughout was low and the equipment is not generally available in most laboratories. The HPLC assays used either a silica column with an aqueous-organic mobile phase or a cyanopropyl column operated in the reverse phase mode.

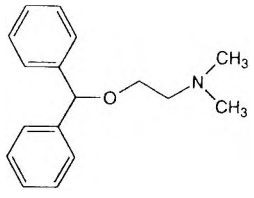
Assay methods for diphenhydramine hydrochloride have included spectrophotometry,[4-9] HPLC[10-13] and GC.[14,15] The HPLC methods are the most common of the procedures reported and have involved the separation of the drug on an octadecylsilane column. The official USP 23 assay for diphenhydramine hydrochloride injection utilizes reverse phase chromatography on a nitrile column.[16]

In this paper, an isocratic HPLC assay is presented that will simultaneously analyze ondansetron and diphenhydramine hydrochlorides in 0.9% sodium chloride injection using a single injection. The compounds are separated on an octadecylsilane column using a buffered aqueous - acetonitrile eluent. The separation is achieved within 22 min. at ambient temperature with sensitivity in the ng/mL range.

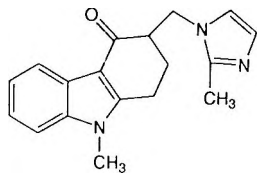
EXPERIMENTAL

Regents and Chemicals

The structure formulae of the compounds studied are shown in Figure 1. Diphenhydramine hydrochloride was purchased from Parke, Davis (Morris Plains, NJ 07950). Ondansetron hydrochloride (Batch C662/116/1) was a gift from Glaxo, Inc. (Research Triangle Park, NC 27709). Methyl paraben and propyl paraben were purchased from Sigma Chemical Co. (St. Louis, MO



DIPHENHYDRAMINE



ONDANSETRON

Figure 1. Chemical structures of compounds studied.

63178). Acetonitrile (J.T. Baker, Phillipsburg, NJ 08865) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, GA 30076). Monobasic potassium phosphate was Baker analyzed reagent.

Instrumentation

The chromatographic separation was performed on an HPLC system consisting of a Beckman Model 110B Solvent Delivery Module (San Ramon, CA 94583), an ABI Model 759A UV-VIS Variable Wavelength Detector (Foster City, CA 94404) and an HP Model 3392A Integrator (Hewlett-Packard Company, Avondale, PA 19311). Separation was accomplished on a 5-μm Spherisorb ODS-1 column (250 x 4.6 mm i.d. Keystone, Bellefonte, PA

16823) equipped with a direct-connect ODS guard column at ambient temperature (23 ± 1°C). The mobile phase consisted of 60:40 v/v 0.1 M monobasic potassium phosphate pH 4.5 - acetonitrile. The mobile phase was filtered through a 0.45 μm Nylon-66 filter (MSI, Westborough, MA 01581) and degassed by sonication prior to use. The flow rate was set at 1.2 mL/min. and the detector was set at 210 nm.

Preparation of Standard Solutions

A combined standard solution containing ondansetron hydrochloride and diphenhydramine hydrochloride was prepared by accurately weighing 1.0 mg of ondansetron hydrochloride and 12.5 mg of diphenhydramine hydrochloride, transferring to a 10-mL volumetric flask, manually shaking for 10 min. and 0.9% sodium chloride injection containing 1.2 mg/mL of methyl paraben and 0.15 mg/mL of propyl paraben added to volume. Dilutions (8:125, 4:125 and 1:250) of the standard solution were made in mobile phase to obtain mixtures containing 6.4, 3.2 and 0.40 μg/mL of ondansetron, and 80.0, 40.0 and 5.0 μg/mL of diphenhydramine hydrochloride. Three point calibration curves were constructed for each analyte. Additional dilutions (1:19.5 and 1:125) of the combined standard solution were prepared in mobile phase to serve as spiked samples for each analyte to determine accuracy and precision of the method. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration in μg/mL.

RESULTS AND DISCUSSION

The goal of this study was to develop an isocratic HPLC assay for the analysis of an ondansetron and diphenhydramine mixture in 0.9% sodium chloride injection. Stability studies of the mixture would require an assay procedure that would detect and quantitate each analyte with reasonable accuracy and precision.

There were no reports in the scientific literature describing a separation of ondansetron and diphenhydramine hydrochlorides in a single injection. To develop a single isocratic HPLC method for these two analytes, our investigation indicated that chromatographic separation of the two compounds was best performed on the octadecylsilane column with a 60:40 v/v 0.1 M aqueous phosphate buffer pH 4.5 - acetonitrile mobile phase.

It was found that the ionic strength of the mobile phase was the

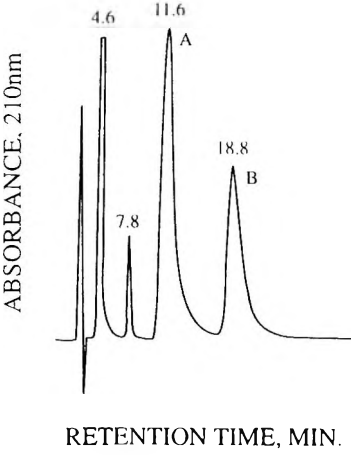


Figure 2. Typical HPLC chromatogram of ondansetron (A) and diphenhydramine (B) on an octadecylsilane column with acetonitrile - aqueous phosphate buffer pH 4.5 mobile phase. The peak at 4.6 min. retention time is methyl paraben and 7.8 min. is propyl paraben. See Experimental Section for assay conditions.

predominant parameter affecting retention of the analytes. Increasing the ionic strength significantly decreased retention times. Since the retention time of the analytes was also affected by the acetonitrile composition, 40% acetonitrile in the mobile phase offered the best separation of ondansetron and diphenhydramine hydrochloride in the shortest run time with no interference from the preservatives methyl paraben and propyl paraben commonly found in some commercial injections. A typical chromatogram of the two analytes is shown in Figure 2.

The HPLC method showed concentration versus absorbance linearity for ondansetron and diphenhydramine hydrochlorides in the 0.40 - 6.4 and 5.0 - 80.0 μg/mL ranges, respectively, at 210 nm. Table 1 gives other analytical figures of merit for each analyte.

Percent error and precision of the method were evaluated using spiked samples containing each analyte. The results shown in Table 2 indicate that the procedure gives acceptable accuracy and precision for the two analytes.

In summary, an octadecylsilane column with an aqueous 0.1 M phosphate buffer pH 4.5 - acetonitrile mobile phase has been shown to be amenable for the separation and the quantitation of an ondansetron - diphenhydramine

Table 1

Analytical Figures of Merit for Ondansetron and Diphenhydramine

Analyte	r^{2a}	System Suitability[b]	LOD[c] ng/mL	k'	Theoretical Plates[d]	Tailing Factor[c]	Rs
Ondan-setron	0.9994	1.8	70	3.7	1767	1.1	2.7
Diphen-hydramine	0.9999	1.3	105	6.6	2209	1.2	

[a]Range examined from 0.40 - 6.40 μg/mL ondansetron (n = 6) and 5.0 - 80.0 μg/mL diphenhydramine (n = 6). Mobile phase consisted of 60:40 v/v 0.1M phosphate buffer pH 4.5 - acetonitrile at 1.2 mL/min. with detection at 210 nm; [b]RSD % of 5 replicate injections at 0.80 μ/mL ondansetron and 10.0 μg/mL diphenhydramine at 210 nm; [c]Limit of detection, S/N = 3; [d]Calculated as N = 16 $(t/w)^2$; [c]Calculated at 5% peak height.

Table 2

Intra- and Inter-day Accuracy and Precision for Analysis of an Ondansetron (OND) and Diphenhydramine (DPH) Mixture

Concentration Added (μg/mL)	Concentration Found[a] (μg/mL)	RSD (%)	Error (%)
OND Intra-day[b]			
0.80	0.81 ± 0.01	1.8	1.0
5.12	5.04± 0.09	1.8	1.6
Inter-day[c]			
0.80	0.79 ± 0.03	3.8	1.2
5.12	5.06 ± 0.14	2.8	1.2
DPH Intra-day[b]			
10.0	9.80 ± 0.14	1.4	2.0
64.0	63.53 ± 1.10	1.7	0.7
Inter-day[c]			
10.0	9.62 ± 0.19	2.0	3.8
64.0	63.80 ± 1.70	2.7	0.3

[a]Mean ± std. dev. based on n =3; [b]n = 5; [c]n = 25.

hydrochlorides mixture in 0.9% sodium chloride injection. The method is free of interference from methyl and propyl parabens. This study suggests that the HPLC method can be used to investigate the chemical stability of the two drugs in sodium chloride injection.

ACKNOWLEDGEMENTS

The authors are grateful to Glaxo Research Institute for financial assistance.

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Received September 22, 1995
Accepted October 10, 1995
Manuscript 3975

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 719–736 (1996)

CHROMATOGRAPHIC METHOD VALIDATION: A REVIEW OF CURRENT PRACTICES AND PROCEDURES. I. GENERAL CONCEPTS AND GUIDELINES

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ABSTRACT

Validation of analytical methodologies is widely recognized as an important aspect of the development/utilization of analytical procedures and is widely required in support of product registration applications. Detailed, specific and comprehensive guidelines for the performance of analytical validations are not universally available. In this manuscript, the role and concept of validation is defined, the necessity for validation is established and published guidelines related to appropriate validation parameters are reviewed. The validation of chromatographic methods for pharmaceutical applications is particularly emphasized.

INTRODUCTION

Chromatographic methods are commonly used for the quantitative and qualitative analysis of environmental and pharmaceutical samples. The object of the analysis is to generate reliable, accurate and interpretable information about the sample. In order to ensure that the analytical method fulfills this objective, it undergoes an evaluation loosely termed validation. Such a

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validation is necessary especially in trade, in regulatory control and in cases of dispute wherein the results of the chemical analysis must be unambiguous and interpretable in only one way.

While the need to validate analytical methods is clear, the mechanics of performing a rigorous validation are not generally well defined. Questions of interest include:

* which validation parameters should be utilized,

* what specific procedures should be used to evaluate a particular parameter, and

* what is the appropriate acceptance criteria for a particular parameter.

Two factors contribute to the uncertainty associated with defining an effective validation protocol. Firstly, the general class of chromatographic methods is sufficiently broad and the applications of the procedure are sufficiently diverse that rigorous, effective, practical and defensible validation protocols are generally technique and application specific. Secondly, while guidelines exist for general classes of applications (e.g., references 1-8), they "are very often vague, sometimes quite inaccurate and misleading and rarely provide the development analyst with guidance on what really should be required of a validation exercise."[9]

In order to "get a handle" on the current state of thinking on the general topic of analytical method validation, a literature review was performed. The result of that review are summarized in a three part series of articles of which this is the first. These articles focus on the following:

* Part I; defining validation, establishing the need for validation, and identifying significant validation parameters.

* Part II: defining, identifying procedures for and summarizing acceptance criteria for specific significant validation parameters.

* Part III; defining, identifying procedures for and summarizing acceptance criteria for secondary validation parameters and related topics (e.g., re-validation and system suitability).

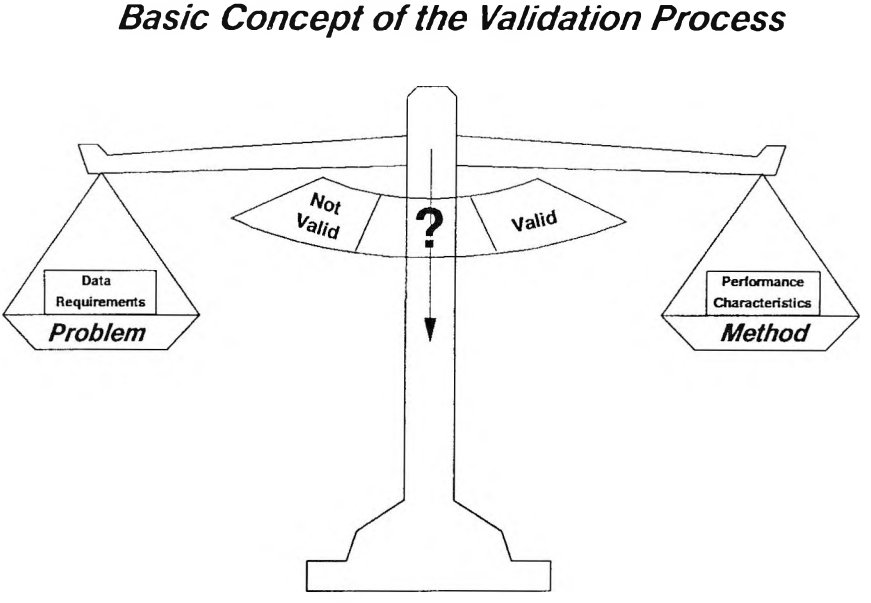

Figure 1. The Basic Concept of Method Validation wherein performance data are compared to pre-determined data requirements to assess whether the method is valid. Concepts suggested by J.K. Taylor (reference 48).

THE REGULATORY PERSPECTIVE ON VALIDATION

Test procedures for the assessment of the quality levels of pharmaceutical products are subject to various requirements. In the United States, the Current Good Manufacturing Practice (cGMP) regulations require that test methods, which are used for assessing compliance of pharmaceutical products with established specifications, must meet proper standards of accuracy and reliability.[2] Pertinent sections in the Code of Federal Regulations (21 CFR, ref. 10) include:

Section 211.165(e): The accuracy, sensitivity, specificity and reproducibility of test methods employed by the firm shall be established and documented.

Section 211.166(e.3): (The written program shall be followed and shall include) ... reliable, meaningful and specific test methods.

Section 211.194(a.2): The statement shall include the location of data

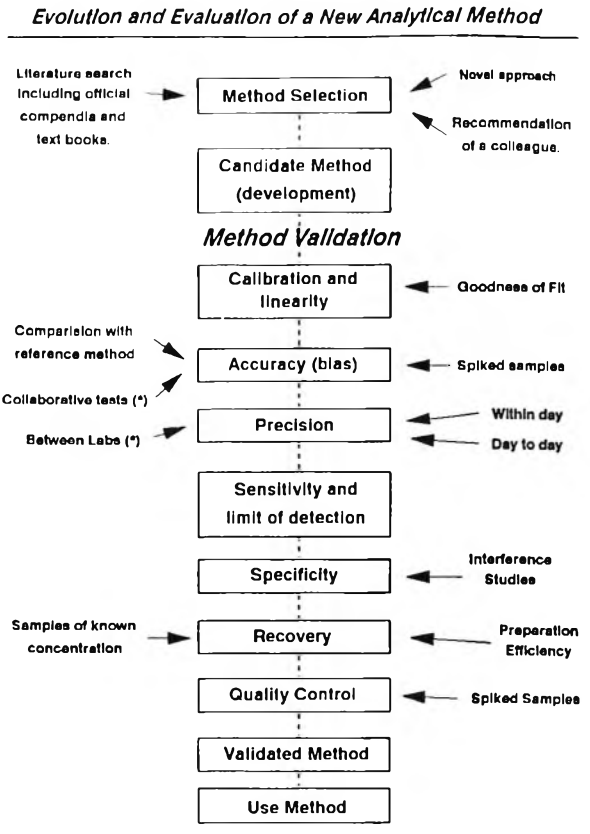

Figure 2. The Evolution and Evaluation of an Analytical Method as suggested by A.C. Mehta (reference 18). After the method has been conceived and operationally developed, its performance is validated with respect to several different performance parameters. Successful completion of the validation results in a method which can reliably be used to characterize "real" samples.

that establish that the methods used in the testing of the sample meet proper standards of accuracy and reliability as applied to the product tested. The suitability of all testing methods shall be verified under actual conditions of use.

Additionally, new product applications (NDA, ANDA, IND) submitted to the federal regulatory agency (FDA) must include method validation data. FDA validation guidelines include those indicated in the CFR regulations as

well as an evaluation of method ruggedness. To wit, "the purpose of validating NDA methodology in FDA laboratories is to make certain that a competent analyst can use the applicant's procedures on the agencies equipment and obtain results that are comparable to those submitted with the NDA."[11]

GENERAL VALIDATION CONCEPTS

Several references provide a working definition of a valid method. These include that the valid method:

* is suitable (reliable) for its intended use;[2,8,12,16,29,30,33,35]

* provides useful analytical data in a specific situation;[15,33,47,48]

* meets the pre-determined requirements (specifications) of the analytical problem;[2,15,16,30,34,48]

* has an established level of performance [accuracy, consistency, reliability];[9,12,13,14]

* does what it is supposed (expected) to do.[9,28]

The term validation thus is relative in the sense that it implies an activity of demonstrating that the process or procedure under examination accomplishes what is claimed or intended. As shown in Figure 1, then, the validation process becomes the action of comparing behavior observed under rigorously defined conditions with pre-determined performance expectations. The extent to which the observed performance agrees with the expectations determines whether the process is valid or not. Additionally, it is clear that the specifics of the validation (which parameters, what procedure, what requirements) are application dependant since the intent of the assay and its performance claims are themselves application specific.

The relationship between validation and the other phases of the method evaluation/utilization process is often unclear. The hierarchical concept of method utilization, wherein the stages of method development, validation and utilization are distinct and sequential (e.g., Figure 2) represents an situation which is practically undesirable and potentailly inefficient. Method development without some rudimentary method validation activities can readily lead to a "vicious cycle" wherein promising methods are subjected to rigorous

Type of Validation Data Reported

(From a Survey of Published LC Analysis of Drug Substances and Dosage Forms)

Parameter		Number of Occurances		
		Tablet and Capsules *	Solid and Liquids *	Liquids
Accuracy	Standard Add.	2	2	2
	Spike, 1 level	11	4	2
	Spike, 2-3 levels	6	4	8
	Spike, >3 levels	8	5	1
	Alt. Method	13	3	3
Precision	Repeatability	17	3	5
	Reproducibility	29	17	7
Linearity		34	12	7
Minimum Quantifiable Level		19	5	2
Specificity	Deg. Products	15	9	7
	Deg. Kinetics	10	10	0
	Forced Degr.	7	7	7
	Analogs	7	3	4
	Impurities	3	1	0
Quantitation	Areas	20	7	11
	Heights	12	10	4
	Internal Std.	16	12	4
System Suitability		18	5	5
Number of References		47	22	15

Figure 3. Type of Validation Data Reported by T.D. Wilson as a result of a 1990 review of published liquid chromatography methods used in the evaluation of pharmaceutical samples (reference 28).

validation protocols only to fail one or more criteria and thus require additional development and re-validation. Thus several authors propose that validation be a two stage process, with rudimentary or pre-validation activities occurring during development and a formal validation assessment occurring after the method development process has been completed.[31,39,45] In this way, the validation specialist has some assurance going into the formal validation study that in fact the validation results will be favorable.

Test Type	Number of Responses Indicating that this Validation Parameter was Used for a Particular Test (maximum is 20)									
	Accuracy	Precision (1)	Precision (2)	Linearity	Sensitivity	Selectivity	LOD	LOQ	Solution Stability	Ruggedness
ID Tests (Specification Tests)										
GC, LC, TLC, CE	0	0	0	0	0	5	1	0	1	1
Related Substances Tests (Specification Tests)										
TLC	14	7	9	9	3	16	16	11	10	9
GC	16	14	8	16	6	14	14	13	12	9
HPLC	19	16	13	19	8	20	18	17	15	12
CE	3	3	3	3	1	3	3	3	3	3
IC	1	1	0	1	1	1	1	1	1	0
Various Assays (Specification Tests)										
GC, LC, TLC, CE	18	17	15	18	10	17	8	8	16	14
Degradation Products (Stability Tests)										
TLC	14	8	8	9	3	14	16	15	11	8
GC	14	11	6	14	6	11	12	11	11	8
HPLC	16	15	12	16	9	17	17	15	16	12
CZE, CE	1	1	1	1	1	1	1	1	1	1

(1) Repeatability (2) Reproducibility

Figure 4. Number of Responses Indicating that a Validation Parameter is Applied to a Test on Bulk Active Ingredient/Synthesis Material at Various Stages in the Product Development Stage. Data is from a Survey of UK Pharmaceutical Companies by G.S. Clark.[49]

Test Type	Number of Responses Indicating that this Validation Parameter was Used for a Particular Test (maximum is 20)									
	Accuracy	Precision (1)	Precision (2)	Linearity	Sensitivity	Selectivity	LOD	LOQ	Solution Stability	Ruggedness
ID Tests (Specification Tests)										
GC, LC, TLC, CE	3	2	2	2	0	16	4	1	4	4
Related Substances Tests (Specification Tests)										
TLC	12	7	6	6	3	13	12	10	10	4
GC	9	8	7	9	4	9	8	8	9	6
HPLC	18	16	12	17	9	19	17	17	18	11
CE	2	2	2	2	1	2	2	2	2	2
Active Ingredient Assays (Specification Tests)										
GC, LC, TLC, CE	16	15	15	15	8	16	7	7	16	12
Preservatives and/or Anti-oxidants (Specification Tests)										
GC, LC, TLC, CE	18	17	13	18	6	14	6	6	18	10
Degradation Products (Stability Tests)										
TLC	12	6	6	7	13	14	11	11	11	5
GC	10	9	8	10	6	9	10	10	9	7
HPLC	17	14	13	17	9	18	16	16	17	10
CE	1	1	1	1	1	1	1	1	1	1
Active Ingredient Assays (Stability Tests)										
GC, LC, TLC, CE	15	16	12	16	9	16	7	6	16	11

(1) Repeatability (2) Reproducibility

Figure 5. Number of Responses Indicating that a Validation Parameter is Applied to a Test on the Finished Product at Various Stages in the Product Development Stage. Data is from a Survey of UK Pharmaceutical Companies by G.S. Clark.[49]

Ongoing validation activities may also be necessary during the routine utilization of an analytical procedure. System suitability determinations, frequently performed as a prerequisite to the generation of "real" data, represent essentially a validation at use. Re-validation of the analytical procedure may also be necessary as certain operational aspects of the method are changed during its routine and continuous application.

The focus of the remainder of this manuscript will be the formal or proper validation of an analytical method. The concepts of system suitability and re-validation are more completely addressed in the third part of this series.

VALIDATION PARAMETER GUIDELINES

Generally, two approaches can be utilized to determine which operational parameters should be included in a formal validation protocol. On one hand, one can look to the chemical literature to assess the practical state of the art among the practitioners of the desired methodology. In the case of chromatographic analyses, numerous reviews of implemented method validation strategies and procedures have been published. On the other hand, one can examine existing guidelines published by organizations with recognized authority within a given industrial arena (e.g., the FDA in the United States pharmaceutical industry). Both approaches are explored in this portion of the manuscript.

Trend Analysis

In 1990, T.D. Wilson, then a member of the Sterling Research Group, suggested that the question of how much and what kind of validation was necessary could be answered by examination of the pertinent literature, specifically published descriptions of liquid chromatographic procedures used in the analysis of drug substances and dosage forms. In a review published in 1990 which included 132 references,[28] Wilson summarized both the types of validation parameters typically reported in the pharmaceutical and analytical literature as well as the general validation approaches approaches employed. This information is further synthesized into Figure 3, which summarizes Wilson's results in the broadest general terms. For the purpose of this categorization, pharmaceutical products were divided into three general categories: drug substance, solid dosage forms; drug substance, solid and liquid dosage forms; and liquid dosage forms alone (including parenterals and aerosols). Thus for example, in 47 references related to the analysis of tablet

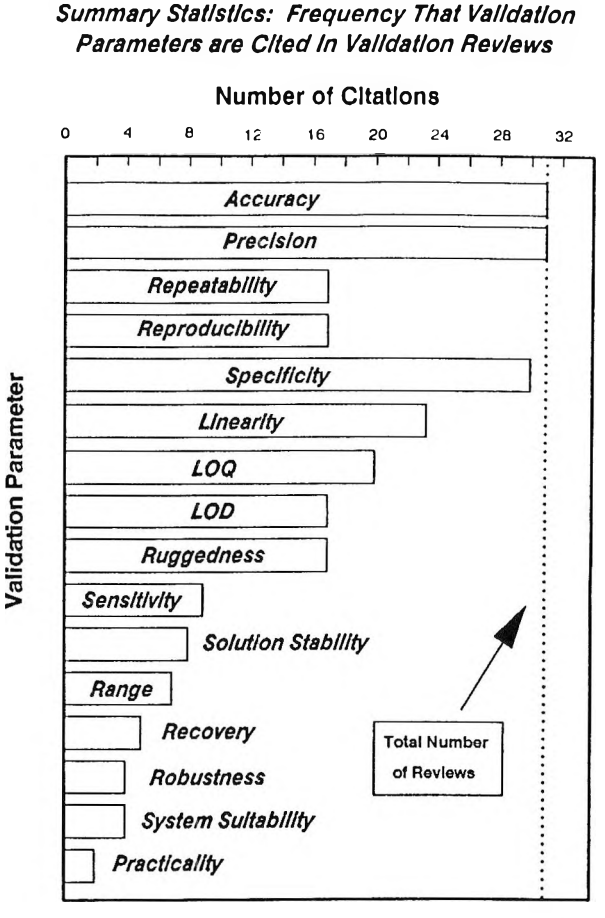

Figure 6. Summary Statistics, Frequency with which Specific Validation Parameters were referenced in General Manuscripts related to Analytical Method Validation.

and capsule dosage forms, accuracy was mentioned as a validation parameter in 40 instances, with 8 of the studies having performed the accuracy assessment by spiking at three different concentration levels.

C.S. Clarke, from Bristol-Myers Squibb, has recently published a survey of method validation procedures used in the testing of drug substances and finished products by many major research based pharmaceutical companies in the UK.[49] Portions of the results of the survey, summarized in Figures 4 and 5,

Data Elements Required for Assay Validation

(United States Pharmacopeial Convention)

Analytical Performance Parameter	*Assay Category I*	*Assay Category II*		*Assay Category III*
		Quantitative	*Limit Tests*	
Accuracy	Yes	Yes	(*)	(*)
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	(*)
Limit of Detection	No	No	Yes	(*)
Limit of Quantitation	No	Yes	No	(*)
Linearity	Yes	Yes	No	(*)
Range	Yes	Yes	(*)	(*)
Ruggedness	Yes	Yes	Yes	Yes

(*) May be required, depending on the nature of the specific test.

Figure 7. Data Elements Required for Assay Validation per the USP. Table 2 from reference 2.

indicate which validation parameters were applied to particular tests at several stages in the product development cycle.

During the course of the literature review performed for this manuscript, 31 references, representing a cross section of authors from government, industry and academia, which specifically considered the issue of method validation from the perspective of which validation parameters were necessary were examined. Figure 6 represents a frequency distribution of the specific validation parameters which were mentioned in these manuscripts. For example, method accuracy was recognized as a universally necessary validation parameter in all 31 references. Validation parameters such as sensitivity were less universally mentioned but have a particular importance in specific applications.

Existing Guidelines

Several governmental bodies have published recommended general vali-

Important Validation Characteristics For Various Assay Types

(International Conference on Harmonisation: Draft Guidelines on ValidationProcedures for Pharmaceuticals; Availability)

Type of Assay / Requirement	Identification	Impurity Test		Content or Potency
		Quantitative	Limit	
Accuracy	-	+	-	+
Precision:				
Repeatability	-	+	-	+
Intermediate	-	+ (3)	-	+ (3)
Reproducibility	-	- (1)	-	- (1)
Specificity	+	+	+	+ (2)
Detection Limit	-	+	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- = not normally evaluated + = normally evaluated

(1) may be needed in some cases (2) may not be needed in some cases

(3) if reproducibility has been performed, intermediate is not needed.

Figure 8. Important Validation Characteristics for Various Assay Types per the International Conference on Harmonization (ICH), reference 8.

dation guidelines. For example, Figure 7 summarizes current validation guidelines established by the United States Pharmacopoeial Convention (USP).[2] The guidelines describe which validation procedures are necessary for compendial methods that fall under three general assay categories. Assay category I includes methods used for the quanititation of major components of bulk drugs or active ingredients (including preservatives). For these types of assays, in which the analyte should be present in relative abundance, parameters such as accuracy and precision are deemed necessary while measures of assay sensitivity (e.g., detection and quantitation limits) are not required. However, for assay category II, those used for impurities in bulk drugs and degradation products in finished product, the possibility that the quantity of analyte may be relatively small increases the importance of sensitivity-type validation parameters. Interesting differences in validation requirements arise in this category depending upon whether the assay is used to quantitate or only as a limit test.

Assay category III represents methods used to measure product perform-

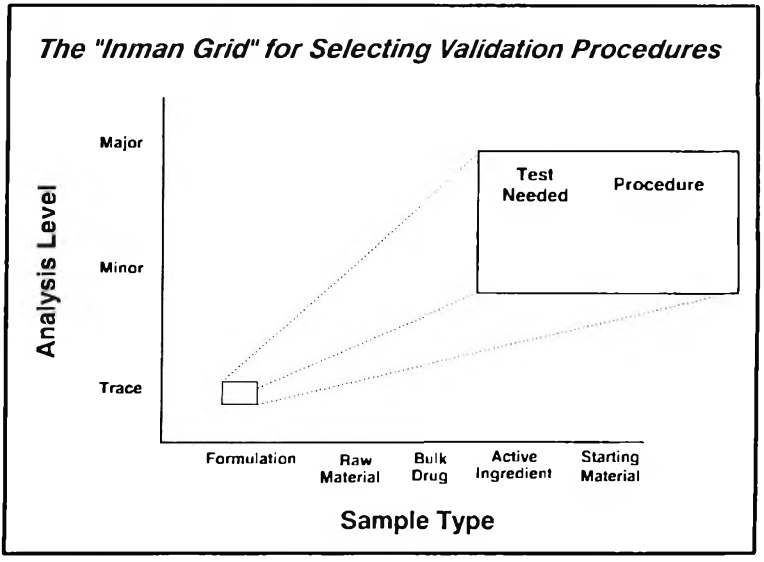

Figure 9. The "Inman" Grid for Selecting Validation Procedures. The assay is defined in terms of analyte level and matrix type and the validation parameters and general procedures are indicated.

perform-ance characteristics such as dissolution and/or release rate. Since this category includes any number of methods for any number of product properties, the general validation requirements are vague.

In a similar vein, the International Conference on Harmonization (ICH) has recently published its own list of important validation characteristics for various assay types[1] which is reproduced in Figure 8. In this classification, assays are categorized with respect to the utilization of the resulting data; e.g., identification [intended to ensure the identity of the analyte in the sample], impurity testing [intended to reflect the purity characteristics of a sample] and content/potency [intended to measure the analyte (active ingredient or major component)] present in a given sample.

In general the classifications of the USP and ICH are consistent, with the USP including ruggedness as a validation parameter due to the potential repeated use of compendial methods at numerous analytical sites.

General method validation guidelines were published by E.L. Inman and

Example of the "Inman Grid"

Validation Parameters; Major Component (>10%)

Sample Type	*Parameter*	*Validation Procedure*
Formulation	Linearity	Wide Conc. Range (10 to 200%)
		Narrow Conc. Range (50 - 150%)
		Both
	Precision	Multiple labs, instrument, analyst
	Recovery	Multiple spikes, multiple levels
	Specificity	Analyte-related substances
	Stability	Following day
	Matrix Effect	Wide Conc. Range (10 to 200%)
		Narrow Conc. Range (50 - 150%)
		Both

Figure 10. Portion of the Inman Validation Table for Major Components (>10%) in Formulation Samples. Based on the analyte level and sample matrix, the Table defines what validation parameters are necessary and suggests appropriate general validation procedures. From reference 19.

associates at Eli Lilly and Company in 1987.[19] The general outline of this manuscript (Figure 9) was to classify assays on the basis of analyte level and sample type. For each position along a two dimensional grid defined by these assay characteristics, specific validation procedures were defined. Thus, for example, for the validation of analytical methods used to quantitate major components in pharmaceutical formulations, these researchers suggest that linearity, precision, accuracy (recovery and matrix effect), specificity and stability are appropriate validation parameters (Figure 10). More specifically, they suggest that the precision determination, for example, would include a consideration of multiple labs, instruments and analysts, with the test for variation including a single run on potentially multiple product lots consisting of a minimum of ten replicates.

CONCLUDING REMARKS

It is clear from a review of the analytical literature that specific and unambiguous validation guidelines on even as general a topic as which validation parameters are appropriate for every specific analytical situation which might be encountered in the industrial and academic environment do not

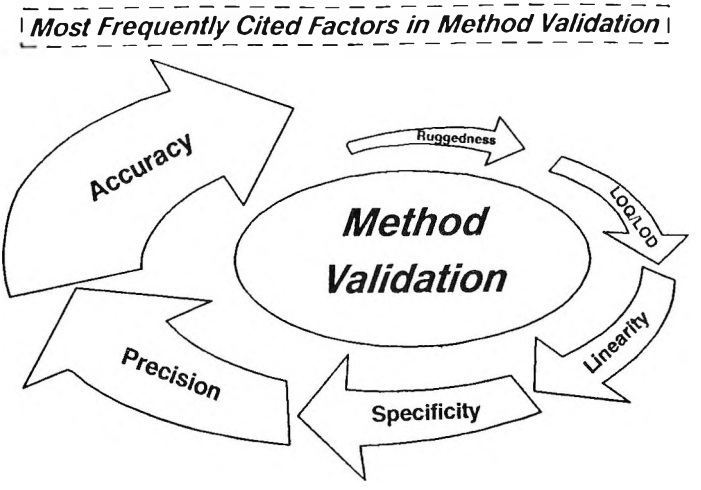

Figure 11. The Most Frequently Cited Validation Parameters in the papers reviewed for this manuscript. The most frequently cited validation parameters (accuracy and precision) would normally be a part of virtually every validation protocol while the use of the less frequently cited parameters would be application specific.

exist. The Holy Grail of one reference applicable for all situations has not surfaced and in a practical sense is impossible to envision. However, analytical professionals can receive meaningful guidance with respect to establishing appropriate validation parameters in particular situations from the research experiences and proposals published by their colleagues. Thus rather than relying on single reference which rigorously establishes invariant outlines of the validation study, the researcher has information which allows one to establish the boundaries of the validation study in a way that is meaningful for a specific analytical situation.

As illustrated in Figure 11, the literature clearly establishes that certain validation parameters (e.g., accuracy and precision) are applicable in virtually every analytical situation. Exclusion of such parameters from a validation protocol would most certainly require an extensive and scientifically rigorous justification.

Other parameters, such as specificity and linearity, are less universally applicable and thus their exclusion from general validation protocols could be somewhat less controversial. However, the need to include even these less common validation parameters in validation protocols under specific analytical

situations is clearly established in the literature.

This discussion represents a rather superficial examination of the analytical literature which considers the topic of primary cheomatogaphic method validation which can provide the validation specialist with potentially useful validation recommendations. The reader is strongly encouraged to examine applicable primary references in greater detail than can be presented herein.

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Received September 23, 1995
Accepted October 15, 1995
Manuscript 3979

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 737–757 (1996)

CHROMATOGRAPHIC METHOD VALIDATION: A REVIEW OF CURRENT PRACTICES AND PROCEDURES. II. GUIDELINES FOR PRIMARY VALIDATION PARAMETERS.

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ABSTRACT

Validation of analytical methodologies is an important aspect of their development/utilization and is widely required in support of product registration applications. In this manuscript, definitions, procedures and acceptance criteria which appear in the pharmaceutical literature are summarized for the more commonly encountered validation parameters. Parameters examined include accuracy, precision, specificity, linearity and sensitivity limits.

INTRODUCTION

Chromatographic methods are used for the quantitative and qualitative analysis of environmental and pharmaceutical samples. The object of the analysis is to generate reliable, accurate and interpretable information about the sample. In order to ensure that the analytical method fulfills this objective, it undergoes an evaluation loosely termed validation. In the first part of this series,[1] accuracy, precision, specificity, linearity and sensitivity were identified as validation parameters which were most frequently cited in general

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manuscripts related to the validation of chromatographic assays in primarily pharmaceutical applications. In this manuscript, these primary validation parameters are examined in greater detail to develop their working definition, to establish specific measurement protocols and to summarize recommended acceptance criteria.

Characteristics of an Effective Validation Parameter

An investigator's ability to understand, evaluate and utilize a validation parameter is influenced by the parameter's description. Descriptors of validation parameters include its definition, its scope (applicability), recommended assessment procedures and its acceptance criteria. An effective validation parameter is one where the user knows what the parameter is, when to use it, how to perform its evaluation and what criteria to use to complete the validation assessment. For a validation parameter to be effectively understood and utilized it must possess the following minimal characteristics:

Definition

The parameter must be defined in a clear, concise and unambiguous manner. Alternate definitions of the parameter should contain a core set of universally acceptable concepts or phrases. As necessary, the definition should be quantitative and mathematically rigorous.

Scope

The acceptability and applicability of the parameter in common situations should be clearly established.

Procedures

The procedures for performing the validation must be presented in a complete, well defined, practical and understandable format. Procedures should be outlined with sufficient detail so that all important experimental variables can be set to defined values. While it is most advantageous for the procedures to be as broadly applicable as possible, exceptions should be clearly and completely stated.

Acceptance Criteria

Once the validation is complete, an investigator must be able to interpret

the results. Acceptance criteria must be available which allows the researcher to unambiuously determine, by comparing method performance data to the criteria, whether the method under evaluation is performing in a valid manner.

To facilitate the evaluation, acceptance criteria should be universally applicable, numerically and mathematically explicit, complete and achievable. Additionally, acceptance criteria should be referenceable in that they should be traceable, through appropriate literature citations, to a rigorous scientific evaluation of their development and justification.

Given these criteria, several validation parameters will be considered with respect to their published descriptions. Trends of conceptual commonality within the literature will be established via nesting of similar literature citations.

Accuracy

Definition

Generally one expects a properly working procedure to produce the expected results when it is performed in a standardized manner. Thus a procedure is validated for accuracy by performing it in a standardized manner and comparing the observed results with the expected behavior. For an analytical method, the accuracy is most commonly defined as follows:

The closeness of agreement between the value found by the method and the value which is accepted either as a conventional true value or a reference value.[2,3,4,5,6,8,19,20,21,24-27,30]

Accuracy can also be defined as the difference between a result and a true or known value.[12,15] The concept of acceptable limits, recognizing that the comparison between observed and true behavior must be statistically based given the inherent variation in the observed behavior, is addressed by several authors.[10]

Procedures

Several procedures appear in the literature for the determination of method accuracy. A commonly referenced procedure involves the fortification of a test solution with a known amount of the analyte of interest. Accuracy is assessed by "applying the analytical method to samples or mixtures of sample

matrix components to which known amounts of the analyte have been added both above and below the normal levels expected in the samples."[3,7,8,11-13,17,18,26,30,36] Method accuracy is the agreement between the difference in the measured analyte concentrations of the fortified and unfortified samples and the known amount of analyte added to the fortified sample. If the solution being fortified is a placebo (an artificially prepared simulation of the sample's matrix alone), the fortification procedure is termed spiking. The method of standard additions is the fortification of a sample which already contains the analyte at its normal level.

In a variation of fortification, Cardone and associates propose a relative response curve method wherein the placebo and standard blank are both spiked at several analyte levels encompassing the method's linear range.[22] Both sets of data are subjected to separate linear regression analyses and the determination of accuracy is performed by comparing the slopes and intercepts of the respective best fit lines.

Other procedures suggested for assessing method accuracy include collaboration, in which data obtained from the candidate method is compared to data generated with a widely accepted (e.g., validated, compendial, standard) method.[5,11,15,35,34,36] In theory, "the best way to determine system bias (accuracy) is to use some definitive method, based on some unique property of the analyte, which eliminates or corrects for every possible source of error."[36] Analysis of reference materials, prepared externally by an approved vendor[7,19,25,36] or internally via spiking,[4,7,12,15,21,29,34] is another procedure for assessing accuracy. Its application is limited by both the availability and stability of the reference materials and the degree of certainty with which the analyte's true concentration in the reference material is known. For chromatographic assays a mass balance approach has been recommended wherein the sample is injected into the chromatographic system both with and without the column and the total peak response in both configurations is compared.[17]

Procedure Guidelines

The following procedural guidelines appeared in the validation literature:

* Accuracy requires six replicate assays.[6,19,27]

* Accuracy is determined over the range from 80% of the lowest expected assay value to 120% of the highest expected assay value[2,4,9,17,26] with triplicate measurements[4] or at five levels.[17]

* Recovery studies should be run at 75% to 125% of label claim.[30]

* Use six samples of drug in the matrix spanning 50 to 150% of the expected content.[6]

* The appropriate standard addition level is 20% of the target analyte level.[8]

* Use replicates,[12,15] minimum of five samples[29] or at least six degrees of freedom[7] at 3 concentrations within the analytical range (extremes and midpoint of expected[7] or near quantitation limit, center of range and upper bound of standard curve).[29]

Acceptance Criteria

For trace level analyses, the following criteria are pertinent:

* Below 100 ppb, 60 to 110% recovery is acceptable; above 100 ppb, 80 to 100% recovery is acceptable.[9,27]

* Below 1 ppm, 70 to 120% recoveries ar acceptable.[19]

* Impurities present at 0.1 to 10% should produce data within ± 5% of actual.[17]

General criteria for pharmaceutical samples included:

* The average recovery of spikes should be 98% to 102% of the theoretical value.[14,17]

* The recovery of the drug (as % of theory) must be within ± 4S of the theoretical value where S is the system (or method) precision.[6,8]

* For standard additions, the plot of assay response versus amount added should have a slope of 0.95 or greater and an intercept equal to the initial concentration.[26,34]

* For spiking, the plot of recovered versus known spike should have a correlation coefficient of 1.00, a slope of 1.00 and an intercept of 0.00.[11,34]

* For the relative response curve method, analyte/matrix interaction effects are absent if the intercepts of the matrix and standard plots are

statistically equal to zero. Proportional systematic error is absent if the ratio of the slopes of the response curves for the matrix and standard is statistically equivalent to one.[22]

In biological samples, method accuracy for discovery phase investigations should be ± 20% of actual, with recoveries of ± 10% being necessary in pre-clinical and clinical studies.[27] Alternatively, it is recommended that the mean recovery value should be within ± 15% of actual except at the quantitation limit where ± 20% is acceptable.[29]

Precision

Definition

A properly performed validated procedure will produce consistent results reflecting those sources of variation inherent in the procedure's steps. Thus precision reflects a procedure's ability to reproduce the same, but not necessarily the correct or expected, result each time it is correctly preformed. In the pharmaceutical literature, precision is commonly defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple analyses of the same homogeneous samples under the prescribed assay conditions.[3,4,5,9,11,18,21,24,25,27,30,33,36] This definition clearly establishes that method precision is sample and procedure specific and emphasizes the role that a uniform sample and a standard procedure protocol have in establishing precision. Since precision has statistical connotations, it can be defined as the distribution of individual test results around their mean.[6,12,15,19,25]

Precision is frequently subdivided into three flavors as a function of the number of locations at which the procedure is performed and the time span over which the precision data is collected. Repeatability (intra-assay or within day precision) reflects the variation in replicate procedures performed within a short time period (same analytical run) with the same operational conditions (operator, instrument, reagents, operating conditions). Intermediate (day to day) precision is related to analyses performed on different days by different analysts on different instruments with different reagents at the same operating facility. Reproducibility (intra-laboratory precision) is related to the procedure being performed at two or more laboratories in, for example, a collaborative study.

Precision can be categorized in terms of its source within a procedure. System precision is related only to the operation of an analytical instrument or

the performance of the analytical test while method precision is related to all aspects of a procedure including sampling and sample preparation.

Procedure Guidelines

Precision is assessed by repetitively performing the procedure in a prescribed manner with a prescribed sample and statistically evaluating the resulting data. Important issues related to the precision determination include the number of replicates required and the type of sample to be tested. Considering the issue of replicates, recommendations in the literature are somewhat discordant. For the determination of repeatability, the following recommendations are noted:

* Five replicates for release or stability assays[4]

* At least six to ten replicates.[4,3,31]

* Duplicate measurements made on ten samples at each of three different analyte levels.[12,15]

* Five replicates at three levels (limit of quantitation, mid-range and upper calibration bound).[29]

* Replicate samples at analyte levels of 80 to 120% of expected for dosage forms and drug substance tests.[2]

* Sufficient data should be generated to ensure more than thirty degrees of freedom.[11]

For intermediate precision, the repeatability experiments should be performed on 2,[4,20,21] 3 to 5[30] or at least 10[12,15] separate days. To assess reproducibility, the repeatability experiments have to be performed in at least two laboratories.[6]

The issue of sample type is of key importance, particularly if material sampling is an important procedure component. Central to this issue is the use of authentic versus artificially prepared samples. While several authors recommend the use of authentic samples,[5,13,20] it is recognized that sample instability or the inability to produce a sufficiently homogeneous authentic sample limits their use. If artificially prepared samples are used, several authors suggest that they be prepared to mimic either freshly prepared or degraded product.[5,21,29,30] Alternatively, several authors argue that authentic or artificially prepared product is not required for the precision evaluation,

suggesting the use of standard solutions[6,18] or any sample which will produce a similar response when the method is applied to it.[25]

Acceptance Criteria:

The following acceptance criteria for precision were found in the literature:

* The desired precision of stability indicating methods should not be more than ± 1.0 % RSD.[16]

* System precision should be ≤ 1% RSD (or higher for low level impurities).[24]

* System precision ≤ 1.5% RSD; method precision ≤ 2.0% RSD.[18]

* % RSD ≤ 2.0%.[9,20,27,30]

* The required discrimination ability must be ≥ the quantity 1.96 σ/n.[26]

* The repeatability is generally 1/2 to 1/3 of the reproducibility.[19]

* For biological samples, a CV of 10% should be acceptable as the minimum precision.[12,15]

* For biological samples a CV of ±15% is appropriate except at the quantitation limit where ±20% is acceptable.[29]

* For discovery samples, a ±20% RSD is acceptable. For pre-clinical and clinical samples, ±10% is more appropriate.[21]

Several authors link precision criteria with the procedure's acceptance range. For example, for acceptance ranges of 95 to 105% and 90 to 110% respectively, the recommended precision is 2% or 4% RSD.[8] A more detailed breakdown of precision versus the procedure's acceptance range is summarized in Table 1.

Specificity

The purpose of performing an analytical determination is to assign an accurate value to some chemical property of a sample. This process is

Table 1

Recommended Precision Acceptance Criteria

Acceptance Range (% of Claim	Maximum Allowable % RSD			
	Single Determinations		Multiple Determinations	
	Method % RSD	System % RSD	Method % RSD	System % RSD
98.5-101.5	0.58	0.41	0.82	0.58
97-103	1.2	0.82	1.6	1.2
95-105	1.9	1.4	2.7	1.9
90-110	3.9	2.8	5.5	3.9
90-115	4.8	3.4	6.9	4.8
90-125	6.8	4.8	9.6	6.8
85-155	5.8	4.1	-	-
75-125	9.7	6.9	-	-
50-150	19.4	13.7	-	-

This table is based on 99% confidence levels, assuming that half of the variance of a method is attributable to system error. (From reference 6)

facilitated if the determination's response to that specific chemical property can be distinguished from its response to any other sample property. In general, specificity relates to the ability of a method to measure only what it is intended to measure[19] even though the sample may contain a sea of excipients and related compounds.[1C] More specifically, specificity is commonly defined as the ability of an assay to assess unequivocally, with a requisite level of accuracy and precision, the analyte of interest in the presence of compounds which might be expected to be in the sample, which for pharmaceutical samples might include inactive excipients, degradation products, synthesis impurities and precursors, container extractables and analytical artifacts.[3,5,9,11,18,21,24,25,30]

Specificity is a measure of the method's sensitivity to potential sample-related interferents[27] and for chromatographic procedures reflects the system's ability to resolve all other sample components that will give a detector response[6,8] from the peak of interest. The specificity determination ensures that the signal measured in the method is not influenced by interfering species or, at least, that the contribution of such substances has been removed.[32]

Procedures

The most commonly cited specificity evaluation procedure is the analysis of a placebo, wherein the sample matrix without the analyte is analyzed and the resulting system response is examined for the presence of responses which interfere or overlap with that of the analyte of interest.[3,6,11,15,17,19-21,25,26,28,29] If the sample matrix is variable either in terms of absolute composition or source of its component raw materials (for example, in the case of a manufactured product), it has been recommended that specificity be established with six independent sources of the sample matrix.[6,18] The method of standard additions can be applied to specificity evaluations.[3,8,13,18,25,27] In this approach, samples (in the matrix) and standards (no matrix) prepared at equivalent analyte concentration levels can be analyzed or, alternatively, both samples and standards can be fortified with equivalent levels of the analyte and re-analyzed. In either case, specificity is the degree of agreement between the sample and standard responses. One author recommends that five different spike levels, encompassing the method's linear range, be used to assess specificity.[13]

Additional procedures for specificity include:

* Peak re-analysis, wherein the peak of interest is collected and re-analyzed by different chromatographic conditions or with methods that are sensitive to analyte structure.[6,18]

* Collaboration in which the sample is quantitatively analyzed using two or more detection/separation strategies and the results compared.[19]

* Use of information-rich detectors (e.g., mass spectrometric[16,21] or multiple wavelength UV[4,6,16,18,21] to assess peak purity.

Procedure Guidelines

A specificity guideline whose use facilitates an effective validation evaluation involves degradation products. Clearly if the assay supports product expiry dating via stability studies, its response must be unaffected by any degradation products (from either the active ingredient or formulation matrix) which could be generated over the entire course of the study. Ideally, the most effective test articles for the specificity evaluation would be stability samples retrieved throughout the study's duration. The duration of most stability studies makes this ideal situation untenable. Thus the analyst is faced with artificially producing degraded samples via accelerated methods. Two issues are encountered; degradation mechanism and the extent of degradation. While it is

recommended that the selectivity of a method be established by forcing drug degradation using acid, alkali, oxidizing agents, temperature and intense light,[4,8,16,20,30] it is clear that the analyst should use methods which are consistent with the product's decomposition under normal manufacturing, storage and use conditions. Since these conditions are application specific, it is impossible to provide general decomposition guidelines; however, the analyst must be able to defend his choice of accelerated decomposition conditions based on a sound scientific understanding of the product's decomposition mechanism under typical use conditions.

Once conditions have been established for producing artificially degraded samples, the issue of how much degradation is adequate must be addressed. The key to a realistic specificity evaluation is to perform the assessment on samples which one might reasonably encounter at extremes in the product's utilization environment. Specificity evaluations performed on samples which have degraded significantly more or less than those which might encountered in worst case applications serve no useful purpose. For active ingredient assays on products whose stability is dictated by the typical pharmaceutical limit of 90% of label claim, peak purity should be performed on stressed samples exhibiting a demonstrable degradation of 10 to 15%.[16] If other product properties limit stability (e.g., the accumulation of a degradate, solution color or solution pH), the accelerated decomposition conditions used to produce specificity samples must result in a sample whose behavior is slightly beyond these product limits.

Acceptance Criteria

Published acceptance criteria include:

1. Placebo, Visual Examination. For chromatographic procedures, there should be baseline separation between the peak of interest and all other analytical responses.[4,6,8] One author suggests that the nearest peak maximum should be separated from the designated analyte peak by at least one full width at half height.[14]

2. Peak Re-analysis. If the peak is collected and re-analyzed on another chromatographic system, it should produce a single response.[6]

3. Low Resolution Mass Spectrometric Detection.[14] The intensities of four diagnostic ions (including the molecular ion) must be measured in the sample and a standard. The relative abundances of all diagnostic ions (expressed as a percentage of the intensity of the base peak) must be the same in the sample and the standard within a margin of ± 10% (EI mode) or ± 20% (CI mode).

4. Peak Purity (Multiple wavelength UV detection). Wavelength ratio techniques should show that the spectrum of the analyte peak matches that of a reference standard.[6] The maximum absorption wavelength of the analyte in the sample must be the same as that of the standard reference material within the resolution of the detector (±2 nm). The spectrum of the analyte in the sample should not be visually different from that of the standard for parts of the spectrum with a relative absorbance larger than 10%.[14] If the peak absorbance ratios at two (or more) wavelengths determined for treated, spiked and non-treated samples are within ± 5%, the chromatographic peak is considered to be pure.[16]

Linearity

Definition. Most analytical procedures do not produce output which is an absolute indication of the sample property being measured. Rather, instrument output must be mathematically transformed into sample property units. In chromatography, peak parameters are related to analyte concentration via standardization procedures. This relationship is then used to convert a sample's peak parameter to its apparent analyte concentration. A linearity assessment establishes the nature of the peak parameter to standard analyte concentration relationship. The linearity assessment determines the procedure's ability to obtain test results which are proportional to the concentration of the analyte in the sample within a given range either directly[5,8,13,17,20,21,27] or via a well defined mathematical transformation.[3,11,24,25,27]

A procedure's range is linked to its linearity. The range is the interval between the lower and upper analyte concentration for which it has been demonstrated that the analytical procedure has a suitable level of accuracy, precision and linearity.[3,5,24,27,30] The range is validated by verifying that the procedure provides acceptable accuracy, precision and linearity at the extremes of the range as well as within the range.[5]

Procedure Guidelines

Establishing the appropriate concentration range is the major issue associated with the linearity assessment. Generally, the appropriate range is application specific. Recommendations noted in the literature for the range include:

* the range of expected concentrations.[3,15,27,29,30]

* 80% of the lowest expected level to 120% of the highest expected level.[2,4,9]

* 50% to 150% of the expected working range.[6,8,11,13,18]

* 25% to 125% of the target range specified.[30]

* 10% to 200% of the expected range.[13,20]

* For impurity tests and dissolution studies, several orders of magnitude.[4,13]

Specific guidelines for linearity ranges, as provided by Carr and Wahlich,[17] are contained in Table 2.

In determining the range, the analyst must balance the requirements of scientific rigor with practical constraints. A method validated for linearity need only produce accurate values in the concentration range in which it is intended to be used.[17] The range selected for validation should not be unrealistically wide, as this may lead to rejection of a method which is really quite suitable for the intended purpose.[25]

Acceptance Criteria

Acceptance criteria provided by various researchers include:

* Data should be plotted to look for dubious points and to visually establish the calibration range.[15]

* The correlation coefficient of the best linear least squares regression model should be between 0.98 and 1.00[8] or greater than 0.999 with the slope and intercept reported.[4]

* The value of n in the equation $y = mX^n + b$ should be between 0.9 and 1.1 and the maximum allowable relative error is 1%.[6]

* Taking the regression line as a mean, a RSD calculated for the data should not be greater than 2.0%.[17]

* The intercept of the regression line should not be significantly different from zero[18,22] or, more specifically, the percentage of the intercept relative to the 100% analyte level should be ± 2%.[17]

* A response factor plot is used to identify concentrations where true proportionality is not observed.[30]

Table 2

Recommended Validation Ranges for Linearity Studies

Purpose of Analysis	Typical Product Range (%)	Recommended Validation Range (%)
Release Specification Assay	95 to 105	80 to 120
Check Specification Assay	90 to 110	80 to 120
Content Uniformity Test	75 to 125	70 to 130
Asssay for a Preservative in a Stability Study	50 to 110	40 to 120
Assay for a Degradant in a Stability Study	0 to 10	0 to 20

From reference 17.

While the correlation coefficient is commonly cited as a test of linearity, its use is not universally accepted (for example, references 10 and 28). These authors suggest a more rigorous statistical evaluation of linearity, including a test of significance for the b_2 term in the equation[22] $y = b_0 + b_1X + b_2x^2$ and the utilization of the residual sum of squares.[17]

In closing, this author notes that there is no unwritten rule that states the relationship between instrumental response and analyte concentration must be directly linear for a procedure to be valid. Rather, the requirement is that the relationship between method response and analyte concentration be rigorously defined over the expected analyte range. The desire to have a linear relationship reflects the practical consideration that a linear relationship can be accurately described with fewer standards than a non-linear relationship and the subjective expectation that a linear relationship is more rugged than a more complicated one.

Limits of Sensitivity

Definitions

Sensitivity is the ability of a method to reliably respond in a consistently recognizable manner to decreasingly smaller amounts of analyte. Frequently utilized measures of sensitivity are the limit of detection (LOD) and limit of

quantitation (LOQ). While similar in concept, their utility is application specific. For example, the LOD is suggested by the USP for qualitative limits tests while LOQ is specified for quantitative impurity determinations.[3] The LOD is usually required for impurity tests, assays for dissolution test samples, limit tests and "absence of" tests.[26] The LOD is the lowest amount of an analyte in a sample which can be detected but not necessarily quantitated.[3,4,5,21,27,30] In practice, it is the lowest concentration of analyte which can be distinguished from the blank with a stated degree of confidence.[11,12,15,17] It is generally the lowest concentration of analyte that is detected at the most sensitive instrument setting[25] and is that point in the response range that a measured value is greater than the uncertainty associated with it.[18] For chromatographic procedures, it is the lowest amount of analyte which can be detected above the baseline detector noise.[20,32]

Alternatively, LOQ is the lowest amount of analyte which can be reproducibly quantitated above the baseline noise.[20] Quantitation implies that the measurement possess a specified accuracy and precision.[4-6,10,16,17,18,21,24,25,27,30,32] LOQ has been variously defined as that quantity of analyte which has a signal to noise ratio of at least 10 and a precision of less than 10% or which has a signal to noise ratio greater than 20 and a precision of 5% or less.[30] In some applications, LOQ is defined as the smallest concentration included in the standard curve.[26]

Procedures

LOD can be determined either directly or from other validation data. Its direct measurement involves an analysis of the method's peak to peak baseline noise[4,11,13,20,26] or an analysis of the variation in the method's blank response[3,15] In either case, LOD is calculated as either 2 or 3 times the variation in measured response, where the factors are associated with the 95 and 99% confidence intervals for a normal distribution. Practically, LOD can be measured by the serial dilution of samples until the peak can no longer be observed.[13,15] LOD can be estimated as the value of the linear calibration curve's y-intercept.[11,12,15] Considering method precision, LOD has been defined as the concentration equal to 3.29 times the injection to injection standard deviation[35] or as that concentration at which the system precision (CV) reaches 20%.[15]

Similarly LOQ can be determined via the precision of replicate blank analyses (ten times the %RSD of the replicates)[3,4,27,35] or by analyzing successively diluted samples until the requisite levels of accuracy and precision are achieved.[20,21] Several authors have suggested procedures for estimating

LOQ based on an analysis of the method's baseline response.[17,26] Such procedures generally require that the chromatogram resulting from a blank injection be examined over a range of twenty peak widths and that the noise be measured as either the largest peak to peak fluctuation or as the largest deviation (positive or negative) from the mean response. The LOQ is then calculated as the product of ten times the measured deviation and the calibration curve slope. The LOQ can also be determined as the lowest analyte concentration for which duplicate injections results in a %RSD $\leq$ 2%.[20]

Regardless of the method employed, it is commonly recommended that the calculated LOQ be confirmed by injecting samples prepared to contain the analyte at or near the LOQ.[3,17,27]

Acceptance Criteria

In general, there exists no specific criteria for what value LOD and LOQ must have. Rather, the requirements are generally stated in terms of the relationship between LOD/LOQ and the concentration of the analyte in the samples to be analyzed. For routine applications involving LOD, it is desired that test samples contain 2 to 3 times the minimum amount detectable.[27] Alternatively, a factor of 5 or 10 is recommended between the LOD and the specification value for an analyte level.[13]

In routine applications it has been recommended that LOQ be within the working linear concentration range[30] and that a specification limit should be no lower than twice the LOQ.[6] For clinical applications, the LOQ should be at least 10% of the minimum effective concentration.[21]

A Survey of Procedures Used

To determine what type of validation protocols were typically being performed in industry, C.S. Clarke of Bristol-Myers Squibb surveyed twenty major research based pharmaceutical companies in the UK. Portions of the results of this survey[37] are shown in Table 3 and document parameters used in the evaluation of specific validation parameters as well as acceptance criteria.

CONCLUDING COMMENTS

The purpose of this manuscript is to provide the reader with a general sense of current procedures used by active investigators or recommended by

Table 3

Criteria for Performing Method Validation Experiments; Median Responses from a Survey of UK Pharmaceutical Manufacturers

Parameter	Number of Samples	Range (1)	Tolerance
		Bulk Drug Assays	
Accuracy	6	50-150	±2%
Repeatability	6	-	±2%
Reproducibility	6	-	±2%
Linearity	6	20-150	r^2>0.999, Intercept
LOD & LOQ		- - - - - - Not Applicable - - - - - -	
		Bulk Drug Impurity Assays	
Accuracy	5	50-150	±20%
Repeatability	6	-	±2%
Reproducibility	6	-	±5%
Linearity	6	20-150	r^2>0.999, Intercept
LOD		3 times the signal-to-noise ratio	
LOQ		10 times the signal-to-noise ratio	
		Finished Product, Active Ingredient Assays	
Accuracy	6	75-125	±2%
Repeatability	6	-	±2%
Reproducibility	6	-	±5%
Linearity	6	25-150	r^2>0.999, Intercept
		Finished Product, Degradant Assays	
Accuracy	6	50-150	±10%
Repeatability	6	-	±2%
Reproducibility	5	-	±3%
Linearity	6	0-2%(2)	Intercept
LOD		3 times the signal-to-noise ratio	
LOQ		10 times the signal-to-noise ratio	

(1) Range is represented as 95% of label claim

(2) As the percent of the active drug level in the formulation.

Intercept criteria is that the 95% confidence interval for the intercept include 0.

From Reference 37.

industrial, academic and governmental experts, to assess the validity of chromatographic analytical methods with respect to several common parameters. While specific details are provided as appropriate, space limitations make it impossible to completely describe the exact application for which the details were appropriate.

Thus in order to gain a greater understanding of how specific details can be applied to a particular validation situation, readers are directed to the references supplied herein. Such an in-depth analysis is particularly necessary when the details seem to be mutually discordant.

In closing, the following key points are offered.

1. The validation strategy is specific for a given application and is influenced by the purpose of the analytical measurement, the analytical procedure used, the nature of the analyte, the concentration of the analyte and the nature of the test sample (matrix).

2. Validation is the systematic comparison of measured performance and pre-determined acceptance criteria. It is absolutely essential that these criteria be clearly established as part of a formal validation plan prior to the initiation of the validation study.

3. Acceptance criteria for validation are not always available from a decision-making third party. In instances where acceptance criteria must be established by the validation team, two concepts are pertinent.

Firstly, the criterion established must be both clearly relevant and applicable to the assay's intended use and scientifically defensible. Secondly, if you set the rules, you had better well follow them.

4. The burden of proof with respect to establishing a method's validity rests with the user/developer.

5. Validation builds quality into the method, ensuring that the method works when needed with no unexpected results.

6. Validation is the insurance policy that assures our customers that our products contain what they should and are capable of doing what they were intended to do.

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Received September 23, 1995
Accepted October 15, 1995
Manuscript 3980

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 759–770 (1996)

SEPARATION STUDY OF PAHs BY HPLC USING A MICELLAR SDS MOBILE PHASE AND SHORT CHAIN COLUMNS

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ABSTRACT

The possibility of using micellar sodium dodecyl sulfate mobile phase modified with n-propanol to separate six PAHs on apolar columns was examined. The large capacity factors found in large-chain stationary phases made the analysis impractical. The use of short-chain stationary phases and the presence of n-propanol in the mobile phase, as a modifier, significantly decreased the capacity factors but also decreased resolution, allowing separation of five PAHs in reasonable analysis times. Conditioning of the column was easy and reproducible but the effect of temperature was quite critical. The gradient technique decreased peak width significantly.

INTRODUCTION

The importance of polycyclic aromatic hydrocarbons (PAHs) in environmental studies is well known.[1,2]

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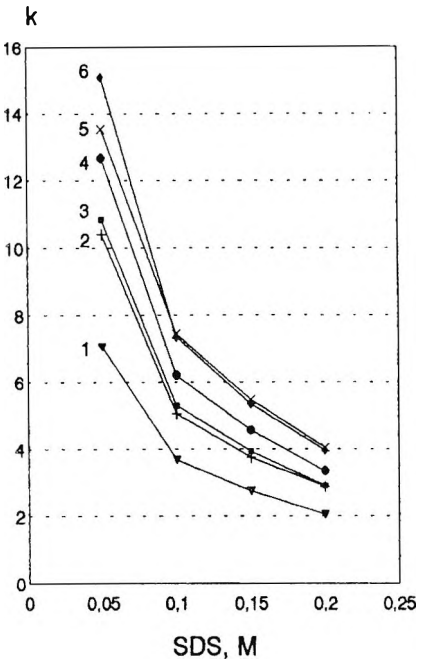

Figure 1. Effect of SDS concentration on capacity factor of PAHs. Conditions: C_4 column; flow-rate, 1 mL/min; temperature 600C; PAHs: 1,naphthalene; 2,acenaphthene; 3,phenanthrene; 4,pyrene; 5,benzo(a)anthracene; 6,chrysene.

Table 1

Characteristics of the Columns

Characteristics	C_{18}	C_4	C_1
Pore size Å	100	300	80
Surface area, m^2/g	350	50	200
Carbon load percentage, %	14	2.0	3.0
Calculated bonded phase coverage, $\mu mol/m^2$	2.06	4.8	4.8
End capping	Yes	Yes	No

The analytical techniques most often used for their determination are gas chromatography (GC) and, particularly, reverse phase high performance liquid chromatography RP-HPLC with fluorimetric detection. The sensitivity of the

latter technique can be increased by a suitable choice of excitation and emission wavelength pairs.[3] Additionally, the sensitivity and selectivity of fluorimetric determination of PAHs increases in micellar solutions.[4,5] Several authors claim that the use of micellar liquid chromatography (MLC) has advantages such as greater selectivity and sensitivity as well as lower toxicity and cost.[6-8] Very few works have been published regarding the separation of PAHs by MLC. Kord and Khaledi studied several, mainly polar, compounds by MLC.[9] Some information on the retention mechanism for non-polar compounds was supplied by Ji.[10] On the other hand, a mobile phase gradient acetonitrile/SDS allowed separation of eleven PAHs.[11] Nine PAHs were separated using a mobile phase (v/v), 0.05 M Brij-35/methanol: 50/50.[12] Solute-micelle association constants of some PAHs were calculated.[13,14]

Accordingly, the separation of PAHs by high performance liquid chromatography using micellar mobile phase (MLC) and short hydrocarbonated chains such as C_1 and C_4 was examined; organic modifiers were used in an attempt to shorten the high retention times in MLC reported in the literature for other compounds.[6,7,15-17]

EXPERIMENTAL

Apparatus and Material

The chromatograph consisting of a high pressure gradient Milton Roy CM 4000 pump, a Rheodyne 7125 sample injector with a 20 μL loop, a Waters 420 fluorimetric detector with the excitation and emission filters of 254 and 375 nm (long-pass), respectively, and a Milton Roy CI 4100 integrator. The columns were a C_{18} Nucleosil 5 μm particulate size (150 x 4.6 mm, Phenomenex) a C_4 Hypersil 5 μm particulate size (100 x 4.6 mm, Phenomenex) and a C_1 Ultremex 3 μm particulate size (100 x 4 mm, Phenomenex). Information about relevant characteristics of the columns are shown in Table I. A P-Selecta Precisterm bath was used for thermostating the columns. A P-Selecta Ultrasons bath was used for preparation all the solutions. A Lida nylon membrane filter with 0.45 μm pore size was used to filter the eluents used to prepare the mobile phase.

Chemicals

Standard stock methanol solutions of 6 PAHs (Sigma) at concentrations in the range 10^{-3}-10^{-4} M were used. More dilute solutions were prepared by dilution with methanol.

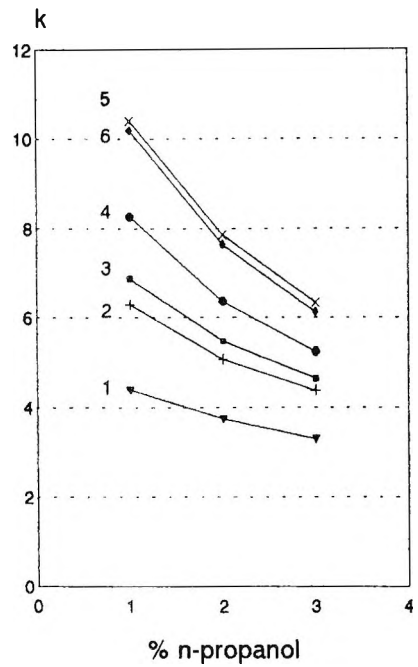

Figure 2. Effect of organic modifier on capacity factor.
Conditions: C_4 column; 0.10 M SDS mobile phase; flow-rate, 1 mL/min; temperature 600C; PAHs are identified in Figure 1.

An aqueous micellar solution of sodium dodecyl sulfate (SDS) ($C_{12}H_{15}NaSO_4$, FW = 288.38-Fluka) was prepared by stirring in an ultrasonic bath to give a final concentration of 0.20M, higher than its critical micellar concentration (CMC) = 8.1×10^{-3} M. More dilute solutions were prepared by dilution with water.

Methanol, n-propanol and n-butanol (Carlo Erba) of chromatographic grade were used. Water was obtained from a Milli-Q system (Millipore). All chemicals were of analytical reagent grade. Before use, all eluents were degassed under vacuum and filtered.

Procedure

In the isocratic mode micellar mobile phases (containig a surfactant concentration in the range of 0.05 M to 0.20 M) were used with small

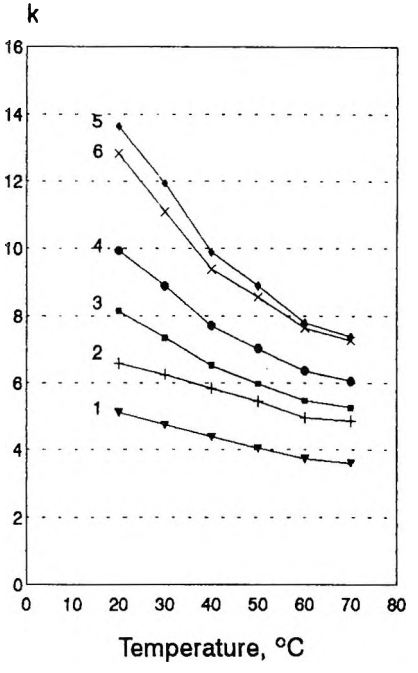

Figure 3. Effect of temperature on capacity factor.
Conditions: C_4 column; 0.10 M SDS with 2% n-propanol mobile phase; flow-rate, 1 mL/min; PAHs are identified in Figure 1.

percentages of n-propanol or n-butanol (in the range 1-3%) as organic modifiers at a flow-rate of 1 mL/min. In gradient chromatography the following gradient was used: 0.15 M SDS containing 12% n-propanol: water, 50:50 (v:v), changing to 0.15 M SDS containing 12% n-propanol in nine minutes, at a flow-rate of 1 mL/min.

Temperatures in the range 20-70°C were tested. Stock solutions of the PAHs were used and their concentrations were adjusted to allow detection in the range of ng/μL by the injection of 20 μL of standard sample. For fluorimetric detection excitation and emission filters of 254 nm and 375 nm (long-pass), respectively, were used. The column was conditioned by applying the following gradient: water for 15 minutes, which changed to 0.20 M SDS containing 2% n-propanol in 75 minutes, at a flow-rate of 1 mL/min. Acetone was used to determine the void time. C_1 Ultremex, C_4 Hypersil and C_{18} Nucleosil were tested stationary phases.

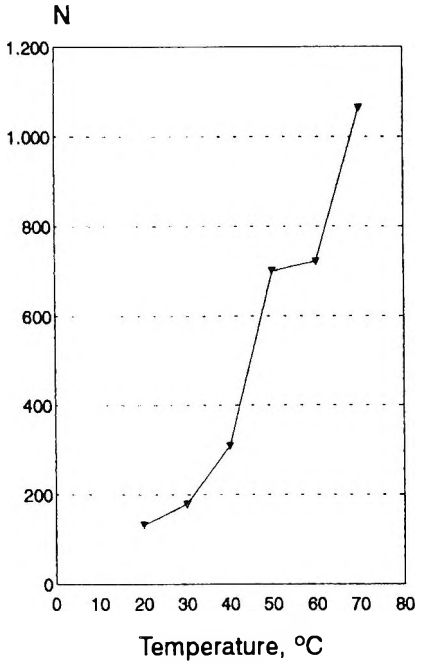

Figure 4. Effect of temperature on efficiency for pyrene. Conditions: C_4 column; 0.10 M SDS with 2% n-propanol mobile phase; flow-rate, 1 mL/min.

RESULTS AND DISCUSSION

Void Time Determination

Sodium nitrate was not appropriate to determine the void time, due to its electrostatic effects on the mobile phase, which contained an ionic surfactant.[16] Various compounds were tested for this purpose and acetone was found to give quite a significant signal due to the change in the refraction index; a 20 μL injection yielded a signal about twice that obtained from 20 μL 0.05 M sodium nitrate in conventional RP-HPLC with spectrophotometric detection.

Void times of 1.07, 1.14 and 1.36 minutes were found for the C_1 , C_4 and C_{18}, stationary phase, respectively, independently of the concentration of the surfactant. These void times were used for all k calculations.

Conditioning of the Column

Because the stationary phases C_1, C_4 and C_{18} are modified in the presence of the SDS[18-20] the column must be conditioned beforehand; this was carried out using water as a mobile phase for 15 minutes, which changed to 0.20 M SDS containing 2% n-propanol in 75 minutes at a flow-rate of 1 mL/min.

The column was thermostated at 60°C. Equilibrium was taken to be reached when the retention time for naphthalene remained constant, in the range 0.1 minutes. No column re-equilibration was necessary due to irreversibility of the SDS retention. The behaviour of C_1, C_4 and C_{18} columns did not change significantly for at least six-month working periods.

Effect of Surfactant Concentration

Figure 1 shows the effect of SDS concentration on capacity factors (k) of PAHs in the C_4 column. Clearly, retention decreased significantly with increasing SDS concentration, due to Micelle concentration elevation. According to the literature[21] k varies inversely with Micelle concentration. Resolution of the six PAHs studied is possible at low SDS concentrations; the best resolution or maximum spread of k values was obtained at 0.05 M SDS concentration. Using a mobile phase containing 0.20 M SDS there is evident overlapping and only four peaks can be observed. When six PAHs were injected, acenaphthene overlapped with phenanthrene, and benzo(a)anthracene with chrysene. The separation of isomers pair, benzo(a)anthracene-chrysene, is only possible using specific column.[3] Overlapping was higher with the C_1 column at the same SDS concentration in the mobile phase. Capacity factors with C_{18} column were very high even in the presence of 20% n-propanol as modifier, giving rise to impractical analysis times.

A reversal of capacity factor of the benzo(a)anthracene-chrysene pair was observed for SDS concentration form 0.10 M using the C_4 column.

Effect of the Organic Modifier

Low concentrations of organic modifiers are used to modify the surface of the stationary phase and provide the wetting needed for good mass transfer.[16] Alcohols such as n-propanol and n-butanol were tested as modifiers. The highest effect was found with n-butanol. The effect of n-propanol on k is shown in Figure 2, where 0.10 M SDS was used. Increasing percentages of the

modifier decreased k values but increased overlapping of the peaks; five peaks were observed in 2% n-propanol mobile phase. The same effect was found using n-butanol but half concentration was neccesary; however, n-propanol was chosen because resolution was better.

Effect of Temperature

Figure 3 shows the effect of temperature on capacity factors for six PAHs mixture. The capacity factors for all the PAHs decreased significantly with increasing temperature, the slope of these changes being higher than those found in RP-HPLC.[7,15,16] The decrease of the mobile phase viscosity with temperature improved the mass transfer, which decreased the C term in the Van Deemter equation. Consequently, the theoretical plate height decreased with the temperature, increasing the efficiency of column. In addition increasing the temperature should enhance the micellar kinetics. A temperature of 70°C was tried, but the column deteriorated clearly after a few runs. Figure 4 shows the effect of temperature on the theoretical number of plates, N, for pyrene. The effect of temperature is critical except in the range 50-60°C. This behaviour is similar for other PAHs. Consequently, a temperature in this range could be recommended.

The sharp decrease in pressure with rising temperature (Figure 5) is another beneficial effect of working at above room temperature. This is due to the decrease of viscosity in the mobile phase. This effect is higher than in conventional RP-HPLC, where changes in pressure drop with temperature are lower.

Effect of Stationary Phase

As indicated above, capacity factors on a C_{18} column were very high; e.g. for naphthalene the value of k was 15 with a mobile phase 0.20 M in SDS; in these conditions k values for naphthalene on C_4 and C_1 columns were 2 and 2.8, respectively, which is due to the carbon load percentage higher of the C_{18} column. In the presence of n-propanol in the mobile phase k values decreased significantly.

On the other hand, as it is shown in Figure 6, the best resoluton is obtained with the C_4 column, using 0.10 M SDS with 2% n-propanol as mobile phase.

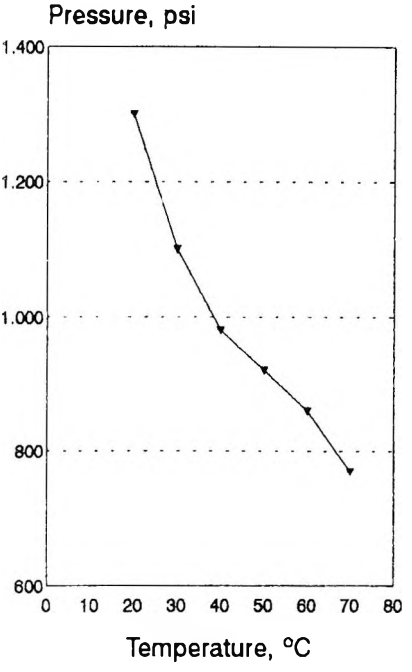

Figure 5. Effect of temperature on pressure.

Gradient Elution Technique

Because the PAHs peaks are quite close in isocratic conditions, gradient elution was tried to increase resolution, thus decreasing the width of the peaks.

The best results were found using a gradient starting with water: 0.15 M SDS and 12% n-propanol, 50:50 (v:v), changing to 0.15 M SDS and 12% n-propanol in nine minutes, at 60°C with a flow-rate of 1 mL/min. Although the decrease in peak width was significant in all the cases, five PAHs were resolved (Figure 7).

Different flow-rate gradients were also tested; starting with 1 mL/min and changing to 0.7 mL/min in 4 minutes gave a separation similar to that found with the concentration gradient, and the same five peaks appeared.

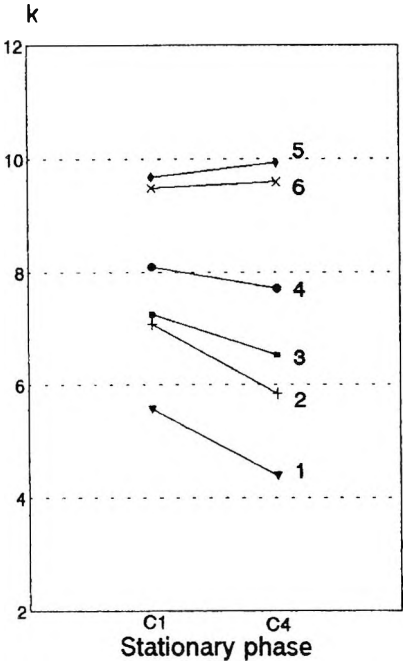

Figure 6. Effect of stationary phase on capacity factor. Conditions: 0.10 M SDS with 2% n-propanol mobile phase; flow-rate, 1 mL/min; temperature 40°C; PAHs are identified in Figure 1.

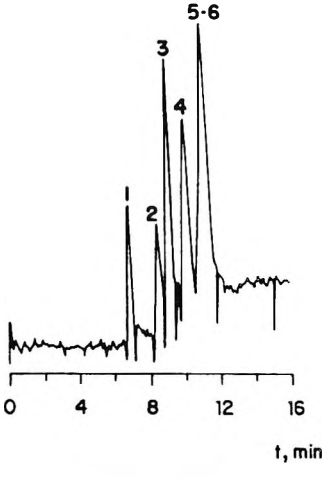

Figure 7. Separation of 6 PAHs using gradient elution.

CONCLUSIONS

The temperature effect is very important in MLC to improve mass transfer, which is an important problem in this technique. It is necessary to control analytical column temperature in the range 50-60°C to improve the PAHs separation.

The use of short alkyl bonded phases and the organic modifiers in the micellar mobile phase provide a more mass transfer and the wetting problem is be less severe.

In conclusion, the use of short columns as well as the addition of alcohols as organic modifiers in the mobile phase and the high temperature of analytical column are necessary for the PAHs separation by MLC.

ACKNOWLEDGEMENTS

The financial support of the Spanish CICYT project PB92-0192 is gratefully acknowledged.

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Received May 30, 1995
Accepted June 8, 1995
Manuscript 3883

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 771–781 (1996)

SEPARATION OF SEVERAL MAIN GLYCOLIPIDS INTO CLASSES AND PARTIALLY INTO SPECIES BY HPLC AND UV-DETECTION

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ABSTRACT

The separation and the estimation of several main glycolipid classes as well as of some of these classes into main subclasses (or species) by reverse phase and normal phase high performance liquid chromatography and UV detection, is described.

Two different gradient elutions onto a C_{18} column and onto a silica column, respectively, and an isocratic elution onto a strong cation exchange column were performed, with detection at 206nm. Separations were achieved within 30min. in the reverse phase and normal phase modes and within 10min. in the cation exchange mode.

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The following glycolipids standard classes were tested: gangliosides, sulfate cerebrosides (sulfatides), digalactosyl-diglycerides, galactosyl-cerebroside as well as ceramides (the backbone of sphingoglycolipids), N-palmitoyl-sphingosine (a synthetic ceramide) and a phospholipid, cardiolipin. Some species of digalactosyl-diglycerides and galactosyl-cerebrosides were also separated.

Application of the present method on the separation of glycolipids from animal tissues is represented.

INTRODUCTION

Glycolipids of plant and animal tissues origin were traditionally isolated from neutral lipids and phospholipids by glass column techniques while thin layer chromatography (TLC), high performance TLC (HPTLC) and high performance liquid chromatography (HPLC) have also been used.

Normal phase HPLC (NP-HPLC) on silica based columns of different type has been used for the separation of several individual glycolipid classes.[1-15] Reverse phase HPLC (RP-HPLC) on C_{18} columns has been used for the classification of glycolipid fractions or for the separation of glycolipid subclasses into molecular species[13-22] while anion exchange HPLC (AX-HPLC) for the separation of gangliosides[23-25] and HPLC on Iatrobeads for glycosphingolipids.[26-30]

NP-HPLC was used for separation of cerebrosides (CER),[1,2,3,5] ceramides (CERA),[3,12] sulfatides (SULF),[4] digalactosyl-diglycerides (DGDG),[5] monogalactosyl-diglycerides (MGDG),[5] sterol-glycosides (STGL),[5] glycosphingolipids (GLSP)[9,10,13] heterocyst type of glycolipids benzoylated derivatives[11] and gangliosides (GANG).[6-8,14,15] The simultaneously separation by NP-HPLC of more than one class of glycolipids only in a few cases have been reported as in the case of the separation of MGDG, DGDG, STGL and CER[5] while other cases have been reported for the species separation of glycosphingolipids.[9,10,13]

RP-HPLC has been reported for the separation of some major GANG subclasses into molecular species,[14,15,21] for the separation of sophorolipids subclasses,[19] for the separation of heterocyst-type glycolipids into subclasses,[22] for the resolution of GLSP[13,17,18] and galactosyl-cerebrosides (GALCER).[20]

AX-HPLC has been used for the separation of GANG.[23-25] Iatrobead separations have been used for GLSP,[26,29,30] GANG[27] and SULF.[28]

The detection of the referred HPLC analysis of the glycolipids were usually performed by techniques other than UV because of the low UV detectability of the underivatized glycolipids. Namely, refractive index (RI) detection,[22] light-scattering detection (LSD),[19] flame ionization detection (FID),[5] fluorescence (FL)[16] have been used.

UV detection for underivatized glycolipids have been reported for GANG,[7,15,21,24,25,27,29] for GLSP,[10,18,31] cyanobacterial heterocyst-type glycolipids[22] while UV- detection for derivatized glycolipids have been reported for GANG,[6] GLSP,[9,13] cyanobacterial heterocyst-type glycolipids[11] and galactosyl-ceramides.[20]

As it can be seen from the above review the simultaneous separation of more than one class of underivatized glycolipids has been reported only in a few cases.[5] This separation was achieved by NP-HPLC while RP-HPLC have been used only for the separation of one particulate glycolipid class or subclass each time into molecular species and AX-HPLC for separation of one particular subclass moiety each time into species.

In the present method the simultaneous separation of four major glycolipid standard classes as well as the separation of these classes into subclasses is represented for the first time by using an easy to run one step gradient RP-HPLC method, by UV-detection at 206nm while an alternative separation by NP-HPLC and a strong cation exchange HPLC (SCX-HPLC) fractionation are also introduced. The present RP-HPLC and NP-HPLC methods are applied to the separation of glycolipids isolated from beef brain.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, purchased from Merck (Darmstadt, G). HPLC solvents were purchased from Ruthburn (Walkerburn, Peebleshire, UK). Lipid standards of HPLC grade were obtained from Supelco (Bellefonte, PA, USA). Bovine brain was also used immediately after the sacrifice of the animal.

Table 1

Retention Times of Individual Standards (min)

Peak No	Glycolipids	RP-HPLC	NP-HPLC	SCX-HPLC
SF	----	3.02	3.02	3.02
1	GANG	8.16	28	----
2	SULF	9.06	10.05	4.21
3	SULF	14.15	SF	5.71
4	(NPSP)	11.41	----	----
5	(NPSP)	15.94	----	----
6	(CARD)	12.87	30.02	----
7	DGDG	13.27	24.05	4.18
8	DGDG	14.37	(24.05)	(4.18)
9	DGDG	15.71	(24.05)	(4.18)
10	GALCER	16.87	26.21	4.32
11	GALCER	21.39	(26.21)	(4.32)
12	GALCER	26.05	(26.21)	(4.32)
13	(CERA)	18.12	SF	4.43
14	(CERA)	23.54	6.05	7.94

Abbreviations: SF, solvent front; GANG, gangliosides; SULF, Sulfatides; NPSP, N-palmitoyl-sphingosine; CARD, cardiolipin; DGDG, digalactosyl-diglycerides; GALCER, galactosyl-cerebrosides; CERA, ceramides.

Standard and Sample Preparation

All standards were prepared as 5% solutions in chloroform/methanol (1:1). Total lipids were isolated from bovine brain by extraction and the glycolipids were separated on a silicic acid glass column chromatography as will be described elsewhere.

Chromatography

HPLC was performed on a dual pump Jasko (Tokyo, Japan) model 880-PU HPLC, supplied with a 330μL loop Rheodyne (P/N 7125-047) injector. A Jasko model 875 UV spectrophotometer was used as detector at 206nm (0.4 a.u.f.s.). The spectrophotometer is connected to a Hewlett-Packard (Avondale, PA USA) Model HP - 3396A integrator - plotter. Three different types of

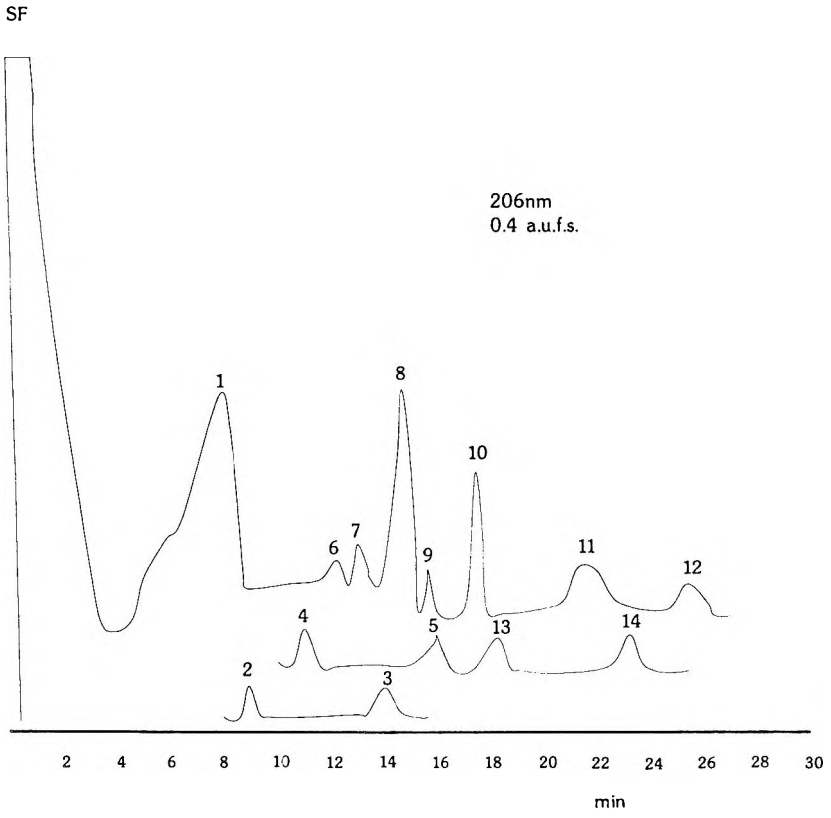

Figure 1. RP-HPLC chromatograms of glycolipid standards. Chromatographic conditions in Results and Discussion section. Peak identification in Table 1.

columns were used: a cation exchange column, SS 10μm Partisil 25cm x 4.6mm I.D., PXS 10/25 SCX from Whatman (Clifton, NJ, USA), an absorption column, Silica 25cm x 4.6mm I.D., from Hichrom H5 (Reading, Berkshire, England) and a nucleosil-300, C_{18} column 7μm, 250 x 4mm I.D. from Analysentechnik (Mainz, G). The flow rate was lmL/min.

RESULTS AND DISCUSSION

Glycolipids analysis was performed by using three different column types, a reverse phase, Fig. 1 and 2, a normal phase, Fig. 3, and a strong cation exchange, Fig. 4.

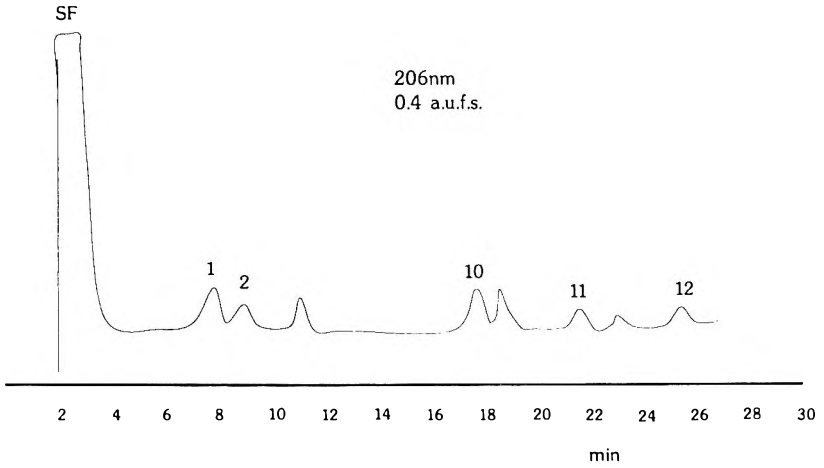

Figure 2. RP-HPLC representative chromatograms of glycolipid fraction from beef brain sample. Chromatographic conditions as in Fig. 1 and in Results and Discussion section. Peak identification in Table 1.

The analysis of standards was performed by using the optimum amounts of each individual substance each time for the UV detection limit of the used instrumentation, as shown in Fig. 1, 3A and 4. Each individual glycolipid standard was injected separately and the resulted peak(s) was collected and co-chromatographed on TLC plates with authentic standards in order to confirm the elution and the retention times (RT's) of the examined glycolipid standard. Similar TLC were also used for the identification of the HPLC peaks from the examined natural sample of beef brain (Fig. 2, 3B).

The mobile phase introduced with the RP-HPLC mode, Fig. 1, was a linear gradient from 100% methanol/water (4:1) to 100% acetonitrile/methanol (7:5) in 10min. and then hold for 15min.. A 25min. elution was sufficient for the separation of four glycolipid classes, as represented in Table 1 with respective RT's. The examined glycolipids were eluted in the following order, GANG as two overlapped species (peak 1), SULF as two species (peaks 2 and 3), DGDG as three species (peaks 7, 8 and 9) and GALCER as three species (peaks 10, 11 and 12). N-palmitoyl-sphingosine (NPSP) was eluted as two species (peaks 4 and 5) and CERA as two species (peaks 13 and 14). Cardiolipin (CARD) was injected in order to compare the RT's of the examined glycolipids with the RT of a relatively non polar phospholipid.

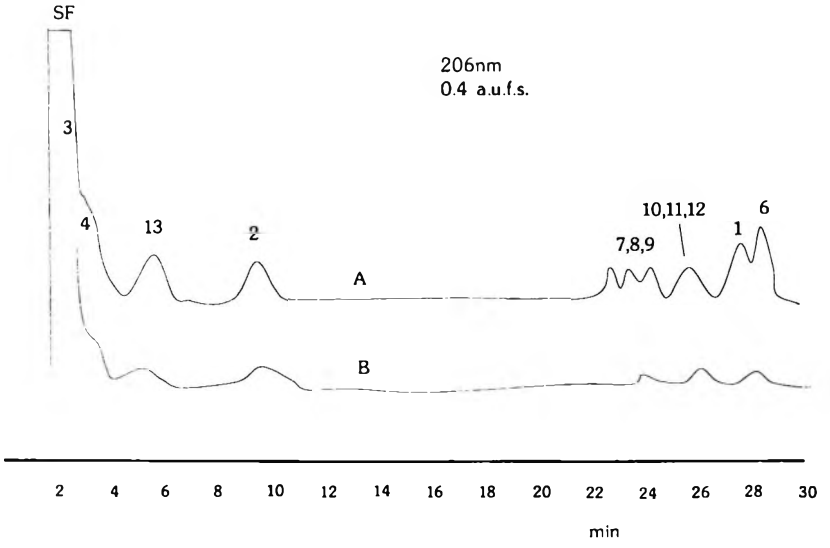

Figure 3. NP-HPLC representative chromatograms (A) of glycolipids standards and (B) of glycolipid fraction from beef brain sample. Chromatographic conditions in Results and Discussion section. Peak identification in Table 1.

Since it has been referred[31] that neutral lipids are also eluted under the above conditions, they should be isolated from glycolipids by other techniques before the analysis performed on HPLC. On the contrary, phospholipids are eluted with the solvent front (SF) (under the above conditions) and they do not interfere in the analysis on the HPLC with the exception of cardiolipin which is eluted among glycolipids.

A glycolipid sample isolated from beef brain was fractionated by this mode as shown in Fig. 2 and revealed the existence of GANG, SULF and GALCER.

A two step isocratic elution was chosen for the separation of glycolipids onto a silica column as shown in Fig. 3. A 15min. elution with 100% acetonitrile followed by a gradient change to 100% methanol within 10min. and hold for 10min., proved to be sufficient for the distinct separation of the relatively low polarity glycolipids from the relatively polar ones, with relatively long intermediate times. Namely, species of SULF (as well as CERA) which were eluted in the first 12min., show a 12 minute difference from DGDG and

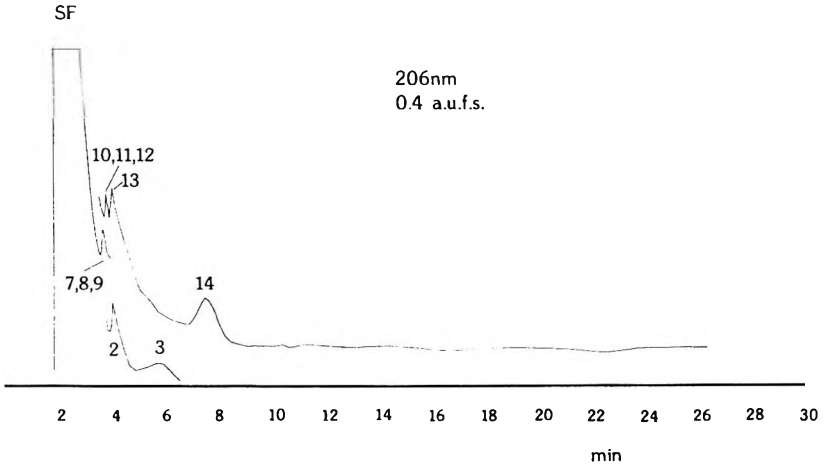

Figure 4. SCX-HPLC representative chromatograms of glycolipid standards. Chromatographic conditions in Results and Discussion section. Peak identification in Table 1.

GANG which were beginning to elute at 23min. The peaks of DGDG and GANG appeared as riders on the "mountain" of the elution solvent change. SULF were elute with solvent front and can be collected and analysed by the RP-HPLC mode. In Fig. 3B is shown the separation of beef brain glycolipids. This method offers an easy collection of the clearly separated glycolipid classes and thus it could be very useful for semi- or preparative purification of individual glycolipid classes.

A strong cation exchange column (Fig. 4) was also used with 100% acetonitrile as elution system. Under these conditions the glycolipids (SULF, DGDG and GALCER) were eluted within 8min. but their separation was insufficient.

The above experimental data show that separation of glycolipids can be achieved with the use of a RP-HPLC as well as with a NP-HPLC but RP-HPLC is superior since it permits also the sufficient separation of glycolipids subclasses and species.

The efficiency of the described method is shown by the analysis of an animal origin sample.

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Recieved June 13, 1995
Accepted July 25, 1995
Manuscript 3904

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 783–797 (1996)

DIRECT DETERMINATION OF ORGANIC ACIDS IN A FERROFLUID (γ-Fe_2O_3) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple HPLC method has been developed for the determination of organic acids adsorbed on particles of maghemite. The advantage of this described technique is the possibility to identify and measure the ratios of differents acids bounded on the same particle. The chromatographic analysis is achieved on a sulphonated copolymer column in acidic form. Good recoveries of the acids were obtained by dosing the free acids in the supernatant and the acids adsorbed on the particles.

INTRODUCTION

Ferrofluids are colloidal suspensions of small magnetic particles (typical diameter of 7 nm) dispersed in a liquid carrier. They are involved in a large number of industrial applications.[1] One of our aims is to synthesize ferrofluids stable in a physiological medium to promote their use in the biomedical field. The ionic ferrofluids studied in the present work are sols of maghemite (γ-Fe_2O_3) particles, stabilized in aqueous medium by adsorption of α-hydroxy

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organic acids at the surface of the particles. The acidic functions of the ligands which are not involved in the complexation with superficial iron ensure the stability of the ferrofluid through electrostatic charges. When different polyfunctional ligands are adsorbed on the same particle, it is possible to fix different biological active molecules on the free organic functions (SH, COOH, NH_2...). For exemple, an antibody could be fixed by the intermediary of a SH free function while on the same particle a drug could be bounded by the intermediary of free carboxylic acid function. However before their use for biomedical applications, it is in particular important to know exactly the quantity of ligand fixed on the particles.

Some indirect methods for the determination of the amount of organic ligands fixed on particles have been reported in the literature. For example, Matijevic *et al.*[2] have used a spectrophotometric method. The concentrations of oxalic and citric acids adsorbed on colloidal spherical hematite (α-Fe_2O_3) particles are deduced from the quantities of ligand present in the supernatant after centrifugation. The iron(III)-5-nitrosalicylate complex presents an absorption peak at 492 nm. When this complex is introduced in the supernatant, the citrate displaces the 5-nitrosalicylate, and so the peak at 492 nm decreases.[3] But organic acids like gluconic or glucuronic acids cannot be determined by this method because of their weak abilities to react with the complex. Furthermore, the linearity range is limited (between 10^{-5}-10^{-4} mol. L^{-1}). A radioactive exchange technique is also used to determine the amount of adsorbed labeled ^{14}C citrate on colloidal silver. The activity of the ^{14}C of this acid remaining in the solution is measured by liquid scintillation. The concentration of the fixed acid is then deduced.[4,5]

By using these methods, the concentration of organic acids is only determined in the supernatant and the amount of organic acids fixed on the particles is then deduced. In this way, validity of the measurements cannot be verified. Precise determination of the amount of ligands fixed on the particles needs a more direct method.

We have developped a simple and specific high-performance liquid chromatography (HPLC) method. This method is usually used for the determination and the separation of mixtures of organic acids in various media.[6-12] In this work, the amounts of several organic acids fixed on maghemite particles and free in the supernatant are quantified and compared with the quantity introduced, a good accuracy is obtained. This method allows also to determine simultaneously the ratios of the different organic acids linked on the particles, which is not possible by previously described methods.

MATERIALS AND METHODS

Apparatus

The chromatographic system used for HPLC analysis is equipped with a Chromatofield Model 501 pump, a Negretti injector and a IOTA refractive index monitor (Precision Instruments, Marseille). The separation is carried out on a 8% cross-linked sulphonated divinyl benzene-styren copolymer in the hydrogen form 300x7.8mm (OA 2216 Benson Polym. Inc NV). The packing particles consists of an impervious core coated with a thin shell of porous material where the exchange occurs. All analysis are carried out at room temperature.

To maintain the column efficiency and so to obtain reproducible results, metallic ions (Fe^{2+}, Fe^{3+}, Na^{+}...) are removed from the samples. They are retained on cations exchange columns (poly-prep) (Biorad AG 50W-X8 resin).[13] These columns are regenerated with three bed volumes of HCl 3 $mol.L^{-1}$ and washed with distilled water until pH is neutral.

The absence of iron in the samples is verified by atomic absorption using a Perkin Elmer 373 spectrophotometer. The calibration curve is done for four concentrations of $(Fe(SO_4)_2(NH_4)_2,6H_2O)$ which are $2\,10^{-6}$, $5\,10^{-6}$, 10^{-5} and $2\,10^{-5}$ $mol.L^{-1}$.

Reagents

HPLC mobile phase

The mobile phase is a sulfuric acid solution prepared from reagent grade sulfuric acid and distilled water. The range of concentration is 0.006-0.02 $mol.L^{-1}$. The mobile phase is used at a flow rate of 0.3 mL / min or 0.5 mL / min (table 1).

Organic acids solutions

Four organic ligands are studied: tartaric acid (pKa: 2.88 ;3.94), citric acid (pKa: 2.79 ;4.30 ;5.65), glucuronic acid (pKa: 3.20 ;12.50), and gluconic acid (pKa: 3.56)[14] (Prolabo RP Normapur). Sodium gluconate is used to prepare the acidic solution because it is easier to handle than the viscous acid. The sodium ions are then exchanged with H^{+} on a cation exchange column to

protect the HPLC column. The original acid solutions are titrated with NaOH 0.1 mol.L^{-1}. They are prepared to obtain a final concentration of about 3.10^{-2} mol.L^{-1}.

Ferrofluid

The ionic aqueous ferrofluids used in this present work are cationic sols of γ-Fe_2O_3 macro-ions. They are synthesized according to a method described elsewhere,[15] by alkalizing an aqueous mixture of iron (II) chloride and iron (III) chloride. The precipitate, consisting in anionic magnetite particles (Fe_3O_4), is isolated by centrifugation and acidified by a solution of nitric acid. The particles are then oxidized in maghemite by ferric nitrate, centrifuged and dispersed in water. The ionic ferrofluid so obtained is composed of magnetic particles positively charged with H^+ at the surface. The molar ratio of superficial protonated sites to total iron is $2.44.10^{-2}$.[16] The iron concentration determined by chemical analysis[17] is 1.03 mol.L^{-1}. The polydisperse system is constituted of roughly spherical particles which the mean diameter, obtained by X-ray diffraction, is 8.3 nm.

Sample Preparation

A known volume of organic acids is added to 4 mL of this ferrofluid, the final volume is adjusted to 100 mL with distilled water. The molar ratio of organic acids added to total iron is noted R in this text. To determine the quantity of ligands adsorbed on the surface of the particles, it is necessary to isolate the particles from the supernatant 10 mL of the sample are centrifuged at 4000 r.p.m during 20 minutes. The supernatant and the solid are then separated.

The solid degraded by 1 ml of HCl 6 mol.L^{-1}, is adjusted to 10 mL with distilled water. 4 mL of this solution are passed two times through the ions exchange columns to eliminate the ferric ions, and the column is then washed with distilled water until a final volume of 10 mL.

Two drops of HCl 6 mol.L^{-1} are added to 3 mL of supernatant to displace the possible complexes of Fe(II) or Fe(III) with organic acids. Then the supernatant is also passed through the ions exchange column, and adjusted to 10 mL.

The absence of iron in these latter samples is confirmed by an atomic adsorption method (iron concentration is lower than 10^{-6} mol.L^{-1}).

Table 1

Retention Times and Operating Conditions, $k' = (t_r - t_0) / t_0$, Capacity Factor $\alpha = (t_{ra} - t_0)/(t_{rb} - t_0)$, Selectivity Factor

Sample Type	pH (Eluent)	Flow (mL/min)	t_r	k'	α
Tartaric Acid	2.24	0.5	11'9"	0.50	
Citric Acid	2.24	0.5	10'19"	0.27	
D-Glucuronic Acid	2.24	0.5	9'40"	0.28	
Gluconic Acid	2.24	0.5	10'50"	0.44	
Citric + Tartaric	2.24	0.5	t_r citric = 10'11" t_r tartaric = 11'09"	0.27 0.49	1.35
Citric + Gluconic	2.57	0.5	t_r citric = 9'05" t_r gluconic = 10'02"	0.25 0.38	1.52
Citric + Glucuronic	2.24	0.5	t_r citric = 10'31" t_r D-glucuronic = 9'40"	0.39 0.28	1.41

The calibration curves are established from four standard solutions for each acid at the concentrations of 3.10^{-4}, 6.10^{-4}, 9.10^{-4}, $1.2.10^{-3}$ mol.L^{-1}. The corresponding heights of the peaks obtained on the chromatograms are plotted versus the concentration of acid. The calibration curves are checked every day with freshly prepared organic acids solutions. For the different organic acids, the calibration curves indicate a linear response over a range of concentration of 5.10^{-5}-$1.2.10^{-3}$ mol.L^{-1}. The curves indicate also that as low as 5.10^{-5} mol.L^{-1} of ligand can be quantified reliably by the HPLC method.

Quantification of Organic Acids in Samples

20 μL of the sample are injected and the amount of organic acid is then obtained directly from the calibration curve. The operating conditions are

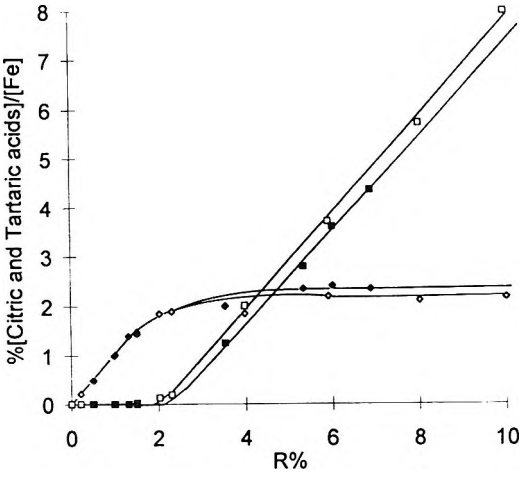

Figure 1. Quantities of adsorbed tartaric acid (◆), citric acid (◇) and free tartaric acid (■), citric acid (□) in the supernatant, versus the quantities of organic acid introduced.

summarized in the Table 1. The organic acids are resolved as a single peak with no interference from other compounds used in this work, which confirm the specificity of the method for organic acids. The identity of the organic acid peaks is assigned by its relative retention time and by spiking with standards.

Recovery

The knowledge of the quantity of organic acids added to the ferrofluid and the determination of adsorbed and free organic acids, allow to calculate the ratios of recovery of ligands from samples. Results are given in the Tables 2-3.

RESULTS AND DISCUSSIONS

The adsorption of organic acids at the oxide surface is based on exchange where the ionizable sites of the particles are replaced by the organic anions which complex the surface iron atoms.[5,18-20] The ligands adsorption on the particles depends on the nature of both oxide surface and ligands. For these acids several complexation models have been proposed in the literature.[21-28]

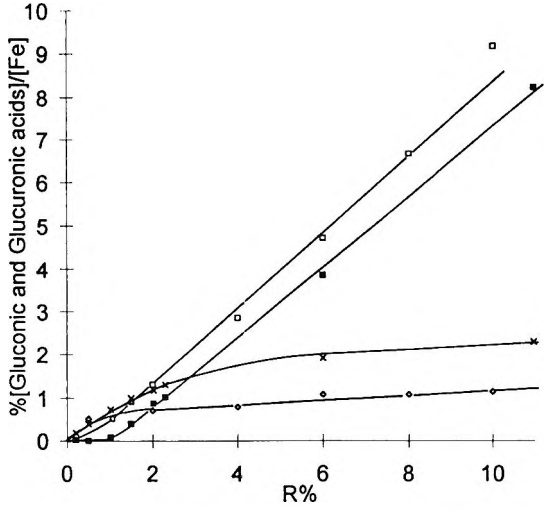

Figure 2. Quantities of adsorbed gluconic acid (✕), glucuronic acid (◇) and free gluconic acid (■), glucuronic acid (□) in the supernatant, versus the quantities of organic acid introduced.

With hydroxy carboxylic acids, both carboxylate and deprotonated hydroxyl groups may participate in the surface complexes. In our experiments, the medium is acidic, the superficial charges of the oxide is then assured by protons and the adsorption of ligands as anionic form is enhanced following the reaction: $MOH_2^+ + L^- \rightarrow ML + H_2O$. The number of ionizable sites (MOH_2^+) on the surface oxide (refered to the total iron) has been determined previously ($[MOH_2^+]/[Fe] = 2.44\%$).[16] In the following section, we will first discuss the results obtained when only one kind of hydroxy acid is adsorbed, and then when two acids are used competitively.

Only One Kind of Ligand Adsorbed on the Particles

The concentrations of organic acids adsorbed on the particles and free are refered to the concentration of total iron, and they are in good accuracy with the introduced quantity as shown in Table 2.

For R lower than 2%, the whole introduced acid is fixed on the particles for tartaric and citric acids (Figure 1). In the case of gluconic and glucuronic

Table 2

Sample Type and Recovery of Organic Acids Introduced

Sample type	R (%)	Recovery (%)	Sample type	R (%)	Recovery (%)
Tartaric acid	0.49	102	Gluconic acid	0.21	100
	0.98	102		0.51	80
	1.31	106		1.02	82
	1.51	99		1.51	93
	3.53	92		2.02	101
	5.32	96		2.30	100
	6.00	97		6.00	96
	6.87	91		10.95	96
	8.96	91		15.47	97
				20.36	96
Citric acid	0.21	100	Glucuronic acid	0.20	95
	0.51	100		1.02	90
	1.01	101		1.51	92
	1.50	98		2.01	100
	2.02	98		4.00	91
	2.30	91		6.00	97
	3.99	96		8.00	97
	5.90	100		10.00	103
	8.00	97		12.51	103
	10.00	101		15.00	101
				17.50	103
				20.10	97
				25.01	106

acids (Figure 2), the introduced acids are partly fixed on the particles, and it remains free ligands in the supernatant. Then the adsorption of anions reachs a maximum. The value of this maximum expressed in adsorbed quantity of ligand refered to iron is about 2.3%. This value agrees with the number of ionizable sites at the oxide surface. The quantity of ligands added to reach this maximum depends on the nature of the ligands. This quantity increases following the order tartaric acid (R=5%) < citric acid (R=6%) < gluconic acid (R=10%) < glucuronic acid (R=20%), indicating that the stability of the surface complexes formed decreases from tartaric to glucuronic acid. This trend is in agreement with the stability constants of the corresponding complexes in

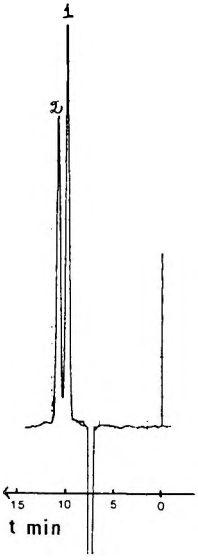

FIGURE 3A

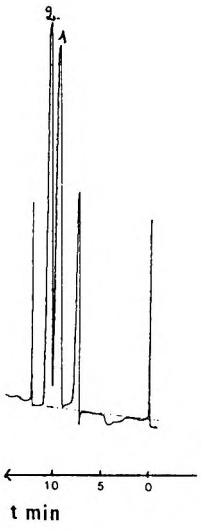

FIGURE 3B

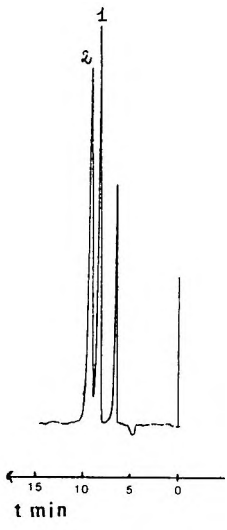

FIGURE 3C

Figure 3. Chromatograms of mixtures of organic acids: A: citric acid (1)- tartaric acid (2) B: glucuronic acid (1)- citric acid (2), C: citric acid (1)- gluconic acid (2).

solution.[27] The weak ability for glucuronic acid to complex superficial iron may be due to its structure. Indeed, among the four ligands studied it is the only one which have no hydroxyl group in a of the carboxylate function. The stability of surface complexe is then lower than with the others ligands which may be coordinated to the surface involving both hydroxyl and carboxylate groups.

Two Kinds of Ligands Adsorbed on the Particles

The following mixtures have been prepared [ferrofluid + citric acid + ligand L] to study the competition for the oxide surface between citric acid and L, L being tartaric, gluconic or glucuronic acid. The molar ratio of ligand added to the particles refered to total iron of the oxide is noted R_1 for citric acid and R_2 for the ligand L. Increasing quantities of citric acid (R_1 varying from 0 to 6%) are added to the ferrofluid and L is then introduced (R_2 = 6%). The quantities of ligands on the particles and free in the supernatant are then

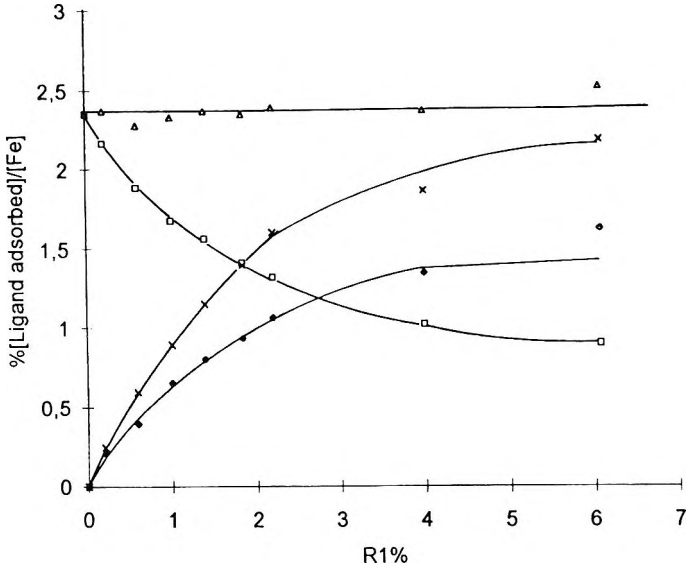

Figure 4. (✕) citric acid adsorbed when only citric acid is added. Quantities adsorbed of: (◆) citric acid, (□) tartaric acid, (△) citric and tartaric versus R_1 when citric acid (R_1=0 to 6%) and tartaric acid (R_2=6%) are added to the ferrofluid.

determined by HPLC in the same way as indicated previously for only one ligand. This HPLC method allows a good separation between two ligands, so the selectivity factor is about 1.4 (Table 1), and good recoveries (Table 3). Typical chromatograms are shown in Figure 3. As shown on the Figures 4-6, the first ligand introduced is adsorbed on the surface and when the second ligand is added, there is a competition for surface sites between the two acids, the second ligand introduced displacing a part of the previously adsorbed ligand. The same observation is reported by Waite and Morel[5] for the competition of citrate and phosphate on lepidocrocite (γ-FeOOH) particles.

Citric acid (1)-tartaric acid (2)

In this experiment (Figure 4), the adsorbed quantity of citric acid increases progressively but is smaller than previously when only citric acid was added to the ferrofluid. The quantity of tartaric acid adsorbed decreases, but the total quantity of ligands on the particles is about the same as ionizable

Table 3

Sample Type and Recovery of Organic Acids Introduced, Ferrofluid + Citric Acid ($0 < R_1 < 6\%$) + A: Tartaric Acid ($R_2 = 6.01\%$) B: Gluconic Acid ($R_2 = 5.88\%$), C: Gluconic Acid ($R_2 = 6.57\%$) Rcv_i Represent the Recovery of the Ligand i

A: Tartaric Acid

$R_1\%$	0.20	0.60	1.00	1.39	1.83	2.10	3.99	6.07
Rcv_1	105	105	107	109	97	97	97	104
Rcv_2	102	106	106	105	95	110	99	106

B: Gluconic Acid

$R_1\%$	0.21	0.63	1.04	1.46	1.91	2.30	4.18	5.99
Rcv_1	124	100	98	86	87	90	85	93
Rcv_2	101	102	106	107	108	103	90	97

C: Glucuronic Acid

$R_1\%$	0.20	0.50	0.82	1.10	1.40	2.51	4.00	6.00
Rcv_1	115	82	106	111	96	97	93	102
Rcv_2	92	91	90	94	93	96	93	102

surface sites. This result indicates that citric acid can displace tartaric acid on the particles.

Citric acid (1)-glucuronic (or gluconic) acid (2)

The gluconic acid comportement in the mixture (Figure 6) is the same as the glucuronic one (Figure 5). For $R_1 < 1.8\%$, all the amount of citric acid added is adsorbed on the particles and when the glucuronic (or the gluconic) acid is added, no deplacement of the citric acid is observed. For $R_1 > 2\%$, the quantity of adsorbed citric acid is lower than for citric acid introduced alone, gluconic (or glucuronic) acid displacing a little part of citric acid. At the beginning of the curves the total organic acids on the particles is smaller than in the precedent mixtures. This agrees with the weak ability of glucuronic (or gluconic) acid to complex the surface iron when it is not introduced in a large excess (10% and 20% for gluconic and glucuronic acids respectively). Therefore, when the quantity of citric acid introduced is higher than 3% the

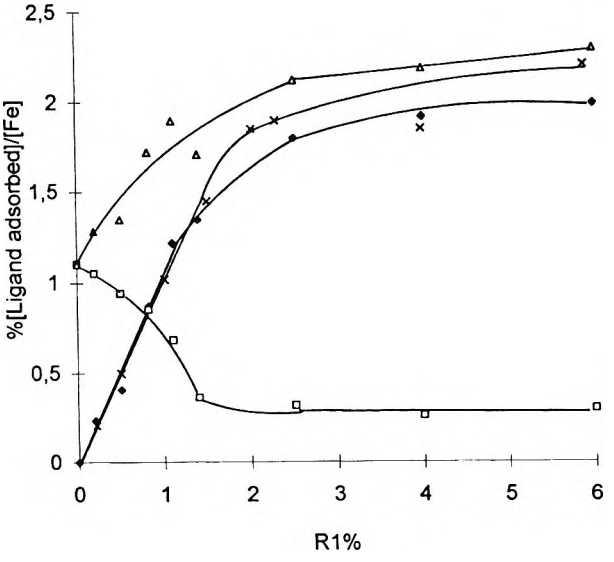

Figure 5. (╳) citric acid adsorbed when only citric acid is added, Quantities adsorbed of (◆) citric acid, (□) glucuronic acid, (△) citric and glucuronic when citric acid (R_1=0 to 6%) and glucuronic acid (R_2=6%) added to the ferrofluid.

total quantity of adsorbed ligands reaches a maximum (2.1% and 2.25% for mixture with gluconic and glucuronic acids respectively) which is a little smaller than for mixture with tartaric acid.

By this method we have shown that it was possible to fix simultaneously two ligands on the same particles. Citric acid is always fixed to the surface, but it can be partly displaced by the others ligands following the order tartaric > gluconic > glucuronic. This trend agrees with the order of stability of surface complexes which has been seen previously.

CONCLUSION

In summary, then, the developed method possesses all the features of a successful analytical method. The recoveries of organic acids from various samples are high, as shown in Tables 2 and 3. By means of this method, the quantity of adsorbed organic acids on the particles of maghemite can be

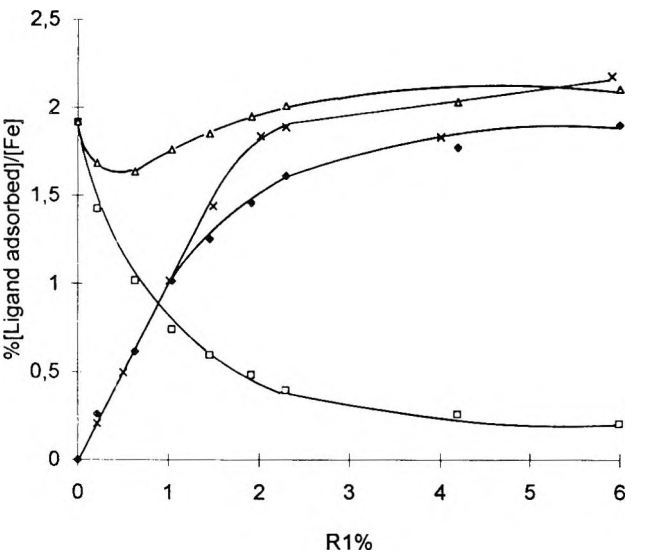

Figure 6. (✕) citric acid adsorbed when only citric acid is added. Quantities adsorbed of: (◆) citric acid, (□) gluconic acid, (△) citric and gluconic when citric acid (R_1=0 to 6%) and gluconic acid (R_2=6%) added to the ferrofluid.

directly determined. The ability ot separate and identify the diffrerent organic acids is an advantage for the analysis of our systems in which two diffrerent ligands are adsorbed on a single particle. The ratio of adsorption for each ligand is a function of its ability to complex the surface.

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Received July 12, 1995
Accepted September 5, 1995
Manuscript 3925

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 799–813 (1996)

CHROMATOGRAPHIC RETENTION OF Ni(II), Co(II) AND Cu(II) AS DIETHYLDITHIO-CARBAMATE COMPLEXES IN PRESENCE OF SURFACTANT/n-PROPANOL/WATER SYSTEMS: DETERMINATION OF MICELLAR BINDING CONSTANTS

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ABSTRACT

This paper presents the values of the micellar binding constants of Ni(II), Co(II) and Cu(II) as complexes with sodium diethyldithiocarbamate, DDTC, in presence of hexadecyltrimethylammonium bromide, CTAB, and sodium dodecylsulphate, SDS, with larger amounts of n-Propanol as organic modifier, by High Performance Liquid Chromatography. Binding constants have been obtained from two equations: a) Arunyanart's treatment, very used for organic compounds but that is not take into account the influence of modifier and b) a multiple regression analysis that permits to consider the high quantities of n-Propanol.

INTRODUCTION

Surfactants which possess both hydrophilic and hydrophobic moieties may

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associate in aqueous media to form dynamic aggregates commonly called normal micelles. Above a certain concentration, termed the critical micelle concentration, CMC, which is unique for every surfactant, the molecules self-aggregate such that the hydrocarbon tails are oriented toward the center of the aggregate and the polar head groups point outward.[1-4] This self-aggregation serves largely to eliminate the hydrocarbon-water interface and is then energetically favorable. The repulsion of the head groups from each other, however, is the force controlling the size and shape of micelles. For these reasons, micelle structure is somewhat dependent on solution properties, changes in ionic strength, addition of an organic solvent, and even some solutes can affect micelle shape and size.[5-8]

Surfactants also can associate in nonaqueous media forming reverse micelles, but now the polar head groups are oriented toward the interior of the aggregate, and the hydrophobic chains are in contact with the solvent.[9-11] The size and characteristics of these structures are critically dependent upon the water content of the solution, the water present tends to accumulate within the core to form an isolate pool of water which may exhibit unique properties. These reverse micelles are more complex and less studied and understood than normal micelles.

The utilization of micellar solutions as the mobile phase in liquid chromatography has generated a new chromatographic variety that is the Micellar Liquid Chromatography, MLC.[12-16] Armstrong et al[12,13] first effectively demonstrated the usefulness of replacing traditional organic modifiers used in reverse phase liquid chromatography with an aqueous micelle solution and developed a three-phase model to allow a theoretical description of MLC

In this model, three equilibria are involved, the first is the solute distribution between the mobile micellar pseudophase and the bulk mobile phase; the second is the solute partitioning between the stationary phase and the mobile micellar pseudophase and the last equilibrium is the distribution of the solute between the bulk mobile phase and the stationary phase.

According to these equilibria, Arunyanart and Cline Love[17] have derived an equation that correlates the capacity factor, k', with the micellized surfactant concentration, C_M, in the form:

$$\frac{1}{k'} = \frac{K_S}{\Phi\,[Ls]\,K_1} C_M + \frac{1}{\Phi\,[Ls]\,K_1} \qquad (1)$$

where K_S is the association or binding constant of a solute to micelles, Φ is the phase ratio (V_S/V_M), V_S and V_M are the total stationary phase volume and the dead

column volume respectively, [Ls] is the stationary phase concentration, K_1 the binding constant for the solute between the stationary phase and the bulk solvent and C_M is given by the total surfactant concentration minus the CMC. If plots of 1/k' vs C_M are lineal it is possible to calculate the binding constant K_S from the slope:intercept ratio.

In the literature, there are many papers in regard to determine the solute-micelle binding constant using normal micelles and micellar systems modified with small percentages of some organic modifiers, like short and medium chain alcohol, for some organic solutes.[18-29] On the contrary, the use of micellar mobile phases in inorganic chromatography, metal-complexes, has been limited.[30-36] In a previous paper,[36] the separation and determination of Co(II), Ni(II) and Cu(II) as diethyldithiocarbamate complexes is achieved by HPLC using a cationic surfactant, hexadecyltrimethylammonium bromide (CTAB), and a larger amount (45% v/v) of n-Propanol in the mobile phase.

In any case, the Arunyanart's expression (equation 1) is not applied when the surfactant mobile phase solutions contain a larger amount of an organic modifier.

In this paper, the equation 1 has been tested using two different surfactants, one cationic as hexadecyltrimethylammonium bromide,CTAB, and one anionic as sodium dodecylsulphate, SDS, in presence of increasing percentages of n-Propanol, PrOH.

EXPERIMENTAL

Materials

All reagents were of analytical grade. The Cu(II), Ni(II) and Co(II) solutions were prepared from the nitrates (Merck) of each one. The ligand, sodium diethyldithiocarbamate (DDTC), surfactants (CTAB, SDS) and n-Propanol (PrOH) from Merck were used as was received.

A Waters liquid chromatograph was used with a pump model 510, UV-Vis detector model 481, an integrator model 740 and an injection valve Rheodyne with an injection volume of 20 μl.

The separation columns were Lichrosorb RP-18, 150x3.9 mm, particle size 10μm from Sugelabor for CTAB and Bondclone RP-18, 150x3.9 mm, particle size 10μm from Phenomenex for SDS.

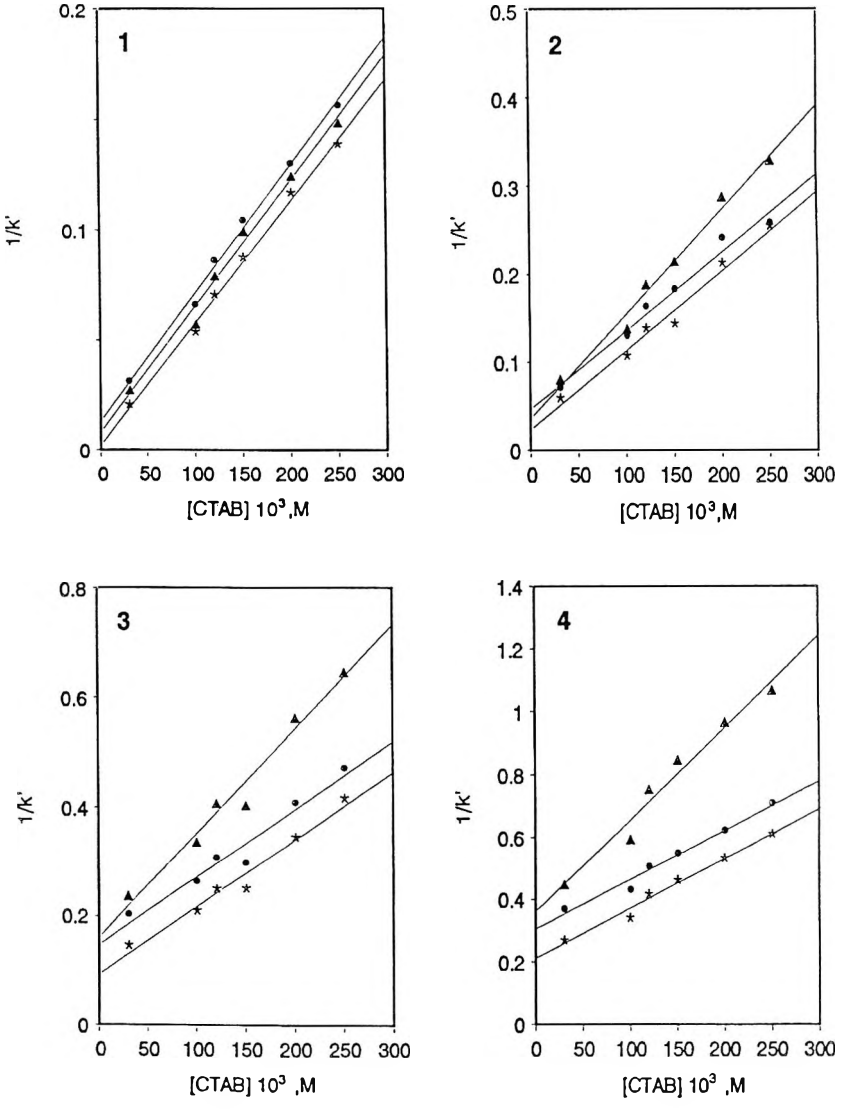

Figure 1. Variation of 1/k' in function of CTAB concentration at different percentages of n-Propanol (1, 20%; 2, 30%; 3, 40%; 4, 50%); • Ni(II); ▲ Co(II); ✳ Cu(II).

Methods

The mobile phases used in this work were prepared with the cationic and

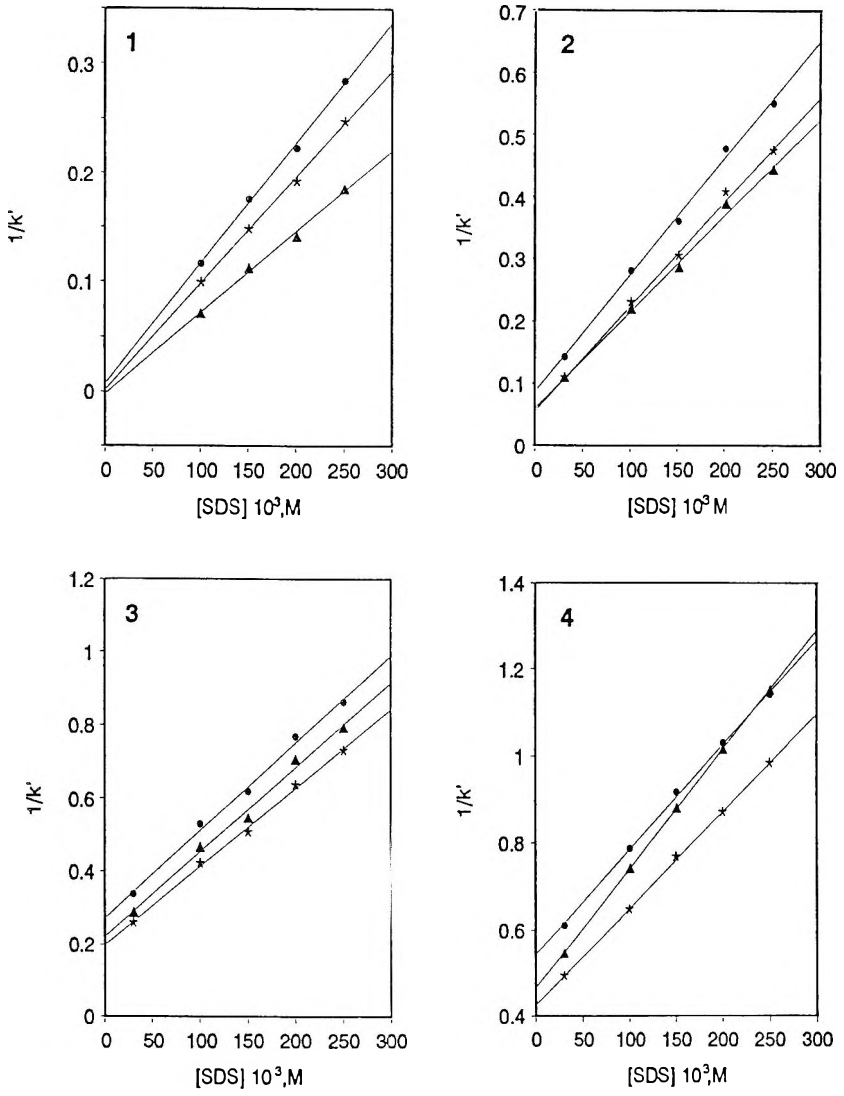

Figure 2. Variation of 1/k' in function of SDS concentration at different percentages of n-Propanol (1, 20%; 2, 30%; 3, 40%; 4, 50%); • Ni(II); ▲ Co(II); ✳ Cu(II).

anionic surfactants (CTAB,SDS) in an appropriate concentration, the ligand (DDTC) and the organic modifier, PrOH, which was needed to reduce the retention

Table 1

Values of the Binding Constant for Metal(II)-DDTC Complexes at Different Percentages of n-Propanol in Presence of CTAB and SDS

K_S, M^{-1} in CTAB

%PrOH v/v	$[PrOH]_T$, M	Ni(II)	Co(II)	Cu(II)
20	2.66	48.9	58.9	147.3
30	3.99	15.5	23.9	36.1
40	5.32	8.1	11.1	12.5
50	6.66	4.8	5.0	6.8

K_S, M^{-1} in SDS

%PrOH v/v	$[PrOH]_T$, M	Ni(II)	Co(II)	Cu(II)
20	2.66	133.8	---	476.1
30	3.99	20.5	24.2	27.6
40	5.32	8.7	10.2	10.7
50	6.66	4.4	5.9	5.2

$[PrOH]_T$ is the total concentration of n-Propanol

times. A buffer wasn't used to modify the pH.

These mobile phases were prepared weighing the necessary quantities of surfactants (at concentrations between 0.03M and 0.25M) and DDTC 10^{-4}M and dissolving them in a mixture of n-Propanol and Milli-Q water with the percentage of alcohol varying from 20 to 50% v/v. All the mobile phases were filtered and placed in an ultrasound bath for twenty minutes for degasification before introduction to the chromatographic system.

The complexes were prepared dissolving the necessary quantity of each one of the salts directly in the mobile phase. These complexes were then injected into the chromatographic system.

The variation of the retention times of the three complexes as a function of the concentration of CTAB or SDS in the mobile phase with different percentages of propanol, as organic modifier, and DDTC in a concentration 10^{-4}M was determined.

Table 2

Equations Obtained for Multiple Regression with Confidence Level of 95% for CTAB in High Performance Liquid Chromatography for the Three Metal Ions ($1/k' = a + b\,[CTAB] + c\,[PrOH]_M$)

	a±C.I.	b±C.I.	c±C.I.	% Agreement
Ni(II)	-0.1552±0.0383	1.1805±0.1817	0.0871±0.0072	96.41

$1/k' = 0.1552 \pm 1.1805\,[CTAB] + 0.0871\,[PrOH]_M$

	a±C.I.	b±C.I.	c±C.I.	% Agreement
Co(II)	-0.3153±0.0847	1.7318±0.4012	0.1412±0.0159	93.28

$1/k' = -0.3153 + 1.7318\,[CTAB] + 0.1412\,[PrOH]_M$

	a±C.1.	b±C.1	C±C.1	% Agreement
Cu(II)	-0.1249±0.0527	1.0866±0.2580	0.0700±0.0103	90.37

$1/k' = -0.1249 = 1.0866\,[CTAB] + 0.0700\,[PrOH]_M$

C.I. = Confidence interval.

The detection was carried out by UV-visible spectrophotometry with a wavelength of 326nm for Ni(II) and Co(II) complexes and 440nm for the Cu(II) complex.

RESULTS AND DISCUSSION

Figures 1 and 2 show the variation of 1/k' vs. C_M for the three DDTC complexes in presence of CTAB and SDS, respectively, at four different percentages of n-Propanol. According to the high quantities of alcohol, the critical micelle concentration, CMC, is practically zero and C_M is the total surfactant concentration. In all cases, exists a good linear regression and thus it is possible to calculate the binding constants, K_S for the three metal complexes at different percentages of PrOH, for CTAB and SDS; Table 1 collects the K_S values.

For the Co(II)-DDTC complex is not possible to calculate the binding constant

at 20% (v/v) PrOH in presence of SDS because in the linear regression, the intercept has a negative value. The found values of K_S show that for elevated percentages of PrOH the binding constants are very different for the three complexes, but at high PrOH concentrations (~up 30% v/v) the K_S values are low and similar for the Ni(II), Co(II) and Cu(II) DDTC complexes in presence of both surfactant systems. This behavior indicates that, the presence of organic modifier, PrOH, at high concentration provokes the same microenvironment to possible complexes interactions.

Despite this good correlation, in equation 1 the binding constant, K_S, according to the pseudophase micellar model,[37,38] is defined by:

$$K_S = \frac{[S]_M}{[S]_W \; C_M} \tag{2}$$

where, S is the solute and the subscripts W and M denote the aqueous and micellar phases. In this equation the PrOH concentration is not considered. However, in presence of alcohol the binding constant can be defined by the following expression:

$$K_S = \frac{[S]_M}{[S]_W \; (C_M + [PrOH]_M)} \tag{3}$$

since one fraction of micellized alcohol is a part of the micellar phase. The $[PrOH]_M$ is calculated from a distribution equilibrium defined by Gettins et al.[39] that gives the following equation:

$$K_{PrOH} = \frac{[PrOH]_M}{[PrOH]_W \; (C_M + [PrOH]_M)} \tag{4}$$

To calculate the micellized concentration of n-Propanol it is necessary to know the equilibrium constants, K_{PrOH}, that according to the literature have the following values: 0.5 M^{-1} for CTAB[39] and 8.0 for SDS.[40]

In order to study the influence of the micellized concentration of PrOH upon the chromatographic retention, a multiple regression analysis has been realized according to the equation:

$$\text{RETENTION} = a + b \; C_M + c \; [PrOH]_M \tag{5}$$

Table 3

Equations Obtained for Multiple Regression with Confidence Level of 95% for SDS in High Performance Liquid Chromatography for the Three Metal Ions ($1/k' = a + b\,[SDS] + c\,[PrOH]_M$)

	a±C.I.	b±C.I.	c±C.I.	% Agreement
Ni(II)	-0.6674±0.0774	2.1015±0.2546	0.19111±0.0129	98.49

$1/k' = 0.6674 + 2.1015\,[SDS] + 0.1911\,[PrOH]_M$

	a±C.I.	b±C.I.	c±C.I.	% Agreement
Co(II)	-0.7689±0.1193	2.0484±0.3920	0.2021±0.0199	96.75

$1/k' = -0.7689 + 2.0484\,[SDS] + 0.2021\,[PrOH]_M$

	a±C.1.	b±C.1	C±C.1	% Agreement
Cu(II)	-0.5099±0.1113	1.7132±0.3645	0.1522±0.0195	95.19

$1/k' = -0.5099 + 1.7132\,[SDS] + 0.1522\,[PrOH]_M$

C.I. = Confidence interval.

where the retention has been expressed by three ways: the capacity factor, k', the logarithm form, log k' and the opposite, 1/k'. The best results have been found by 1/k' for the three metal-DDTC complexes and both surfactants, CTAB and SDS.

Tables 2 and 3 show, for CTAB and SDS respectively, the obtained values of parameters a, b and c with the corresponding confidence intervals. As can be observed in these tables, the agreement of experimental values of 1/k'to Equation 5 is, in all cases, greater than 90%. To confirm this behavior, Figures 3,4 show the agreement between the experimental and calculate values in presence of CTAB and SDS, respectively.

According to these results and the linear relation between 1/k' and C_M (Figures 1,2), it is possible to arranged equation 5 in form:

$$\frac{1}{k'} = a' + b\,C_M \qquad (6)$$

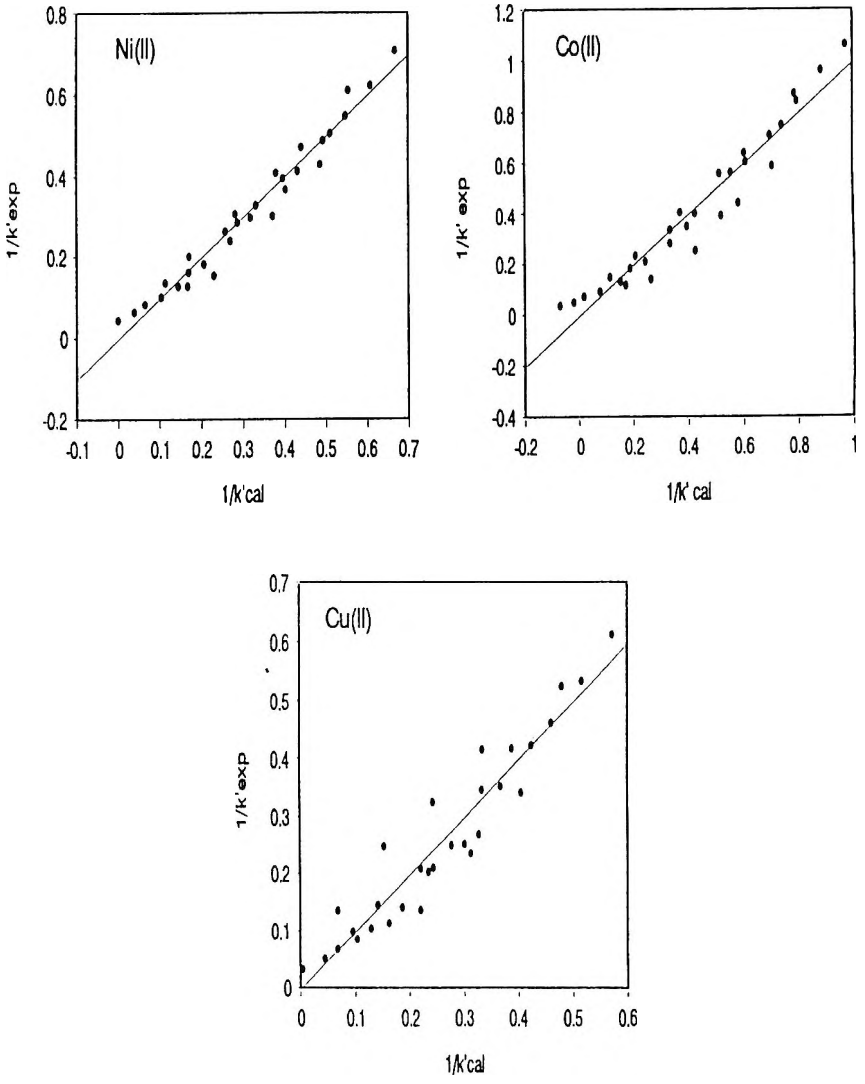

Figure 3. Experimental 1/k' *vs* calculated 1/k' using multiple regression equation in CTAB media.

so that the parameter a' is expressed like:

$$a' = a + c\,[PrOH]_M \qquad (7)$$

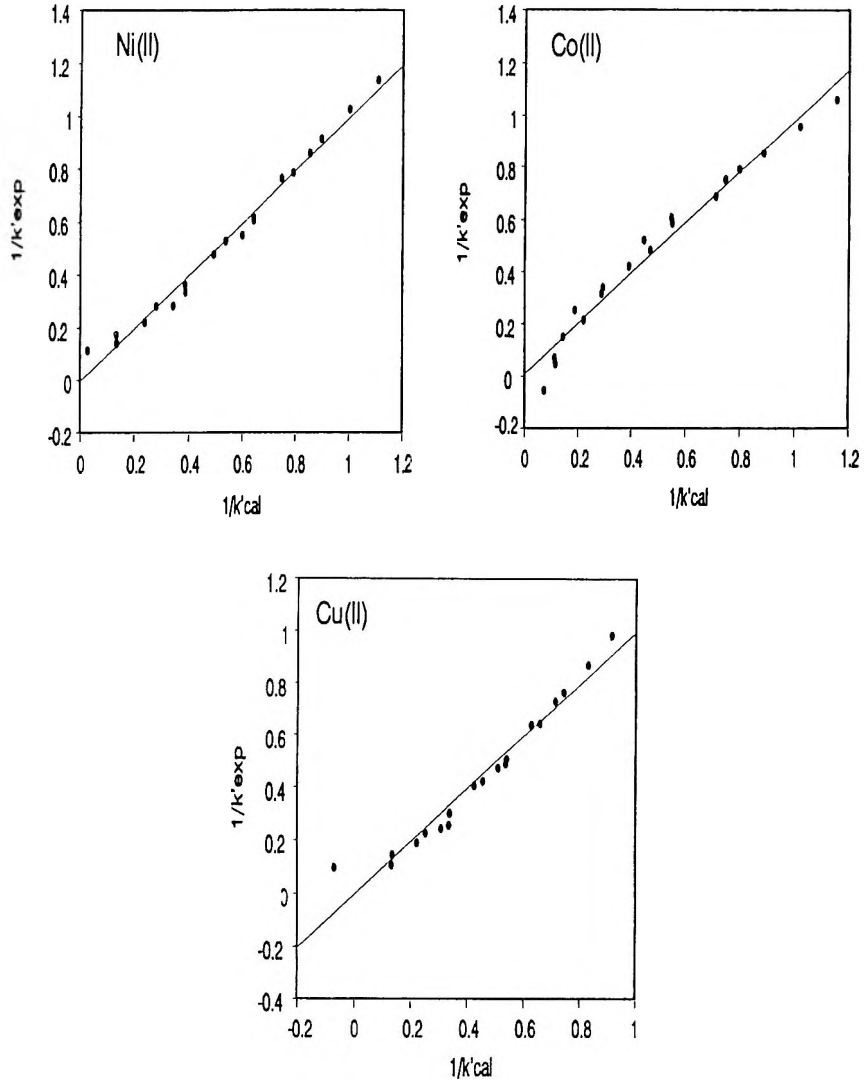

Figure 4. Experimental 1/k' *vs* calculated 1/k' using multiple regression equation in SDS media.

and in comparison to the Arunyanart's equation the parameter b would be K_S / a', so that a' includes the distribution constant of the complexes between the stationary phase and the hydroalcoholic extramicellar phase. In this way, it is possible to calculate the binding constants, K_S, but taking into account the micellized

Table 4

Binding Constants for the Complexes with CTAB and SDS at Different Concentrations of n-Propanol Using the Multiple Regression

K_S, M^{-1} (CTAB)

$[PrOH]_M$, M	Ni(II)	Co(II)	Cu(II)
2	62.1	----	72.0
3	11.1	16.0	12.8
4	6.1	6.9	7.0
5	4.2	4.4	4.8
6	3.2	3.3	3.7
7	2.6	2.6	3.0

K_S, M^{-1} (SDS)

$[PrOH]_M$, M	Ni(II)	Co(II)	Cu(II)
2	----	----	----
3	----	----	----
4	21.7	51.9	17.3
5	7.3	8.5	6.8
6	4.4	4.6	4.3
7	3.1	2.7	3.1

concentration of n-Propanol. For this calculation has been necessary to obtain, from the $[PrOH]_M$ calculated by equation 4, the total range of micellized concentration of n-Propanol for each surfactant and some values, have been introduced into equations 6,7 with the aim to obtain the K_S values.

Table 4 shows the calculated values of the binding constants for Ni(II), Co(II) and Cu(II) as DDTC complexes in CTAB and SDS. In some cases, it is not possible to obtain the K_S values because in equation 6 the parameter a' gives a negative value.

In presence of CTAB, if it is compared with Arunyanart's equation (Table 1), the multiple regression analysis gives, in all cases, K_S values more lower and at high micellized PrOH concentration, up to 4 M (~ 30% v/v) the three metal-DDTC complexes present the same values. According with the results obtained in our laboratory[41] that, confirm the presence of aggregates in these systems

CTAB/PrOH/water it is possible to think that the complexes interact in the same way with them, showing a similar behavior.

The results in SDS, indicate similar K_S values for the DDTC complexes, at more higher micellized PrOH concentrations than CTAB, $[PrOH]_M \geq 6$ M (~ 50% v/v). At micellized PrOH low concentrations there is a dispersion of K_S data, that agrees with the presence of mixed aggregates for this system SDS/PrOH/water.[41] Anyway, comparing the obtained results with those by Arunyanart's equation, the multiple regression analysis gives low binding constant values.

CONCLUSIONS

The variation of the opposite capacity factor, as chromatographic parameter, is linear with surfactant concentration in presence of larger percentages of n-Propanol, for Ni(II), Co(II) and Cu(II) as DDTC complexes.

According with this behavior, it is possible to obtain the micellar binding constants, K_S, by a multiple regression analysis where, it has been considered the micellized n-Propanol concentration.

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Received June 18, 1995
Accepted June 23, 1995
Manuscript 3891

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 815–821 (1996)

DETERMINATION OF PLATINUM AND RHODIUM IN Pt-Rh CATALYSTS BY TLC IN SITU

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ABSTRACT

Favourable chromatographic conditions for separation of Pt and Rh were achieved on microcrystalline cellulose with solvent system methylisobutyl ketone - conc. HCl (40+60, v/v) and KI in ethanol as detection reagent; narow, compact and coloured bands on white background (R_F 0.44 for Rh and 0.76 for Pt) were obtained.

Convenient conditions for photometry in situ (maximum absorption at 480 nm for Rh and 510 nm for Pt; range of linearity from 0.06 μg/cm to about 1.5 μg/cm) were also established.

Worked out procedure was employed to analyze standard solutions and samples of catalysts (ingots). Results obtained have shown that the method is sufficienthly accurate and suitable for simultaneous quantitative determination of Pt and Rh.

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INTRODUCTION

Platinum and rhodium alloys are applied in the industry of fertilizers as catalysts. Their manufacturers analyze various samples of catalysts (ingots, powder, filter mass and net). Gravimetric methods[1,2] are the most employed, but are time-consuming, complicated (comprise a whole range of operations) and costly (high quantities of samples, large number and volume of reagents).

Present investigation was aimed to study the convenience of TLC in situ for simultaneous determination of platinum and rhodium in the aforementioned samples of catalysts. Several authors studied the applicability of paper chromatography[3] and TLC on Al_2O_3 and silica gel layers[4] for separation and detection of the group of platinum metals. In this work we used microcrystalline cellulose.

MATERIALS AND METHODS

The experiments were carried out on TLC plates precoated with microcrystalline cellulose, (E. Merck), 20x20 cm, thickness of layer 0.1 mm.

Preparation of Standard Solutions

Standard solutions were prepared from H_2PtCl_6 and $RhCl_3x3H_2O$ (E. Merck) by dissolution of required amounts in HCl solution, c(HCl) =0.1 mol/L. The concentration of Pt and Rh in standard solutions were within the range 0.001 to 2 mg/mL.

Application of Standard and Sample Solution

Was performed with the CAMAG LINOMAT IV: 10 μL in band along 10 mm; 10 mm distance between bands; 4 bands of standards and 5 bands of samples were applied to the plate.

Development

Was carried out in CAMAG chromatographic chamber (saturated with developer for 30 minutes). Developer: methyl-isobutyl ketone - HCl conc. (40+60 v/v).

Visualisation

Was performed by spraying with 1% solution of KI in ethanol (sprayer with compressed air). Preparation of KI solution: dissolve 1.0 g of pure KI in a small volume of water and add ethanol to 100 mL. After spraying with KI solution, warm the plate in draying oven at 100 ^{o}C for 30 minutes.

A Camag TLC/HPTLC scanner (scanning rate: 3 mm/s, slit: 4/5, sensitivity: 50) was used for densitometry of Pt and Rh bands at 510 nm and 480 nm respectively.

Preparation of Sample Solution from Pt-Rh Ingot:

Weigh (using analytical balance) 0.20-0.25 g of the ground sample, place in a glass vessel and add 20-25 mL of aqua regia. Cover with the watch glass and warm on the hot plate (at 85-90°C). When dissolved, uncover and evaporate to almost dry. Add 1.2 mL of concentrated HCl (to cover the syrup precipitate) and evaporate again to almost dry. Add HCl, c=0.1 mol/L, to the precipitate to dissolve completely. Transfer the solution quantitatively into a 1000-mL volumetric flask, add the solution of HCl, c=0.1 mol/L and shake vigorously.

RESULTS AND DISCUSSION

Optimal conditions for separation of rhodium and platinum by TCL, as well as their direct and simultaneous determination on a thin-layer were established experimentally. Out of the several solvent systems studied, methyl-isobutyl ketone - HCl conc. (40+60 v/v) gave the most satisfactory results. That solvent system produced separated, narrow, compact Rh and Pt bands. The obtained R_F values for Rh and Pt were 0.44 and 0.76 respectively. Developing time for 10 cm length was around 3.5 hours.

Studied were several reagents for detection of Rh and Pt (PAN, NaDDTC, cinchonine, KI). Ethanol solution of KI, showed superiority for having produced intensely coloured bands against white background: dark brown for Rh and pink-brown for Pt. That selective reagent allowed uniform and fine spraying when the sprayer was connected to compressed air.

The most convenient conditions for photometry on TLC/HPTLC Scanner were determined experimentally. Detected were wavelengths of maximum

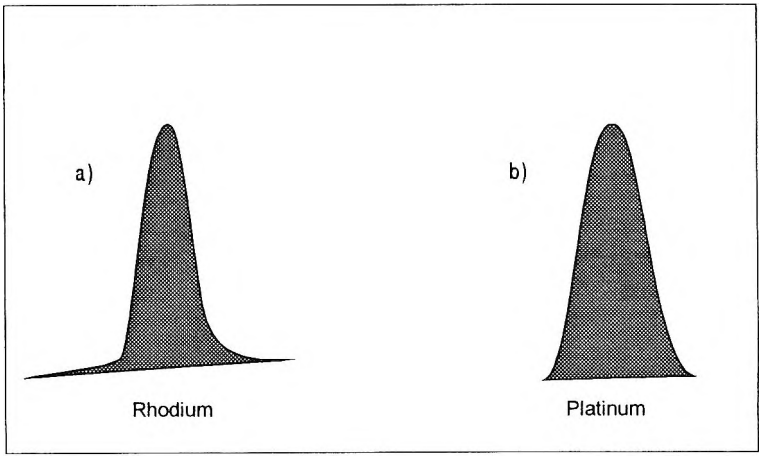

Figure 1. Photometric curves of rhodium and platinum bands a) γ (Rh)=0.469 μg/cm, R_F=0.44; b) γ (Pt)=0.52 μg/cm, R_F=0.76; thin layer: microcrystalline cellulose; developer: methyl-isobutyl ketone - HCl conc. (40+60, v/v); reagent for detection: 1% solution of KI in ethanol.

absorption: λ = 480 nm for Rh and λ= 510 nm for Pt. The lowest recordable limit concentrations of the solutions were about 0.006 mg/mL (i. e. 0.06 μg/cm with the application of 10 μL) for both Rh and Pt. Established range of solution concentrations, most convenient for measuring, was from 0.006 mg/mL (0.06 μg/cm) to approx. 0.14 mg/mL (1.4 μg/cm) for Rh and up to approx. 0.16 mg/mL (1.6 μg/cm) for Pt. That concentration range showed linearity between the areas under photometric curves and the quantity of studied ions in the band (correlation coefficient is 0.999). Photometric curves were almost symmetrical Gaussian curves (Figure 1).

The developed TLC in situ method gave sufficiently accurate and reproducible values, as compared with the known quantities of rhodium and platinum standards applied to the layer (Table 1).

The results obtained by this method for Rh and Pt in the samples of catalysts were well in agreement with those obtained by gravimetry (Table 2). Consequently, it is possible to employ the diveloped TLC in situ method for simultaneous quantitative determination of rhodium and platinum in Rh-Pt-catalysts, applied in the industry of fertilizers. The method is simple, cost-saving and rapid (unlike gravimetry). Its particular advantage is in that it allows simultaneous separation and determination of rhodium and platinum

Table 1

Comparison of Rh and Pt Values Obtained by TLC In Situ and Amounts Applied to Thin Layer

Rh/(μg)		Pt (μg)	
Applied	**Determined**	**Applied**	**Determined**
	0.8600		0.0970
	0.0865		0.0968
0.088	0.0870	0.0975	0.0980
	0.0876		0.0960
	0.0885		0.0969
	0.4650		0.5180
	0.4630		0.5185
0.469	0.4710	0.520	0.05176
	0.4670		0.5230
	9.4730		0.5180

which, in turn, simplifies and shortens the procedure itself and duration of analysis. The analysis can be performed on one sample solution if the mass fraction of one element in the sample is below 65% and of the other over 5%. In such instances it is necessary to weigh 0.20-0.25 g of the sample, dissolve it and make with HCl solution, c=0.1 mol/L, to 1000.0 mL. Rh and Pt concentrations in the obtained solution are within the range of linearity. The solution should then be applied to chromatoplate in 5 bands and parallely 4-5 bands of standards; results of determination are mean values of parallel determinations for both Rh and Pt. If the sample composition is other than the stated (i. e. if the ratio of these elements exceeds 13:1), two sample solutions should be prepared. Since the Rh and Pt content limits being known for each catalyst sample during the production, sample weight and solution volume can be calculated. In that way the concentration of the element contained in the sample in a lower quantity is brought to the linearity limits (solution 1). The aliquot of this solution has then to be diluted to a specified volume, in which the concentration of the element having higher mass fraction, is now brought to the linearity range of the method (solution 2). Three bands of solution 1 and three bands of solution 2 are applied to chromatoplate and chromatography and densitometry carried out. Mean values of three parallel determinations for every element represent results of the determination.

Table 2

Comparison of Quantitative Determination of Rh and Pt in PT and Rh Alloys (Ingots) by TLC In Situ and by Gravimetry

	TLC In Situ		Gravimetry	
Sample (Ingot)	**Rh/(%)**	**Pt/(%)**	**Rh/(%)**	**Pt/(%)**
1	13.89	85.56	13.80	85.34
2	14.01	85.60	14.30	84.99
3	13.98	85.87	13.70	85.50

This TLC in situ method gives satisfactory results, the standards and samples being chromatographied and photometered under equal conditions and simultaneously, which annuls the errors. It is important that the standards be very pure chemicals and that the analysis be carried out with care and accuracy. Dissolution of samples represents major difficulties. Using aqua regia for dissolution is very time-consuming (takes four and more hours). If rhodium content in a sample is high, the sample is then slightly soluble in agua regia. Therefore, an indissoluble part has to be melted with $NaHSO_4$ or $Na_2S_2O_7$ and the melt dissolved in HCl.

Compared to gravimetry, this TLC method requires analytical samples of significantly smaller weight, contained in the big volume of the solution. Therefore small amounts of silicon, which may occur in some samples of catalysts (as filter mass), will not produce noticeable errors. One of the advantages of TLC method for Rh and Pt determination is in that it does not require removal of the impurities in the catalyst samples (Cr, Ni, Fe, Mn and Pd) from the sample solution, as they do not interfere with determination procedure.

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Received June 12, 1995
Accepted June 23, 1995
Manuscript 3907

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 823–836 (1996)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH FLUORESCENCE DETECTION FOR THE ANALYSIS OF 1954U89, 1,3-DIAMINO-7-(1-ETHYLPROPYL)-8-METHYL-7H-PYRROLO-(3,2-f)-QUINAZOLINE, IN PLASMA

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ABSTRACT

1954U89, 1,3-diamino -7 - (1-ethylpropyl) -8- methyl-7H-pyrrolo(3,2-f)-quinazoline, is a potent, lipid-soluble inhibitor of dihydrofolate reductase that is under preclinical evaluation as an anticancer agent. A rapid and selective high performance liquid chromatographic assay with fluorescence detection was developed for the quantitation of 1954U89 in rat and dog plasma. The compound was removed from plasma by solid phase extraction, and the extracts were chromatographed on a Hypersil C_1 column (4.6 mm x 15 cm) under isocratic conditions. The HPLC mobile phase consisted of methanol and 0.02 M ammonium acetate buffer (pH = 4.5) delivered in a ratio of 70:30 and at a flow rate of 1.0 mL/min. The compound was quantitated by fluorescence detection with excitation and emission wavelengths set at 335 and 460 nm, respectively. The quantitation range of the assay was 0.01 to 2.0 μg/mL. The intra- and interassay precision of the method were approximately

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4 and 6%, respectively, in rats, and approximately 7 and 4%, respectively, in dogs. The accuracy (% bias) ranged from -10 to +2% across the concentration range in both species. This assay has been used to support nonclinical pharmacokinetic and bioavailability studies of 1954U89 in rats and dogs.

INTRODUCTION

The importance of folate antagonists in cancer chemotherapy has been recognized since the introduction of methotrexate (MTX) in the 1950s, and the role of nonclassical, especially lipid-soluble, antifolates in overcoming the limitations presented by MTX has been the subject of extensive research during the past four decades.[1-3] Lipid-soluble inhibitors of dihydrofolate reductase (DHFR) continue to show potential in cancer chemotherapy, and compounds that may show clinical utility based on their favorable disposition profile or their ability to overcome some forms of resistance have been identified.[4,5]

The diaminoquinazoline, 1954U89, 1,3-diamino-7-(1-ethylpropyl)-8-methyl-7H-pyrrolo-(3,2-f)-quinazoline (Figure 1), is a potent DHFR inhibitor that arose from an extensive research program that also produced pyrimethamine, metoprine, and piritrexim.[6] It is a small (MW 283), lipophilic (log P 2.7) compound with a K_i against human DHFR of 1.4 pM. The free base has limited aqueous solubility (<0.1 mg/mL), but the mesylate salt is soluble up to approximately 2.0 mg/mL in aqueous solutions and buffers. The compound achieves relatively high brain to plasma and lung to plasma concentration ratios in mice, rats, and monkeys, and shows activity against several tumor cell lines in culture.[6]

In conjunction with preclinical development of the compound, an analytical method with sufficient sensitivity, specificity, accuracy, and precision to support pharmacokinetic, disposition, and toxicology studies was required. Previous assay methods in our laboratory for compounds of this type included quantitative thin layer chromatography (TLC),[7] high performance liquid chromatography (HPLC),[8] and competitive protein binding.[9]

For the quantitation of 1954U89 in rat and dog plasma, an HPLC method with fluorescence detection was developed, validated, and applied successfully to rat and dog pharmacokinetic studies. The method and the results of the validation are reported here.

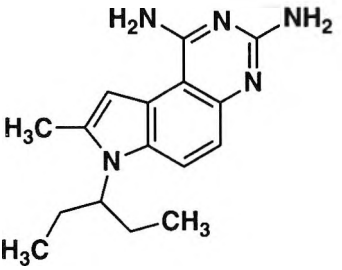

Figure 1. Chemical structure of 1954U89.

MATERIALS AND METHODS

Chemicals, Solvents, and Solutions

The mesylate salt of 1954U89 was obtained from Compound Registration, Burroughs Wellcome Co., Research Triangle Park, NC. Methanol and water were HPLC grade (Omnisolv, EM Science, Cherry Hill, NJ). Ammonium acetate and hydrochloric acid (37%) were A.R. grade (Mallinckrodt, Paris, KY). Rat plasma was obtained from male CD rats (Charles River Laboratories, Raleigh, NC) housed in the Central Animal Facility (Burroughs Wellcome Co.). Dog plasma was obtained from Environmental Diagnostics (Burlington, NC) or the Central Animal Facility. Ammonium acetate buffer (0.02 M, pH = 4.5), prepared by dissolving ammonium acetate (1.54 g) in distilled water (1000 mL) and adjusting the pH with HCl, was used to buffer plasma and to reconstitute extracts after evaporation. Ammonium acetate in methanol (0.02 M), prepared by dissolving ammonium acetate (1.54 g) in methanol (1000 mL), was required to elute the compound from the extraction columns. The HPLC mobile phase consisted of methanol and ammonium acetate buffer in a ratio of 70:30. Stock solutions of 1954U89 in ammonium acetate buffer were used to prepare spiked plasma calibration standards; separate stock solutions were used to prepare the spiked plasma controls. Eight concentrations of calibration standards (0.01 to 2.0 μg/mL) and three spiked plasma controls (2.0, 0.2, 0.02 μg/mL) were prepared, divided into 1-mL portions, and stored at 80°C until assayed.

Experiment

A Perkin Elmer AD-2 analytical balance was used to weigh the compound for the preparation of stock solutions. Micropipettors with glass tips (Scientific

Manufacturing Industries, Emeryville, CA) were used to prepare standard solutions and plasma controls. 1954U89 was extracted from plasma with 100-mg C_2 Bond Elut solid-phase extraction cartridges (Varian Associates, Sunnyvale, CA) and a multiple-cartridge vacuum apparatus (Vac Elut, Varian). Plasma extracts were evaporated in an N-Evap analytical evaporator (Organomation Associates, South Berlin, MA). The HPLC system consisted of a Waters 600 multisolvent delivery system and a Waters 712 WISP auto injector (Waters Associates, Milford, MA).

Samples were injected onto a Hypersil C_1 analytical column (4.6 mm x 15 cm, Phenomenex, Torrance, CA) with a 2-μm precolumn filter (Upcurch Scientific, Oak Harbor, WA) and a C_1 guard cartridge (Keystone Scientific, Bellefonte, PA). Sample fluorescence was quantitated with a Shimadzu RF-530 fluorescence detector (Shimadzu Scientific Instruments, Inc., Columbia, MD). The excitation (or absorption) spectrum of 1954U89 was determined initially, then the excitation wavelength was set at its maximum and the compound was scanned to determine the emission spectrum. The excitation and emission maxima were determined to be 335 and 460 nm, respectively. The mobile phase was delivered at a flow rate of 1.0 mL/min.

Chromatographic data acquisition and peak area analyses were accomplished with VG Multichrom software (Fisons Instruments Inc., Beverly, MA) and a VMS operating system (U.S. 5-20) on a VAX 6320 (Digital Equipment Corp., Maynard, MA).

Sample Preparation and Assay

Portions (0.1 mL) of plasma samples, calibration standards, or plasma control samples were combined with ammonium acetate buffer (0.5 mL) in 12 x 75 mm glass test tubes and vortexed. For each sample, standard, and control sample, an individual solid-phase extraction cartridge was preconditioned with methanol (1.0 mL) followed by ammonium acetate buffer (1.0 mL).

The buffered samples were loaded onto the cartridges while the packing was wet, and washed sequentially with water (1.0 mL) and methanol (1.0 mL). 1954U89 was eluted from the cartridges with ammonium acetate in methanol (1.0 mL). The eluates were evaporated under nitrogen at 50°C, and the residues were reconstituted with ammonium acetate buffer (0.2 mL) and vortexed. Reconstituted extracts were loaded into autosampler vials, and the autosampler was programmed to inject 0.1 mL at 7-min intervals.

Calculations

A least-squares linear regression model (weighted $1/c^2$) was selected and fitted to the peak area and concentration data obtained from the calibration standards. The concentrations of 1954U89 in plasma control samples were calculated from the equation of the regression line.

Assay Validation

The calibration model was selected after the analysis of calibration standards at eight concentrations (0.01 - 2.0 μg/mL). Standards were assayed in triplicate during six assay runs (rat plasma) and in duplicate during twelve assay runs (dog plasma), and a least-squares linear regression model with four weighting schemes (unweighted, $1/c$, $1/c^2$, and log-log transformed) was fitted to the concentration-peak area data. The residuals (the difference between the observed peak area and the peak area predicted by the regression equation) at each concentration were calculated and plotted as a function of concentration. The residuals plots were inspected for heteroscedasticity and for random distribution of the residuals around a residual value of zero.[10] The weighting scheme that resulted in homogeneous and normally-distributed variance of the residuals was chosen as the calibration model.[11,12] Assignment of the upper and lower limits of quantitation of the assay, defined as those concentrations at either end of the calibration curve that maintained the variance characteristics of the rest of the calibration curve, were made from the plot of the residuals.

The extraction efficiency of the assay was assessed by the comparison of extracted samples to unextracted calibration standards. Spiked rat plasma controls (0.02, 0.2 and 2.0 μg/mL) were extracted and assayed. Measured concentrations were determined with a calibration curve derived from the direct injection of 1954U89 stock standards and compared to their theoretical (nominal) concentrations to estimate the recovery from plasma.

Plasma from rats (n = 6) and dogs (n = 6) without added 1954U89 was extracted and assayed as described to ascertain that the method was specific for 1954U89. Chromatograms from these experiments were examined to determine if endogenous substances would interfere significantly with the integration of the compound peak. In addition, extracts of *in vitro* incubations of 1954U89 with hamster liver homogenates, which contained five (as yet unidentified) metabolites of 1954U89, were injected onto the HPLC system to check the retention times of the putative metabolites relative to the unchanged compound.

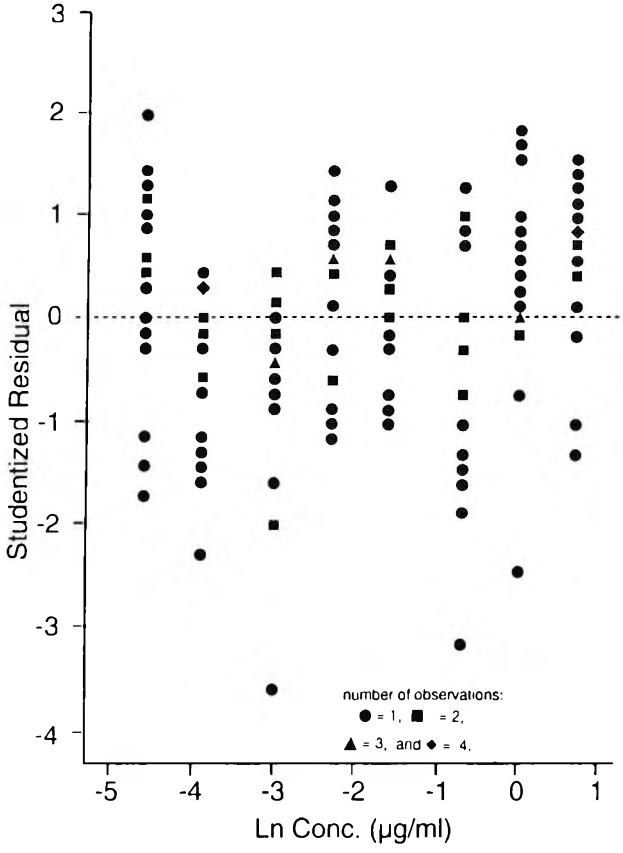

Figure 2. Plot of the Studentized residuals for the weighted ($1/c^2$) least-squares linear regression on the calibration standards in rat plasma (n = 3 x 6 at each concentration).

Intra- and interday precision and accuracy of the assay also were determined. Rat plasma was spiked with 1954U89 at three concentrations (0.02, 0.2, and 2.0 µg/mL). Each sample was divided into 1.0-mL portions and stored at -80°C. Eighteen samples from each group were analyzed over a six-week period. Analysis of variance was used to partition the total observed variance of the assay into its two components, intra-assay variability (random error) and interassay variability, or error associated with differences in day to day conditions.[13] Accuracy (% bias) was calculated as the percentage difference between the mean measured concentrations for each group of control samples and their theoretical (nominal) values.

Table 1

Extraction Efficiencies of 1954U89 from Rat Plasma

Normal Concentration (μg/mL)	Assayed Concentration[a] (μg/mL)	Recovery (%)
0.02	0.020 ± 0.0011[b]	100 ± 6
0.20	0.204 ± 0.0119	102 ± 6
2.00	2.027 ± 0.0804	101 ± 4

[a]n = 9 at each concentrtion
[b]Mean ± S.D.

Several studies were conducted to observe the effect of various storage conditions and experimental treatments on the stability of 1954U89. The stability of an analytical standard solution of 1954U89 stored at approximately 25°C in the dark was evaluated by assaying portions (50 μL, 0.5 μg, n = 4) at two, four, and six weeks after preparation. The concentration of the standard solution was determined with a standard curve derived from a freshly prepared standard solution. The stability of 1954U89 in plasma stored at -80°C was determined with rat plasma spiked at three concentrations (0.02, 0.2, and 2.0 μg/mL). Three replicates from each concentration were assayed on the day of preparation, frozen, and then re-assayed at one, three, and six weeks after preparation with freshly prepared calibration standards. The stability of the compound in plasma that underwent repeated freeze-thaw cycles also was studied. Dog plasma was spiked with 1954U89 (2.0 μg/mL), and duplicate portions were assayed immediately. The remainder was stored at -80°C, allowed to thaw, and then reassayed; this freeze-thaw-assay cycle was repeated three times. The stability of 1954U89 during the period between extraction and injection of the samples also was investigated. Fresh calibration standards and a spiked dog plasma sample (5.0 μg/mL) were prepared and extracted as described above. The extract was divided into six autosampler vials, and injected at 4-h intervals over a 21-h period, which was the maximum expected assay run time.

RESULTS AND DISCUSSION

The approach taken in the development of this method reflected both the

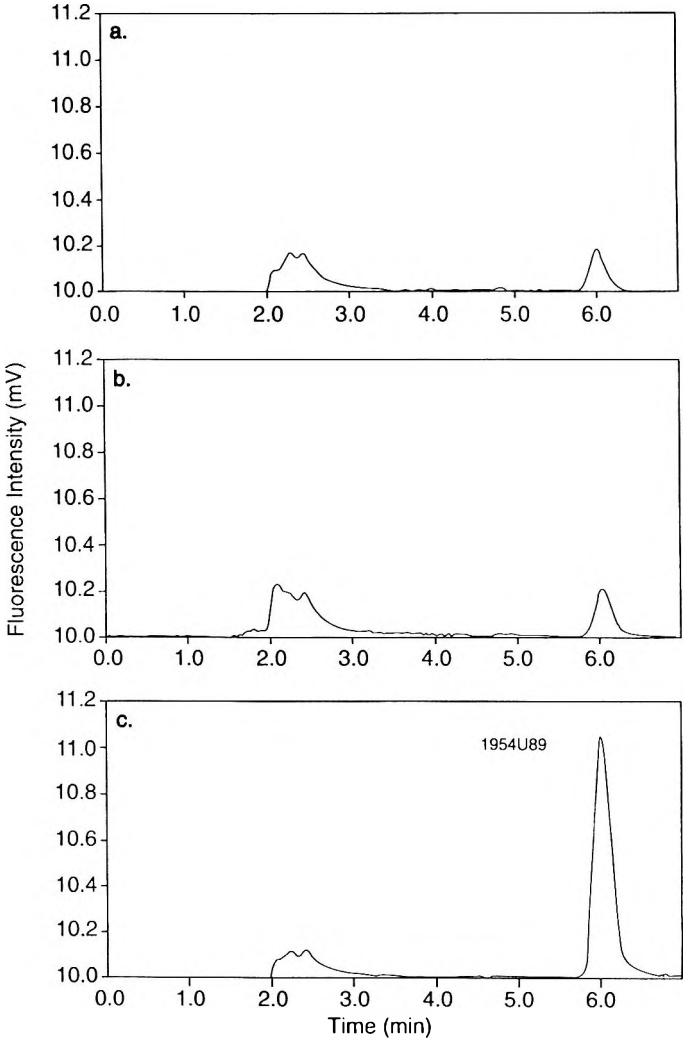

Figure 3. Concentration-peak area profiles of extracted (a) rat plasma, (b) dog plasma and (c) 0.01 μg/mL (LLOQ) calibration standard prepared in rat plasma. The retention time of 1954U89 was 6.0 min.

anticipated requirements of the assay as well as the early stage in the development of 1954U89 for which the assay was being used. Previous

Table 2

Accuracy and Precision for the Analysis of 1954U89 in Rat Plasma

Nominal Concentration (μg/mL)	Assayed Concentration[a] (μg/mL)	Bias (%)	Intraday CV (%)	Intraday CV (%)
0.02	0.018 ± 0.0010[b]	-10.0	3.5	5.3
0.20	0.202 ± 0.0118	+ 1.0	3.5	5.4
2.00	2.024 ± 0.803	+ 1.2	2.9	3.1

[a]n = 9 at each concentration
[b]Mean ± S.D.

Table 3

Stability of 1954U89 in Standard Solution

Time (Week)	Concentration (μg/mL)	Ratio
2	0.52 ± 0.002	1.04
4	0.48 ± 0.005	0.96
6	0.49 ± 0.002	0.98

[a]Mean ± S.D.

experience with compounds having similar physico-chemical properties (lipid solubility, strong chromophore/fluorophore), biochemical properties (potent DHFR inhibition), and pharmacokinetic properties (high volume of distribution and extensive metabolism), suggested that assay limits of detection below 0.1 μg/mL would be required; previous toxicity studies with similar compounds suggested that concentrations greater than 2 μg/mL would be near a toxic range of 1954U89. Consequently, the projected analytical range of 0.01 to 2 μg/mL was set for the method. Previous experience in assaying this class of compounds included quantitative TLC,[7] HPLC,[8] and competitive protein binding assays.[9] Although each of these approaches had potential, HPLC was chosen for its versatility and applicability. Preliminary development work on the method incorporated an internal standard, but the accuracy and precision of

the assay was not improved significantly when internal standarization was used. The excellent accuracy and precision of the data obtained without an internal standard, coupled with the potential problems of internal standard stability, specificity, and precision,[14] made internal standardization unnecessary.

Data from calibration curves were compiled from six assay runs (rats) or twelve assay runs (dogs) and examined to determine the most appropriate model selection and weighting scheme. A plot of the Studentized residuals from the least-squares linear regressions of the rat calibration curve data (weighted $1/c^2$) is shown in Figure 2. A similar plot was obtained for the residuals analysis of the dog calibration curve data. The plot demonstrates that the residuals were distributed normally around a residual value of zero and that the variance was homogeneous over the concentration range. This showed that $1/c^2$ weighted least-squares linear regression was an appropriate model to use for the estimation of 1954U89 concentrations in plasma.[10] Unweighted and 1/c weighted least-squares linear regression were unable to correct for heteroscedastic variance; log-log transformed weighting yielded results similar to the $1/c^2$ weighted regression. The upper and lower limits of quantitation were assigned as 2.0 and 0.01 μg/mL, respectively, based on this analysis of the residuals.[12]

The recovery of 1954U89 from rat plasma is shown in Table 1. The extraction efficiency of the assay ranged from 100 to 102%. Similar results were observed in the recovery of the compound from dog plasma (data not shown). Analysis of variance indicated that the recovery of 1954U89 did not differ significantly at different concentrations.

The specificity of the assay was assessed with plasma samples from untreated animals. Figure 3 contains concentration-peak area profiles from untreated rat and dog plasma, and the concentration-peak area profile of the lowest calibration standard (0.01 μg/mL). Although a small endogenous peak present in blank rat and dog plasma had a retention time similar to 1954U89, the contribution of this peak to the area of the lowest calibration standard was less than 10% and therefore did not present a significant interference to the quantitation of 1954U89. This endogenous peak was not present in human plasma samples.

Five putative metabolites of 1954U89 obtained from *in vitro* incubations with hamster liver homogenates were examined to determine their retention times relative to unchanged 1954U89. All metabolites eluted before, and were

Table 4

Stability of 1954U89 in Rat Plasma Stored at -80°C

Time (Week)	Concentration[a] (μg/mL)	Ratio
0.02μg/mL		
0	0.019 ± 0.0058[b]	0.94
1	0.023 ± 0	1.15
3	0.022 ± .0029	1.10
6	0.021 ± 0.0012	1.05
0.20 μg/mL		
0	0.206 ± 0.0096	1.03
1	0.213 ± 0.0040	1.06
3	0.208 ± 0.0070	1.04
6	0.19 ± 0.0058	0.96
2.00 μg/mL		
0	2.016 ± 0.075	1.01
1	2.038 ± 0.084	1.02
3	1.984 ± 0.031	0.99
6	2.094 ± 0.046	1.05

[a]n = 3 at each concentration
[b]Mean ± S.D.

resolved completely from, the parent compound. After intravenous administration of 1954U89 to rats and dogs, several (three to five) metabolites were present in plasma. These metabolites eluted before the parent compound and did not interfere with quantitation of 1954U89.

The intra- and interday precision and accuracy data for the assay of 1954U89 in rat plasma are shown in Table 2. The intraday CV was less than 4% over the examined concentration range in rats, and less than 7% in dogs. The interday precision ranged from 3 to 5% in rats, and from 1 to 6% in dogs. The percent bias ranged from -10.0 to +1.2 in rats, and -5.5 to +1.0 in dogs.

The concentration of 1954U89 in a standard solution stored at approximately 25°C remained stable during a six-week period. Table 3 shows

Table 5

Stability of 1954U89 After Freeze-Thaw Cycles

Cycle	Asseyed Concentration[a] (μg/mL)	Ratio
Initial	1.95 ± 0.06[b]	0.98
1	2.05 ± 0.16	1.02
2	1.92 ± 0.13	0.96
3	2.02 ± 0.04	1.01

[a]n = 4; nominal concentration = 2.00 μg/mL
[b]Mean ± S.D.

Table 6

Stability of 1954U89 in Processed Samples

Time[a] (h)	Assayed Concentration[b] (μg/mL)	Ratio
1	4.88	0.98
5	4.80	0.96
9	4.83	0.97
13	4.88	0.98
17	4.89	0.98
21	4.80	0.96

[a]Time elapsed between sample preparation and sample analysis
[b]Nominal concentration = 5.00 μg/mL

the average measured concentrations of the compound and the ratios of the assayed spiked concentrations to the nominal concentration on the days the assay was run. The mean concentrations of 1954U89 and the assayed concentration/nominal concentration ratios obtained from the assay of spiked plasma stored frozen (-80°C) during a six-week period are presented in Table 4. No trend in concentration during the time period was apparent. Likewise, no change was evident in the measured plasma concentrations of 1954U89 after

three freeze-thaw cycles (Table 5). The results of the experiment to determine the stability of the compound in processed samples (Table 6) indicated that 1954U89 remained stable at room temperature for at least 21 h after extraction from plasma.

In summary, a rapid, precise, and specific HPLC method for measuring 1954U89 concentrations in rat and dog plasma was developed and validated. The compound was removed from plasma by solid-phase extraction, chromatographed by isocratic reversed-phase HPLC, and quantitated by fluorescence. Least-squares linear regression with $1/c^2$ weighting was used as the calibration model. This assay is useful for the measurement of 1954U89 concentrations in plasma from nonclinical studies, and preliminary work suggests that it could also provide the basis for a bioanalytical method should the compound proceed to clinical trials.

ACKNOWLEDGEMENTS

The authors wish to thank Suzanne Joyner for preliminary assay development of 1954U89, Pisal Chandrasurin for the *in vitro* synthesis of 1954U89 metabolites, and Carl Sigel for helpful suggestions in the preparation of the manuscript.

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Received August 28, 1995
Accepted September 18, 1995
Manuscript 3957

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 837–842 (1996)

RETENTION BEHAVIOR OF VITAMIN D N-ACETYLGLUCOSAMINIDES DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention behavior of N-acetylglucosaminides of vitamin-D_2 and -D_3, and those of provitamin-D_2 and -D_3 are examined using reverse phase high performance liquid chromatography. Inclusion chromatography using cyclodextrin as the mobile phase additive is also used for this purpose. The addition of methyl-β-cyclodextrin to the mobile phase is effective in separating the pair of N-acetylglucosaminides of vitamin-D_2 and -D_3 or those of provitamin-D_2 and -D_3 .

INTRODUCTION

In the previous paper of this series, we clarified the retention behavior of vitamin D (D) and related compounds including the glucuronides or sulfates during high performance liquid chromatography (HPLC) and found that the inclusion chromatography using heptakis-(2, 6-di-*O*-methyl)-β-cyclodextrin (Me-β-CD) as the mobile phase additive is effective in separating the pair of D_2 (**1a**) and D_3 (**1c**) or related compounds.[1,2] Recently, the occurrence of bile acid N-acetylglucosaminide (*N*AG) in biological fluids[3] and the synthesis of

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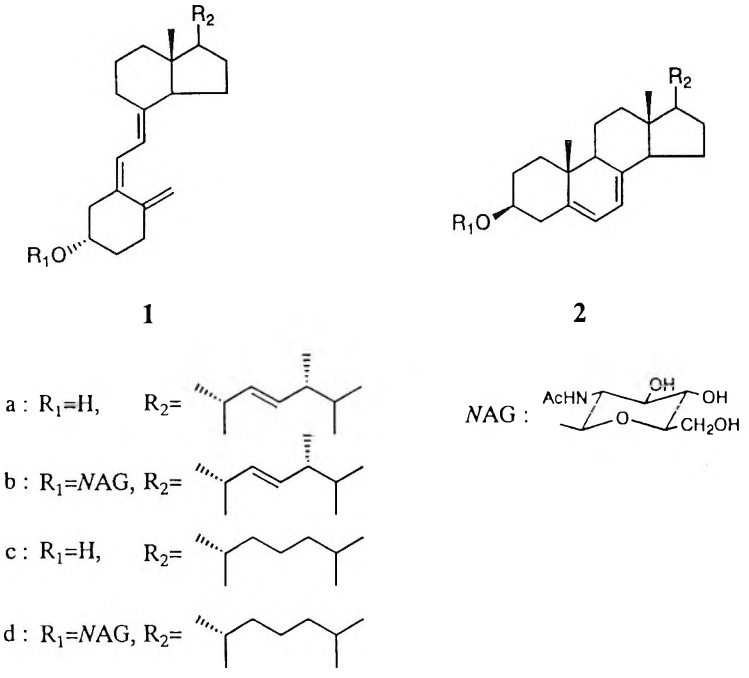

Figure 1. Structures of D, pro D and its *N*AGs

estrogen NAG as a reference compound for the determination of the compound in biological fluids[4] have been reported. These data prompted us to synthesize NAGs of D_2 (**1b**), D_3 (**1d**), pro D_2 (**2b**) and pro D_3 (**2d**) in order to examine their retention behavior during HPLC (Fig. 1).

MATERIALS AND METHODS

Materials

Me-β-CD was prepared and donated by Kao (Tokyo, Japan). D_2 (**1a**), D_3 (**1c**) and ergosterol (pro D_2, **2a**) were purchased from Tokyo Kasei Kogyo (Tokyo). 7-Dehydrocholesterol (pro D_3, **2c**) was obtained from Wako Pure Chemical Ind. (Osaka, Japan).

Preparation of N-Acetylglucosaminides of D and Pro D

The preparation of NAGs of D_2 (1b), D_3 (1d), pro D_2 (2b) and pro D_3 (2d) using respective pro D (2a,c) as a starting material was done in these laboratories and the details will be reported elsewhere in the near future. Some of the physical data are shown below. Proton nuclear magnetic resonance (^{1}HNMR) spectra were measured using a JEOL JNM-EX 270 (270 MHz) spectrometer (JEOL, Tokyo) and Me_4Si was used as the internal standard. Chemical shifts and *J*-values are given in ppm and Hz, respectively. The following abbreviations are used: s=singlet, d=doublet and m=multiplet. Fast atom bombardment mass (FABMS) spectra were measured using a JEOL JMS-DX 303 mass spectrometer. Pro D_2*N*AG (**2b**): ^{1}HNMR (CD_3OD-$CDCl_3$)δ: 2.03 (3H, s, CH_3CO-), 4.64 (1H, d, *J*=7.3 Hz, H-1'), 5.37-5.56 (2H, m, H-6, 7). FABMS *m/z* : 622 $(M+Na)^+$. D_2*N*AG (**1b**): FABMS *m/z* : 622 $(M+Na)^+$. pro D_3*N*AG (**2d**): ^{1}HNMR (CD_3OD-$CDCl_3$)δ: 4.62 (1H, d, *J*=7.6 Hz, H-1'), 5.38, 5.56 (1H each, m, H-6, 7). FABMS *m/z*: 610 $(M+Na)^+$. D_3*N*AG (**1d**): ^{1}HNMR (CD_3OD) δ: 1.89 (3H, s, CH_3CO-), 4.56 (1H, d, *J*=7.6 Hz, H-1'), 4.73 (1H, s, H-19E), 5.02 (1H, s, H-19Z), 6.04 (1H, d, *J*=11.2 Hz, H-7), 6.21(1H, d, *J*=11.2 Hz, H-6). FABMS *m/z* : 610 $(M+Na)^+$.

HPLC

HPLC was carried out using a TOSOH CCPD chromatograph (TOSOH, Tokyo) equipped with a JASCO UVIDEC- 100-II ultraviolet detector (UV) (JASCO, Tokyo). Reverse phase columns [Develosil ODS-5, 5 μm, 15 x 0.46 cm i.d. (Nomura, Seto, Japan), Inertsil ODS-2, 5 μm, 25 x 0.46 cm i.d. (GL Sciences, Tokyo), CAPCELL PAK C_{18}, 5 μm, 25 x 0.46 cm i.d. (SHISEIDO, Tokyo) and TSKgel Super-ODS, 2 μm, 10 x 0.46 cm i.d. (TOSOH)] were used under ambient conditions at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Separation using Conventional Method

Initially, efforts were directed at the separation of the pair of *N*AGs of D_2 (**1b**) and D_3 (**1d**) and those of pro D_2 (**2b**) and pro D_3 (**2d**) during reverse phase HPLC. MeOH was superior to MeCN as an organic modifier during the separation of the D related compounds as previously reported.[2] The separation

Table 1

Separation of *N*AGs

	Column and Solvent System (t_R:min)				
Compounds	**1**	**2**	**3**	**4a**	**4b**
D_2*N*AG (**1b**)[1]	18.7	15.2	18.7	8.8	
D_3*N*AG (**1d**)	19.9	16.0	19.6	9.5	
Resolution (Rs)	0.88	0.74	1.21	1.77	
pro D_2*N*AG (**2b**)[2]	25.6				10.9
pro D_3*N*AG (**2d**)	27.3				11.9
Rs	1.04				1.78

1. Develosil ODS-5 [MeOH-H_2O (10:1)]. 2. Inertsil ODS-2 [MeOH-H_2O (10:1)]. 3. CAPCELL PAK C_{18} [MeOH-H_2O (10:1)]. 4a. TSKgel Super-ODS [MeOH-H_2O (8:1)] 4b. TSK gel Super ODS (MeOH-H_2O (10:1)], Detection: UV 1) 265 nm 2) 254 nm.

of D_2- and D_3-*N*AG (**1b,d**) is summarized in Table 1, in which TSKgel Super-ODS gave the best results with shortest t_R and greatest Rs as shown in Fig. 2a. The separation of pro D_2- and pro D_3-*N*AG (**2b,d** :Rs 1.78) was also done using this column (Table 1, Fig. 2b).

Separation using Inclusion Chromatography

We next applied the inclusion chromatography using Develosil ODS-5 and Me- β -CD as the column and the mobile phase additive, respectively, for the separation of the pair of these *N*AGs. Both pairs (**1b,d: 2b,d**) showed satisfactory results (Rs 3.44, 3.23 with the shorter t_R, respectively) with the addition of 5 mM of the host compound. The elution order of 1d and 2d became faster than that of **1b** and **2b** with the addition of the host compound, respectively (Table 1, Fig. 3a,b). These data are compatible with the previously obtained results.[1,2]

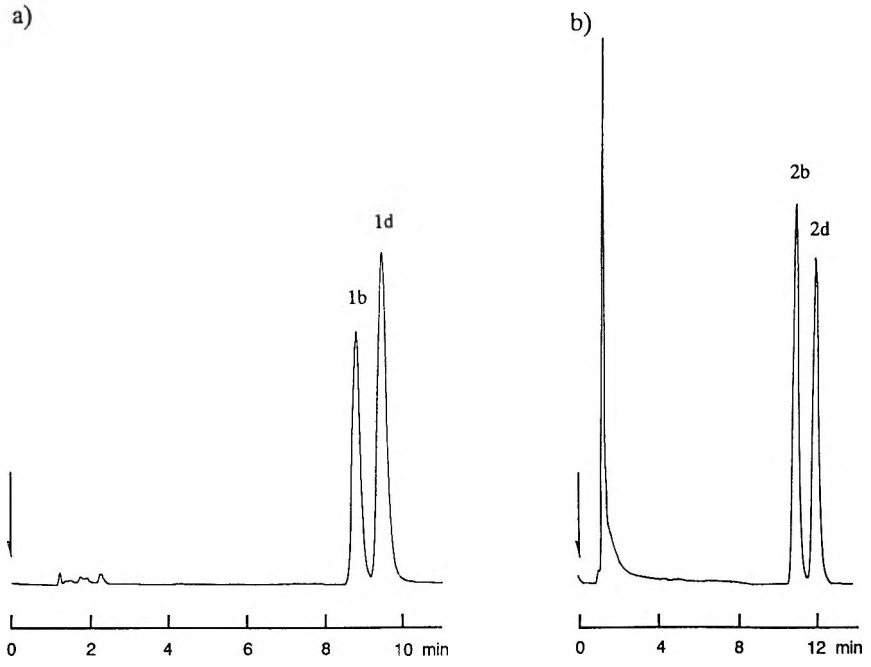

Figure 2. Separation using conventional method. Conditions: column, TSKgel Super-ODS; mobile phase, a) MeOH-H_2O (8:1) b) MeOH-H_2O (10:1); detection: UV a) 265 nm b) 254 nm.

All the above data showed that TSKgel Super-ODS gave the best results among the examined reverse phase columns. The inclusion chromatography using Me- β -CD as an additive is also effective for the separation of these compounds.

ACKNOWLEDGEMENTS

The authors thank Mr. T. Nemoto (Kao Company, Tokyo) for providing Me-β-CD. Our thanks are also due to Dr. Koukwa Yamashita (Nippon Kayaku Co., Tokyo) for the mass spectrum measurement.

This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

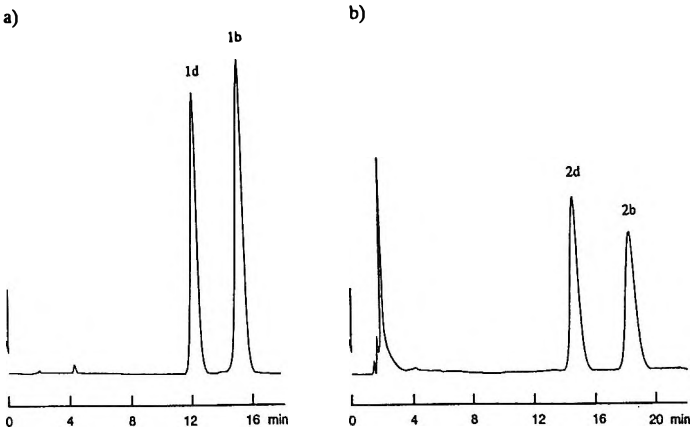

Figure 3. Separation using inclusion chromatography. Conditions: column, Develosil ODS-5; mobile phase, MeOH-H_2O (10:1) containing Me-β-CD (5 mM); detection: UV, a) 265 nm b) 254 nm.

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Recieved September 4, 1995
Accepted September 22, 1995
Manuscript 3959

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 843 (1996)

ERRATUM

corrected 27 Jun 96 /AP

M. C. Gennaro, D. Giacosa, **Separation Of Triazine Herbicides By Ion-Interaction HPLC And Application To Surface Waters**, J. Liquid Chrom., **19(1)**, 149-160 (1996).

In the first line of the third paragraph of the **ABSTRACT**,

. . . (around 1.0 mg/L) . . . should read: . . . (around 1.0 μg/L) . . .

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 845 (1996)

ANNOUNCEMENT

WORKSHOP ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC AND CAPILLARY ELECTROPHORETIC TECHNIQUES APPLIED TO FOOD ANALYSIS

March 8, 1996
Area della Ricerca di Roma
Montelibretti, Italy

The state-of-the-art and the new developments on high-performance liquid chromatographic and capillary electrophoretic techniques for the analysis of fats, vitamins, carbohydrates and molecular markers in food will be presented and discussed.

Fundamentals, instrumentation and applications concerning the various techniques will be covered.

Internationally recognized scientists, including Prof. Csaba Horvath (Yale University, USA), Prof. Ziad El-Rassi (Oklahoma State University, USA) and Prof. Heinz Engelhardt (Universitat des Saarlandes, Germany) will report on trends and future developments over the above subjects.

The registration fee will be 240,000 Italian Liras (US $150) plus value added tax and will include the seminar folder with scientific documents, lunch, coffee breaks and transportation Rome → Montelibretti → Rome. For further details, contact Dr. Claudio Corradini, Istituto di chromatografia C.N.R, Area della Ricerca di Roma, P. O. Box 10, 00016 Monterotondo Stazione, Italy. Tel.: 0039-6-90572258; FAX: 0039-6-90625849; Email: nicolet@mlib.cnr.it.

J. LIQ. CHROM. & REL. TECHNOL., 19(5), 847–851 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

MARCH 3 - 8: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 8: Workshop on HPLC and CE Techniques Applied to Food Analysis, Area della Ricerca di Roma, Montelibretti, Italy. Contact: Dr. Claudio Corradini, Istituto di Cromatografia CNR, Area della Ricerca di Roma, P. O. Box 10, 00016 Monterotondo Stazione, Italy. Email: nicolet@mlib.cnr.it.

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

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography & Extraction, Indianpolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

APRIL 17 - 19: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Université de Nice, Fracnce. Contact: Prof. W. R. G. Baeyens, University of Ghent Pharmaceutical Institute, Lab or Drug Quality Control, Harelbekestraat 72, Ghent, Belgium. Email: willy.baeyens@rug.ac.be.

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

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 1 - 3: International Symposium on Polymer Analysis and Characterization, Keble College, Oxford University, U.K. Contact: Prof. J. V. Dawkins, Dept. of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE11 3TU, U.K.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-

La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact:

ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 14 - 19: Genes and Gen 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.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Mtgs, 1155 16th Street, NW, Wash., DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

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1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

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

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J. LIQ. CHROM. & REL. TECHNOL., 19(5), (1996)

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Volume 19, Number 5, 1996

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