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Aims and Scope. The journal publishes papers involving the applications of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all models of liquid chromatography.

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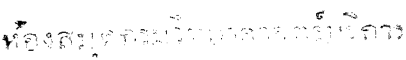

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HPLC SEPARATION OF THE ZZ, ZE, EZ, AND EE GEOMETRIC ISOMERS AND EE ISOMER ENANTIOMERS OF A SUBSTITUTED PENTADIENYL CARBOXAMIDE USING ACHIRAL/CHIRAL COLUMN SWITCHING

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ABSTRACT

The four geometric isomers of a substituted pentadienyl carboxamide were separated on an achiral aminopropyl column coupled with a silica precolumn. The R and S enantiomers of the biologically active EE isomer (RO 24-0238 and RO 24-2099) were resolved (R_s 1.65) on a cellulose-based chiral stationary phase (Chiracel OF). The coupled silica and aminopropyl columns were connected to the Chiralcel OF column through a six port switching valve that enabled transfer to the EE isomer to the chiral phase for enantiomer resolution. By examining the selectivity for separation of the geometric isomers of various achiral stationary phases using hexane-isopropanol mobile phases, a method was developed which linked the achiral separation of the geometric isomers with the chiral separation of the EE enantiomers.

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INTRODUCTION

Platelet activating factor (1-O-alkyl-sn-glycero-3-phosphocholine, PAF) is a phospholipid mediator produced in inflammatory and allergic reactions. The biological activity consists of hypotension and increased vascular permeability, bronchoconstriction, coronary vasoconstriction and platelet aggregation. As a result of these actions, PAF is a mediator in septic shock, asthma, coronary artery disease, and stroke (1-4). RO 24-0238, 5-(4-methoxyphenyl)-N-[1-methyl-4-(3-pyridinyl)-butyl]-2,4-decadienamide, is a pentadienyl carboxamide PAF antagonist with a stereoselective bioactivity profile (see Fig. 1). The R enantiomer (RO 24-0238) not only causes a greater inhibition of PAF induced bronchoconstriction, but also has a substantially longer duration of action than the S enantiomer (RO 24-2099) (5). Both enantiomers have the EE configuration. The remaining geometric isomers shown in Fig. 1 are biologically inactive.

Column switching in HPLC has been reported in the literature for such applications as sample preparation, direct injection of serum samples and for the separation of drugs and metabolites. Oda et al separated verapamil from its metabolites on a reversed phase octadecylsilane column and then transferred the drug via column switching to an ovomucoid chiral stationary phase (CSP) for separation of the enantiomers (6). Stalcup et al used column switching to determine enantiomeric purity of scopolamine on a beta cyclodextrin

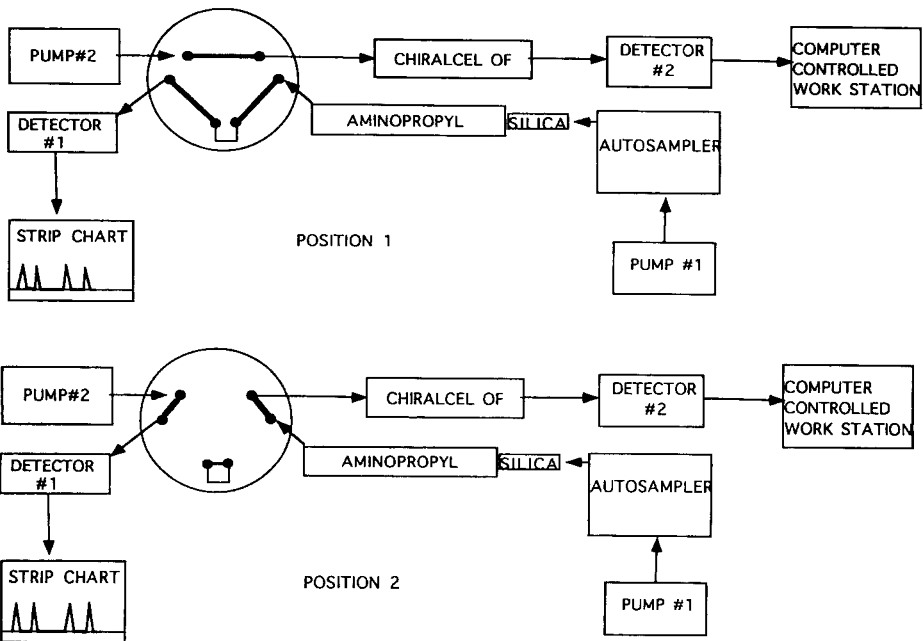

Figure 1 - The chemical structure of a substituted Pentadienyl Carboxamide and its ZZ, ZE, EZ and EE Geometric Isomers and the R and S enantiomers of the EE isomer.

column after separation of the drug from other alkaloids and biological material in a vegetal extract (7).

Individual methods for the separation of the four geometric isomers (ZZ, ZE, EZ and EE) and chiral resolution of the R and S enantiomers of the EE isomer have previously been performed by Aggarwal et al (8). The geometric isomers were separated on a cyanopropyl column using a mobile phase of 60:35:5:0.2 v/v/v/v

hexane-methylene chloride-ethyl acetate-triethylamine. The enantiomers of the EE isomer (RO 24-0238 and RO 24-2099) were separated on a Chiralcel OF column using a mobile phase of 70:30 v/v hexane-isopropanol. Since the mobile phase used in the normal phase separation of the geometric isomers was not compatible with the Chiralcel OF column, other stationary phases were investigated that might be useful in the separation of the geometric isomers and yet be compatible with the solvent restrictions of the Chiralcel OF column. In this paper, a column switching method is reported for the on-line analysis of the four geometric isomers of a substituted pentadienyl carboxamide including a chiral separation of the R and S enantiomers of the EE isomer.

EXPERIMENTAL

Reagents and Chemicals

The R and S enantiomers of the EE isomer were provided as RO 24-0238 and RO 24-2099 respectively, by Hoffmann-LaRoche (Nutley, NJ). The geometric isomers RO 24-6106 (ZZ), RO 24-6105 (ZE) and RO 24-1553 (EZ) as racemates were also supplied by Hoffmann-LaRoche. HPLC grade hexane and isopropanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ). Diethylamine was obtained from Eastman Kodak Co., (Rochester, NY).

Instrumentation

Figure 2 shows schematics of the two HPLC systems used. HPLC system #1 for the achiral separation consisted of a Spectra-Physics

RO 24-0238, EE (R)
RO 24-2099, EE (S)
RO 24-6106, ZZ (R,S)
RO 24-6105, ZE (R,S)
RO 24-1553, EZ (R,S)

Figure 2 - Schematics of the achiral HPLC system #1 and the chiral HPLC system #2.

Model SP 8800 Ternary pump (San Jose, CA), a Lambda-Max Model 400 UV Spectrophotometer (Waters Associates, Milford, MA) set at 322 nm, a Kipp and Zonen Model BD41 chart recorder, and a Perkin-Elmer Model 1SS 100 autosampler set for a 10 μL injection. HPLC System #2 consisted of a Beckman Model-110B pump (Fullterton, CA), a Waters Model 440 UV detector set at 322 nm, and a Nelson Model 2600 Chromatography Data System to accumulate data from the chiral separation and relay a signal to activate the Rheodyne Model 7010 six port pneumatic actuated column switching valve (Cotati, CA).

The chiral stationary phase was cellulose tris(4-chlorophenyl carbamate) bonded to silica gel commercially available as Chiralcel OF

(Daicel Inc., Fort Lee, NJ, USA). The achiral stationary phase was a Zorbax 4 cm x 60 mm i.d., 3 μm particle size silica column coupled to a μ-Bondapak aminopropyl 30 cm x 3.9 mm i.d., 10 μm particle size column (Waters Inc., Milford, MA). The HPLC pump in system #1 delivered a mobile phase of hexane-isopropanol (92:8) containing 1 mM diethylamine to the achiral coupled columns and the HPLC pump in system #2 delivered a mobile phase of hexane-isopropanol (70:30) containing 1 mM diethylamine to the chiral column. A sample containing the four geometric isomers was initially injected onto the coupled silica-aminopropyl stationary phases. Retention times for each geometric isomer were monitored by the detector in system #1 and the chromatograms recorded on the chart recorder with the switching valve in position #1(see Fig. 2). The valve was switched to position #2 just before the EE geometric isomer peak eluted from the column (approx. 40 min). This allowed for transfer of the EE isomer to the chiral column for separation of the R and S enantiomers. The valve was then switched back to position #1 for the remaining detection of the EZ peak (approx. 44 min).

Preparation of Standard Solutions

Approximately 5 mg of each geometric isomer (ZZ, ZE and EZ) and the R and S enantiomers of the EE isomer were dissolved in 70:30 hexane-isopropanol in a 10 mL volumetric flask and the solvent was added to volume. The samples were sonicated for 10 min and then

filtered through a 0.5 μm Millex SR syringe filter (Millipore Co., Rutherford, NJ) attached to a 20 mL glass syringe (Becton Dickinson Co., Rutherford, NJ).

RESULTS AND DISCUSSION

An achiral/chiral column switching HPLC method was developed to separate the biologically active EE isomer from its three other geometric isomers including a transfer of the EE isomer to a CSP for resolution of the R and S enantiomers. It had been previously shown that a chiral separation of the R and S enantiomers of the EE isomer was achieved on a cellulose tris(4-chlorophenyl carbamate) CSP using hexane-isopropanol (70:30) containing 1 mM diethylamine. Since the choices of mobile phase solvents with the Chiracel OF CSP are essentially limited to hexane, isopropanol and small amounts of diethylamine, various achiral stationary phases applicable to normal phase chromatography were initially examined for geometric isomer separation prior to transfer and separation of the EE enantiomers on the Chiracel OF column. It was found that a silica column used in the normal phase mode provided a partial separation of the geometric isomers. A beta cyclodextrin stationary phase, which had been reported to separate geometric isomers in the normal phase mode (9), was also investigated. However, when this stationary phase was used in our laboratories, coelution of the EE and EZ isomers occurred. An aminopropyl stationary phase with various combinations of hexane-

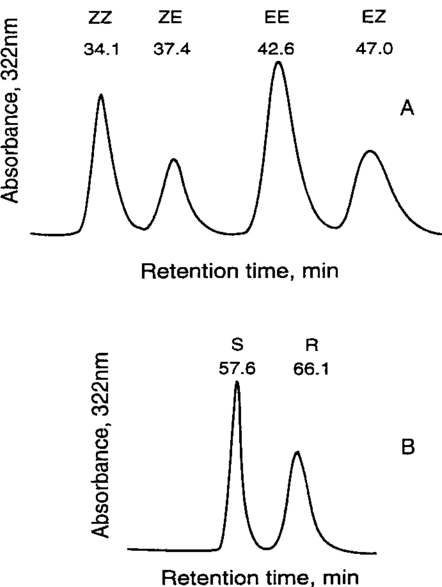

Figure 3 - (A) Chromatogram of the HPLC separation of the ZZ, ZE, EE and EZ isomers of a substituted pentadienyl carboxamide on tandem silica-aminopropyl columns using a mobile phase of 92:8 hexane-isopropanol.

(B) Chromatogram of the HPLC separation of the R and S enantiomers of the EE isomer of a substituted pentadienyl carboxamide on a chiracel OF column using a 70:30 hexane-isopropanol mobile phase.

isopropanol as mobile phases was unable to provide satisfactory separation of the four geometric isomers. However, when the aminopropyl column was coupled in tandem with a silica precolumn, all of the geometric isomers were well-resolved. A thorough examination of different mobile phase compositions of hexane and isopropanol

containing 1 mM diethylamine finally resulted in baseline resolution of the four isomers. By varying the diethylamine concentration, silanol interactions with each analyte were controlled such that excellent resolution was obtained. Increasing the diethylamine concentration from 0.5 to 1.0 mM significantly improved the peak asymmetry. Therefore, the final composition of the mobile phase used for the achiral separation of the four geometric isomers was 92:8 hexane-isopropanol containing 1 mM diethyl amine. Thus, it was now possible to transfer the EE isomer via column switching from achiral System #1 to the chiral System #2 for resolution of the R and S enantiomers of the EE isomer (see Fig. 3a and 3b). An R_s of 1.65 was obtained for the separation of the EE enantiomers on the Chiralcel OF column.

In summary, an HPLC achiral/chiral column switching method was developed for the achiral separation of the geometric isomers of of a substituted pentadienyl carboxamide including transfer and chiral resolution of the R and S enantiomers of the EE isomer. This multidimensional chromatographic approach to the analysis of geometric isomers and enantiomers permits two HPLC systems to be linked together to solve a complex analytical problem.

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Dr. Roger Blain and Ms. Janice Schmidt of Roche Laboratories are thanked for technical assistance and for use of the pneumatic activated column switching valve and the Nelson chromatography data system.

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STUDY AND OPTIMIZATION OF COLUMN EFFICIENCY IN HPLC: COMPARISON OF TWO METHODS FOR SEPARATING TEN BENZODIAZEPINES

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ABSTRACT

To understand the influence of mobile phase composition, its flow rate and column temperature involved in high performance Liquid Chromatography, an experimental design was used. The observed responses were the theoretical plate number, the linear velocity of the mobile phase and a new chromatographic resolution function which provided the most efficient separation of ten compounds as ten benzodiazepines. Optimum conditions obtained were compared with another optimization method.

INTRODUCTION

The column efficiency in high performance liquid chromatography (HPLC) has been widely studied and is generally represented by the height equivalent to a theoretical plate (HETP). HETP was first related to the mobile phase flow rate by Knox (1). To optimize the column efficiency, the traditional approach would be to study separately the mobile phase composition, its flow rate and column temperature which influences HETP (2). In this research, an experimental design (3, 4), assisted with a simplex method was used to study the simultaneous variation of the mobile phase composition, its flow rate and column temperature. An equation relating the column efficiency with these three factors was proposed. A new chromatographic

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resolution function (CRF) (5, 6, 7, 8) was studied for the separation of several compounds and this method was compared with that developed in a recent work (9).

MATERIALS AND METHODS

CHROMATOGRAPHIC CONDITIONS

APPARATUS : The HPLC system consisted of a HPLC Waters pump 501 (Saint Quentin en Yvelines, France), an Interchim rheodyne injection valve Model 7125 (Montluçon, France) fitted with a 20μl sample loop, a Merck L 4000 variable wavelength UV spectrophotometer detector and a Merck D 2500 chromato integrator (Nogent-sur-Marne, France). A Waters 150 mm × 3.9 mm ID. RP 18 column (Nova pak, 5 μm particle size) was used with a controlled temperature by an Interchim crococil oven TM N° 701 (Montluçon, France). Overall temperature control was maintained within ± 1° C with a variation from 26° C to 50° C. The detection wavelength was 254 nm. The flow rate used varied from 0.6 to 1.6 mL/min. The mobile phase was a mixture methanol-water with varied percentages of methanol from 50 % to 80 %. Weaker percentages were not used because of the excessively high column pressure obtained with 50 % of methanol with a flow rate of 1.6 mL/min.

REAGENTS AND SAMPLES : Methanol was HPLC grade determine analytical. Naphtalene obtained from Merck (Nogent-sur-Marne, France) was used to determine the theoretical plate number (N). The linear velocity (u) was measured by timing the passage of an unretained peak such as sodium nitrate purchased from Merck (Nogent-sur-Marne, France). (1) Bromazepam (2) Nitrazepam (3) Flunitrazepam (4) Clobazam (5) Lorazepam (6) Oxazepam (7) Tofisopam (8) Chlordiazepoxide (9) Chlorazepate dipotassic and (10) Diazepam were obtained from HOFFMANN LA ROCHE (Basel, Switzerland). These were diluted in methanol in a concentration range of 10-80 mg/mL.

METHODS

EFFICIENCY : In chromatography, the column plate number (N) is used to study the sharpness of a peak. N depends on the mobile phase composition, its flow rate and column temperature. N is given by the following equation :

Table 1. Experiments required for a three variable experimental design

Experiments N	Methanol (% v/v)	Flow rate (mL/min)	Temperature (C)
1	50	0.6	36
2	50	1.0	50
3	50	1.6	36
4	50	1.0	26
5	63	0.6	26
6	63	0.6	50
7	63	1.6	50
8	63	1.6	26
9	80	1.0	50
10	80	0.6	36
11	80	1.0	26
12	80	1.6	36
13	63	1.0	36

$$N = \left(\frac{t_R}{s}\right)^2 \quad [1]$$

where t_R is the retention time of naphtalene, s its standard deviation considering the peak as gaussian. N is calculated using the peak width ($W_{0.5}$) at half height using :

$$N = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2 \quad [2]$$

The theoretical plate number is directly proportional to the column length (L). Thus using : the height equivalent to a theoretical plate HETP can be calculated:

$$HETP = \frac{L}{N} = \frac{L}{5.54}\left(\frac{W_{0.5}}{t_R}\right)^2 \quad [3]$$

LINEAR VELOCITY : A fundamental parameter affecting the separation speed and the column plate number is the linear mobile phase velocity (u) in millimeters per second. It was measured by timing the passage of an unretained peak such as sodium nitrate (t_m) along the length of the column L.

$$u = \frac{L}{t_m} \quad [4]$$

CHROMATOGRAPHIC RESOLUTION FUNCTION : The quality of each separation of ten compounds was assessed at the end of the chromatogram by calculating the value of a chromatographic resolution function CRF which describes the separation quality. CRF is usually given by the following equation :

$$CRF = \alpha F_{obj} + \beta m^a \quad [5]$$

where F_{obj} = objective function

m = detected peak number

α, β and a are constants.

F_{obj} is expressed in terms of resolution Rsij between two peaks i and j (10, 11, 12). But Rsij is insensitive to the relative quantity of solutes in the mixture. For a badly separated pair of compounds injected into a diluted solution, the difference between the solute quantity which is represented by the amplitude difference between peaks leads to an error in the compound quantification particularly for the minority compounds. Although the resolution is constant, this error increases drastically when the amplitude ratio is high. Some authors have tried to find another separation function which takes into account the amplitude ratio of peaks by a direct measurement on the chromatogram : Kaiser ratio (13) Christophe ratio (14). More recently EL Fallah and Martin (15) have introduced a discrimination factor $d_0 = \frac{h_p - h_v}{h_{..}}$ where h_p - h_v is the highest difference between the smallest peak (h_p) and the valley (h_v) separating the two peaks.

In this paper, a new chromatographic resolution function was studied where Fobj is a function of d_0 given by the following equation

$$F_{obj} = \sum \ln (1 + d_{ij}) \quad [6]$$

where d_{ij} is the discrimination factor between peak i and j. The sum is extended to all the peak pairs on the chromatogram.

CHEMOMETRIC METHODOLOGY : A chemometric approach based on the use of Box and Benhken matrix experiments (3) [Fig.1] was used to study simultaneously the variations in all the factors. These models can be used for regression analysis and for three factors takes the form of:

$$y = a_0 + a_1 \ln x_1 + a_2 \ln x_2 + a_3 \ln x_3 + a_{11}(\ln x_1)^2 + a_{22}(\ln x_2)^2 + a_{33}(\ln x_3)^2 + a_{12}(\ln x_1)(\ln x_2) + a_{13}(\ln x_1)(\ln x_3) + a_{23}(\ln x_2)(\ln x_3)$$

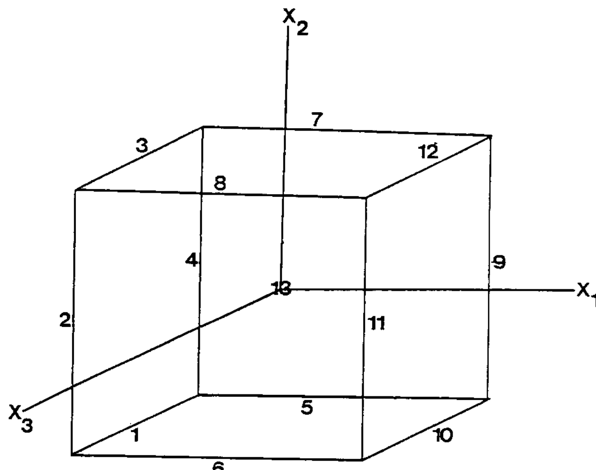

Figure 1. Modified Box and Benhken experimental design.

where y is the response studied and x_1, x_2, x_3 are respectively the mobile phase composition, the flow rate and the column temperature. In our case these variables were coded to have a variation of -1 to +1.

SIMPLEX OPTIMIZATION : To optimize the mathematical model y given by the experimental design, a simplex method was always used. This way, the y value was calculated for m sets of starting condition where m was given by the number of factors to be optimized plus 1. In this case therefore, m was 4. The point corresponding to the lowest value of y was then reflected about the surface defined by the three other points to give a fifth set of starting conditions. Once again, the point with the lowest y was reflected and the process repeated sequentially until an apparent optimum was obtained.

RESULTS AND DISCUSSION

The key chromatographic parameters used as response criteria were the theoretical plate number N, the linear velocity u and the chromatographic resolution function CRF. The data acquired from the design were analysed using software developed in our laboratory.

PLATE NUMBER STUDY : The estimates parameter generated for the regression model are given in table 2. The difference between the predicted and the actual value obtained experimentally was used as a criterion for evaluation of the regression model. This generated model can be assessed statistically (16) using a Fischer Snedecor test (F-test) and the coefficient of multiple determination R^2. The F ratio tests the validity of the model and the value of R^2 is an indicator of the explanatory power model and assumes values from 0, indicating that the variables in the model perfectly explain the variation in the dependent variable N. These criteria were equal to 80, 11 for F and 0.9639 for R^2. This value shows that this model is able to explain a high proportion of the variation present in the theoretical plate number. Calculated values of N are given in Table 3. From the full regression model it was interesting to exclude these variable terms that had no significant effect on the plate number. For this, a student test (T-test) was used to provide the basis for a decision as to whether or not the model coefficients were significant or not. The results of this test are given in Table 4. This reduced model excludes the variable x_2^2 and the interaction $x_1.x_2$. As the matrix of the

Table 2. Regression parameter estimates for the three regression models

Independent variables	Parameter terms	N/L[a]	u[b]	CRF[c]
intercept	a_0	+44.671	+2.747	+36.656
x_1	a_1	-11.641	-0.052	-21.735
x_2	a_2	+5.531	+1.393	+1.021
x_3	a_3	+7.412	+0.006	+1.322
x_1^2	a_{11}	-10.200	-0.021	-1.257
x_2^2	a_{22}	+0.391	+0.285	-1.260
x_3^2	a_{33}	-5.752	+0.023	-0.640
x_1x_2	a_{12}	+0.251	-0.033	+0.536
x_1x_3	a_{13}	-7.052	-0.030	-2.091
x_2x_3	a_{23}	+11.831	-0.011	-1.400

[a] : inverse of the height to a theoretical plate (mm^{-1})
[b] : linear mobile phase velocity (mm/s)
[c] : chromatographic resolution function

Table 3. Response functions calculated for the 13 experiments

Chromatographic conditions							
Experiment N°	Methanol (% v/v)	Flow rate (mL/min)	Temperature (° C)	N/L[a]	u[b]	Fobj[c]	CRF[d]
1	50	0.6	36	41.213	1.636	5.183	55.398
2	50	1.0	50	54.811	2.837	4.940	57.265
3	50	1.6	36	51.780	4.489	5.329	56.352
4	50	1.0	26	25.895	2.765	5.233	55.725
5	63	0.6	26	38.187	1.645	2.349	33.666
6	63	0.6	50	29.358	1.678	2.855	33.822
7	63	1.6	50	64.069	4.443	2.736	33.047
8	63	1.6	26	25.595	4.452	3.091	38.491
9	80	1.0	50	17.437	2.673	0.407	9.610
10	80	0.6	36	17.442	1.599	0.598	10.856
11	80	1.0	26	16.709	2.721	1.456	16.438
12	80	1.6	36	28.991	4.319	1.074	13.953
13	63	1.0	36	44.665	2.747	2.809	36.657

[a] : see Table 2

[b] : see Table 2

[c] : objective function

[d] : see Table 2

Table 4. Student T test used to study the effects of variables of each model

Independent variables	calculated t[a]	
	N/L[b]	u[c]
intercept		
x_1	9.56	4.77
x_2	4.54	127.47
x_3	6.08	0.56
x_1^2	4.48	1.04
x_2^2	0.17	13.92
x_3^2	2.52	1.11
x_1x_2	0.14	2.15
x_1x_3	4.09	1.96
x_2x_3	6.87	0.70

[a]: studentT test
[b] : see Table 2
[c] :see Table 2

experiments is orthogonal the coefficients of the two models are similar thus, it is not necessary to obtain the new coefficients of the reduced model using the two well known "forward" and "backward" methods.

The reduced model excludes the variable x_2^2. This would suggest on the one hand that in the range of variation of the flow rate 0.6-1.6 mL/min, the mobile phase flow rate has no influence on the degree of curvature of the response surface. On the other hand, the non significance of the interaction $x_1.x_2$ shows that it might have been possible to use a univariate approach to optimize these two factors. The retention of a number of second oder terms in the reduced model eg x_1^2, x_3^2, demonstrates that the mobile phase composition and column temperature influence the degree of curvature of the response surface. Thus, for a constant mobile phase composition and flow rate, when the column temperature increases, the solute mass transfer from the mobile phase to the stationary phase increases producing an increase in column efficiency. Over an optimal temperature, the decrease in the capacity factor with the

temperature hides the first phenomena and produces a decrease in the column plate number. By derivation of the model equation, it is shown that the optimum temperature is given by the following equation

$T_{OP} = \exp(-0.85 \ln P + 0.69 \ln D + 7.35)$ [7]

where P (%) and D (mL / min) are the percentage of methanol in the methanol-water mixture and the flow rate.

For p equals 63.24 % and for D equals 0.6 mL/min ; 0.8 mL/min and 1 mL/min, the optimum temperature is respectively equal to 32.22° C; 39.30° C, 45.83° C (Fig.2). According to Horwath and Melander (17) when the percentage of methanol increases the superficial tension between mobile and stationary phases decreases. The consequence is a higher rate of mass transfer of the hydrophobic solute (naphtalene) in the stationary phase and equally in the mobile phase by decreasing the solvent polarity. The factor peak band broadening due to mass transfer decreases. Thus, the plate height decreases. But rapidly, the decrease in the capacity factor with an increase in the percentage of methanol necessitates a higher plate number. In this case beyond an optimal mobile phase composition, this second effect supplants the increase of the column efficiency referred to above and the plate number decreases.

By derivation of the model equation the optimum mobile phase composition is given by the following equation :

$P_{OP} = \exp(0.006 \ln D - 0.246 \ln T + 4.90)$ [8]

For a mobile phase flow rate of 1 mL / min and a column temperature of 26° C, 36° C and 50° C the optimum mobile phase composition was respectively equal to 60.25 %, 55.61 %, 51.30 % methanol in the methanol-water mixture(Fig. 3).

The simplex method was employed to find optimum contitions when the three factors vary simultaneously. The four sets of starting conditions were :

[1] D = 1.00 mL/min T = 30.00° C p = 70.00 %

[2] D = 1.12 mL/min T = 30.47° C p = 71.88 %

[3] D = 1.47 mL/min T = 30.47° C p = 70.47 %

[4] D = 1.12 mL/min T = 31.88° C p = 68.74 %

Twenty five interative processes were performed by computer and the results are given in Table 5. The optimum conditions were a mobile phase flow rate of 1.6 mL/min with a 50.00 %

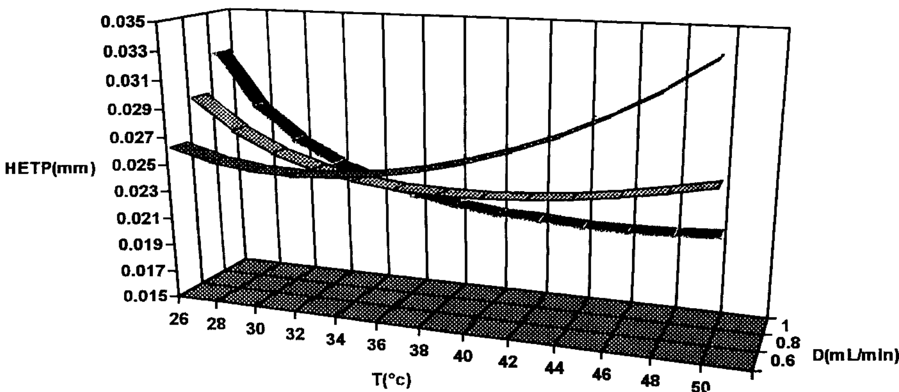

Figure 2. Plots of the height to a theoretical plate vs column temperature (percentage of methanol kept at 63.24 %)

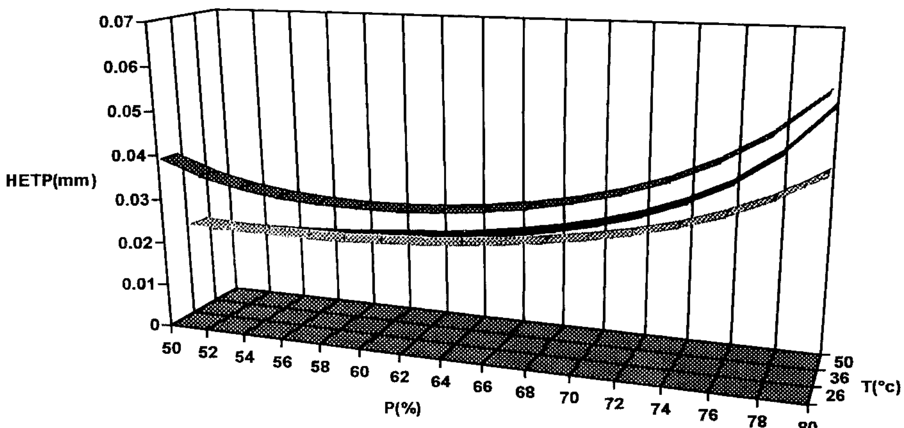

Figure 3. Plots of the height to a theoretical plate vs the percentage of methanol (flow rate kept at 1mL/min)

Table 5. Results of the simplex process for HETP

Experiment N	Percentage of methanol (%)	Flow rate (mL/min)	Temperature (C)	HETP (mm)
1	70.00	1.00	30.00	0.0299
2	71.88	1.12	30.47	0.0314
3	70.47	1.47	30.47	0.0300
4	70.47	1.12	31.88	0.0285
5	68.74	1.27	31.10	0.0277
6	69.00	0.70	31.52	0.0280
7	68.81	1.12	33.02	0.0262
8	67.24	1.00	31.86	0.0263
9	67.52	1.47	32.46	0.0249
10	66.97	1.13	33.78	0.0245
11	68.30	1.48	34.30	0.0236
12	66.39	1.60	34.02	0.0225
13	66.92	1.33	35.61	0.0225
14	65.21	1.23	34.26	0.029
15	65.47	1.31	32.40	0.0243
16	69.83	1.60	33.29	0.2520
17	66.29	1.60	34.83	0.0217
18	61.46	1.60	35.54	0.0196
19	62.76	1.60	40.37	0.0175
20	57.93	1.60	39.07	0.0171
21	59.22	1.60	43.90	0.0157
22	54.42	1.60	42.60	0.0155
23	55.71	1.60	47.44	0.0145
24	50.90	1.60	46.14	0.0146
25	50.01	1.60	49.95	0.0138

percentage of methanol and a column temperature of 50° C. The maximum theoretical plate number was 10846 and the height equivalent to a theoretical plate 0.0138 mm.

LINEAR VELOCITY STUDY : Flow rate varied from 0.6 to 1.6 mL/min. The corresponding average linear velocity u was modeled. The parameter estimates generated for the regression model are given in Table 2. The value of F was 5527 giving an excellent validity for the model. The R^2 value shows that 99.78 % of the linear velocity mean square was explicated. The calculated u values for the 13 experiments are given in Table 3. The results of the student T test are given in Table 4. The mobile phase flow rate had the strongest influence. The retention of the second order term x_2^2 on the reduced model shows that flow rate influenced the degree of curvature of the response surface. For p = 63.24 % and T = 26° C and for a flow rate variation range of 0.6 to 1.6 mL/min the corresponding linear velocity u are plotted on the y axis in Figure 4. The plots show a slight curvature. The reduced model excludes the variable x_1^2 but not x_1 showing that the mobile phase composition did not influence the degree of curvature of the response surface and did not greatly affect the value of u. The terms x_3 and x_3^2 are suppressed from the model. The column temperature does not affect either the curvature of the response surface or the intensity of the value of u. The suppression of the term x_2x_3 from the model would suggest that the interaction is not important. It might have been possible to use a unvariate approach to study these two variables.

CHROMATOGRAPHIC RESOLUTION FUNCTION : As indicated above, the chromatographic resolution function is given by equation [5] where α, β, a are constants which must be determined to have the most efficient CRF to separate the ten benzodiazepines . The constant a is often taken to be equal to 1.4 (10). Calculated values of Fobj (equation [6]) given by the model for the 13 experiments are given in Table 3. The coefficient of multiple determination R^2 corresponding to CRF is given by the well-known equation :

$$R^2 = 1 - \frac{(n-k-1)}{(n-1)}\frac{s_1^2}{s_2^2} \qquad [9]$$

where s_1^2 : residual variance

s_2^2 : Total variance

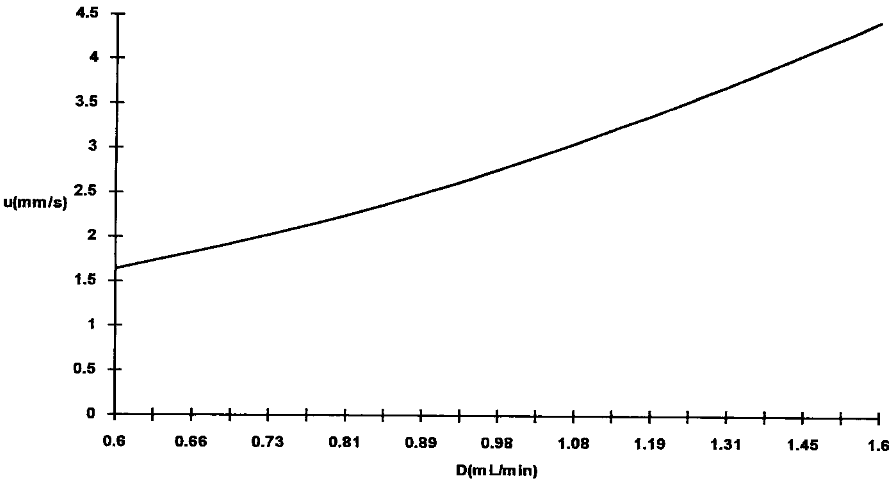

Figure 4. Plots of linear velocity vs the flow rate (percentage of methanol and column temperature kept respectively at 63.24 % and 26 C)

n : N of experiments

k : N of parameters

s_1^2 and s_2^2 are given by the two equations :

$s_2^2 = 3.1599\,\alpha^2 + 43.5930\,\beta^2 + 21.9389\,\alpha\beta$ [10]

$s_1^2 = 0.0285\,\alpha^2 + 1.4795\,\beta^2 + 0.01909\,\alpha\beta$ [11]

eq [9] is a second order equation in α.

Eq [9] is rewritten as :

$a\alpha^2 + b\alpha + c = 0$ [12]

where

$a = 37.9188\,R^2 - 37.6624$

$b = (263.2668\,R^2 - 263.4386)\,\beta$

$c = (523.1160\,R^2 - 509.8005)\,\beta^2$

Eq [12] has roots if

$\Delta = b^2 - 4ac$

Table 6. Results of the simplex process for CRF

Experiment N	Percentage of methanol (%)	Flow rate (mL/min)	Temperature (C)	CRF
1	55.00	0.80	30.00	48.141
2	59.71	0.85	31.18	41.589
3	56.18	0.99	31.18	47.312
4	56.18	0.85	34.62	46.941
5	52.00	0.88	33.71	53.526
6	55.41	1.02	36.82	48.520
7	59.72	1.00	34.95	41.995
8	56.92	0.90	39.01	45.903
9	53.01	0.84	39.03	52.083
10	57.00	0.80	39.20	47.489
11	60.20	0.80	40.00	40.411
12	58.95	0.80	43.02	42.224
13	56.67	0.84	43.22	46.022
14	57.00	0.88	44.23	45.485
15	57.02	0.87	45.81	45.374
16	56.00	0.80	46.00	46.944
17	53.81	0.81	47.80	50.651
18	52.88	0.83	49.10	52.260
19	51.82	0.81	50.00	54.122
20	50.00	0.82	50.00	57.427

is positive. The maximum value to obtain $\Delta > 0$ was $R^2 = 0.9940$

For $R^2 = 0.994$ Roots of Eq [12] are :

$$\frac{\alpha}{\beta} = k$$

where

$k = 6.5071$ or $k = 54.291$

So that the weight of $\sum \ln (1 + dij)$ is not too high in relation to the weight of $N^{1.4}$ $k = 6.5071$ for $\beta = 1$ was used.

$$CRF = 6.5071 \sum \ln (1 + dij) + N^{1.4}$$

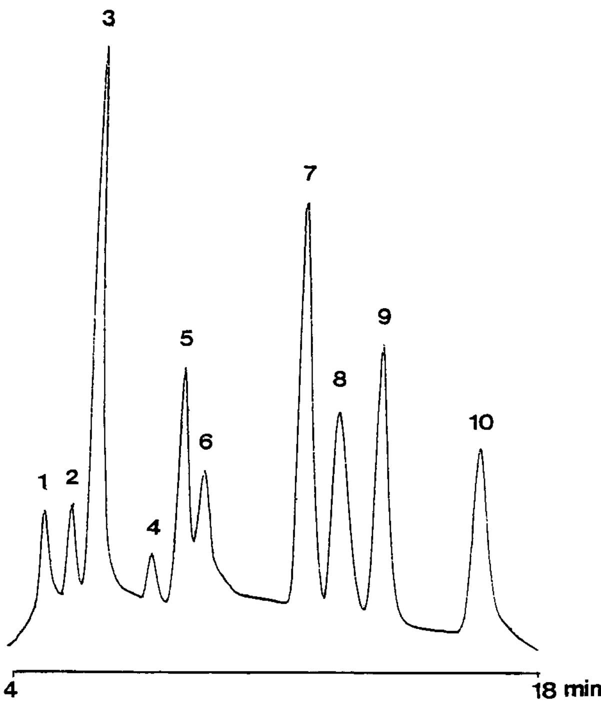

Figure 5. Benzodiazepines chromatogram in the optimum conditions : Methanol = 50 % v/v - Flow rate = 0.82 mL/min - Temperature = 50 C. Number above peaks refers to the benzodiazepines see paragraph reagents and samples.

The parameter estimates generated for the regression model are given in Table 2. CRF calculated values for the 13 experiments are given in Table 3.

In a recent work (9) using another criterion of separation (Ln (1 + Rs) method) it has been demonstrated that the optimum values which gives the most efficient separation conditions are a flow rate of 0.77 mL/min with a percentage of methanol of 49.95% and a column temperature of 51.62° C. In this present work, the highest value of CRF was used as a criterion of separation. Using the sequential simplex method, the four sets of starting conditions were :

[1] D = 0.80 mL/min   T = 30.00° C   p = 55.00 %

[2] D = 0.85 mL /min   T = 31.18° C   p = 59.71 %

Table 7. Optimum conditions found with two different methods

Methods	D(mL/min)	T(C)	P(%)
"Ln(1 + Rs)"	0.77	51.62	49.95
"CRF"	0.82	50.00	50.00

[3] D = 0.99 mL/min T = 31.18° C p = 56.18 %

[4] D = 0.85 mL/min T = 34.62° C p = 56.18 %

Twenty iterative processes were performed by computer and the results are given in Table 6. The optimum conditions were a mobile phase flow rate of 0.82 mL/min, a 50.00 % percentage of methanol and a column temperature of 50° C. The maximum value of CRF was 57.427. The chromatogram for these conditions is given in Figure 5. The same optimum values were obtained with these two methods (Table 7) for column temperature and mobile phase composition. The relative deviation for the mobile phase flow rate was 23 %. Optimum column efficiency and optimum separation were only similar in relation to the mobile phase composition and column temperature.

CONCLUSION

Optimizing temperature, flow rate (linear velocity), and mobile phase composition can improve the speed and quality of a separation. The effects of changing column temperature are important as the results of flow rate changes especially if similar compounds are present. It must also be recalled that column efficiency can be greatly improved if column temperature is increased. Agreement between these two separation methods used is good.

Results demonstrate the importance of temperature. Thus, the central design has been shown to be a useful tool for method development when used with a powerful statistical package.

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COMPARATIVE BEHAVIOUR OF DIFFERENT REVERSED-PHASE PACKINGS WHEN EQUILIBRATED WITH CETYLTRIMETHYLAMMONIUM BROMIDE

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ABSTRACT

The saturation of different reversed-phase HPLC C_{18} packings with the cationic surfactant cetyltrimethylammonium bromide (CTA) and its subsequent elution in net aqueous eluants, has been studied. The distribution of the surfactant between the mobile and stationary phases indicates a mixed retention mechanism. Under conditions of saturation, the total amount of CTA retained in different commercial packings is inversely related to the density of coating (μmoles C_{18}/m^2), and for a given packing depends on the temperature and degree of ageing. When deionised water or a phosphate buffer was passed through the column saturated with CTA, part of it was eluted with the mobile phase, but there was always an amount of CTA that did not elute with aqueous eluants; this uneluted CTA was a constant quantity depending on the eluant, packing characteristics and temperature. When columns were equilibrated with CTA, there was a shift in the eluant pH which was related with the life of these packings working in the presence of CTA.

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INTRODUCTION

The importance of silica type[1] and especially the packing surface characteristics, (residual hydroxyl groups, pore-size and surface alkyl concentration), on the chromatographic performance has been reported[2]. Differences in the stability of bonded-silicas, under hard eluent conditions, have been demonstrated for silicas of different procedences[3], and for the same silica using different alkyl substituents[3,4] or different chemistry for graft attachment[3-5].

Columns preequilibrated with CTA have been used in RP-HPLC for the separation and determination of compounds such as pterins[6], benzoates, phenylacetates[7], and inorganic anions[8]. Moreover, CTA has been used to dinamically modify bare silica gel columns to get reverse phase separations[9]. With aqueous eluants, the retention of CTA onto unmodified silica surfaces is mainly due to an ion exchange mechanism[9]. Ammonium quaternary salts have been used to block the effect of residual silanol that causes mixed-mode retention in reverse phase chromatography[10]. CTA itself is retained by both ion-exchange and hydrophobic interactions in RP-HPLC stationary phases[11,12]. The relative importance of both retention mechanisms has been discussed previously[13].

When reverse phase columns are equilibrated with ionic surfactants, the bound C_{18} chains are rearranged on

the surface of packings[14,15], with the consequent variation on the retention of analites, including neutral compounds[14]. Also, during CTA adsorption, the occurrence of pH-shifts in the eluant have been observed[9,11,16].

We present data on the adsorption of CTA by different commercial RP-HPLC packings, and a qualitative comparison of the stability of these packings when working in the presence of CTA.

MATERIALS AND METHODS

Reagents

Xanthopterin (X), isoxanthopterin (I), pterin (P), biopterin (B) and neopterin (N), were purchased from Sigma (St. Louis, MO, USA), and were of chromatographic grade. CTA was obtained from Serva (Heidelberg, Germany).

Sodium dihydrogenphosphate and disodium hydrogenphosphate, analytical-reagent grade, from Merck (Darmstadt, Germany). HPLC-grade methanol was purchased from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Before use, all eluants were degassed under vacuum and filtered with an all glass apparatus through 0.45-μm filters (Millipore, Bedford, MA, USA). Other chemicals were of analytical reagent grade.

Apparatus

A Waters (Milford, USA) liquid chromatograph, consisting of a 590 model solvent-delivery pump, a 481 model UV variable-wavelength detector, a 420 model fluorescence detector and a 730 model data module, was used. The injector, with a 10 μl loop was from Rheodyne (Cotati, CA, USA). A Selecta (Barcelona, Spain) thermostated bath was used to control the column temperature. For pH measurements, a Crison (Barcelona, Spain) digital pH-meter with an Ingold 10-402-253 glass combined electrode was used.

Sep-Pak C_{18} cartridges were obtained from Waters. Nucleosil-CN 10μm (250x4 mm) was from Knauer (Berlin, Germany), μBondapack 10μm (300x4 mm) from Waters, Ultracarb 5μm (250x4.6 mm) from Phenomenex (Torrence,CA,USA), Nucleosil-120 5μm (120x4 mm) from Knauer, Hypersil 5μm (100x4 mm), Nucleosil-100 5μm (150x4 mm) and a Spherisorb-ODS2 10μm (300x4 mm) were packed by Technokroma (Barcelona, Spain). Other technical characteristics from these columns are summarized in Table I.

Procedures

To saturate the columns with CTA [the term saturation is used to specify the CTA adsorpted on the

TABLE I.

Technical Characteristics of the C_{18} Packings Used.

	A	B	C	D	E	F
Total volume, ml	4.15	3.58	3.58	1.88	1.51	1.26
Dead volume, ml	3.00	2.72	2.10	1.38	1.05	1.26
Porosity	0.72	0.76	0.58	0.73	0.70	0.64
Mass (g)	2.53	1.89	3.26	1.10	1.01	0.98
Particle size, μm	5*	10**	10*	5*	5*	5*
% Carbon	22	10	12	14	11	8.8
Surface area (m^2/g)	370	350	220	350	220	200
Coating, μ-mol C_{18}/m^2	4.05	1.55	3.26	2.33	3.03	2.70
Coating, μ-mol C_{18}/g	1567	542	673	815	606	467

A: Ultracarb ODS; B: μ-Bondapak C_{18}; C: Spherisorb ODS-2; D: Nucleosil 100-C_{18}; E: Nucleosil 120-C_{18}; F: Hypersil C_{18}.
* Spheric. ** Irregular.

stationary phase when it is in equilibrium with the CTA in the mobile phase at the critical micellar concentration (CMC)], a 5 mM aqueous solution of this compound was passed through them at a flow rate of 1.5 ml/min, until CTA was present in the efluent. The amount of CTA retained was called CTA-T (i.e. the maximum quantity of CTA that can be retained by a column at a given temperature). Then 200-300 ml of water or 1.5 mM phosphate buffer (pH 6.5) without CTA were passed through the columns at the same flow-rate. After this aqueous washing there was some uneluted CTA that was called CTA-P

(the amount that remains in the column when there is no CTA in the mobile phase).

CTA was determined chromatographically with UV indirect photometric detection. For this procedure[17] a Nucleosil-CN column thermostated at 20°C was used; the mobile phase was (55:45) methanol:water containing 5 mM p-toluensulphonic acid at a flow-rate of 1 ml/min. The UV detector was set at 250 nm. To determine CTA at concentrations below 0.1 mM, the solution was concentrated using a C_{18} Sep-pak cartridge. The diluted aqueous solution, up to 450 ml, was passed through the cartridge; CTA was eluted with 5 ml of (90:10) methanol:1.2 M HCl.

The distribution isotherm of CTA between the mobile and stationary phases was studied at 22°C with a Hypersyl column using different aqueous solutions of CTA ranging from 0.1 to 1.5 mM. When the CTA concentration was the same in the mobile phase and in the efluent, the retained CTA was calculated. The amount of CTA retained was obtained by measuring the difference between the total CTA passed through the column and the CTA in the efluent.

Pterins were separated using a Hypersil C_{18} column, pre-equilibrated with 50 mg of CTA; 1.5 mM phosphate buffer (pH 6.5) was used as mobile phase at a flow rate of 1.5 ml/min. Fluorescence detection was used (excitation filter: 365nm; emision filter: band pass 420 nm).

RESULTS AND DISCUSSION

Saturation of C_{18} packings

The distribution isotherm (Fig.1) between mobile and stationary phases reached saturation at 0.8-0.9 mM CTA, which is the CMC of CTA. This has been described before[13,18]. Table II shows the amount of CTA retained at different temperatures for a new and old Hypersil 5-ODS column, depends on temperature and degree of ageing of the column. It is observed that for both, new and old packing, the retention of CTA changed very little with temperature variations around 20°C (room temperature). The new packing retained at 18°C an amount close to 30 % higher than the old packing. Since there was no void volume in this Hypersil column, the decrease in CTA retention by the old column must be due to a decrease in C_{18} coverage. There were important variations in the amount of CTA retained when the temperature was lowered to 14^{0}C.

The amounts of CTA-T retained by the packings tested were very different (Table III) and were mainly related to the specific C_{18} coating of packings. From the comparison between CTA-T, in $\mu mol/m^2$, and the specific C_{18} coating of the packing, also expressed as $\mu molC_{18}/m_2$, the total CTA retention can be explained by taking into

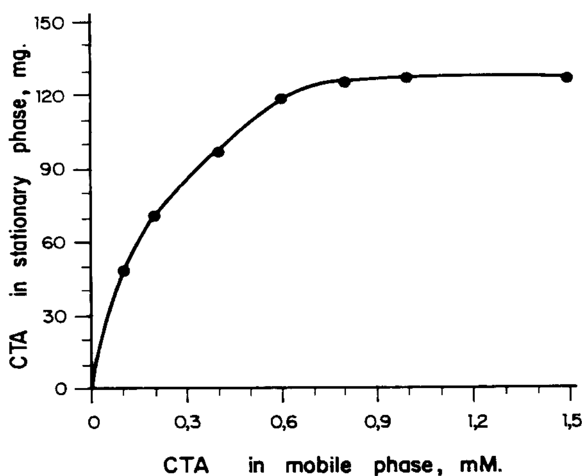

FIGURE 1. Distribution isotherm of CTA at 22°C using a Hypersil column and different aqueous solutions of CTA.

TABLE II

Influence of the Temperature and Degree of Ageing on the Retention of CTA on Reversed-Phase Hypersil 5-ODS (100 mm x 4 mm i.d.) Packing.

	T,°C	CTA, mg
New column	14	170
	20	127
	22	125
Old column	10	168
	17	109
	28	102

TABLE III

CTA Retained by Different Reversed-phase Packings Under Saturation (CTA-T) and After Equilibration with 1.5 mM Phosphate (pH 6.5) (CTA-P).

CTA-T	mg	μmol	$\mu mol \cdot m^{-2}$	$\mu mol \cdot g^{-1}$
Ultracarb	545	1487	1.59	587
μ-Bondapak	432	1185	2.09	626
Spherisorb	377	1034	1.44	317
Nucleosil-100				
- New	291	798	2.07	725
- Aged	256	702	1.82	638
Nucleosil-120	151	414	1.86	410
Hypersil ODS	127	348	1.76	355

CTA-P	mg	μmol	$\mu mol \cdot m^{-2}$	$\mu mol \cdot g^{-1}$
Ultracarb	360	960	1.02	379
μ-Bondapak	168	460	0.69	243
Spherisorb	250	658	0.95	210
Nucleosil-100				
- New	180	494	1.28	449
- Aged	135	369	0.96	335
Nucleosil-120	55	150	0.68	148
Hypersil ODS	50	137	0.69	140

account steric effects, i.e. Ultracarb, with higher C_{18} coverage, retained lower CTA-T/m^2 of packing. However, the μ-Bondapack packing, which has lower density of coverage, retained higher CTA-T/m^2 . These results were in agreement with other studies about the steric effects that limit the maximun C_{18} coating for a given packing[19].

The elution of CTA from C_{18} packings

Once the different packings were saturated with CTA, a mobile phase without CTA was passed through the column,

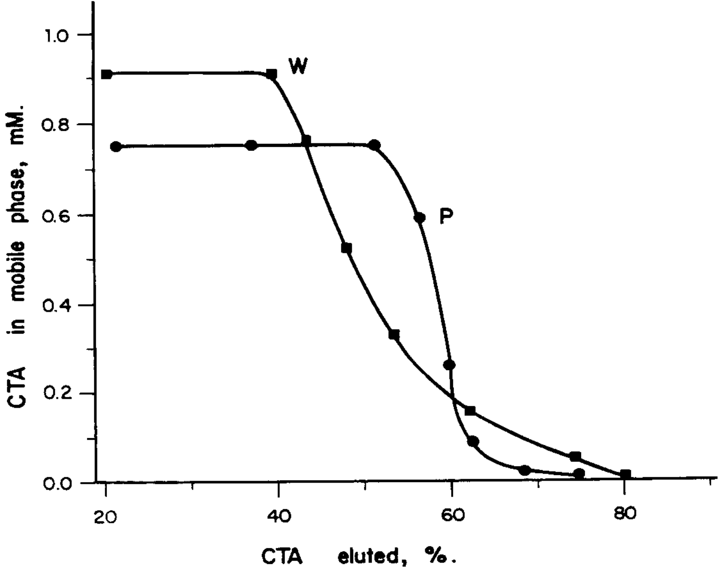

FIGURE 2. Dynamics of CTA elution from a Hypersil column, when CTA was suppresed from the mobile phase. W: Deionised water and P: 1.5 mM phosphate buffer (pH 6.5) mobile phases.

and the elution of CTA was studied. Two mobile phases were used: deionised water and a 1.5 mM phosphate (pH 6.5) buffer. The results are shown in Fig. 2 for a Hypersil column and a similar pattern was observed for the other columns studied. In these curves it is possible to distinguish three intervals. In the first one the CTA concentration that eluted from the column remained constant and equal to the CMC of CTA in these eluents. This indicates that the aqueous mobile phase is able to remove CTA from the stationary phase, but without

exceeding the CMC. In other words, the association of CTA with the stationary phase is more stable than the intermolecular association of CTA to form micelles.

In the second interval the slope is higher with the phosphate buffer than with deionised water as mobile phase. This result could be expected, since CTA is more soluble in water than in this buffer. This different solubility can be used to manipulate the selectivity of the column: small amounts of CTA can be eluted from the column with water until the desired separation is obtained with the buffer.

In the third interval the elution of CTA from the column with buffer was negligible and the CTA amount remaining in the stationary phase was constant. So, a reverse phase columnn, that also works as an anion exchanger, without adding CTA to the mobile phase, is obtained. When this equilibrium was reached, large volumes of mobile phase (2-3 liters) could be passed through the column and the retention time of the analites remained constant.

The amount of CTA retained by the stationary phase without eluting was called CTA-P (phosphate buffer as mobile phase) and was characteristic for each packing (Table III). CTA-P was related to the packing's superficial C_{18} coating, i.e., the more coated packing (Ultracarb), retained higher amount of CTA, and the lower

value of CTA-P corresponded with the less coated packing (μBondapak) (Table III). However, other factors must contribute because there is not a good correlation between these two magnitudes. In three of the columns used, CTA-P is one third of CTA-T, while in the others it is about two thirds. A different organization of the C_{18} chains must be responsible of these differences.

pH effects during the saturation and equilibration of the packings

When the C_{18} packings were saturated with 5 mM CTA in water, the pH of the eluent was measured. During the saturation process there was a decrease in the pH of the efluent. There was a displacement of hydronium ions from the acidic silanols of the silica matrix by CTA, with a consequent acidification of the mobile phase. So, it is possible to understand why, using these columns with CTA, it was necessary to wait for a long time to obtain reproducible retention times for ionic compounds. A mixture of pterins was used to illustrate this phenomena. Fig.3 shows the variation of the retention times of these compounds during the equilibration of the column with phosphate buffer. For all pterins, after passing 200 ml of phosphate buffer, the retention times and peak heights were stabilized. Xanthopterin(X) and Isoxanthopterin(I),

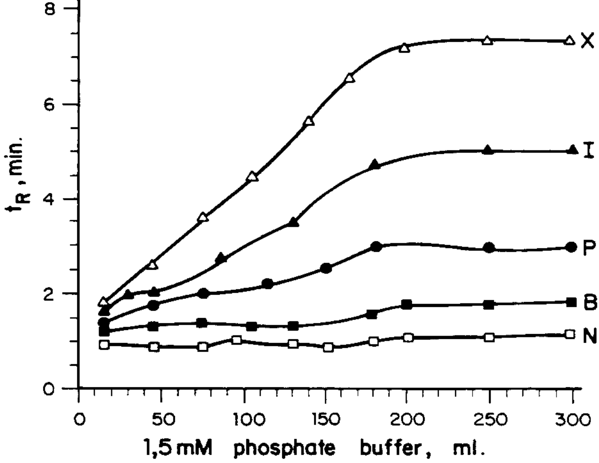

FIGURE 3. Variation of the t_r of pterins during the Hypersil column equilibration with 1.5 mM phosphate buffer (pH 6.5), when the column was previously equilibrated with 50 mg of CTA.

with lower pK values than the other pterins, were the most affected compounds by this equilibration process. That means that the real pH of the separation during the equilibration process was lower than the pH of the mobile phase. Using different flow rates it was observed that this process was not affected by kinetic parameters, but only by the total volume of eluant passing through the column.

Table IV shows the hydronium ions liberated, by the different columns used, during their saturation with CTA. These values were different and depend on the packing used. It was possible to observe:

TABLE IV.
Hydronium Interchange During the Saturation of Different C_{18} Packings with CTA, Using an Aqueous Solution.

Packing	Surface area $m^2.g^{-1}$	H^+ μmol	H^+ $\mu mol \cdot m^{-2}$	H^+ $\mu mol \cdot cm^{-3}$	H^+ $\mu mol \cdot g^{-1}$
Ultracarb	370	100	0.107	33.3	39.5
μ-Bondapack	350	80	0.121	29.4	42.3
Spherisorb	220	43	0.060	20.5	13.2
Nucleosil-100	350	33	0.086	23.9	30.0
Nucleosil-120	220	11	0.050	10.5	10.9
Hypersil	200	4	0.020	4.9	4.1

i) the packings with higher specific surface area (m^2/g) exchanged more hydronium ions per volume that the packings with lower specific surface-area; so, the packings with lower specific area needed less volume of eluant for their equilibration.

ii) When the hydronium ions liberation was related to the surface area of the packing, the differences between packings still remained, and those with higher specific surface area liberated more hydronium ions per m^2 than the packings with less specific surface area.

The stability and chomatographic performance of reverse-phase packings vary depending on the surface population of acidic and hydrogen bond associated silanols groups[20,21]. Acidic silanols have been described to cause peak tailing for basic compounds[20]. Moreover, silicas with an homogeneous distribution of associated

silanols have better stability and performances[21]. According to the results herein presented, packings with lower surface area seemed to have a better distribution of silanol groups than those with higher surface area, since they exchanged less quantity of hydronium ions when they were equilibrated with CTA. This can be due to accesibility problems: packings with a high specific surface area have a small pore-size and, due to steric effects, the C_{18} coating reached in the silanization process may be less homogeneous than the coating in higher pore-size materials. This kind of coating may provide a higher amount of isolated silanol groups and, as a consequence, the modified silica has higher acidity.

Duration of the packings used with CTA

The special aggressivity of cationic surfactants for silica packings has been described[10,22]. This behaviour was corroborated herein. Table V shows the qualitative results about the relative deterioration of packings which were conditioned with CTA. There was a group of packings which suffered a ready deterioration because silica was dissolved with formation of a void volume; its uses with CTA are restricted. The second group presents a decrease in the capacity factor (k′) during the early uses with CTA. After that, the capacity factor values

TABLE V.
Estimation of the Duration of Different C_{18} Packings when Used with CTA.

Packing	Surface area $m^2.g^{-1}$	H^+ $\mu mol \cdot cm^{-3}$	Durability
Ultracarb	370	33.3	short (void volume)
μBondapack	350	29.4	short (void volume)
Spherisorb	220	20.5	long
Nucleosil-100	350	23.9	medium (k'decrease)
Nucleosil-120	220	10.5	long
Hypersil	200	4.9	long

were stabilized and there was no variation with time. The third group presents better stability: there were no void volumes and the capacity factor values were constant after iterative processes using CTA and only after a long time did the capacity factor values decreased.

With the packings tested, there is a correlation between stability and specific surface-area. When they are equilibrated with CTA, those with less specific surface area used to be the more stable. This relation between pore-size and stability has been described before[23]. That implies that the higher production of hydronium ions during the equilibration with CTA, may be responsible of the lower stability of the high specific surface area packings.

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A STUDY OF PRECOLUMN AND ANALYTICAL COLUMN DIMENSIONS FOR ON-LINE SAMPLE PRETREATMENT IN HPLC

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ABSTRACT

A systematic study has been made of the effect of precolumn size and shape in relationship to the size of analytical column for column switching systems in HPLC. It is found that, even for miniature precolumns (volumes 30 - 500 μl) the volume does not affect the efficiency achieved nor the recovery of a set of six steroids from aqueous solution when 4.6 and 2 mm diameter analytical columns are used. Coupling of these precolumns with 1 mm diameter analytical columns results in decreased efficiency although in the systems tested sensitivity increased as analytical column diameter decreased. Such coupled systems could be applied to analysis of selected steroids in matrices of urine and saliva with little difficulty. Deterioration in both efficiency and recovery were found when extracting from a plasma matrix although linear calibrations were obtained.

INTRODUCTION

Solid phase extraction is most frequently carried out as an off-line procedure and is capable of providing effective removal of endogenous species from biological

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samples. Preconcentration of the analyte(s) is also possible which can improve detection limits. On line, the solid extractant is contained in a closed precolumn and switching valves are used for transfer of analytes. The perceived advantages of the latter are simplification of the overall analysis and improvement of detection limits by utilising all of the sample available. The principles of this approach have been reviewed [1]. An advantage often claimed for such on-line column switching methods is the longevity of the column[2,3]. This is perhaps surprising in terms of the contradictory evidence that cartridges, used off-line, incorporating such stationary phases (most frequently C-18) are not reusable[4]. A survey of the literature since the above review showed that a wide variety of precolumn dimensions are in use and also reveals that many such column switching methods incorporate an additional off-line pretreatment procedure. No systematic study has been reported concerning the interrelationship between precolumn and analytical column dimensions. In addition, while many publications report the number of injections possible without adversely affecting the performance of the different columns, it is not always possible to generalise from these data since sample size and prior pretreatment may vary.

Such a systematic study is of particular interest when so called microbore analytical columns are employed. Such columns have been shown to provide advantages in concentration sensitivity when large sample volumes are injected in weak chromatographic solvents[5]. The performance of such columns of 1 and 2 mm diameter may well depend upon the band broadening introduced during the column switching. Relatively few publications have been located which report such data[6,7]. The work of Goewie et al[8] showed that for a 250 x 4.6 mm analytical column, precolumn dimensions were not critical in terms of efficiency of resultant peaks. On the other hand, it has been shown[9] that appreciable band broadening due to extra column effects results when microbore analytical columns are employed in column switching mode.

In the light of the above, the purpose of the present work is to:

1. Make a systematic study of the effect of precolumn/analytical column

dimensions with respect to analyte recovery from aqueous solution, plate number and sensitivity of detection using a set of six steroids as test solutes.

2. Evaluate the practical utility of such systems in the pretreatment of the common biological matrices of plasma, urine and saliva with respect to recovery, ruggedness and effectiveness of sample clean-up and compare the findings with reports in the literature.

EQUIPMENT AND MATERIALS

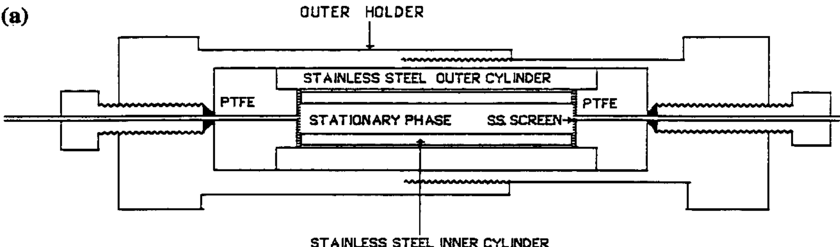
Chromatographic equipment consisted of Varian 2510 and Shimadzu LC5A pumps coupled with a Jasco 875-UV variable wavelength detector. Analytical columns were stainless steel, 100 mm long of diameters 1,2, and 4.6 mm. The loading valve was a Rheodyne 7125 fitted with a 20 or 1000 μl loop and column switching was via a Rheodyne 7010 valve. The analytical columns were slurry packed in the laboratory using 5 μm ODS Hypersil. Precolumns were constructed as in Figure 1A. having internal diameters of 1, 2,3 and 4 mm with a standard length of 10 mm. This design allowed variation of column length and diameter within a standard connecting holder which facilitated incorporation into the chromatographic system. An additional set of precolumns was made having a standard diameter of 3 mm and lengths of 2.5, 5, 7.5 and 10 mm incorporating suitable PTFE spacers. The precolumns were dry filled using 40 μm octadecyl silica obtained by removing the packing from Bondelut sample pretreatment cartridges. A simplified diagram of the column switching system is shown in Figure 1B. The set of test steroids used were obtained from Sigma and consisted of progesterone (P), 17α-hydroxyprogesterone (17P), 20α-hydroxy-4-pregnen-3-one (20P), androstenedione (A), testosterone (T) and norethindrone (N). These were chosen since the separation of these compounds required a ternary solvent of methanol, acetonitrile (Rathburn Chemicals) and water (25:25:50)[10] and thus represented a stringent test of resolution.

RESULTS AND DISCUSSION

Performances of the analytical columns used alone

The data in Table 1 provide information as to the chromatographic performance of the system for different diameters of chromatographic columns used alone

(a)



(b)

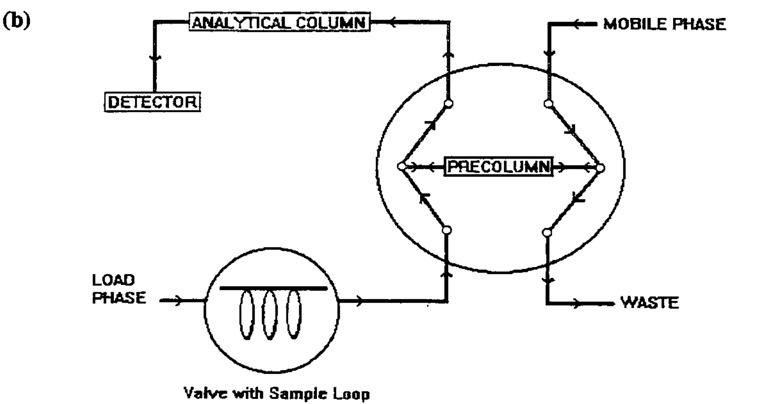


Figure 1. (A) Construction of Precolumn (B) Column switching system.

and, as such, constitute baselines from which the effects of the inclusion of precolumns into the system may be assessed. These results were obtained by injecting an aqueous solution of the six steroids at a concentration of 2 μgcm^{-3} each via a 20 μl loop directly on to the analytical column. To maintain constancy of linear flow rate, volumetric flow rates of 1.0, 0.2 and 0.05 cm^3min^{-1} were used for the 4.6, 2.0 and 1 mm diameter columns respectively. The data show that, as column diameter decreases, the overall system efficiency decreases. At the same time the absorbance data show an overall increase in sensitivity as column diameter decreases. The average sensitivity increase on going from a 4.6 to a 1

TABLE 1 Comparison of Plate Number and Absorbance Maxima for the Analytical Columns used alone

Steroid	Plate Number			Absorbance x 100 (RSD%)		
	Column Diameter			Column Diameter		
	4.6	2	1	4.6	2	1
T	7557	3036	2034	0.671 (1.8)	2.61 (2.9)	3.60 (1.9)
A	8185	3228	2089	0.550 (1.5)	2.28 (1.6)	3.29 (0.83)
17P	7210	3191	2144	0.365 (3.1)	1.54 (3.4)	2.22 (1.0)
20P	7068	3363	1998	0.257 (2.4)	1.17 (2.9)	1.87 (1.4)
P	7023	3677	2331	0.178 (2.0)	0.798 (2.6)	1.28 (1.8)
N	6720	3980	2242	0.132 (3.6)	0.589 (3.4)	0.948(2.9)

mm diameter column being 13 taking into account the decreased path length used with the 1 mm column. It can be seen that the absorbance ratio increases with retention time of the solute being measured which also indicates the existence of extra- column band broadening with the 1 mm diameter column. Such extra column band broadening effects are well established [11,12] The maximum sensitivity increase nevertheless is similar to that reported previously[10].

General characteristics of the column switching system

A 1 x 10 mm precolumn was incorporated with a 100 x 2 mm analytical column and a 1cm^3 loop in a column switching system designed to operate in the backflush mode as represented in Figure 1B. The absorbance of each steroid and the plate number of the corresponding peak were determined as follows. After filling the loop with an aqueous solution containing the six steroids at concentrations of 40 ngcm^{-3}, these were loaded on to the precolumn using different volumes of water, transferred to the analytical column and eluted with the chromatographic solvent.

Two loop volumes were required for maximum loading. It was also found that less than 2% loss was incurred by using 20 loop volumes which can allow extensive

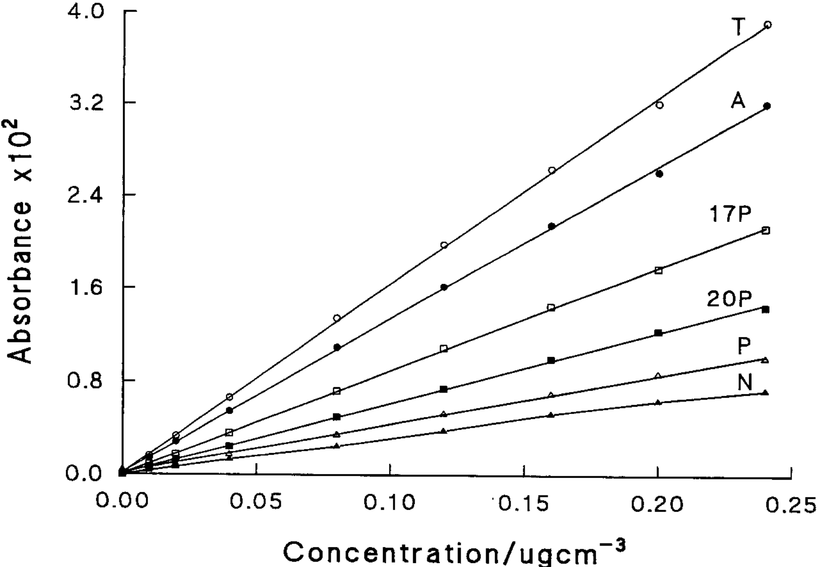


Figure 2. Plots of peak absorbance against concentration for six steroids using a 1x10 mm precolumn and a 4.6x100 mm analytical column. Injection loop volume 1000 μl. Compound identification as in figure.

removal of water soluble endogenous components from biological samples. The linearity of calibration was determined by injecting 1 cm^3 samples of the steroid solution ranging in concentration from 10 - 240 ng cm^3 for each compound. The linearity of plots of absorbance against concentration injected is shown in Figure 2. Regression analysis yielded correlation coefficients greater than 0.998 with a mean relative standard error of the gradients of 1.05%. This confirms the quantitative utility of such preconcentration systems at least for aqueous solutions.

Coupled systems

Each of the three analytical columns were, in turn, coupled with each of the precolumns. Samples of the six steroid test mixture (1 cm^3) at concentrations of 40 $ngcm^{-3}$ were injected and the absorbances and efficiencies of all peaks were

recorded as the mean of 4 replicate injections. The percentage recovery of each steroid was also determined by relating the response to that obtained in the absence of a precolumn by injecting 20 μl of 2 $\mu g cm^{-3}$ solution.

With the 4.6 and 2 mm diameter columns one way analysis of variance showed no significant difference at the 5% level in the overall system efficiency nor in the maximum peak absorbance for any of the test solutes as a result of incorporating a precolumn in the system and operating in the backflush mode. This was true for all of the precolumn geometries tested. Also, recoveries among all steroids and precolumns were uniformly high. For the 4.6 mm diameter column the mean recovery of all steroids from all precolumns used was 99.3% (sd 1.8%) and for the 2 mm column 99.6% (sd 1.9%). Significant differences in the efficiency and maximum peak absorbance were observed with the 1 mm column. However, when recoveries were based on peak area measurements a mean recovery of 96.5% (sd 2.2%) was obtained.

The effects of precolumn volume on absorbance and theoretical plate number are demonstrated graphically in Figures 3 and 4. In Figure 3 it is evident that coupling any size of precolumn with either a 4.6 or 2 mm diameter analytical column has no effect upon the absorbance values and the same is seen for the effect on theoretical plate number in Figure 4. The plots in these Figures for the 1 mm column show initial drops in both absorbance and efficiency. This appears to be due to extra column band broadening in the column switching system rather than to the precolumn volume since, after this initial drop, both variables appear to be substantially independent of column volume.

The effect of precolumn volume on precolumn load capacity was determined by removing the analytical column and connecting the precolumn directly to the detector. A solution containing 3 $\mu g cm^{-3}$ of each steroid was pumped through the precolumn at 1 $cm^3 min^{-1}$ and the absorbance monitored with time. Breakthrough volumes were determined for each precolumn and the corresponding mass of each steroid calculated. The results are shown in Table 2 where it can be seen that the ratio of mass loading to precolumn volume is approximately constant at

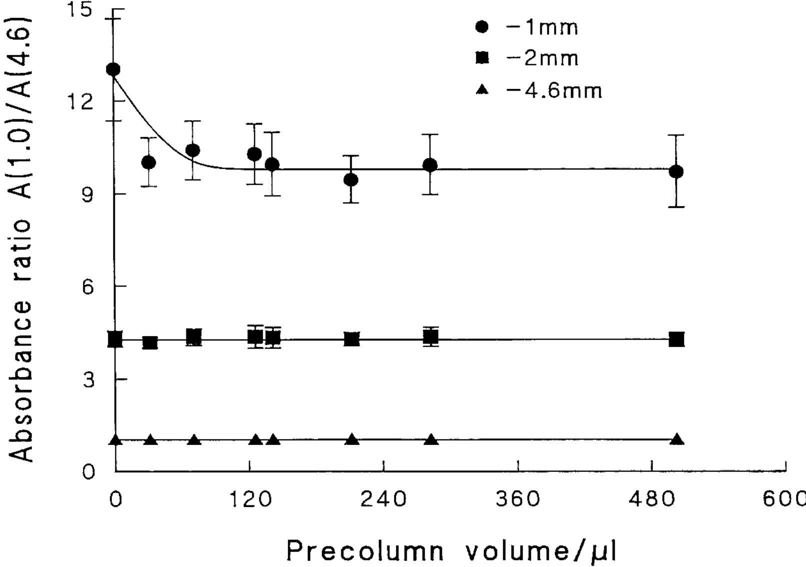


Figure 3. Plots of the mean peak absorbance for all solutes obtained for different analytical column diameters divided by the corresponding value obtained using a 4.6 mm diameter column as a function of coupled precolumn volume.

1.2 - 1.3 for all precolumns other than the two whose diameter to length ratio exceeds 0.5. These precolumns show a significantly lower mass loading. This is attributed to the flow pattern within the column not allowing full saturation of the stationary phase. For all the precolumns investigated, however, the mass loading was in excess of 40 µg of each steroid representing a total analyte loading of 240 µg.

From the above it would appear that analytes can be preconcentrated to an appreciable degree on such coupled column systems using 4.6 and 2 mm diameter columns without appreciable loss of overall system efficiency. The use of 2 mm diameter analytical columns confers a similar increase in sensitivity to that observed with conventional valve and loop injection. When 1 mm diameter

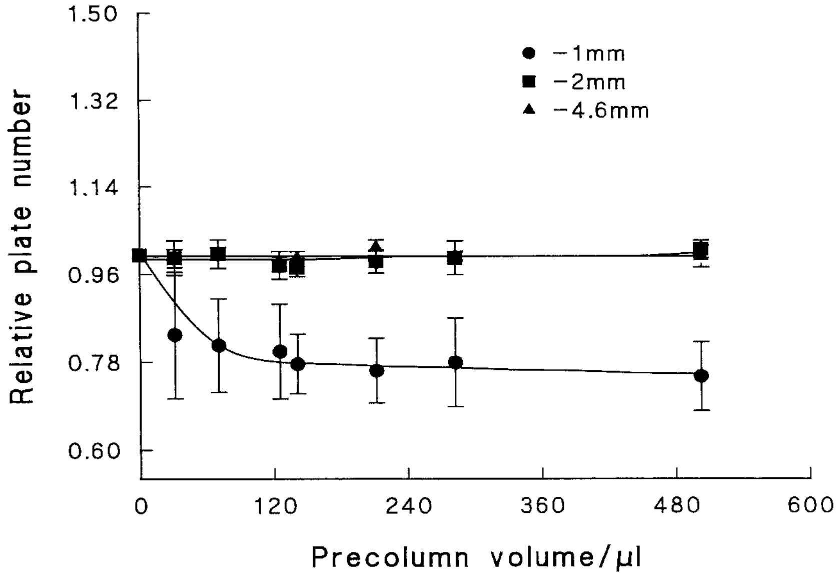


Figure 4. Plots of the relative theoretical plate number for all solutes using different analytical column diameters as a function of coupled precolumn volume.

TABLE 2 Analyte Load Capacity of Different Precolumns

Precolumn Size/ mm	Volume/ mm^3	Mass of each steroid/ μg	Mass/Volume / $\mu g mm^{-3}$	Precolumn Diam./Length
1 x 10	31.4	40	1.27	0.1
2 x 10	125.7	149	1.19	0.2
3 x 10	282,7	369	1.31	0.3
4 x 10	502.7	612	1.22	0.4
3 x 2.5	70.7	64	0.91	1.2
3 x 5	141.4	150	1.06	0.6
3 x 7.5	212.1	248	1.17	0.4

columns are incorporated there is an appreciable drop in the theoretical plate number which is independent of precolumn dimensions but which is associated with the extra column band broadening inherent in the coupled system. This results in lower gains in sensitivity over conventional diameter columns than were realised in the absence of on-line preconcentration.

The above refers only to the preconcentration aspect of column coupling from aqueous solutions which lack matrix interferents. In practice, for such techniques to be useful for the analysis of drugs in biological fluids, the effect of endogenous components present in such complex matrices on recovery and efficiency as well as on the general ruggedness of the system is of considerable importance. For this reason, the performances of the coupled systems were examined for the steroid set in matrices of urine, plasma and saliva. In the present study, the effects of the endogenous species present in such matrices on the recovery, sensitivity and longevity of the system is examined. The equally important aspect of sample clean-up by effective removal of endogenous components which could potentially interfere and thus adversely affect the specificity of the analysis is not addressed. The rationale for this is that any clean-up procedure will be particular for the analyte. That is, more drastic wash procedures may be employed the greater the capacity factor of the analyte in the chromatographic solvent and, conversely, it may not be possible to obtain adequate removal of endogenous materials from weakly retained analytes. The effect of matrix components upon the quantitative aspects of recovery, sensitivity and plate number are, however, likely to be a general characteristic of that matrix and therefore operative for all analytes.

Extraction from urine

The 40 ngcm^{-3} solution used in previous experiments did not allow reliable detection of the first three steroids eluted (T, A and 17P) on the 1 or 2 mm diameter columns due to the increased solvent front following injection of 1 cm^{3} of spiked urine and washing with water only. For this reason in the examination of urine matrix, concentrations 0.3 μgcm^{-3} in each of two steroids, T and 17P,

were used. Absorbance, efficiency and recovery of each steroid were determined from water and from urine using 1 x 10 and 4 x 10 mm precolumns coupled in turn with 4.6, 2 and 1 mm diameter analytical columns.

No significant differences were found between matrices of water or urine for absorbance or efficiency and the recovery from urine was identical with that from water alone. The main effect of the urine matrix was an increase in back pressure of approximately 10 bar on loading. The magnitude depended on the concentration of the urine in terms of endogenous components.It was independent of steroid concentration and was reduced following aqueous flushing of the precolumn. Up to 5 cm^3 aliquots of urine could be applied by repeated operation of the 1 cm^3 loading valve and loop. Each precolumn was found to be capable of accommodating at least 20 repeat applications of urine with no deterioration in performance or permanent rise in back pressure. These findings are consistent with the general experience that urine as a matrix is readily pretreated for drug analysis and that direct, on column injection of urine is often possible[13,14].

Extraction from Plasma

Plasma samples spiked with 0.3 μgcm^{-3} of the two steroids were injected via the precolumn and the peak efficiencies and sensitivities were compared with the corresponding values in aqueous solutions for the precolumns and analytical columns specified above. In contrast to the situation in urine, there was an appreciable decrease in overall system efficiency and sensitivity even taking into account band broadening produced with the 1 mm diameter column. Also, the recovery from plasma was significantly lower than from aqueous solution. The recoveries from plasma diluted with 20% water are shown in Table 3. The dilution was found necessary in order to allow loading of plasma samples without excessive increases in system back pressure. It can be seen from Table 3. that the decrease in efficiency and recovery is common to both precolumns but is more marked for the smaller precolumn. The efficiency and thus sensitivity

TABLE 3 Peak Areas of Testosterone and 17α-Hydroxyprogesterone in Water and 80% Plasma and Recoveries from Plasma

		Testosterone			17α-OH-progesterone		
		Peak Area		Percent	Peak area		Percent
Column Size/ mm	Precolumn Size/ mm	Water	Plasma	Recovery	Water	Plasma	Recovery
4.6 x100	1 x 10	501	327	65.3	432	262	60.6
	4 x 10	498	437	87.7	440	350	79.5
2 x 100	1 x 10	625	405	64.8	541	330	61.0
	4 x 10	628	549	87.4	545	415	76.1
1 x 100	1 x 10	1006	684	68.0	899	575	64.0
	1 x 10	1016	842	82.9	890	671	75.4

continue to decrease with successive injections to a value of 40% of the original. Recovery based on peak area measurements remained constant over approximately 10 injections. It was confirmed that the decrease in recovery was due to plasma constituents by recording the peak areas obtained by injection of the above concentration of the two steroids in solutions containing different proportions of plasma to water. The results are shown in Figure 5. where it is seen that for both precolumns tested the peak area decreases monotonically with increasing proportion of plasma in the sample matrix injected. This decrease is more marked for the smaller precolumn. In spite of the decrease in recovery, the linearity of calibration in plasma remains high and plots of peak area against steroid concentration over a range of concentration from 150 - 600 $ngcm^{-3}$ are shown in Figure 6. The lines for plasma show the lower sensitivity consequent on the lower recoveries obtained. It was found also that recovery could not be improved by substituting C-8 for C-18 in the precolumn and that reducing the particle size merely exacerbated back pressure problems.

It is not possible on the basis of the present study unequivocally to specify the reason for the deterioration in recovery when plasma is injected. It is known that

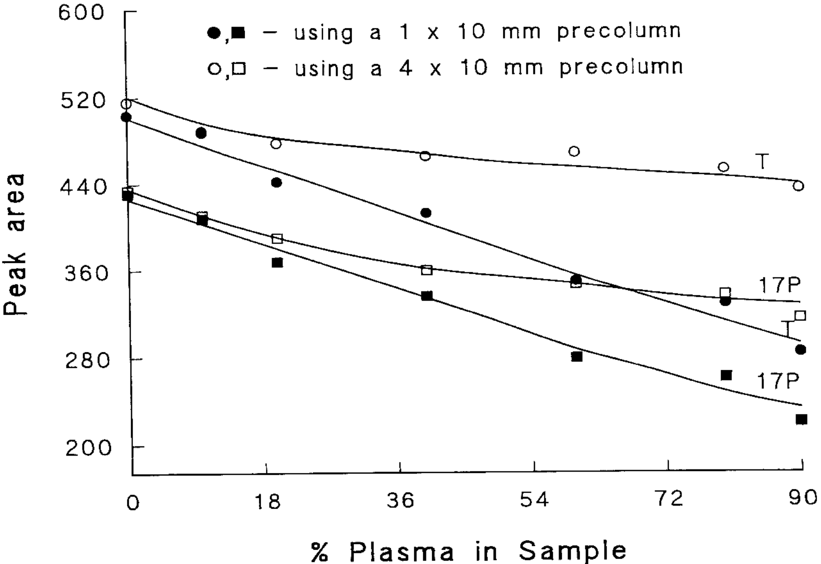


Figure 5. Variation of peak area with proportion of plasma in injected sample for testosterone (T) and 17α-hydroxyprogesterone (17P)

these steroids are appreciably protein bound[15] but it is generally accepted that the interactions of drugs with C-18 stationary phases are sufficiently strong to effect complete recovery. It appears more likely from our experiments that the addition of plasma components alters the C-18 surface irreversibly so that the load capacity is considerably reduced. This is at variance with reports that drug adsorption on C-18 columns is enhanced following several injections of plasma and that such columns should be conditioned by plasma injection before use[16] In the present study the reverse was found and prior injection, even of drug free plasma, was found to produce low efficiencies on subsequent injections of steroids in plasma or in purely aqueous solution. This would appear to indicate some permanent modification of the C-18 surface.

While many reports in the literature suggest that such on-line pretreatment of plasma samples for chromatography is effective[17-19] and that large numbers of

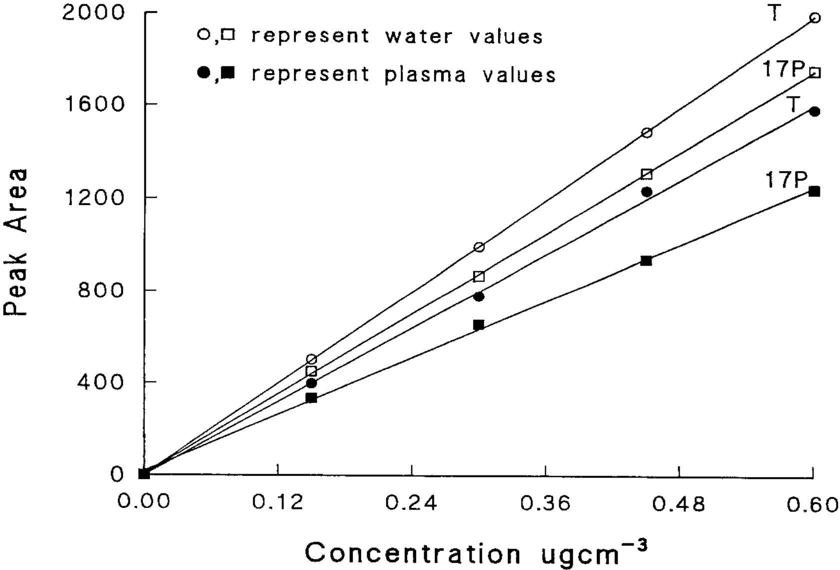


Figure 6. Plots of peak area against concentration for testosterone (T) and 17α-hydroxyprogesterone (17P) injected in water and plasma matrices.

samples may be processed using a single precolumn, there are also many reports in the literature which indicate that additional pretreatment of biological samples is required before a sample is injected into the precolumn[19-21]. A study of the literature of on-line sample pretreatment reveals that about 65% of methods reported use some form of off-line plasma sample pretreatment e.g. protein precipitation before on-line preconcentration by an integral precolumn. In addition, many assays involve the injection of small <100 μl volumes of plasma achieving sample clean-up but no appreciable preconcentration.

Extraction from Saliva

Saliva offers a useful alternative matrix for the assay of drugs for many clinical purposes. It has the advantage of being non invasive and good correlations have

been obtained between saliva and plasma concentrations for some drugs[22,23]. The above experiments were repeated using spiked saliva. Absorbance, efficiency and recovery of each steroid were determined. It was found that injection of samples in the saliva matrix resulted in only slight peak broadening and lowered absorbance while recovery, calculated on the basis of peak area, was identical to that obtained from water. No appreciable pressure rise on saliva injection was observed. As with urine, up to 5 cm^3 aliquots of saliva could be applied by repeated operation of the loading valve and at least 20 repeat samples could be applied to a single precolumn with no deterioration in performance.

GENERAL CONCLUSIONS

It has been shown that, when a column switching sample pretreatment system is operated in the backflush mode, the precolumn dimensions are of little importance with respect to peak broadening or sensitivity when coupled with either 4.6 or 2 mm diameter analytical columns. When coupled with 1 mm diameter analytical columns the increased extra column broadening adversely affects efficiency with consequent loss of sensitivity. From aqueous solutions the recovery is uniformly high. Precolumn concentration coupled with use of narrow bore columns has been shown to improve sensitivity. The analyte loading of the precolumn is proportional to its volume and is in the region of 6 - 7 $\mu g\ \mu l^{-1}$ of stationary phase. On this basis the optimum system among those tested is a 4 x 10 mm precolumn coupled with a 1 mm diameter analytical column although a more rugged system would incorporate a 2 mm diameter analytical column. It has also been shown that such sensitivity advantages can be realised for the biological matrices of urine and saliva and that the endogenous components of these do not appreciably adversely affect the performance of such coupled systems. For the steroid set in plasma, however, it has been shown that such a matrix reduces the efficiency and recovery of the overall system and severely restricts the lifetime of the precolumn. The apparent inconsistency between these findings and more positive reports in the literature concerning on-line plasma pretreatment are explained on the basis that in many reports additional pretreatment is used or that small plasma volumes only are injected.

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STUDY AND OPTIMIZATION OF CITRUS FLAVANONE AND FLAVONES ELUCIDATION BY REVERSE PHASE HPLC WITH SEVERAL MOBILE PHASES: INFLUENCE OF THE STRUCTURAL CHARACTERISTICS

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ABSTRACT

A high-performance liquid chromatographic procedure for the analysis of the different flavonoids found in *Citrus aurantium* tissue extracts has been developed. It employs a C_{18} reverse phase column and an elution isocratic-gradient system with a mixture of water, methanol, acetonitrile and acetic acid. We make an exhaustive description of the optimisation process by studying the quantitative chromatographic parameters: k', w, α, N, *HETP* and R. The use of different extraction solvents for flavonoids in *Citrus aurantium* tissues was also studied, DMSO presenting the best results. Twelve compounds were detected, isolated and their spectral structures characterized.

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INTRODUCTION

Citrus species are of great interest because they accumulate large amounts of flavanone glycosides in fruit and young vegetative tissues [1, 2, 3], whose aglycones are early intermediates in the flavonoid biosynthetic pathway [4]. Naringenin and hesperetin are widely found in *Citrus* species in their glycoside forms: naringin, neohesperidin (neohesperidosides), narirutin and hesperidin (rutinosides) [5, 6, 7] being the most common. *Citrus aurantium* is characterized by the accumulation of these four flavanones [5, 6, 8]. We recently demonstrated and quantified the glucosides of the flavanones naringin and hesperetin, prunin and hesperetin 7-*O*-glucoside, respectively in the same natural source (in press). The presence of the flavanones, neoeriocitrin [9] and poncirin [10], and the flavones rhoifolin [11] and neodiosmin [12] has also been described.

In recent years, high pressure liquid chromatography (HPLC) has become the method of choice for the separation of diverse natural flavonoids mixtures [13, 14, 15]. However, only very specific methods have been described for particular extracts of certaines plant materials, in which such a high number of flavonoids does not occur simultaneously.

The present work has three objectives: (I) to optimize the analytical technique used for the elucidation of all the above mentionated flavonoids. (II) To characterize the most suitable agent for the extraction of flavonoids from the plant material. (III) To isolate and analyse the flavonoids found in the variety of *Citrus aurantium* studied by using semipreparative liquid chromatography and to identify them by spectroscopic techniques and their quantification.

MATERIALS AND METHODS

Plant Material

Young leaves (10-130 mm in length) and immature fruits (3-61 mm diameter) were obtained from 5-year-old *Citrus aurantium* cv Sevillano trees, grown in greenhouses of the University of Murcia.

Chemicals

Naringin, hesperidin, prunin, hesperetin 7-*O*-glucóside, neohesperidin, neodiosmin, naringenin and hesperetin were obtained from Zoster S.A., Murcia, Spain. Neoeriocitrin, narirutin, rhoifolin and poncirin were obtained from Extrasynthese S.A, Genay, France. The structures of these flavonoids are shown in Table 1. Dimethylsulphoxide (DMSO) was used as solvent.

Extraction of Flavonoids

Leaves and fruits were collected, immediately dried at 50°C [16] and ground. The flavonoids were extracted with several solvents at different concentrations in order to establish the most suitable. The solvents used were: methanol, dioxane-methanol (1:1), 0.01 % sodium hidroxide (aq), 0.1 and 0.01 % potassium hidroxide (methanolic solution), pyridin, dimethylformamide and dimethylsulphoxide. The plant materials were extracted in three differents ratios: 2 mg/ml, 20 mg/ml and 200 mg/ml. The solutions were filtered through a 0.45 μm nylon membrane.

Chromatographic Analysis

For the elucidation of the flavonoids present in the *Citrus aurantium* leaf and fruit, we used a μBondapak C_{18} (250 x 4 mm ID) analytical column with an average particle size of 5 μm. The flow rate was 1 ml/min at room temperature.

HPLC analysis was performed using a Beckman liquid chromatograph with a Model 110B solvent-delivery module and a System Gold Module 168 diode array detector (Beckman Instruments, Inc, CA., USA). The absorbance change was monitored at 280 nm.

In order to carry out a quantitative study, the elucidation capacity of the different mobile phases was verified by determining the following parameters [17] for each flavonoid:

a) Retention time (t_{Ri}= experimental value)

b) Capacity factor $k' = (t_{Ri} - t_o)/ t_o$

t_o= mobile phase interstitial volume (void volume)/flow rate.

c) Selectivity factor $\alpha = (t_{Ri+1} - t_o)/(t_{Ri} - t_o)$

d) *Number of theoretical plates*: $N = 16(t_{Ri}/ w_i)^2$

w= width of the peak.

e) The *height equivalent to a theoretical plate (HETP)* $HETP = L/ N$

L= column length (mm).

f) Resolution

$$R = 1/4(N)^{1/2}((\alpha-1)/\alpha)(k'/(k'+1))$$

Isolation of Flavonoids

For the isolation of different flavonoids found in DMSO extracts of *Citrus aurantium* leaves and fruits the following semipreparative column was used: Nucleosil C_{18}, 5 μm (250 x 10 mm ID), eluted with water-acetic acid-methanol (75-0.5-25) and a flow rate of 3 ml/min. All flavonoids whose retention times were similar to the flavonoid standard were isolated by several chromatographs in this semipreparative column, and the

fractions were collected with a Pharmacia FRAC 100 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

All the compounds isolated were identified by their melting points (Gallemkamp, England) and their ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra (Bruker, Bremen, Germany) in hexadeutero dimethylsulphoxide (DMSO-d_6).

RESULTS AND DISCUSSION

Chromatographic Analysis Optimisation. A Structural Study of Flavonoid Elucidation.

HPLC elucidation of the flavonoids contained in the standard mixture (see Table 1) was optimized in a first step by using isocratic systems in which only the proportions of the components of the mobile phase were varied.

Mobile Phases with Water and Methanol.

This has traditionally been one of the most commonly used combinations for flavonoid elucidation [18, 19, 20].

An analysis of the variation in the k' values for the different flavonoids according to the percentage of methanol in the mobile phase reveals the influence of their structures on their separation capacity (Table 2). Three structural aspects can first be considered: a) glycosilation in position 7; b) the B-ring substitution pattern maintaining a similar type of 7-glycosilation and c) the relation between the flavanone structures and their corresponding flavones. In principle, it seems that any direct influence of the molecular weight on the order of elucidation can be discarded (Table 2).

a) Figure 1 shows variations in ln k' according to variations in the percentage of methanol in the mobile phase for the flavonoids hesperidin, neohesperidin and hesperetin 7-*O*-glucoside. An analysis of the data

TABLE 1

Flavonoid Structures in Standard Mixture.

	Radical Positions			
Flavonoids	5	7	3'	4'
Flavanones				
Neoeriocitrin	OH	O-NEO[a]	OH	OH
Naringin	OH	O-NEO		OH
Narirutin	OH	O-RUT[b]		OH
Hesperidin	OH	O-RUT	OH	OCH_3
Neohesperidin	OH	O-NEO	OH	OCH_3
Poncirin	OH	O-NEO		OCH_3
Prunin	OH	O-GLU[c]		OH
Hespt.7-*O*-glu.	OH	O-GLU	OH	OCH_3
Naringenin	OH	OH		OH
Hesperetin	OH	OH	OH	OCH_3
Flavones (double bond $C_2 = C_3$)				
Rhoifolin	OH	O-NEO		OH
Neodiosmin	OH	O-NEO	OH	OCH_3

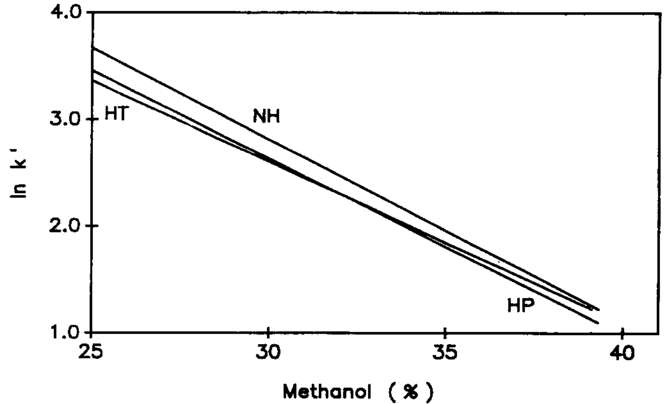

FIGURE 1. Variation of ln k' versus methanol percentage of mobile phases with 0.01M phosphoric acid-methanol. NH: Neohesperidin; HT: Hesperetin 7-*O*-glucoside; HP: Hesperidin.

TABLE 2

k' Values for Flavonoids of Standard Mixture Elucidated with Mobile Phases: Methanol-0.01 M Phosphoric acid.

MeOH (%)[a]	50	40	35	30	25
Flavonoid			k' values		
Neoeriocitrin	0.2	1.3	2.5	5.1	14.4
Prunin	0.8	2.3	4.1	7.8	20.3
Narirutin	0.8	2.1	4.1	7.8	22.0
Naringin	0.8	2.3	4.5	9.6	27.3
Hesp.7-*O*-glu.	1.4	3.4	5.5	12.0	32.8
Hesperidin	1.2	3.0	5.5	12.0	36.2
Neohesperidin	1.4	3.4	6.4	14.4	44.7
Rhoifolin	2.2	5.3	8.2	19.0	57.6
Neodiosmin	2.2	7.5	10.8	27.2	88.5
Naringenin	2.7	9.6	16.7	31.4	77.6
Poncirin	2.2	9.6	19.6	44.7	135.8
Hesperetin	3.4	13.4	23.1	47.1	124.3

[a]Methanol percentage of mobile phase.

corresponding to the flavonoids isonaringin (narirutin), naringin and naringenin 7-*O*-glucoside (prunin) shows similar results to those represented in this figure.

The glycosilation of a 7-OH group produces a much greater hydrophilic interaction with the mobile phase solvent, as has been described by other authors [21]. The hydrophilic character of the flavonoid molecule increases with the number of sugars in the side chair [22], however, it must be borne in mind that this hydrophilic character is not only marked by the number of sugars in the side chain but also by their nature. Thus, for a particular flavonoid k'-glucoside < k'-arabinoside < k'-rhamnoside [22].

TABLE 3

Variation of k' Values for 7-*O*-glycosides of Naringenin and Hesperetin with Reference to Methanol Percentage Changes in the Mobile Phase.

Methanol (%)	k' values
50-40	RUT[a] = GLU[b] = NEOH[c]
40-35	RUT < GLU = NEOH
35-25	RUT = GLU < NEOH
< 25	GLU < RUT < NEOH

[a] Rutinoside; [b]glucoside; [c]neohesperidoside

Table 3 shows how the k' value of glucosylated structures undergoes a greater relative change than the corresponding rhamnoglucosides. From Figure 1, it can be seen that hesperidin and neohesperidin show a similar decrease of their k' values as the percentage of methanol in the mobile phase increases. The k' of the rutinosides is always below that of their corresponding neohesperidosides. This clearly contradicts the experimental data obtained when the solubility of these compounds was analysed, as shown in Table 4, where it can be seen that the solubility of neohesperidin is always greater than that of hesperidin in all the mobile phases tested. It seems likely, then, that the cause of this alteration in the k' values of both glycosylated flavanones has a structural origin, which particulary affects the reverse phase-flavonoid interaction. The flavanones are stabilized by the formation of an intramolecular hydrogen bond between the *per*-hydroxyl group of C-5 and oxigen of 4-keto group [23]. This speacial structure between the A and C rings is planar except in the C_2- C_3 bond [24]. The

TABLE 4

Hesperidin and Neohesperidin Solubility (gr/l and Room Temperature) in Chromatographic Mobile Phases with Phosphoric Acid and Methanol.

Methanol (%)	Hesperidin	Neohesperidin
25	0.010	0.575
30	0.011	0.754
35	0.013	0.992
40	0.016	1.123
50	0.023	1.680

substitution of the hydroxyl group in position 7 by a glycosidic group (rhamnoglucoside) might affect this configuration. Given its special "lineal" type configuration (rhamnose-glucose bond type 1-6), the presence of a rutinoside type substitution would not affect the mentioned intramolecular hydrogen bond, thus maintaining the highly conjugated planar system of the aglycon portion of the flavanone [25]. However, the presence of 7-O-neohesperidoside substitution (rhamnose-glucose bond type 1-2) would affect the stability of the intramolecular hydrogen bond, and the neohesperidoside structures can be non planar to rutinosides [24]. These would certainly be a greater hydrophobic interaction between the neohesperidoside molecules and those of the stationary phase of the column which, in spite of the enormous difference in the solubility of the phases studied, would increase the k' value of these compounds compared with their respective rutinosides.

b) The different substitution in the B ring of these flavonoid structures strongly affects the order

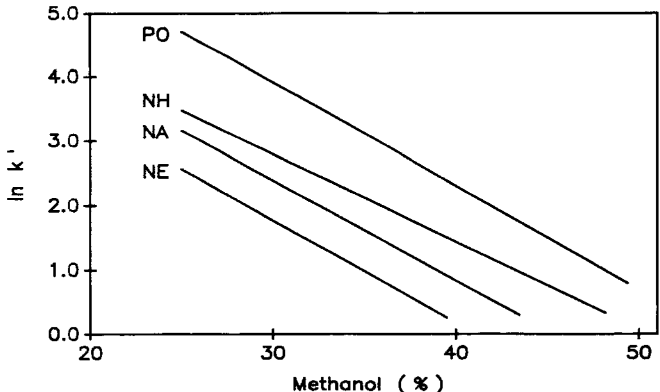

FIGURE 2. Variation of ln k' versus methanol percentage of mobile phases with 0.01M phosphoric acid-methanol. PO: Poncirin; NH: Neohesperidin; NA: Naringin; NE: Neoeriocitrin.

of these compounds elucidation. In Figure 2, it can be seen how this order and the consequent ln k' values are basically affected by the degree of polarity of these flavonoids. The enormous influence on the order of the chromatographic resolution, of the number of hydroxyl groups in the B ring of its structure is clear. It is interesting to note how the addition of a methoxyl group to the neoeriocitrin molecule (neohesperidin) decreases the polarity of the flavonoid. The influence of this effect is increased when the hydroxyl radical in the monohydroxylated molecule (naringin) is replaced by a methoxyl radical (poncirin).

c) The comparison between the flavanones naringin and neohesperidin with their flavones, rhoifolin and neodiosmin, respectively, reveals the effect of insaturation between positions 2 and 3 upon elution behavior. The flavones eluted much later than

respective flavanones, and their k' values are higher (see Table 2). One factor operating to make flavones much less polar than respective flavanones is a larger electron density on the oxigen atom of the 4-keto group of flavones resulting from resonance structures where the keto oxigen assumes a negative charge [21].

The larger electron density will make the hydrogen bond between the 5-hydroxyl group and the 4-keto group stronger and make both funcional groups appear less polar to the solvent. However, this may not be the only possible explanation for the greater polarity of flavanones over flavones. In flavanones, the 4-keto group may be out of the plane of the adjacent phloroglucinol ring, thus making a hydrogen bond with the *peri*-hydroxyl group weaker and exposing both functionalities to stronger interactions with the solvent. Flavones because of their total planarity, simply may be more difficult to solvate than the partially planar flavanones. The planar flavones would require a more ordered solvent structure and thus a larger entropy term in solvation than would flavanones [21].

Figure 3 shows ln k' versus variations in the methanol percentage of the mobile phase. A decrease in the k' value with increases in the methanol percentage results in a similar slope for the flavanones and their respective flavones.

Acid must be present in the mobile phase for reverse phase HPLC, and it has been included for sound reasons, such as the suppression of ionisable acidic groups, to decrease the tailing of peaks or for unspecified improvements in separation [21, 26].

In this paper we haved tested the influence of the degree of acidification of the mobile phase by observing variations of k' and w values in all the

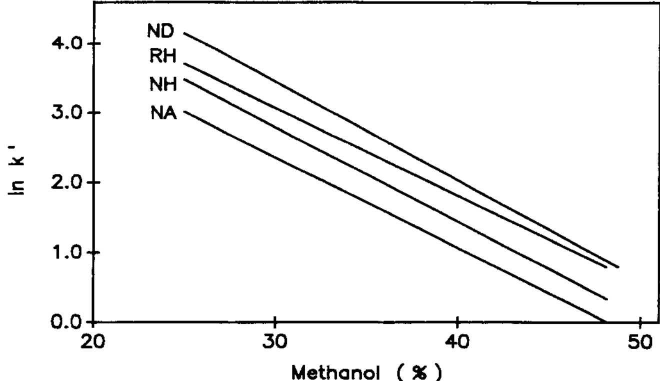

FIGURE 3. Variation of ln k' versus methanol percentage of mobile phases with 0.01M phosphoric acid-methanol. ND: Neodiosmin; RH: Rhoifolin; NH: Neohesperidin; NA: Naringin.

flavonoid studied (Table 5). Best results were obtained with 0.5 % acetic acid in the mobile phase (the pH of aqueous fraction was 2.46) as against the use of 0.01 M phosphoric acid (pH solution is 3.24). For this reason, acetic acid was used in subsequent experiments with new ternary and quaternary phases. Using acetic acid at concentrations in excess of 0.5 %, did not result in a better elucidation of the compounds under study and even produced new overlaps. In addition, the stability of silica-based bonded phase packings towards most solvents in the pH 2.0-7.5 range. Below pH 2.0, the silicon-carbon bond of the stationary phase is subject to nucleophilic attack [27].

Finally, the total resolution of all the flavonoids contained in the standard mixture was only attained in the analytical conditions studied when the percentage of methanol in the mobile phase was equal or less than

TABLE 5

k' and w Values of the Flavonoid Standard Mixture Elucidated with Mobile Phases containing 30 % of Methanol and different Acid Agents.

Mobile Phase	A[a]		B[b]		C[c]	
Flavonoid	k'	w	k'	w	k'	w
Neoeriocitrin	6.0	2.1	5.1	1.1	5.0	1.1
Prunin	8.2	2.1	7.8	1.8	7.8	1.8
Narirutin	8.2	2.1	7.8	1.1	7.8	1.8
Naringin	12.4	2.5	9.6	2.2	9.4	2.2
Hespt.7-*O*-glu.	12.6	3.1	12.0	2.9	11.8	2.3
Hesperidin	12.6	3.1	12.0	2.9	11.8	2.3
Neohesperidin	15.2	3.1	14.4	2.9	14.2	2.5
Rhoifolin	20.8	4.5	19.0	4.0	18.0	3.6
Neodiosmin	30.6	6.5	27.2	5.0	26.1	5.0
Naringenin	33.7	7.5	31.4	6.5	32.3	5.4
Poncirin	46.5	10.2	44.7	7.6	44.2	7.9
Hesperetin	49.6	12.3	47.1	10.4	47.0	7.9

[a]Methanol-water (30:70); [b]Methanol-0.01 M phosphoric acid (30:70); [c]Methanol-water-acetic acid (30:70:0.5).

25%. The high k' value for most of the flavonoids elucidated means that this technique needs further optimisation. However it does represent a valuable aid to semipreparative and preparative chromatography in the isolation of this type of flavonoids, because it adequately separates the different substances present and permits the easy and correct programming of the fraction collection systems.

Mobile Phases with Water and Acetonitrile.

Mobile phases containing acetonitrile have been widely used in the HPLC elucidation of flavonoid compounds of low polarity and high molecular weight,

such as flavone, flavanone aglycons and a large number of polymethoxylated flavonoid structures [20, 28, 29].

Using this type of mobile phase to elucidate the standard mixture considerably reduces the retention times of all the flavonoids contained in it, which produces a simultaneous overlapping of many and impedes accurate elucidation. This overlapping particularly affects the flavanone-glycosides and some of these are not resolved until the percentage of acetonitrile in the mobile phase drops below 25 %. Of note are the different results obtained for the separation of the 7-*O*-glycosylated derivatives of hesperetin and naringenin. In the former case, the flavonoids are not resolved, while in the case of naringenin, the three 7-*O*-derivatives are resolved. This must be related with the lower degree of polarity of the 3'-hydroxylated-4'-methoxylated B ring structures compared with those which are merely 4'-hydroxylated, since the former are more affected as regard, the diminution in k' values as the proportion of acetonitrile in the mobile phase increases.

When the percentage of acetonitrile in the mobile phase falls below 20 %, the glycosylated flavanones are better resolved, although still never completely, and the other chromatographic parameters are adversely affected. In particular w and k' values increase, which produces excessively high flavone and aglycone retention times. When the acetonitrile content of the mobile phase is 10 %, neoeriocitrin shows a retention time of 64 minutes, which explains why scant viability of this method.

The use of mobile phases containing acetonitrile does provide useful information for the elucidation of this and other flavonoid mixtures of a greater or

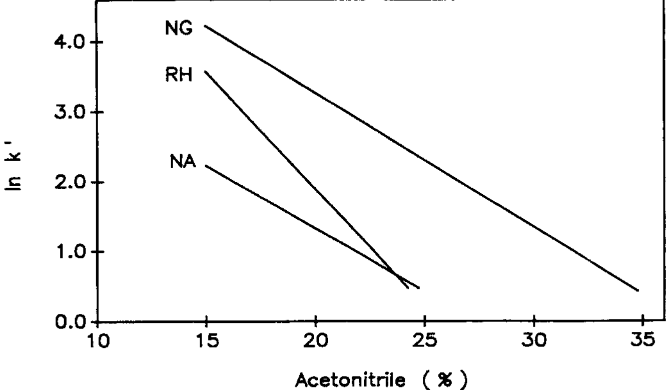

FIGURE 4. Variation of ln k' versus acetonitrile percentage of mobile phases with water-acetonitrile-acetic acid. NG: Naringenin; RH: Rhoifolin; NA: Naringin.

lesser complexity. In the first place there is a general decrease in the k' values of all the flavonoids when compared to phases containing a similar proportion of methanol. This pattern of behaviour is not the same for all the flavonoids, since the flavones, compounds with a very low polarity, show a much more pronounced decrease in k' than do the flavanone aglycones and 7-*O*-glycosides of flavanones, which both show a similar behaviour (Fig. 4). This general fall in k' values, the most pronounced characteristic of the use of these mobile phases, generally hinders the correct elucidation of the most polar flavonoids, glucosides and rhamnoglucosides although it does permit the good resolution of flavanone aglycones and the slightly polar flavanone poncirin, especially within the 20-25 % acetonitrile range. The considerable reduction in the w values of all flavonoids analysed is also of not.

Mobil Phases with Water, Methanol and Acetonitrile.

In order to attain a better elucidation of the different flavonoids studied by optimising the analytical parameters mentionated, mobile phases containing water, acetic acid, methanol and acetonitrile were used. Because of the strong influence of acetonitrile on the resolution of these compounds, increasing quantities of this solvent were introduced into the mobile phases containing water and methanol at the expense of the methanol proportion. This was does until the proportion of both in the mobile phase was identical, the water and acetic acid proportions remaining unaltered.

The mobile phases with less than 70 % water did not improve the elucidation of these compounds, despite the substitution of methanol by acetonitrile.

An analysis of the ln k' values against variations in acetonitrile, shows that the resolutions obtained with 70 and 75 % water both permit similar structural interpretations. For this reason, Figures 5, 6 and 7 depict variations in ln k' against increases in the proportion of acetonitrile with 70 % water in the mobile phase. The parameters providing most information for the elucidation of these compounds, k' and R, are shown in Table 6.

Figure 5 shows that, similar to that which occurred in phases containing only methanol and water, the k' values for a neohesperidoside are always higher than their rutinoside counterpart, whatever the composition of the mobile phase. This increases the influence of the esterospacial structure on the order of elucidation of these compounds compared with their solubility in this phases, which in phases with a light acetonitrile content favour the neohesperidosides. Increases in the percentage of acetonitrile affects rutinoside

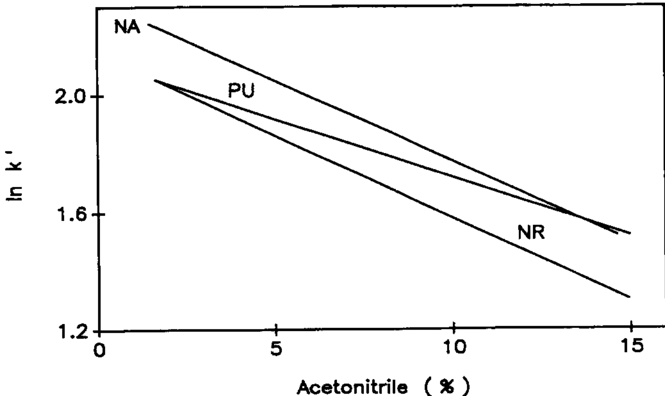

FIGURE 5. Variation of ln k' versus acetonitrile percentage of mobile phases: water-acetonitrile-methanol-acetic ac. NA: Naringin; PU: Prunin; NR: Narirutin.

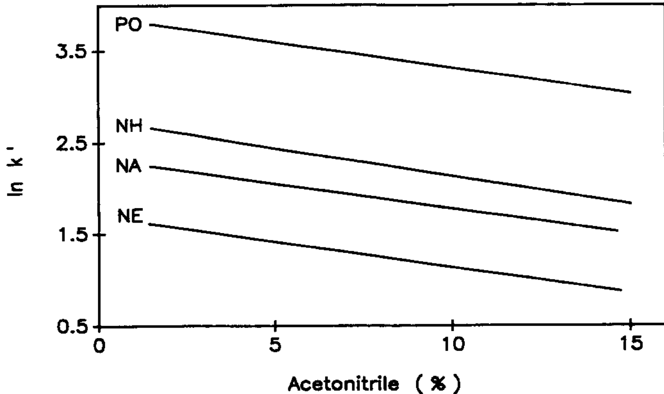

FIGURE 6. Variation of ln k' versus acetonitrile percentage of mobile phases:water-acetonitrile-methanol-acetic ac. PO: Poncirin; NH: Neohesperidin; NA: Naringin; NE: Neoeriocitrin.

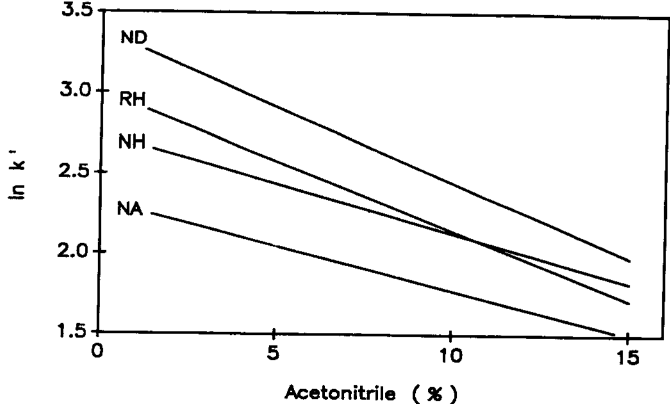

FIGURE 7. Variation of ln k' versus acetonitrile percentage of mobile phases: water-acetonitrile-methanol-acetic ac. ND: Neodiosmin; RH: Rhoifolin; NH: Neohesperidin; NA: Naringin.

TABLE 6

k' and R Values of the Flavonoid Standard Mixture Elucidated with Quaternary Mobile Phase containing 70 % Water.

Mobile Phase	A[a]		B[b]		C[d]	
Flavonoid	k'	R	k'	R	k'	R
Neoeriocitrin	4.9	3.7	2.8	2.8	2.4	2.8
Prunin	7.8	1.1	5.2	0.0	4.6	0.0
Narirutin	7.8	0.0	4.4	1.3	3.7	2.1
Naringin	9.3	1.9	5.2	1.1	4.6	0.7
Hespt.7-*O*-glu	11.2	1.5	7.9	0.0	6.1	0.0
Hesperidin	11.2	0.0	6.4	1.7	5.1	0.6
Neohesperidin	13.6	1.9	7.9	0.0	6.1	1.4
Rhoifolin	16.3	3.1	7.9	1.5	5.5	0.8
Neodiosmin	22.6	4.0	10.7	5.3	7.1	6.5
Naringenin	33.6	2.9	23.0	1.0	20.5	2.2
Poncirin	43.2	1.2	25.4	1.8	20.5	0.0
Hesperetin	46.6	1.2	32.1	1.2	27.7	2.2

[a]water-acetic acid-methanol-acetonitrile:(70:0.5:25:5).
[b]water-acetic acid-methanol-acetonitrile:(70:0.5:20:10).
[c]water-acetic acid-methanol-acetonitrile:(70:0.5:15:15).

(narirutin) and neohesperidoside (naringin) equally, while glucoside (prunin) shows a more pronounced decrease as the polarity of the mobile phase falls. In the 2-14 % range of acetonitrile concentration, prunin at first shows the lowest k' value and then the highest. This shows once again that the most esterospacial polar flavanones are less influenced as regards their decrease in k' values by increases in the proportion of acetonitrile.

Figure 6 confirms the close relationship between the k' value and the flavonoid polarity due to the B-ring substitution pattern, with an identical 7-O-glycosilation type. The k' values are inversely proportional to the polarity of the flavonoids elucidated. Variations in these values show similar slopes for the four neohesperidosides represented, which confirms that the B-ring substitution pattern does not modify the relationship between the k' values, whatever the mobile phase over the ranges studied.

Figure 7 illustrates how the substitution of methanol by acetonitrile in the mobile phase brings about a sharper drop in the k' value of the flavones than of their corresponding flavanones as a direct consequence of the decrease in polarity of the mobile phase. Thus, when the proportion of acetonitrile reaches or surpasses 11 %, rhoifolin, shows a k' value lower than that of neohesperidin. Such effects as this must he borne in mind when choosing solvent mixtures to elucidate extracts or solutions, whether known or not, of flavonoids and for the their isolation and identification.

Isocratic-Gradient Quaternary System.

From the results obtained in the analysis of the different mobile phases used in the optimisation of the

simultaneous elucidation of these twelve compounds, it is possible to design a system which permits the complete resolution of all the flavonoids found in the standard mixture in a much shorter time than hitherto possible, at the same time allowing the location and identification of these flavonoids in *Citrus aurantium* extracts.

Since experiments carried out with continuous lineal gradient systems, some of which are mentioned in the bibliography [13], did not permit the complete elucidation of all the flavonoids analysed, the system finally used was a combination of two isocratic regime periods and one intermediate lineal-gradient step: 1) water-acetic acid-methanol-acetonitrile (70:0.5:23:7) for 40 minutes, 2) lineal gradient to water-acetic acid-methanol-acetonitrile (55:0.5:25:20) in 15 minutes, and 3) isocratic water-acetic acid-methanol-acetonitrile (55:0.5:25:20) for 10 minutes and reequilibrate. An analysis of all the chromatographic parameters obtained with this system is to be found in Table 7.

Figure 8 shows the chromatogram of the flavonoids of the completely elucidated standard mixture.

<u>Optimization of the Flavonoid Extraction Solvent in Plant Material</u>.

In order to determine the most suitable solvent for flavonoid extraction in the *Citrus aurantium* tissues analysed (leaves and fruits), the extraction capacity of several solvents was studied (see Materials and Methods) in these tissues. In all cases, the extraction capacity of each solvent (in the above indicated concentrations) was estimated according to the extraction of the major flavonoids in *Citrus aurantium*, naringin and neohesperidin [1]. Three weight/volume

TABLE 7

Chromatographic Parameters of the Flavonoid Standard Mixture Elucidated with Mobile Phase: Water-Acetic Acid-Methanol-Acetonitrile in an Isocratic-Gradient System.

Flavonoid	k'	α	w	N	*HETP*	R
Neoericitrin	3.2	1.5	1.0	1644	0.15	2.7
Narirutin	5.0	1.1	1.6	1331	0.19	0.8
Prunin	5.6	1.1	1.9	1194	0.21	0.6
Naringin	6.1	1.2	2.1	1127	0.22	1.2
Hesperidin	7.3	1.1	2.3	1337	0.19	0.8
Hespt.7-*O*-glu	8.1	1.1	2.4	1438	0.17	0.7
Neohesperidin	8.8	1.1	2.7	1289	0.19	0.9
Rhoifolin	9.9	1.4	3.3	1113	0.22	2.0
Neodiosmin	13.5	1.5	4.1	1234	0.20	2.8
Naringenin	20.7	1.0	2.6	7123	0.04	0.6
Poncirin	21.3	1.1	1.8	15137	0.02	1.7
Hesperetin	22.6	1.1	1.7	20142	0.01	2.1

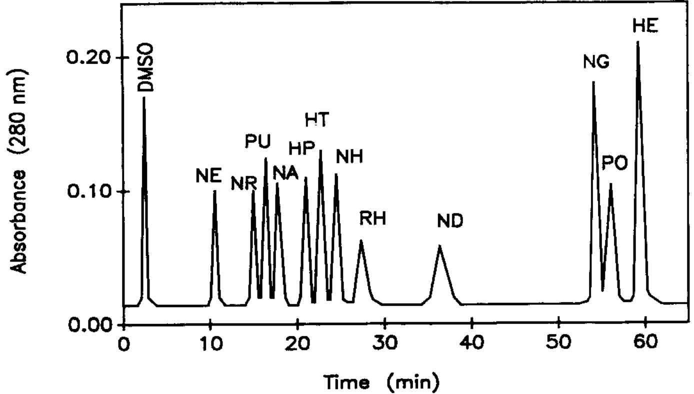

FIGURE 8. Chromatogram of flavonoid standard mixture elucidated by mean of isocratic-gradient system with mobile phase: water-acetonitrile-methanol-acetic acid. NE: Neoeriocitrin; NR: Narirutin; PU: Prunin; NA: Naringin; HP: Hesperidin; HT: Hesperetin 7-*O*-glucoside; NH: Neohesperidin; RH: Rhoifolin; ND: Neodiosmin; NG: Naringenin; PO: Poncirin; HE: Hesperetin.

TABLE 8

Relative Content of Naringin and Neohesperidin in *Citrus aurantium* Extracts using Different Solvents.

CONCENTRATION SOLVENTS	2 mg/ml NAR[a]	2 mg/ml NEOH[b]	20 mg/ml NAR	20 mg/ml NEOH	200 mg/ml NAR	200 mg/ml NEOH
Methanol	97	95	97	96	75	60
Diox/MeOH[c]	100[d]	100[d]	99	99	89	86
NaOH(aq)0.01%	86	87				
KOH(MeOH)0.01%	92	92	90	90	77	68
KOH(MeOH)0.1%	87	89				
Pyridin	66	71				
DMF[e]	96	95	96	94	95	85
DMSO[f]	100	100	100	100	100	100

[a]Naringin; [b]neohesperidin; [c]dioxane/methanol (1:1); [d]maximum signal obtained (chromatographic area) for naringin and neohesperidin; [e]dimethylformamide; [f]dimethylsulphoxide.

ratios were used in the extraction: 2, 20 and 200 mg/ml. See Table 8.

Solvents of an organic nature gave the best results for both flavonoids, except pyridin. The alkaline hydroxides commonly used for flavonoid solution gave clearly poorer results, basically because in these media the flavanones are in equilibrium with their respective chalcones, which are susceptible to rupture (forming phloroacetophenones).

The most effective solvent for flavonoid extraction at all concentrations was DMSO, not only in the case of naringin and neohesperidin but also all the other flavonoids in these extracts. For this reason DMSO was used as the extraction agent of the *Citrus aurantium* flavonoids.

Analysis, Isolation and Identification of Flavonoids in Citrus aurantium Leaves and Fruits.

After the complete resolution of all the flavonoids which, according to the bibliography, can be found in the tissues of *Citrus aurantium*, with the characteristic of having a hydroxil or methoxyl radical in one or more of the 5,7.3' and/or 4' position of their structure, the DMSO extracts of leaves and fruits of this plant material were analised in order to confirm the presence of these compounds and to isolate and identified them.

HPLC analysis of the extracts of young leaves and fruits of *Citrus aurantium* shows the presence of many flavonoid structures. Figure 9 shows a characteristic chromatogram of an extract from fruits (5-6 mm in diameter). The chromatogram of the leaf extracts showed the same peaks although with different relative proportions.

Peaks whose spectra are similar or identical to the flavanones or flavones referred to in this work (standard mixture) are numbered (1 to 12). In the chromatogram of Figure 9, compounds 1-12 present retention times in identical order to those of all flavonoids referred to in Table 7, and coincide with those shown by the other chromatographic systems: water-acetic acid-methanol (75:0.5:25) and similarity, these compounds (1-12) were isolated in order to confirm their identity.

These componds were isolated with a semipreparative column (see Materials and Methods) and 12 products were obtained. Their melting points and characteristic UV absortion spectra maxima are described in Table 9. The principal characteristics of both ^{1}H NMR and ^{13}C NMR spectra for compounds 1-12 are also described in Table 9, [12, 30, 31, 32, 33]. All these data can be used to

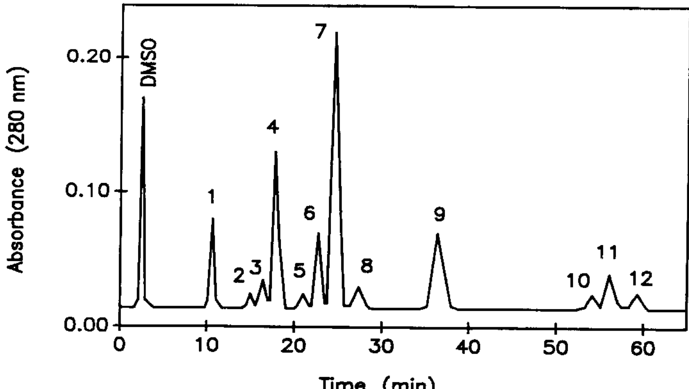

FIGURE 9. Characteristic flavonoid chromatogram of DMSO extracts of young leaves (20 mm in length) and immature fruits (5-6 mm diameter). Peaks whose spectra are similar or identical to flavanones and flavones are numbered (1 to 12).

TABLE 9

Melting Points, Spectral Characteristics and Flavonoid Structures of 12 Products Isolated by Semipreparative Chromatography from DMSO of *Citrus aurantium* Extracts.

P[a]	MP[b]	UV[c]	NMR SP[d]	GLY[h]		STRUCTURES
1	192	285,330	FAG[e]	7-NEO[i]	[30, 31]	Neoeriocitrin
2	181	284,328	FAG	7-RUT[j]	[30, 31]	Narirutin
3	156	283,330	FAG	7-GLU[k]	[31, 32]	Prunin
4	172	284,328	FAG	7-NEO	[30, 31]	Naringin
5	262	285,330	FAG	7-RUT	[30, 31]	Hesperidin
6	243	284,330	FAG	7-GLU	[31, 33]	Hespt.7-*O*-glu
7	245	285,330	FAG	7-NEO	[31, 33]	Neohesperidin
8	246	268,335	FOG[f]	7-NEO	[30, 31]	Rhoifolin
9	268	253,268 344	FOG	7-NEO	[12, 31]	Neodiosmin
10	252	289,326	FA[g]		[30, 31]	Naringenin
11	212	286,330	FAG	7-NEO	[30, 31]	Poncirin
12	227	283,326	FA		[30, 31]	Hesperetin

confirm the nature of compounds 1-12, isolated from extracts of the young leaves and and immature fruits of *Citrus aurantium*.

In short, the present work shows that it is possible to determine simultaneously by means of HPLC the quantitative presence of a large number of flavonoids in *Citrus aurantium*, which until now have only been described independently or in closed structural groups.

This work not only intends to describe a specific methodology for the elucidation, isolation and identification of the flavonoids found in *Citrus aurantium* extracts, but also to suggest a way of varying this methodology for the study of flavonoids of a similar nature in other extracts.

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DETERMINATION OF WATER SOLUBLE VITAMINS BY LIQUID CHROMATOGRAPHY WITH ORDINARY AND NARROW-BORE COLUMNS

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ABSTRACT

A reversed phase high performance liquid chromatographic method is described for the simultaneous determination of nicotinic acid, nicotinamide, pyridoxine, thiamine, folic acid and riboflavin. The vitamins were separated on a C_8 bonded phase column and eluted with a dihidrogen phosfate buffer, hexanesulphonate and methanol as the eluent. A rapid spectral detector Diode Array was used to optimise the detection of each vitamin. All vitamins were separated in less than 12 minutes. This method was applied to the determination all the vitamins in multivitamin pharmaceuticals. Recovery studies showed good results for all solutes (93.4%-106.5%) and some day coefficients of variation ranging from 0.91% to 4.95%.

Narrow bore columns were recommended because this alternative provided a good separation efficiency, plus greater economy and sensibility. Finally, the performance of an ordinary UV detector and that of a rapid spectral detector in this type of determination were critically compared.

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INTRODUCTION

The current increased interest in good eating habits has meant greater awareness of the vital role vitamins play in normal growth, development and health. Their measurement is of interest to those involved in biochemistry, pharmaceuticals and the food sciences. These considerations, together with regulations about food labelling, lead us to the necessity to have available rapid and reliable analytical methods for the simultaneous analysis of vitamins.

Vitamins are determined separately using widely different techniques-chemical, physicochemical, microbiological and biological (1-3). These methods are frequently time-consuming and are often limited by the number of interferent compounds found in the sample matrix. HPLC techniques could offer advantages of specificity and speed using the adequate equipment. In fact, the use of HPLC for water soluble vitamin analysis is not new; on the contrary, various liquid chromatography procedures have been described for their determination (4-6). Some workers have used amino-bonded phases (7,8) or a cation exchange column for the determination of nicotinic acid and its derivates (9) owing to the ionic nature of these water soluble vitamins. However, reversed phase high performance liquid chromatography methods are employed most generally, and its use for the analysis of formulations containing vitamins has gained wide acceptance from Quality Assurance laboratories of pharmaceutical industries (10-12).

The procedure proposed for the separation of the several water soluble vitamins in this paper makes use of hexanesulphonate as ion interaction reagent, since the high water solubility and structural dissimilarity (Figure 1) of these analytes makes it necessary to use the ion-pair technique (13-15) sometimes involving gradient elutions.

The objective of the present work was to develop a method for the simultaneous determination of B_1, B_2, B_6, nicotinic acid, nicotinamide and

Folic Acid

Nicotinic acid

Thiamine

Nicotinamide

Pyridoxine

R = OH
Riboflavine

R = O — P(=O)(OH) — OH
Flavin Mononucleotide
(FMN)

FIGURE 1. Chemical structures of the investigated water soluble vitamins.

folic acid and was applied to the analyst of multivitamin pharmaceuticals. Alternatively these separations could been carried out over narrowbore columns with the aim of reducing the actual cost and increasing the sensibility.

EXPERIMENTAL

Apparatus and conditions

The HPLC system consisted of a Hewlett-Packard HP 1090 liquid chromatograph, a Rheodyne 7010 injection valve with a 5 µL loop, a Hewlett-Packard (HP) 79881A filter photometric detector, a HP 85B personal computer and a HP 3390A integrator. Column effluents were monitored at 254 nm for the nicotinic acid, nicotinamide, thiamine and riboflavin, and 280 nm for pyridoxine and folic acid.

Alternatively, a LKB (Bromma, Sweden) liquid chromatograph was used. The chromatograph was equipped with a model 2150 pump, a Rheodyne 7125 injection valve with a 20 µL loop, a LKB 2140 rapid spectral detector and IBM PS/2 data station.

The HPLC columns used were as follows: Lichrosorb RP-8 (250 x 4 mm i.d., 5 µm), Lichrospher 100 RP-18 (125 x 4 mm i.d., 5 µm) and Spherisorb ODS-2 (100 x 2.1 mm i.d., 3 µm).

For pH measurements, a PW 9422 Philips pHmeter equipped with a combined glass-Ag/AgCl electrode was employed.

Materials and reagents

All solvents used were HPLC grade and were employed as supplied by manufacturers. High purity water was obtained through a Millipore Milli-Q system.

Gradients were performed between two solvent mixtures. Solvent A was composed of 5 mM sodium hexanesulphonate (HSA) (Sigma, Saint

Louis, MO) by adjusting the pH value of the solution to 2.8 through addition of 10^{-2} M potassium dihydrogen orthophosphate/phosphoric acid (Merck, Darmstadt, Germany). Solvent B was methanol (Romil Chemicals, Sps). Before being used both solvents were vacuum-filtered to 0.45 µm nylon filter and degassed with helium.

Analytical grade nicotinic acid, nicotinamide, pyridoxine, thiamine, riboflavin, flavin mononucleothide (FMN), folic acid and theobromine (IS) standards supplied by Sigma were used. Individual stock solutions of each vitamin were prepared every third day in water to provide a concentration of 1 mg mL^{-1}, except riboflavin (0.1 mg mL^{-1}) and folic acid (prepared in sodium bicarbonate 1 M). These solutions were degassed with helium and stored in dark glass flasks, in order to protect them from light, under -18°C refrigeration (folic acid solutions were stored at 3°C).

The working standard was prepared by adding aliquots of individual stock solutions and diluting with water. Then, an aliquot was taken and diluted with internal standard solution to give a concentration about 50 µg mL^{-1} of vitamins. The solution was filtered through a 0.45 µm membrane (Millex-HV_{13}, Millipore) before being injected into the system.

The samples of the liquid multivitamin products were injected immediately after adequate dilution and filtration (Millex, 0.45 µm) to remove impurities that might be present. The application of Sep Pak C_{18} clean-up cartridge in sample preparation for these formulations was not essential.

Complete triplicate analysis was performed on all samples to allow the calculation of average deviations as a measurement of chromatographic reproducibility.

RESULTS AND DISCUSSION

Standards of the vitamins were chromatographed separately in order to determine the retention time for each of them. The vitamins were then

chromatographed as a mixture and chromatographic conditions (pH, ionic strength, hexanesulphonate concentration, percentage of the organic modifier, temperature and flow-rate) were altered to maximize peak resolution.

Using previous studies as a basic, preliminary separations were obtained on a C_{18} column, but, as consequence of ion-pair polarity the C_8 column offered best resolutions.

On the basis of resolution the following operating conditions for the HPLC system were chosen as the optimum:

Flow rate: 1 mL min^{-1}
pH=2.8
Ionic strength: 10^{-2}M KH_2PO_4
Hexanesulphonate concentration: 5 mM
λ detection: 272 nm

The gradient run conditions were programmed as follows using methanol as phase B:

Time in minutes	0	4	5.5	12	17	22
%B	10	28.2	28.2	50	50	10

Figure 2 demonstrates the separation achieved from a standard vitamin mixture. As can be seen, the majority of vitamins present in the mixture have a baseline separation and elute as sharp peaks. Using the system described the retention times were found to be very consistent from one chromatogram to another. Table 1 gives the retention times of investigated vitamins and the coefficients of variation based on eleven sequential runs of standards.

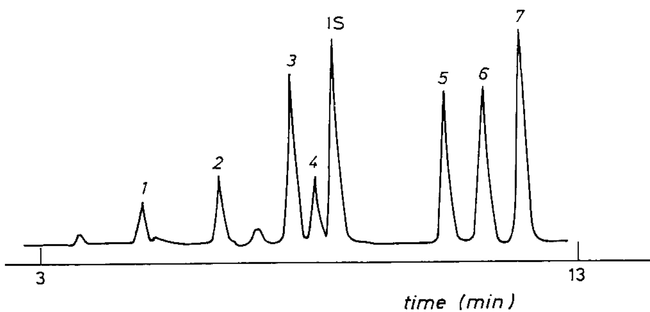


FIGURE 2. Chromatogram obtained from standard solutions of vitamins by using a Lichrosorb RP-8 (250x4 mm I.D., 5 µm) column. For gradient programme and conditions, see text. Peaks; 1.- Nicotinic acid; 2.- Nicotinamide; 3.- FMN; 4.- Pyridoxine; 5.- Thiamine; 6.- Folic acid; 7.- Riboflavin; I.S.- Teobromine.

TABLE 1

Retention Times and its Repetibility in the investigated Vitamins.

Vitamin	Retention time (min)	CV %
Nicotinic acid	4.73	0.36
Nicotinamide	6.05	0.22
FMN	7.30	0.28
Pyridoxine	7.65	0.31
Thiamine	9.90	0.41
Folic acid	10.49	0.69
Riboflavin	11.12	0.97

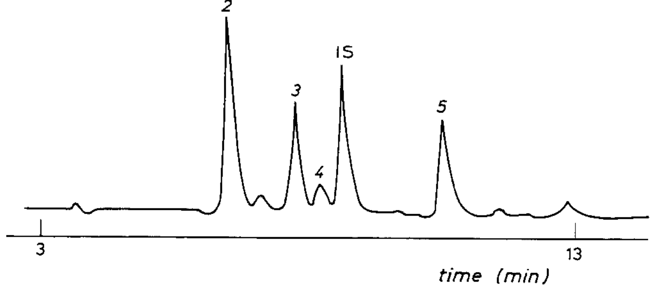


FIGURE 3. Typical chromatogram of water soluble vitamins in an oral liquid multivitamin product. Column and chromatographic conditions as in Figure 2. For peak identification see also Figure 2.

Figure 3 illustrates a typical chromatogram of the water soluble vitamins in an oral liquid multivitamin product, obtained using the operating conditions specified. The sample peaks were identified by comparing either the relative retention times and the spectrum of each one with those of the standard reference vitamins. Four vitamins were present in the sample and they were quantitatively determined.

The quantification of vitamins was achieved by using the internal standard method. The calibration curve data were generated by repeated injections of a fixed volume, 20 µL, of standard solutions of vitamins covering a broad concentration range. The resulting peak areas data ($A_{vitamin}/A_{IS}$) were determined, plotted against concentration and stored in the data module. Injection volumes of 20 µL were employed to analise the multivitamin sample and the amount of vitamins was directly obtained from the data module. In order to determine the accuracy of the method, recovery studies were carried out. Known amounts of each vitamin were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. All analytes were carried out in triplicate at four concentrations levels. The results are given in Table 2. The

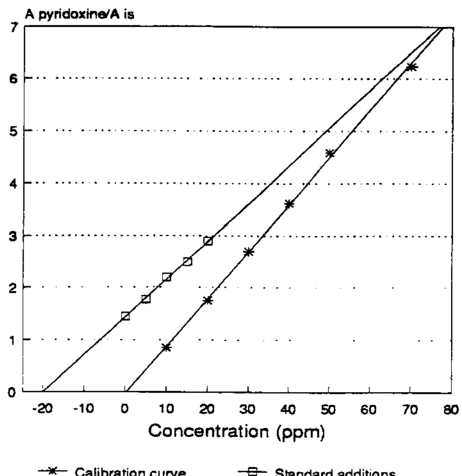
TABLE 2

Recovery Studies of Water Soluble Vitamins added to Multivitamin Sample

Vitamin	Amount in sample µg/ml	Amount added µg/ml	Amount found µg/ml	%Recovery
Nicotinamide	455.2	50	511.4	101.2 ± 0.1
		100	555.6	100.3 ± 0.1
		150	619.5	104.3 ± 0.5
		200	661.8	100.8 ± 0.2
FMN	32.4	5	37.7	106.0 ± 1.5
		10	42.6	102.2 ± 0.3
		15	48.0	104.0 ± 2.1
		20	53.9	106.5 ± 1.7
Piridoxine	18.0	5	22.6	93.4 ± 2.7
		10	27.4	94.1 ± 2.3
		15	32.4	96.0 ± 3.5
		20	38.7	103.5 ± 2.9
Thiamin	61.5	10	71.9	104.1 ± 1.1
		20	82.4	104.8 ± 0.7
		30	92.6	100.3 ± 1.3
		40	100.8	99.5 ± 0.9

recovery data for the pyridoxine were obtained using 290 nm as wavelength of detection, because with the values corresponding to 272 nm (optimised for all vitamins) matrix effects were present, as can be seen in Figure 4 which presents the calibration graph with standards and the standard additions graph with the multivitamin sample. The average recoveries between 93.4 and 106.5 indicate that the method has an adequate degree of accuracy for the analysis of these substances. The results obtained were in accordance with the values specified by the manufacturer, as the maximun deviation was 10% (in the case of pyridoxine).

In order to increase the quality of the proposed method, with respect to sensibility and cost, microcolumns with 2.1 mm I.D. for the vitamin separation were used.



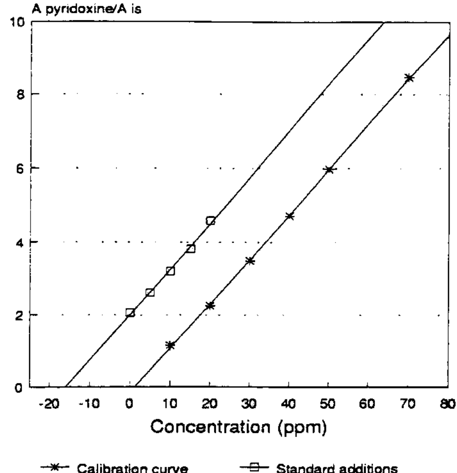
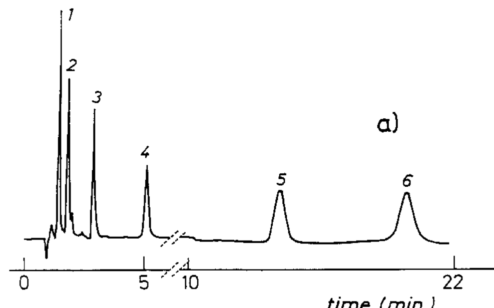


FIGURE 4.(*) Calibration graph with standards and (□) the standard additions graph with the multivitamin sample for the pyridoxine.



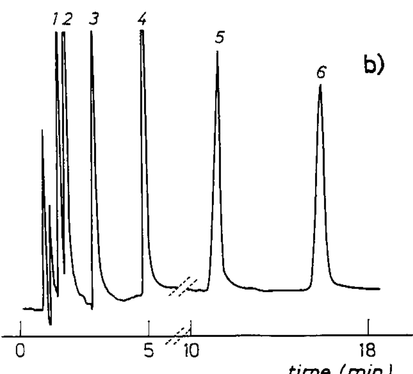


FIGURE 5. Chromatograms obtained from standard solutions of water soluble vitamins by using the following columns:
a) Lichrospher 100 RP-18 (125 x 4 mm i.d., 5 μm).
b) Spherisorb ODS-2 (100 x 2.1 mm i.d., 3 μm).
Mobile phase: 5 mM HSA, 20% Methanol, 0.1% Triethilamine and 0.01 M KH_2PO_4/H_3PO_4 pH=2.8.
Flow rate: a) 1 mL min^{-1}
b) Flow 0.2 and 0.3 mL min^{-1}
Peaks: 1.- Nicotinic acid; 2.- Nicotinamide; 3.-Pyridoxine; 4.- Thiamine; 5.- Folic acid; 6.- Riboflavin.

TABLE 3

Detection Limits (ng) of Water Soluble Vitamins determined by using Narrow-Bore and Normal-Bore Columns with Ordinary Photometric and Diode Array Detection.

Vitamin	Narrow-bore column	Normal-bore column	
	Photometric detection	Photometric detection	Diode array detection
Nicotinic Acid	0.125	0.38	16.3
Nicotinamide	0.185	0.56	21.0
Pyridoxine	0.260	0.62	22.7
Thiamine	0.430	1.26	26.9
Folic Acid	0.465	1.70	23.6
Riboflavin	0.465	1.80	32.5

To do this, and because of our instrumental limitations, the separations were carried out in isocratic mode according to the following conditions:

Mobile Phase: 5 mM HSA, 0.01 M KH_2PO_4/H_3PO_4 pH=2.8, 20% Methanol, 0.1% triethilamine

Temperature: 25°C

Flow gradient:

t(min)	Flow (mL min^{-1})
0	0.2
5	0.2
5.5	0.3
18	0.3

λ detection: 254 and 280 nm

Figure 5 compares a chromatogram on the 125 x 4.6 mm I.D. column (A) and on the 100 x 2.1 mm I.D. column (B) and shows the benefits of the microcolumn packed with 3μm particles. Figure 5 also shows an increase in sensitivity for the microcolumn.

Table 3 compares the detection limits of nicotinic acid, nicotinamide, pyridoxine, thiamine, folic acid and riboflavin, obtained with these columns by using the ordinary filter detector and the diode array detector (DAD). As can be seen, the limits affored by narrow-bore column are lower than those by ordinary column. Analysts are increasingly using DAD in order to facilitate optimisation of separation and detection of analytes. However, the detection limits provided by the DAD used with the fotodiodes at room temperature, were between 14 and 43 times higher than those provided by an ordinary photometric detector.

Finally, the use of less solvent (flow = 0.25 mL min^{-1}) decreases the cost of disposal.

CONCLUSIONS

This method provides a reliable means of analysing a number of water soluble vitamins simultaneously in multivitamin pharmaceuticals. Good results were obtained with baseline resolved peaks and chromatograms without interferences in less than 12 min.

Reversed-phase chromatography with narrow-bore columns packed with 3 μm particles makes an interesting alternative to the separation and determination of water soluble vitamins, through its higher sensibility and lower analysis time and cost.

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SIMULTANEOUS DETERMINATION OF NOREPINEPHRINE, DOPAMINE, AND SEROTONIN IN HIPPOCAMPAL MICRODIALYSIS SAMPLES USING NORMAL BORE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: EFFECTS OF DOPAMINE RECEPTOR AGONIST STIMULATION AND EUTHANASIA

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ABSTRACT

A sensitive and selective conventional chromatographic method has been developed for the simultaneous measurement of norepinephrine, dopamine, and serotonin from in vivo rat brain microdialysis samples. Ion-pair reversed-phase high performance liquid chromatography was used in combination with coulometric electrochemical detection to generate femtogram-level chromatograms reliably and routinely. Analysis is completed within 10 minutes making it ideally suited for the in vivo study of acute pharmacological manipulation in the rat brain to monitor the effects of drugs on and the potential interactions between monoaminergic systems. System performance was evaluated using in vivo microdialysis perfusate samples obtained from the right ventral hippocampus of the anesthetized rat before and after administration of the D_2/D_1 receptor agonist apomorphine as well as post-euthanasia. Basal levels and pharmacological effects are reported and discussed.

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INTRODUCTION

In order to improve the understanding of normal brain function, it is especially important to study the in vivo changes in regional extracellular neurotransmitter release and metabolism. Microdialysis is now a routine sampling technique for the collection of many compounds within the extracellular space in many tissues [1]. It has found particular popularity in combination with HPLC and electrochemical detection for the examination of small changes in neurotransmitter levels in brain tissue [2,3]. Only recently with improvements in analytical equipment, techniques, and the introduction of high efficiency microdialysis probes have the low-level analyses of biogenic amines in microdialysis samples been possible [3-6]. However the majority of current established chromatographic methods typically allow the analysis of only one particular neurotransmitter/system per sample [7-11].

Rather than investigating individual neurotransmitters as single extracellular biochemical events, a deeper understanding would result from the study of several neuronal systems simultaneously. Consequently the important interplay and interdependency between the distinct monoaminergic systems could be better defined not only intra-regionally [12] but between brain regions as well [13], as implied by evidence for inter-regional neuronal networks [14]. Accordingly, the effect of a drug on normal brain activity could be more accurately assessed as the understanding of normal brain function is further refined.

A suitable analytical method must be completed well within the time constraints imposed by sampling and sensitive enough to allow depletion studies in regions containing low levels of neurotransmitters, without the need for artificially elevating basal levels of the monoamines by either increased calcium levels [15-17] in artificial CSF (aCSF) or by localized introduction of reuptake blockers [18, 19] by way of the aCSF. In this way the analysis can be performed under as near normal physiological conditions as possible. Presented here is a new, highly sensitive, stable and selective conventional-HPLC system for the simultaneous analysis of NE, DA, and 5HT from rat brain microdialysis samples.

The hippocampus was chosen to validate the method since the analysis of the low-level of monoamines found there represents a significant challenge. One specific objective was to verify and establish the presence of NE, 5HT and particularly DA in the ventral hippocampus. A second objective was to examine levels of these monoamines following a drug intervention designated to decrease their release. The non-specific D_2/D_1 receptor agonist apomorphine was chosen to reduce DA levels, based, in part, on its known effects in the striatum [20]. The consequence of its action following peripheral administration will be discussed.

METHODS

Chemicals And Reagents

The purest grades of chemicals available were obtained and used in all solutions and mobile phases. Disodium ethylenediaminetetraacetate (EDTA) and phosphoric acid were purchased from Fisher Scientific Co. (Fairlawn, NJ, U.S.A.). Sodium dihydrogen phosphate, acetonitrile, and methanol were purchased from EM Science (Gibbstown, NJ, U.S.A.), while triethylamine (TEA) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Perchloric acid (PCA) was purchased from J.T. Baker Inc. (Phillipsburg, NJ, U.S.A.) and ethanol from Pharmco (Dayton, NJ, U.S.A.). The monoamines norepinephrine (NE), epinephrine (E), dopamine (DA) and serotonin (5HT), and R(-)-apomorphine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Apomorphine solutions were prepared in 0.1μM ascorbate (Gibco Laboratories, Chagrin Falls, OH, U.S.A.) to ensure stability. Sodium dodecyl sulfate (SDS) was obtained from American Bioanalytical (Natick, MA, U.S.A.). All aCSF components (sodium chloride, calcium chloride, magnesium chloride, potassium chloride, and sodium dihydrogen phosphate) were procured from Fluka Chemical Corp. (Ronkonkoma, NY, U.S.A.).

Water Treatment

Milli-Q water (18 MΩ) was passed over a C18 solid-phase extraction cartridge (Sep-pak, Millipore Corp., Milford, MA, U.S.A.) prior to use in every aqueous stock solution, dilution, and mobile phase preparation introduced onto the HPLC-ECD system.

Standard Stock Solutions

Individual stock solutions of the monoamines (5 mM or 1 mg/ml) were prepared in 0.2 M PCA, 100 µM EDTA and stored at 4 °C.

Microdialysis Probe Preparation

The 3 mm loop-type regenerated cellulose probe (ESA Inc., Bedford, MA, U.S.A.) was perfused with 70% ethanol overnight (0.3 µl/min) by a microdialysis syringe pump (Model 22, ESA Inc.) to remove the storage solution. On the day of implantation, the probe was flushed with treated Milli-Q water at 5 µl/min for 30 minutes. Artificial CSF [15, lacking ascorbate] was then perfused through the probe (1.5 µl/min) and immersed in a fresh monoamine standard mixture (20 nM) diluted from 1mg/ml stocks with aCSF. In vitro recovery was determined daily prior to implantation with flow at 1.5 µl/min at 25 °C, to verify probe consistency and system stability. In vitro recoveries were not used to determine extracellular fluid (ECF) levels as this would underestimate the actual ECF concentrations [21-23].

Microdialysis

Male Sprague-Dawley rats (275-350 g) were anesthetized with urethane (2 g/kg i.p.) and stereotactically implanted with a precalibrated 3 mm probe into the right ventral

hippocampus (coordinates from bregma: AP -5.8, LR 4.8, and DV -7.5 mm from the dura surface). The probe was perfused with aCSF at 1.5 µl/min. Samples were collected every 20 minutes into a tube containing 5 µl 0.2 M PCA/100 µM EDTA to minimize monoamine decomposition. Body temperature was monitored rectally and was maintained at 37 ^{o}C by a heating pad. After allowing a period of 2h for stabilization of 'injury-mediated' neurotransmitter release resulting from probe implantation [2, 23], basal samples (defined as three consecutive samples in which monoamine levels varied by less than 10%) were then collected. Animals then received either physiological saline or apomorphine in 0.1µM L-ascorbic acid (0.5 mg/kg) administered subcutaneously (sub. cut.). Samples post-euthanasia (induced by anesthetic overdose) were also collected to verify that monoamine release increased upon death.

Microdialysis Probe Storage And Reuse

The storage conditions of the probes were dependent upon the length of time between experiments. If the probe was to be used the next day then at the completion of an experiment the syringe was washed and filled with water while the probe was washed with and immersed in water. The flow was maintained at 0.3 µl/min overnight. If the probe was not to be used the following day then it was rinsed with water followed by 70% ethanol and stored in that solution for a period of up to 3 weeks. If the latency period exceeded 3 weeks, the probe was washed with water and then filled with 5% glycerol prior to storage.

HPLC Analysis

The chromatographic system was modified to optimize performance for electrochemical detection. A low stroke volume (10 µl) dual piston pump (Model 580) equipped with PEEK (polyetheretherketone) tubing was fitted with two in-line serial

PEEK pulse dampeners. An inert Rheodyne 9125 injector with fixed 20 µl PEEK sample loop was used to introduce perfusates onto an ESA HR-80 column (3µ, C18, 8.0 cm x 4.6 mm i.d.). The analytes were detected on a Coulochem II electrochemical detector equipped with a dual electrode analysis cell (Model 5014 Microdialysis Cell: E_1 -175 mV; E_2 +175 mV vs Pd reference electrode) and a Model 5020 Guard Cell (E_{GC} +300 mV), all from ESA Inc. The mobile phase, pumped at 1.0 ml/min, consisted of 75 mM NaH_2PO_4, 1.5 mM SDS, 100 µl/l TEA, 20 µM EDTA, in 15% acetonitrile and 13% methanol. The pH of the aqueous phase was adjusted to 5.6 before addition of organic modifiers. Final concentrations and amounts are reported. The separation was affected at 25 °C. Hydrodynamic voltammograms were generated for optimization of the electrode potentials.

RESULTS

The optimal applied potentials, according to the hydrodynamic voltammograms, were determined to be 75 mV for DA, 100 mV for NE, and 125 mV for both E and 5HT. A standard chromatogram representing 800 fg injected on column for NE, DA, and 5HT is shown in Figure 1. The analytical run time was less than 10 minutes and the retention times with day-to-day variability were 2.83 ± 0.07, 5.20 ± 0.16, and 9.16 ± 0.38 minutes for NE, DA, and 5HT respectively. The on-column limit of detection at s/n=4 was estimated from external standards to be approximately 400 fg for NE and DA, and 700 fg for 5HT.

The in vitro recovery of these monoamines using the 3mm 'loop-type' probe at 1.5 µl/min and 25 °C was 25-30%. No interferences were derived from the aCSF. However, as a matter of observance it is reported that small non-interfering chromatographic artifacts occasionally appeared in the baseline after the NE and DA peaks and appeared to be associated with the potassium and magnesium components in the

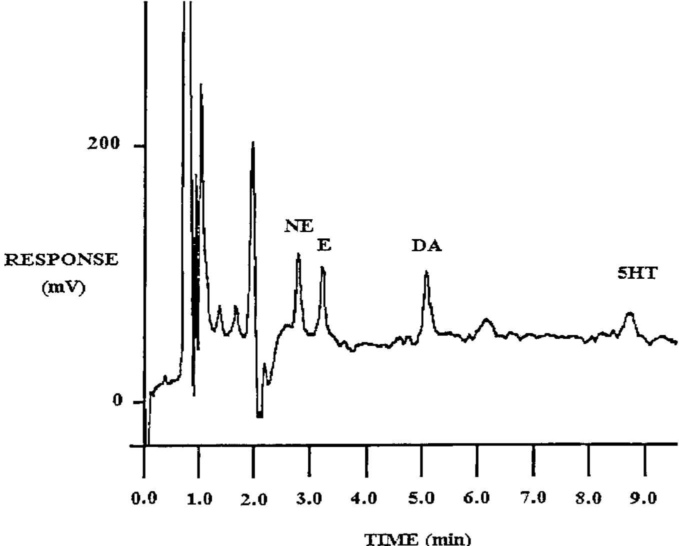

FIGURE I

A low-level standard (800 fg of each NE, E, DA, and 5HT) was injected onto the column with chromatographic conditions as described in the text to generate the above chromatogram. The detector current range is 2 nAFS with a 5 second filter time constant.

aCSF. A basal sample from the right ventral hippocampus of the anesthetized rat is shown in Figure II. In that chromatogram, the on column amounts represent 2.1, 0.49, and 0.95 pg of NE, DA, and 5HT, respectively. The overall basal levels of monoamines per 20 minute collection (30 µl microdialysis sample) in the rat right ventral hippocampus were calculated to be 4.3 ± 0.78, 2.2 ± 0.94, and 8.1 ± 3.58 pg of NE, DA, and 5HT, respectively (n=17). Treatment with the D_2/D_1 receptor agonist apomorphine induced a maximal decrease (56%) in the level of DA in the hippocampal ECF after 100 min with concurrent decreases in both NE (34%) and 5HT (40%) (n=7) (Figure III). Marked increases (typically greater than 15 fold) in all three monoamines were

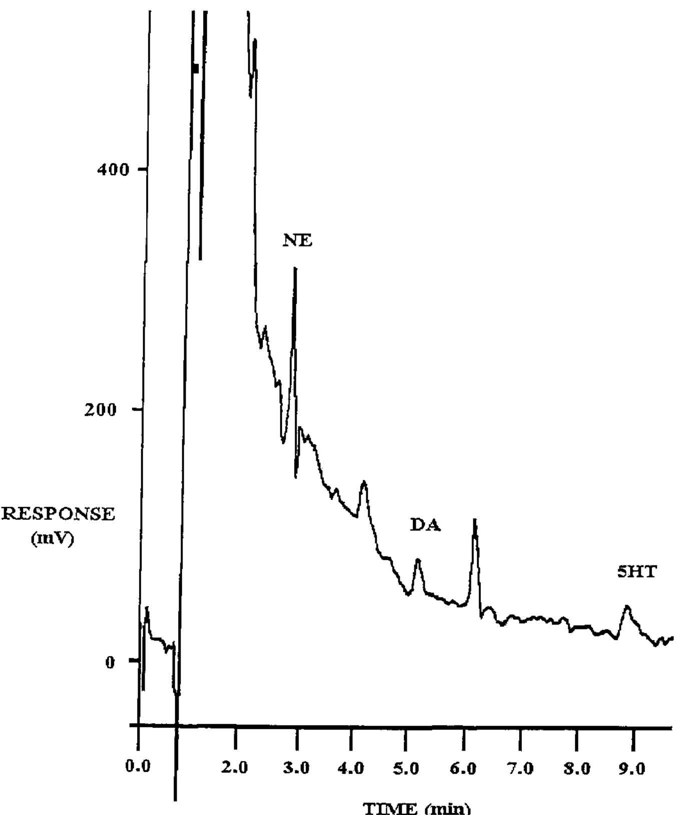

FIGURE II

This chromatogram represents a 20 µl injection of a 30 µl basal microdialysis sample (plus 5 µL PCA) from the right ventral hippocampus of the anesthetized rat. The on-column amount of each NE, DA, and 5HT shown is 2.1, 0.49, and 0.95 pg respectively. Chromatographic conditions are described in the text. The detector current range is 2 nAFS with a 5 second filter time constant.

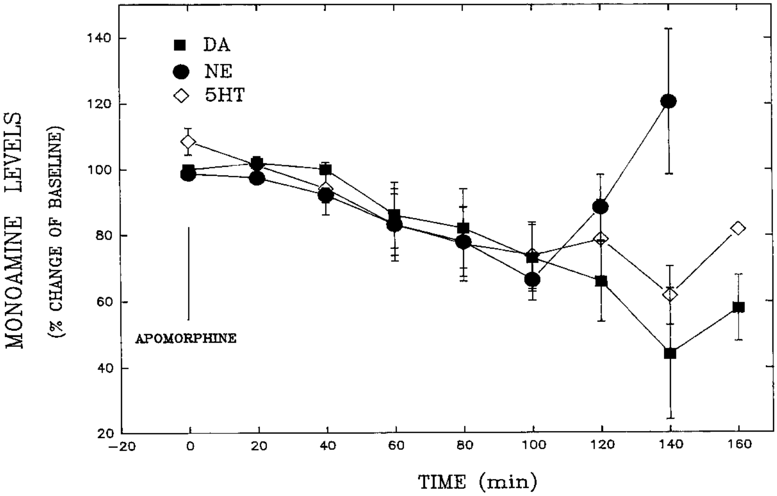

FIGURE III.

The effect of peripherally administered apomorphine (0.5 mg/kg sub. cut.) on the hippocampal levels of NE, DA, and 5HT in the anesthetized rat. The drug was introduced at t=0 min. Changes in the concentration of the monoamines are expressed as a percentage of the mean of the basal levels. Mean values are plotted with the s.e.m. indicated by vertical bars.

observed after administration of an overdose of anesthetic for euthanasia (n=3). The i.p. administration of saline (control) had no significant effect on any of the monoamines measured, except for 5HT which tended to slowly decrease with time (data not shown). Although E standards were analyzed, no equivalent peak was measured in any of the hippocampal samples, even post-mortem. Additionally, no unknown sample components were found to co-elute with any of the monoamines in the microdialysis samples.

All data are reported uncorrected in regard to the in vitro recovery. Probes were typically reused in 10-20 animal experiments before inefficient in vitro recovery compromised analytical sensitivity.

DISCUSSION

Chromatography

Reversed-phase chromatography has long been employed for the general analysis of biogenic amines. However, the chromatographic system presented here was specifically designed to measure the monoamines NE, E, DA, and 5HT only, from microdialysis samples within the time constraint imposed by sampling. Monoamines were selectively retained as potential interferences present in the perfusate (such as DOPAC, HVA, and 5HIAA) were intentionally placed in the void.

Alkylsulfate ion-pair agent forms a strong ion-pair complex with monoamines, at this pH [24]. The relatively high level of SDS in this mobile phase was required to enhance selectivity and separation in the front of the chromatogram. However the strong retaining effects of this long chain ion-pair agent on the late-eluting 5HT had to be moderated by a high content of strong organic modifier, particularly acetonitrile. Methanol was also included to refine the retention of the monoamines but also to provide greater solubility for the buffer and ion-pair reagents. Thus pH, column selection, choice and level of ion pair agent, and organic modifier percentage were manipulated to exert control over metabolite and monoamine-SDS pair placement and retention time.

The fairly clean chromatogram and system stability that results is due in large part to the filtration through the 6000kD molecular weight cutoff membrane of the dialysis probe. However several other components were added to the mobile phase to further enhance the stability of the system as well as peak symmetry, system sensitivity. The amine modifier TEA was included in the mobile phase to reduce tailing problems associated with secondary-retention effects [25]. The resultant improvement in peak symmetry provided better peak resolution and quantitation. The inert PEEK components substituted for stainless steel pieces in places along the fluid path improved system performance. However free metals from such apparent sources as

column frits, column walls, and irreplaceable stainless steel tubing were chelated by EDTA in the mobile phase. Additionally, EDTA stabilizes the catecholamine structure [26].

Increased background currents and random contamination peaks were also limited by the use of ultrapure water in mobile phase preparations. Commercial water purification systems effectively produce water of quality 18.2 MΩ/cm^3, yet the quantity of trace organic material from bacterial sources is unknown. These organic compounds are not removed by the traditional purification cartridges but are easily eliminated by passage over a C18 solid phase extraction cartridge. This 'polished' water was used in all solutions introduced onto the HPLC system to ensure that the HPLC system was kept clean and metal-free.

Finally, the buffer concentration was maintained at 75 mM to provide sufficient buffering capacity to the mobile phase as well as to serve as an adequate electrolyte for electrochemical detection to provide a stable baseline.

Detection

The electrochemical detector employed here was comprised of low noise potentiostats and a high-efficiency porous graphite analytical cell, designed specifically for the analysis of microdialysis perfusates. The application of fairly low potentials and the use of the guard cell resulted in extremely low background currents (typically below 3 nA) and ultimately better sensitivity. This HPLC-ECD system coupled with the use of high efficiency microdialysis probes yielded excellent selectivity and sensitivity for monoamines in the in vivo samples for a period of greater than 1 year.

Monoamine Analysis

The monoamine metabolites were deliberately excluded from the assay. In the past, levels of the metabolites were used to estimate monoamine release (especially when

measurement of the parent monoamine itself was difficult), but their levels are now thought to be more a reflection of intraneuronal metabolism rather than an accurate indicator of neurotransmitter release per se [27-29]. Although we are not the first to measure a monoamine with the exclusion of its metabolites [30], we have developed this assay to measure three monoamine neurotransmitters simultaneously and with high temporal resolution.

Basal monoamine levels of NE from the rat hippocampus were comparable to those found elsewhere [11, 31]. To date, the role of DA in the hippocampus has remained obscure. In the past, support for the presence of DA in the hippocampus has mainly come from evidence of DA innervation [32], tissue levels [33], and release from synaptosomes [34]. Previously basal hippocampal ECF DA levels have usually only been detected in the presence of the reuptake blocker nomifensine [19], probably due to analytical system constraints. However, only recently has DA been quantitated in the hippocampus using in vivo microdialysis without reuptake blockade [35]. With our system, not only were hippocampal basal levels of DA routinely observed and measured but ECF levels of NE and 5HT could also be examined concurrently. Levels of 5HT were only slightly higher than previously published [36] but this may be due, in part, to differences in animal models and diffusion efficiencies across the dialysis membranes.

The sensitivity of the system allowed measurements of diminished release of all three monoamines following pharmacological intervention. As in the striatum [20], peripheral administration of the D2/D1 agonist apomorphine lowered the ECF levels of DA, however the magnitude and the time course were different. Since apomorphine is known to act at both D2 and D1 receptors, studies are ongoing in an attempt to qualify which receptor subtype was affected. It is unclear at present why apomorphine also affected ECF levels of NE and 5HT in the hippocampus. It is unlikely that apomorphine is acting directly on noradrenergic receptors. Another question is whether DA is acting as a neurotransmitter per se, or whether it just constitutes a NE precursor pool which happens to be co-released when the NE neuron is depolarized. Future research might include lesioning studies and the use of enzyme inhibitors to better characterize the source and function of DA in this region.

As has been previously reported for DA in the striatum [37], death caused a major increase (greater than 15 fold) in hippocampal ECF levels of NE, DA, and 5HT, suggesting that each may be neuronal in origin. Since the epinephrine standard was chromatographically resolved from the other monoamines using this method, and no peak was found to correspond to its capacity factor, it was concluded that E was either not present in this region of the brain or that its level was below the limit of detection of this system.

Although possibly present in the microdialysis sample and selectively retained with the other monoamines, 3MT was not observed in the chromatogram. Firstly, the potential applied to the working electrode was too low for the oxidation of this amine so that it was intentionally screened from the chromatogram. Secondly, if similar to the striatum the extracellular level of 3MT is approximately 1/3 that of DA [38, 39] then the level would probably be below the detection limit as currently established. Also present in the microdialysis sample and absent from the chromatogram were the amino acids. Even though most need to be derivatized for detection by ECD [10], those amino acids that are inherently electroactive (such as tyrosine and tryptophan) are not observed since the applied potential was not optimum for their oxidation. In this way these compounds were also intentionally screened from the analysis.

This method is presently being employed to study the possible interaction between these monoamines in the hippocampus before and after administration of known receptor agonists. Since there were no late eluting compounds, this method could be used for on-line analysis if desired. Although adequate for the majority of current analyses, the sensitivity might be further improved by substituting a 3 mm i.d. column in place of the 4.6 mm column, with only minor adjustment in the flow rate.

CONCLUSION

This analytical system delivers routine and sensitive measurement of monoamines in *vivo* microdialysis samples. The achievement of femtogram level sensitivity is

possible without having to employ microbore methods. This method successfully demonstrates detection of basal NE, DA and 5HT in the hippocampus without the addition of reuptake blockers or increased calcium in the aCSF. Additionally, because of its conventional nature, this system is easy to use, stable, and appropriate for routine analysis. Therefore, it might also be useful in the pharmaceutical industry or in clinical laboratory, for the analysis of monoamines in other biological matrices such as plasma.

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DETERMINATION OF THE STABILITY OF DOPAMINE IN AQUEOUS SOLUTIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Methods for the ananlysis of dopamine and its degradation products in aqueous solutions is described. The technique of reverse phase chromatography with electrochemical detection is used to investigate the stability of dopamine in various aqueous solutions. In neutral and basic solutions, dopamine is rapidly oxidized by dissolved oxygen to form degradation products. The results demonstrate that dopamine is stable in 0.1 N HCl solution, pH < 1. The study indicates that EDTA can slow down the oxidation process. The detection limit for the analysis of dopamine is 0.1 μM with 100 μl injection.

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INTRODUCTION

Dopamine is a well-established neurotransmitter in the mammalian central nervous system. [1], [2], [3], [4] Dopamine may be the inhibitory neurotransmitter released from interneurone in autonomic nervous system ganglia. [1] The major metabolites of dopamine include dihydroxyphenylacetic acid, homovanillic acid (HVA) and 3-methoxytyramine. Within the terminal, a free dopamine is first deaminated and oxidized, and then it is methylated to form HVA after leaving the terminal.

In neutral and basic conditions, as it was found in hydroquinone previously, [5], [6] dopamine can be rapidly oxidized by oxygen dissolved in aqueous solutions. The main products are hydrogen peroxide and dopamine orthoquione, with superoxide serving as a chain-propagating radical in the autoxidation of dopamine. [7] Because the number of samples in biological experiments is usually very large, and the analysis can take a whole day and sometimes the extracted samples may be stored for days before analysis, it is vital to preserve the analyte during the sample extraction and analysis. It is considerably interest to determine the stability of dopamine in various aqueous solutions. The investigation was carried out to determine the optimal conditions for (1) the protection of dopamine against degradation and (2) the separation of dopamine and its degradation products. The method developed for the analysis of dopamine and degradation products is reverse phase chromatography with electrochemical detection. The detection limit for

dopamine is 0.1 μM with 100 μl injection. This study indicates that the initial oxidation products of dopamine can undergo further oxidation or reduction.

EXPERIMENTAL

The instrumentation used included a Waters 6000-F pump, a Rheodyne 7125 injection valve with a 20 μl loop, an LC-4B/17(D) amperometric detector for liquid chromatography, and a Fisher recordall Series 5000 chart recorder. Separations were accomplished using a stainless steel column (3 mm x 100 mm) prepacked with 5 μm Biophase octadecyl silica and a guard column (4.5 mm x 20 mm) packed with Waters 40 μm C18/Corasil. The detector consisted of a thin-layer glassy-carbon electrode, a Ag/AgCl reference electrode mounted downstream from the glassy-carbon electrode, and a LC-4B amperometric controller for electrochemical detection. The flow rate was 0.8 ml/minute and the injection volume was 100 μl. The detection potential was 800 mV and the current was 2 nA for the detection of dopamine at the concentration below 0.4 μM and 5 nA at the concentration above 0.4 μM.

The mobile phase was 16.67 mM sodium phosphate, 0.41 mM 1-heptanesulfonic acid, 0.08 mM ethylenediaminetetraacetate disodium salt dihydrate (EDTA) and 3% methanol. The pH value of this mobile phase was 6.5. The mobile phase was filtered with 0.45 μm filter and then sparged by helium at 10 psi for 20 minutes before use. The solution pH was determined with a Corning 610A pH meter calibrated with VWR buffers. A 5 μM dopamine

stock solution was prepared in 0.1 N HCl. Three dopamine solutions were prepared by diluting the stock solution with 0.1 N HCl, mobile phase, or mobile phase without EDTA.

Sodium phosphate (monobasic), 1-heptanesulfonic acid (sodium salt) and EDTA were purchased from Aldrich, methanol from Baxter Healthcare Corporation, 3-hydroxytyramine hydrochloride (dopamine hydrochloride) from Sigma Chemical Company. All solutions were prepared with triply distilled water purified by a Millipore Milli-Q reagent system.

RESULTS AND DISCUSSIONS

Dopamine solutions preserved with 0.1 N hydrochloric acid solution and mobile phase with EDTA were analyzed at various times. In comparison, dopamine solution at neutral pH without preservatives was also analyzed at various times. Figures 1A and 1B show the chromatograms of 1 μM dopamine in 0.1 N HCl solution ($pH < 1.0$). A freshly prepared sample was injected into the HPLC (Figure 1A). The sample was stored in a refrigerator (4 $^{\circ}C$) for 10 days and injected into the HPLC (Figure 1B). The only peak found in the chromatograms (Figures 1A and 1B) was dopamine, which indicated that dopamine was stable and no oxidation occurred in the strong acidic solution. Figures 2 show the chromatograms of 1 μM dopamine, which was made with mobile phase containing 0.2 M EDTA at pH 6.5. The sample was injected into HPLC as fresh (2A), after 5 days (stored at 4 $^{\circ}C$, 2B), 8 days (4 $^{\circ}C$, 2C) and 12

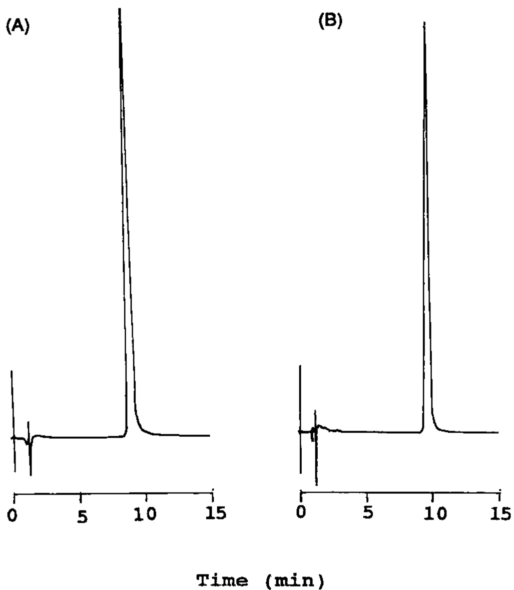

Figure 1 Chromatograms of 0.01 μM dopamine in 0.1 N HCl. Injected after a few minutes (A); after 10 days (B).

days (4 °C, 2D). The chromatograms show that the signal of dopamine (peak a) decreases and the signal of the degradation product (peak b) increases as the time increases. The indication is that dopamine is oxidized in a neutral solution. The oxidation is even more significant in 1 μM dopamine solution made with mobile phase without EDTA at pH 6.5, stored at 4 °C (Figures 3). As shown in Figure 3C, the dopamine peak was completely disappeared after 8 days while in Figure 2D dopamine was still found after 12 days, which suggested that EDTA somewhat protected dopamine from oxidation. It was suggested by Poirier *et al* [7] that a trace amount of transitional metal must

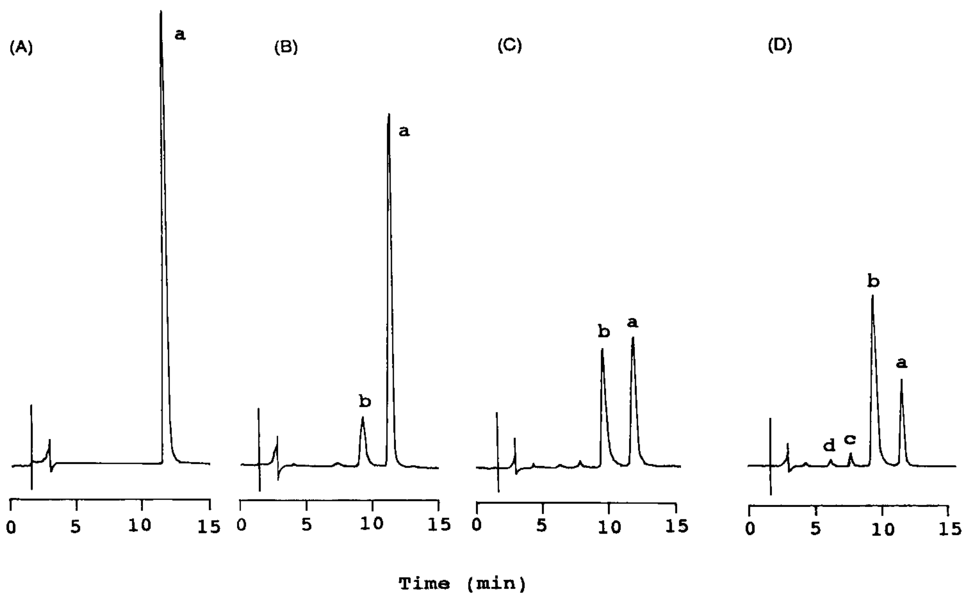

Figure 2 Chromatograms of 0.01 μM dopamine in mobile phase (with 0.2 M EDTA) at pH 6.5, injected after a few minutes (A); after 5 days (B); after 8 days (C); after 12 days (D).

have been present in a quantity sufficient to potentiate the autoxidation of dopamine through the generation of superoxide, hydroxyl radicals, hydrogen peroxide and reactive semiquinones. It is well known that EDTA can scavenge metal by forming an EDTA-metal complex. The catalysis of the degradation of dopamine by cadmium is presently investigated in our laboratory and the result will be published in the next paper. The experimental results suggest that in neutral solution dopamine is rapidly oxidized by dissolved oxygen as in the case of hydroquinone, catechol and resorcinol. [5], [6]

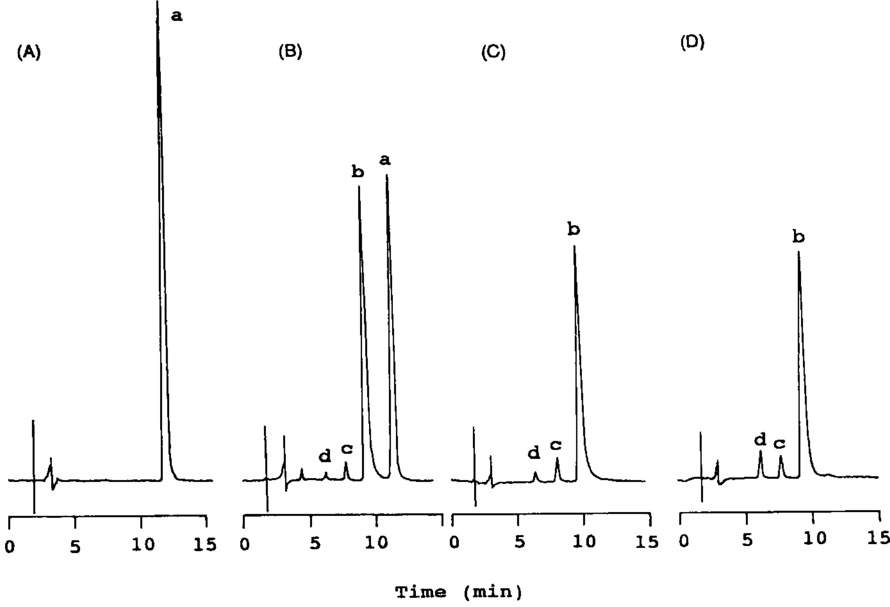

Figure 3 Chromatograms of 0.01 μM dopamine in mobile phase without EDTA at pH 6.5, injected after a few minutes (A); after 5 days (B); after 8 days (C); after 12 days (D).

$CH_2CH_2NH_2$ (OH, $O:^-$) $\xrightarrow{-e^-}$ $CH_2CH_2NH_2$ (OH, O·) $\xrightarrow[-H^+]{-e^-}$ $CH_2CH_2NH_2$ (O·, O·) (1)

$CH_2CH_2NH_2$ (O·, O·) $\rightleftharpoons$ $CH_2CH_2NH_2$ (O, O) (2)

Several other products are also observed (peaks c and d in Figure 2D, peaks c and d in Figures 3B, 3C and 3D), which can be attributed to the further oxidation or reduction of the initial oxidation product. The possible candidates of these oxidation products could be 5, 6-dihydroxyindoline, dopaminochrome and 5, 6-dihydroxyindole, as suggested previously. [8], [9] Since the standards of these degradation products are not available commercially, to the authors' knowledge, it is not possible to identify them.

An electrochemical detector has very high sensitivity for the detection of dopamine and some of its degradation products. Using an electric potential of 800 mV and a current of 2 nA, the detection limit was determined to be 0.1 μM (3 x baseline noise). Although dopamine has stronger signal at higher electric potential, because many molecules are oxidized at potential over 1,000 mV, measurements performed at such high potential will result noisy background and poor signal separation.

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SIMULTANEOUS HPLC ANALYSIS OF CARBAMAZEPINE AND CARBAMAZEPINE EPOXIDE IN HUMAN BRAIN MICRODIALYSATE

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ABSTRACT

Intracerebral microdialysis has become a standard method for neurochemistry studies and is becoming recognized as an important new method for pharmacological studies. The technique permits repeated measurement of drug concentrations in the brain extracellular fluid with minimal disturbance of the neuronal environment. The sample volumes are exceedingly small, on the order of tens of microliters. This is countered by the purity of the samples, reducing the need for extraction prior to assay. We present a HPLC assay capable of reliably measuring the concentrations of the antiepileptic drug carbamazepine and its metabolites carbamazepine-10,11-epoxide and carbamazepine-10,11-*trans*-diol.

INTRODUCTION

Microdialysis is a promising technique for studying drug pharmacokinetics. However, the small sample volumes require new assays or modifications of standard assays. We present a technique which has proven satisfactory for the analysis of antiepileptic drug concentrations from microdialysis catheters implanted in epilepsy patients undergoing intracranial electrical recording for seizure localization in preparation for epilepsy surgery.

To measure clinical concentrations of antiepileptic drugs (AEDs) in human cerebral microdialysate, we have modified an assay previously used in our laboratory for the measurement of carbamazepine (CBZ) and the active metabolite carbamazepine-10,11-epoxide (CBZE) concentrations in human serum (1). That method, an adaptation of the methods of Sawchuck (2) and Szabo (3), required the addition of cyheptamide as an internal standard, followed by an alkaline extraction into methylene chloride. Microdialysis provides protein free samples of sufficient purity that extraction is unnecessary.

MATERIALS AND METHODS

Microdialysis is conducted in patients undergoing depth electrode evaluation for the localization of seizure foci prior to epilepsy surgery. The

details of the implantation and microdialysis apparatus itself have been presented elsewhere (4). The microdialysis catheter is made of regenerated cellulose, (300 μm o.d., molecular weight cutoff 5000, Cuprophan, Enka Glanstoff, Germany); inflow and outflow tubing is made of fused silica. The catheter is perfused with an artificial extracellular fluid (ECF) composed of a sterile solution of NaCl 135 mMol, KCl 3 mMol, $CaCl_2$ 1.2 mMol, MgCl2 1 mMol, and ascorbate 200 uMol buffered with sodium mono- and di-phosphate to pH 7.35. The perfusion rate is typically 2.5 μl/min, but may be varied from 0.25 to 5.0 μl/min.

Chemicals

Water, acetonitrile, and methanol, all HPLC grade, were obtained from Fisher Scientific (Springfield, NJ, USA). CBZ was obtained from Supelco Inc. (Bellefonte, PA USA), CBZE was a gift of Ciba-Geigy Corp. (Summit, NJ USA), carbamazepine-10,11-*trans*-diol (CBZD) was a gift of George Szabo (Boston V.A. Medical Center, Boston MA, USA).

Chromatography

The analytical column is an Econosphere C18, 3 micron (10 cm × 4.6 mm I.D.) (Alltech Associates, Deerfield, IL, USA). A Spectra Physics 8880 autosampler is used to inject 20 μl of sample to overfill a 10 μl sample loop (the surplus is necessary to ensure sufficient injected volume and to avoid injection of air onto the column). To minimize volume loss, samples are

not processed prior to injection. A Spectra Physics SP8800 ternary pump (Spectra Physics, San Jose, CA, USA) is operated in isocratic mode with a mobile phase of water, acetonitrile, and methanol 60/23/17 (v/v). The column temperature is maintained at 40°C, and the flow rate is 0.6 ml/ml.

Peaks are detected at 210 nm by a Spectra Physics Spectra 100 variable wavelength detector; peak areas are computed with a Spectra Physics Chromjet 2 channel integrator.

Spiked samples are used to generate external standard curves. A stock solution of 4 μg/ml CBZ, CBZE, and CBZD in mobile phase is further diluted with mobile phase to 2.0, 1.0, 0.5, 0.2, 0.1, and 0.05 μg/ml concentrations.

Standards are injected at the beginning and end of each assay run. The standard curve is generated by a linear regression of the peak areas of the first set of standards. The second set is used to verify assay stability over the course of the run. Standards and samples are injected every 20 minutes, with mobile phase blanks alternating with samples as a precaution against unanticipated late-eluting peaks.

In vitro within run C.V.'s were computed on replicates of 10 samples, between run C.V.'s were computed on replicates of 5 samples performed

on 3 days. *In vivo* C.V.s were calculated on replicates of 4 within run and 4 between run injections of pooled dialysate from a patient receiving CBZ. A portion of the pool was spiked with CBZ and CBZE to assess assay repeatability in perfused dialysate at higher concentrations.

RESULTS

A calibration chromatogram is presented in figure 1.

In vitro:

CBZ and CBZD concentrations as low as 0.02 μg/ml, and CBZE concentrations of 0.01 μg/ml, were readily quantifiable. C.V.s are presented in table 1.

Patient samples:

Patient microdialysate CBZ and CBZE concentrations were 0.26 and 0.08 μg/ml respectively. C.V.s were 8.9% and 1.6% within run, and 6.8% and 7.9% between run for CBZ and CBZE. The pool was then spiked with CBZ and CBZE resulting in concentrations of 3.41 and 2.21 μg/ml. C.V.s were 1.7% and 1.3% within run, and 1.6% and 2.0% between run. The CBZD peak, visible on the chromatogram, was not quantified. A patient chromatogram is shown in figure 2.

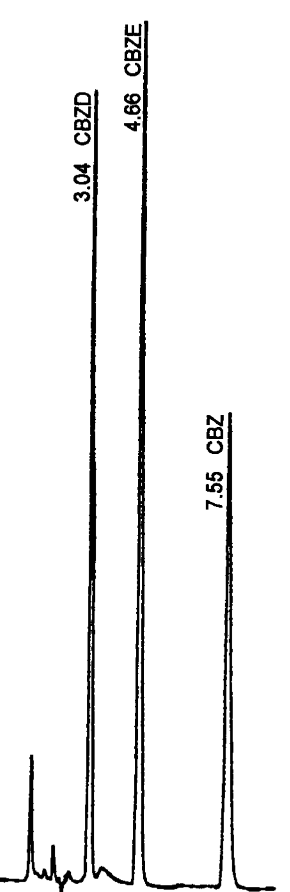

Figure 1 Calibration chromatogram (1.0 μg/ml). (Retention times in minutes.)

TABLE 1

In Vitro C.V.'s

Drug	Concentration (ug/ml)	C.V. within run	C.V. between run
CBZ	2.0	0.7 %	0.9 %
	1.0	0.3	2.0
	0.5	3.8	2.1
	0.2	1.4[a]	
	0.1	0.5[a]	
	0.05	3.9[a]	
	0.02	6.3[a]	
CBZE	0.3	0.9 %	1.8 %
	0.15	0.7	3.0
	0.075	0.5[b]	5.9
	0.03	1.6[a]	
	0.015	7.2[a]	
	0.0075	5.4[a]	
	0.0038	10.5[a]	
CBZD	0.6	2.1 %	3.4 %
	0.3	0.8	4.1
	0.15	0.5	7.7
	0.06	1.8[a]	
	0.03	3.3[a]	
	0.015	0.0[a]	
	0.0075	25.5[a]	

[a] n = 4
[b] n=6

DISCUSSION

Microdialysis of human epileptic tissue offers a unique opportunity to study the concentrations of AEDs in the vicinity of their putative receptors. The technique, although invasive, does not add significantly to the risks of electrode implantation being performed for seizure localization.

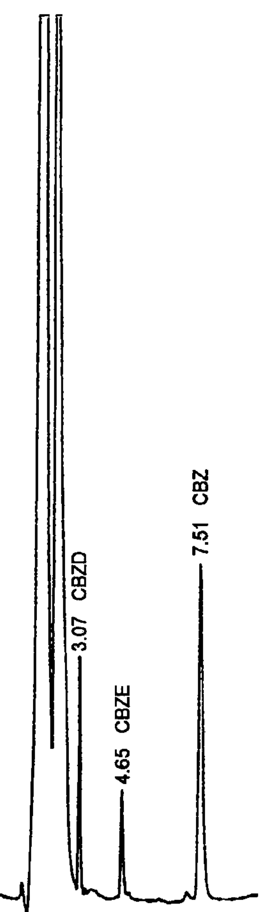

Figure 2 Patient chromatogram.
Direct injection of brain dialysate.

At usual perfusion rates, the sample volumes obtained are quite small, typically about 30 μl (5), but assay sensitivity has been sufficient to quantify CBZ and CBZE concentrations in cerebral microdialysate volumes of <20 μl. A CBZD peak was visible in some patient chromatograms but was not quantified as it is not believed biologically active.

The microdialysis membrane removes large molecules (> approximately 5000 Dalton), and replaces the extraction procedures required by standard assays. The small volumes would make such extractions difficult. To reduce the effect of measurement errors when transferring small volumes, no internal standard is added. This places extra demands on the stability of the analytical column. Repeat controls at the end of each chromatographic run check for possible drift. An internal standard could be added to the ECF prior to perfusion. If this were done, a fraction of the standard (dependent on perfusion rate) might diffuse out of the catheter. This would complicate its use as a chromatographic reference, as well as adding safety concerns and altering the neuronal environment.

Despite the absence of an internal standard, our data have been very consistent. The assay has proven suitable for assessment of brain concentrations of the antiepileptic drug CBZ and its active metabolite CBZE.

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SIMPLE AND FAST HPLC METHOD FOR THE DETERMINATION OF TRIAMTERENE AND HYDROXYTRIAMTERENESULPHATE IN PLASMA AND URINE

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ABSTRACT

Determination of triamterene (T) and hydroxytriamterenesulphate (HTS) in plasma and urine requires a very sensitive and selective method. As both of the substances are highly hydrophilic, it is hardly feasible to carry out an extraction. The method described obviates the need for pre-cleansing of samples. Plasma and urine samples, diluted with water, are directly injected into the HPLC-column and analysed. For the detection of both of the substances fluorescence was used. The use of a Spherisorb-NH2-column with a mobile phase, consisting only of a buffer solution in water, makes it possible to dispense with protein precipitation of plasma. Both substances were analysed within 2 minutes in a single run. Detection limits of 1 ng T and 20 ng HTS/ml plasma as required in practice, were obtained without any difficulties. Referring to the precision of this method with plasma samples, the variation coefficient was below 3 % with HTS in the range of 20 to 1100 ng HTS/ml. Day to day variation showed with T in plasma values of smaller than 7 % in the range of 1 to 100 ng/ml.

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INTRODUCTION

Triamterene is often administered with combined diuretic drugs, because it efficaciously preserves potassium in the body. As the main metabolite HTS is also effective (1), it should also be determined in bioavailability studies or clinical controls (Fig. 1). Several studies on the determination of T and/or HTS (2) have been published, some of them determine the T only (3) or HTS under varying chromatographical conditions (4,5).

DC is frequently used with fluorodensitometry, a method based upon a study dating back to the year 1976 (2). However there are also HPLC methods using fluorescence detection (3,4,5,6). Most of these studies use extraction steps, which requires a longer period of time.

This study describes the determination of T and HTS by HPLC during a single analytical run. Except for dilution, no other preparation of the urine and plasma samples is necessary. Besides, only very small quantities of as little as 50 mcl of plasma are required.

Both substances were analysed within 2 minutes, thus permitting a high number of samples to be analysed per day.

EXPERIMENTAL

Chemicals

Triamterene was supplied by Sagitta (Feldkirchen, FRG).
Hydroxytriamterenesulphate and some Hydroxytriamterene was isolated from human urine in our laboratory. Reagents of GR quality were supplied by E. Merck (Darmstadt, FRG).

Apparatus and Chromatographic Equipment

The chromatographic system consisted of a LC 420 pump (Kontron, Zürich, CH), a Rheodyne injection valve type 7125 with a 20 μl loop (Cotati, CA, USA) and a fluorescence detector F 1000 (Merck-Hitachi, Darmstadt, FRG) (excitation 360 nm/

Triamterene:	- R = -H
Hydroxytriamterenesulphate	- R = $-OSO_3H$

FIGURE 1: **Structure of Triamterene and Hydroxytriamterenesulphate**

emission 436 nm). The analytical column 125 x 4 mm i.d., (SRD-Pannosch, Vienna, Austria) was filled with Spherisorb-Amino 5 μ (Phase Separation, USA). The mobile phase consisted of water with 0.01 M perchloric acid, 0.002 M triethylamin and 0.1 M ammoniumacetate. Chromatography was carried out at ambient temperature. The flowrate was 3.0 ml/min.

Methods

Preparation of plasma-samples

0.2 ml plasma was diluted with 0.6 ml water. 20 μl was injected with a loop.

Preparation of urine-samples

10 μl urine was diluted with 2 ml water. 20 μl was injected with a loop.

Preparation of Calibration Samples

Plasma: Pool-plasma was spiked with T in the range of 1-100 ng/ml and with HTS in the range of 20 - 1100 ng/ml. Dilution was carried out as indicated above.

Urine: Pool-urine was spiked with T in the range of 0.1 - 1.7 μg/ml and with HTS in the range of 5-54 μg/ml urine. Dilution was carried out as above.

RESULTS

Recovery

As only a single dilution process was carried out it amounts to 100 %.

Reproducibility and accuracy for plasma

The within-day standard deviation (Precision) for HTS in calibration samples was in the range of 0.2 - 2.2% (CV) and the day to day variation for T in calibration samples was between 1.8 - 6.8% (CV) (Table 1 and Table 2).

REPRODUCIBILITY of volunteer-samples showed the following results:

T in plasma

concentration range	n	CV % range	median (CV %)
1- 10 ng/ml	7	0,0 - 17,9	3,8
20- 40 ng/ml	13	0,0 - 8,8	2,4
50-160 ng/ml	17	0,1 - 9,6	1,6

HTS in plasma

concentration range	n	CV % range	median (CV %)
5- 100 ng/ml	6	0,1 - 18,5	3,2
100- 500 ng/ml	16	0,3 - 8,8	1,3
500-1000 ng/ml	17	0,0 - 5,8	2,0

Linearity

In the calibration series, the linear regression between the spiked plasma concentrations and the peak areas was determined after analysis of the calibration samples. When multiple analysis was carried out over the range from 1-100 ng T/ml plasma a typical correlation coefficient value of 0,9983 was obtained. With HTS in the range of 20-1100 ng/ml plasma a typical value of r was 0,9999. In practice, a detection limit of 1 ng/ml T and 5 ng/ml HTS was fixed, but this could be lowered without difficulty.

Proof of Method Specificity

Hydrochlorothiazide, Furosemide, Chlorthalidone, Bendroflumethiazide, Butizide,various ß-blocking agents, Nifedipine and Hydroxytriamterene within therapeutical ranges did not influence the determination of T and HTS in plasma and

TABLE 1

REPRODUCIBILITY and ACCURACY of plasma T determinations (day to day variation over 3 days)

Spiked value (ng/ml)	Number of samples	Assay value (ng/ml)	CV %	Accuracy day to day (3 days)
1.0	6	0.87	4.2	- 12.6 %
5.0	6	4.91	6.8	- 1.8 %
30.0	6	28.90	4.3	- 3.7 %
100.0	6	99.90	1.8	- 0.1 %

TABLE 2

PRECISION and ACCURACY of plasma HTS determinations (within day)

Spiked value (ng/ml)	Number of samples	Assay Value (μg/ml)	CV %	Accuracy within day
21.4	3	18.7	2.2	- 12.5 %
107.0	3	107.0	0.8	0.0 %
428.0	3	435.7	1.1	+ 1.8 %
1070.0	3	1067.0	0.2	- 0.3 %

urine. Some drugs have no fluorescence (EX 360 nm/EM 436 nm) and/or different retention times. Amiloride might interfere but was not tested in this pharmacokinetic study on volunteers.

Chromatography

Different types of columns were tested. On C18 and C8 the elution order of T and HTS was changed and both substances could not be determined within one run. On the other hand a certain percentage of methanol is necessary for elution in reversed phase, as ion-pairs or as free bases. For this reason plasma may not be injected without protein precipitation.

The retention times for HTS and T on Spherisorb-Amino were relatively stable but after injection of about 100 samples (plasma or urine), the column was cleaned with 60% methanol.

After injection of plasma and urine samples taken from 12 different volunteers before drug administration no interfering peaks were observed. Also after injection of volunteer-samples following oral administration of T, there were no subsequent interfering eluting peaks.

Precision for urine

Table 3 shows the results for HTS (0.9 - 2.1% CV) for calibraton samples and Table 4 for T (1.2 - 4.1% CV) for calibration samples.

In volunteer samples the reproducibility was lower than 5 % for HTS and for T..

TABLE 3

PRECISION and ACCURACY of urine HTS determinations (within day)

Spiked value (μg/ml)	Number of samples	Assay Value (μg/ml)	CV%	Accuracy
5.35	3	5.40	1.0	+ 0.1 %
21.4	3	21.02	2.1	- 1.8 %
53.5	3	53.53	0.9	+ 0.1 %

TABLE 4

PRECISION and ACCURACY of urine T determinations (within day)

Spiked value (μg/ml)	Number of samples	Assay Value (μg/ml)	CV%	Accuracy
0.109	3	0.112	2.5	+ 2.8 %
0.436	3	0.432	4.1	- 1.0 %
1.744	3	1.745	1.2	0.0 %

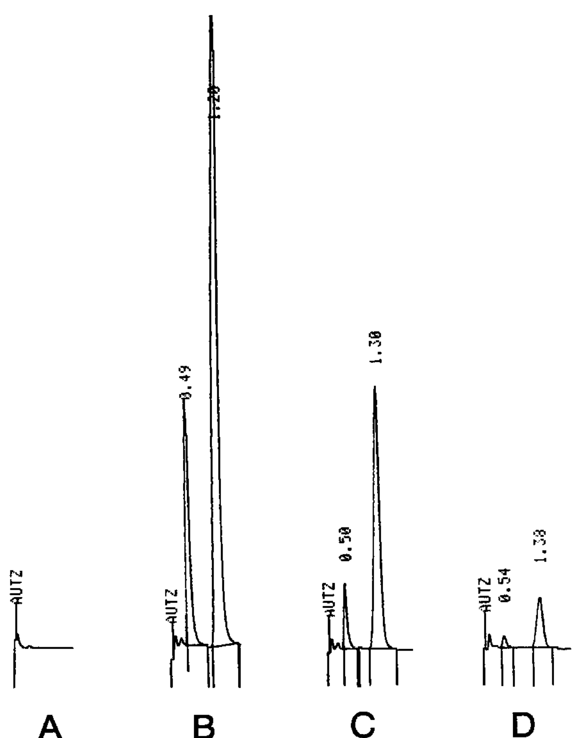

FIGURE 2:

Plasma levels after oral administration of 50 mg Triamterene (in a combination with Hydrochlorothiazide) are shown:

- A: before drug administration
- B: 20 min after drug administration (69.9 ng T and 376 ng HTS resp./ ml plasma)
- C: 4 hours after drug administration (19.4 ng T and 169 ng HTS resp./ ml plasma)
- D: 12 hours after drug administration (3.7 ng T and 31 ng HTS resp./ ml plasma)

HPLC conditions:

mobile phase:	0.01 M perchloric acid/ 0.004 M triethylamine/ 0.1 M ammoniumacetate
column:	Spherisorb-Amino, 5 μm, 125 x 4 mm ID
detection:	Fluorescence 360 nm excitation, 436 nm emission

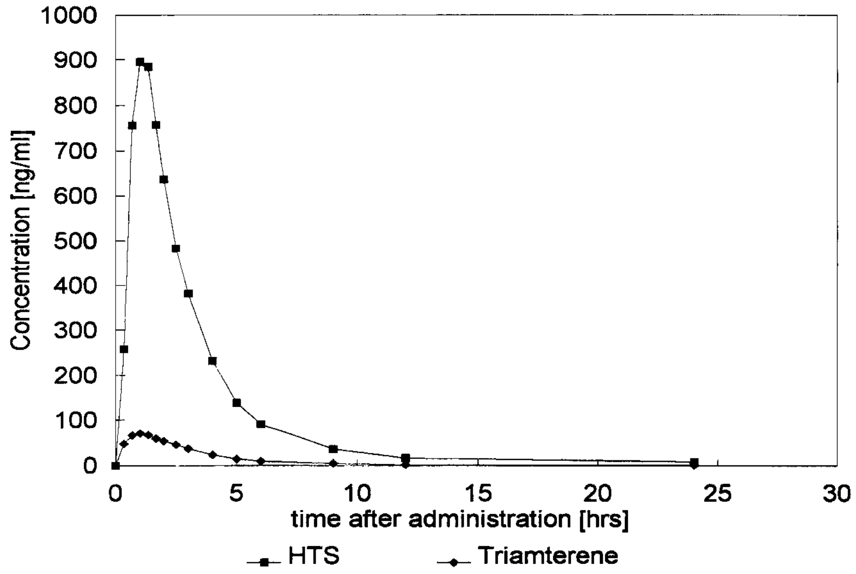

FIGURE 3:

Mean plasma levels of Triamterene and HTS from 12 volunteers. Oral dosage was 50 mg Triamterene (in combination with 25 mg Hydrochlorothiazide)

Chromatography

Figure 2 shows that the retention time for T is approx. 0.5 minutes and for HTS approx. 1.3 minutes. Determination limits as required in practice can be easily obtained.

Plasma levels after oral administration

Figure 3 shows mean plasma levels of T and HTS from 12 volunteers after oral administration of 50 mg T.

CONCLUSION

The method of analysis described above shows fast and accurate determination of T and HTS in plasma and urine, with high precision, reproducibility and accuracy. As most of the published papers analyse only one of the substances concerned, in a single chromatographic run, and/or require preliminary cleansing of the samples, the method described in this paper may be regarded as a real improvement.
The method described was used in a bioavailability study on 12 volunteers with 2 different oral galenic formulations.

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DETERMINATION OF BENZIODARONE IN HUMAN PLASMA AND TABLETS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A rapid HPLC method is described for benziodarone assay in human plasma using a LiChrosorb RP-18 column and UV detection at 240 nm. Protein precipitation is followed by extraction of benziodarone and khellin (internal standard). Extraction of the active substances with diethyl ether at pH ca. 4.8 ensures fairly good recovery (91.04%, mean). Detection limit for this method of determination is 20 ng using a 1 ml sample. The method is specific and can also be used for the determination of benziodarone in pharmaceuticals (tablets).

INTRODUCTION

Benziodarone, 2-ethyl-3-benzofuranyl 4-hydroxy--3,5-diiodophenyl ketone is a drug effective in the treatment of cardiovascular disorders. It has also been shown to have uricosuric activity and is proposed for treatment of primary hyperuricemia; in this case benziodarone can be administered in combination with allopu-

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rinol. The both substances have different mechanisms of action but they act in synergism. Several studies on the properties of benziodarone in UV and visible spectral regions have been published (1-3) but there are no publications about the assay of benziodarone in pharmaceuticals and biological fluids.

MATERIALS

Reagents

Benziodarone was obtained from Sanofi-Labaz (France), "Uricodue" - tablets (50 mg of benziodarone and 100 mg of allopurinol) were obtained from Istituto Farmacoterapico Italiano S.p.A. (Italy), khellin was purchased from Fluka AG, Buchs SG (Switzerland). Heparinized human whole blood was obtained from the District Blood Centre in Lublin. All other reagents and solvents were of analytical grade.

Apparatus

A type LC-730 liquid chromatograph (Laboratorni Přistroje Praha, Czechoslovakia) with a variable - wavelength UV detector and a 250 x 4 mm steel column packed with LiChrosorb RP-18 (dp = 5 μm) was used. A reciprocating shaker, type 327 (Premed, Poland) and a high - speed centrifuge, type T 52.1 (Zentrifugenbau Engelsdorf, Germany) were applied.

METHODS

The mobile phase was acetonitril-phosphate buffer pH 2.6 (0.067 M potassium dihydrogen phosphate adjusted to pH 2.6 with phosphoric acid, pH tolerance ± 0.1), 9:1 (v/v).
The flow rate was 1 $ml \cdot min^{-1}$. Detection was by UV absorption at 240 nm, detector output range was 0.04

AUFS for assay in plasma, 0.08 AUFS for assay in tablets, recorder chart speed was 0.3 $cm \cdot min^{-1}$.

Solutions

Stock solutions (1 $mg \cdot ml^{-1}$ and 10 $mg \cdot ml^{-1}$) of benziodarone and khellin were prepared by dissolving appropriate amounts of the substances in methanol. Working dilutions of 0.01 $mg \cdot ml^{-1}$ and 0.1 $mg \cdot ml^{-1}$ of benziodarone and 0.005 $mg \cdot ml^{-1}$ and 0.1 $mg \cdot ml^{-1}$ of khellin were prepared from the stock solutions.

Linearity Test for the Plasma Samples

From the working solution of benziodarone (0.01 $mg \cdot ml^{-1}$) following volumes were pipetted: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 ml and 0.2 ml of the solution of khellin (0.005 $mg \cdot ml^{-1}$) was then added to each sample and made with mobile phase up to an equal volume of 1.0 ml. 20 µl of each sample was injected into the column. All measurements were repeated three times at each concentration. The calibration curve based on the peak height ratios of benziodarone to that of internal standard was constructed. It was then used to calculate the results.

Extraction Procedure

To the glass centrifuge tubes containing 1.0 ml of blood plasma (obtained from heparinized human blood) 0.2 - 1.2 ml of working solution of benziodarone (0.01 $mg \cdot ml^{-1}$) and 0.4 ml of working solution of khellin (0.005 $mg \cdot ml^{-1}$) were added. Then, acetonitril was added to a final volume of 3.0 ml and the mixture was centrifuged for 20 min at 1100 g. Afterwards 1.5 ml of supernatant plasma from each sample was pipetted to the funnels and 4.0 ml of phosphate buffer pH 4.8 (0.067 M

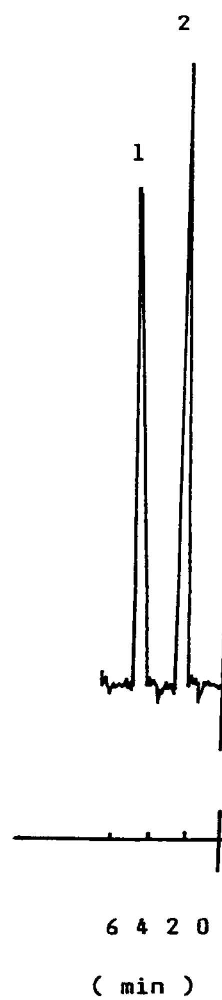

FIGURE 1. Typical chromatogram of benziodarone (1) and internal standard - khellin (2). Peaks correspond to 3.0 and 1.0 μg per ml of benziodarone and khellin, respectively.

potassium dihydrogen phosphate - 0.067 M disodium hydrogen phosphate, 9.75:0.25 v/v adjusted to pH 4.8 with phosphoric acid) and 3.0 ml of freshly distilled diethyl ether were added and then extracted by shaking for 10 min. The ether extract was separated and the aqueous phase was extracted once more with 3.0 ml of diethyl ether. The mixed ether extracts were evaporated to dryness in a stream of nitrogen. After dissolving each sample in 1.0 ml of mobile phase, 20 µl was injected into the column.
Fig. 2 shows typical chromatograms of extracted plasma.

Linearity Test for Tablets

From the solution containing 0.1 $mg \cdot ml^{-1}$ of benziodarone 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml were pipetted into 10-ml measuring flasks. Then, 0.2 ml of the solution of khellin (0.1 $mg \cdot ml^{-1}$) was added to each sample and made with acetonitril up to 10.0 ml. 20 µl of each sample was then injected into the column. The calibration was calculated in the same way as described above for plasma.

Determination of Benziodarone in Tablets

Tablets of benziodarone were ground to a fine powder and amounts equivalent to 2 - 12 mg (after a declaration) of the compound were extracted with methanol in 100-ml volumetric flasks. Filtered 1.0 ml volumes of the extracts were transfered into 10-ml flasks, 0.2 ml of khellin solution (0.1 $mg \cdot ml^{-1}$) was added and made with acetonitril up to 10.0 ml.
Then, 20 µl of each sample was injected into the column.

RESULTS AND DISCUSSION

Khellin, proposed here as an internal standard (i.s.), is a model compound for cardiovascular drugs of benzofuran and benzopyran type.

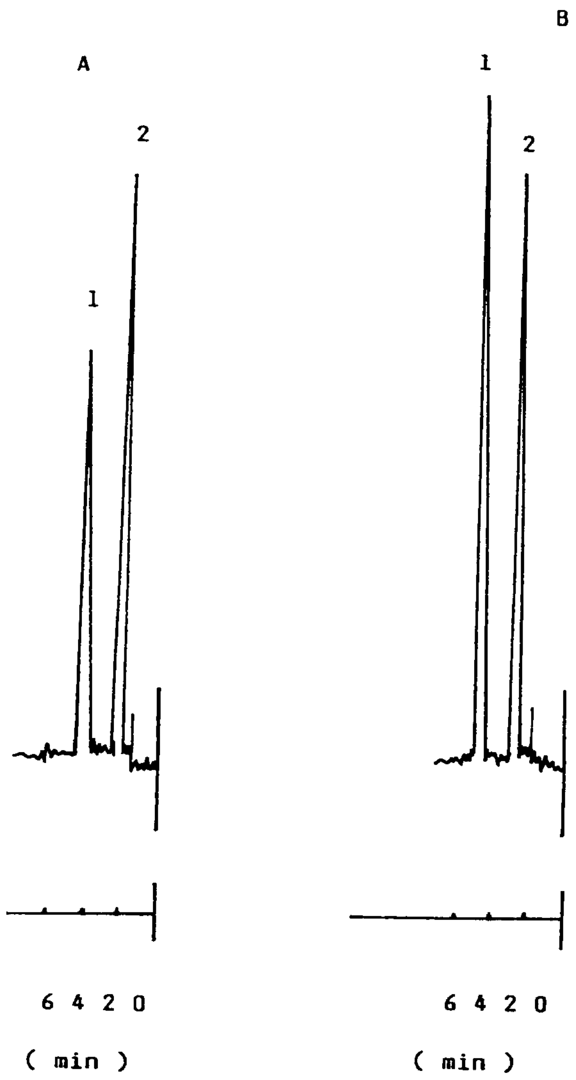

FIGURE 2. Typical chromatograms of extracted plasma treated as in the text containing:
A - 3.0, B - 5.0 μg per ml of benziodarone (1) and both 1.0 μg per ml of khellin (2).

TABLE 1

Concentration of Benziodarone (added) in Plasma (n = 3, at each level of addition)

No.	Benziodarone content (ng) in 20 µl of mobile phase	h/h'		Recovery (%)
		in working solutions of benziodarone and khellin	after extraction from plasma	
1	20	0.25	0.27	93.36
2	40	0.54	0.45	86.03
3	60	0.75	0.68	90.77
4	80	1.0	0.91	93.14
5	100	1.20	1.10	91.07
6	120	1.41	1.32	91.88

h = height of benziodarone peak, h' = height of khellin peak

In a preliminary study different pH conditions: 2.3 - 7.0 and various organic solvents were used for the extraction of benziodarone and i.s. from plasma. Diethyl ether at pH ca 4.8 gave the best reproducibility and a fairly good recovery (91.04%, mean). The data about concentration of benziodarone in plasma of patients treated with therapeutic doses of the drug have not been published yet. In the quantitative HPLC procedure described here benziodarone was reproducibly determined in plasma at concentration 1.0 - 6.0 $\mu g \cdot ml^{-1}$. Comparatively in plasma taken from patients treated with structurally similar drug - benziodarone (2-ethyl-3-benzofuranyl 4-hydroxy-3,5-dibromophenyl ketone) peak plasma concentrations of 1.4 - 2.9 $\mu g \cdot ml^{-1}$

were attained in about 3 hours following a single oral dose of 100 mg to 7 subjects (4); benziodarone is administered in intial dose of 600 mg daily and maintenance dose of 300 - 400 mg daily (4). In the range 1.0 - 6.0 $\mu g \cdot ml^{-1}$ the calibration curve for benziodarone assay in plasma was linear (r = 0.9983) and a regression line through the data points was y = 0.01147x + 0.0553 (x = concentration of benziodarone in ng per 20 µl of the mobile phase, y = h/h'). The precision of analysis was determined at six different concentrations of the drug.

The method described above makes it possible to determine of benziodarone also in pharmaceuticals. The calibration curve for benziodarone assay in tablets was linear (r = 0.9982) in the range 2.0 - 12.0 $\mu g \cdot ml^{-1}$ (regression line was y = 0.00764 x - 0.03). Recovery of benziodarone after extraction of tablets with methanol was found to be 98.30%, mean. Allopurinol (a component of "Uricodue" tablets) was found not to interfere in the assay (its retention time was 1.83 min).

Under the conditions used for the HPLC system, benziodarone and khellin had retention times 4.75 and 2.66 min, respectively. The method is simple, reliable and fairly sensitive and it is thought to be used in clinical and pharmaceutical analysis.

ACKNOWLEDGEMENTS

We wish to thank the firms Sanofi-Labaz for supplying benziodarone standard and Instituto Farmacoterapico Italiano for sending us "Uricodue" tablets.

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APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN THE DETERMINATION OF TAUROMUSTINE IN PRESENCE OF ITS METABOLITES AND DEGRADATION PRODUCTS

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ABSTRACT: A reversed phase high performance liquid chromatography (HPLC) method was developed for the determination of tauromustine (TM) in presence of its metabolited in biological fluids and its degradation products in tablets. The proposed HPLC method was conducted using a lichrosorb C_{18} column [250 X 4 mm], with acetonitrile - water - acetate buffer pH5 (40 : 55 : 5) eluent, the detection was affected at 235 nm. The detector response was linear in the range 0.2-4 μg/ml for TM with minimum detectability (S/N=2) of 2 ng/ml. The proposed HPLC method was applied to the determination of TM in presence of its metabolites in biological fluids. The percentage recoveries of TM and its metabolites from spiked urine range from 93.1 to 92.9 and for plasma 90.3 to 89.6 . The proposed HPLC method was used to study the kinetics of degradation of TM in standard solution as a function of temperature and alkalinity. TM degraded followed first-order kineties in agreement with the Arrhenius theory. The proposed

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HPLC method was applied in tablets and the results obtained were in good agreement with those obtained by the reference method.

Introduction

$$(H_3C)_2N-SO_2-CH_2-CH_2-NH-CO-N(NO)CH_2CH_2Cl$$

Scheme I

Tauromustine,1-(2-chloroethyl)-3-[2-(dimethylamino-sulphonyl)ethyl] 1-nitrosourea (TM) (scheme I), is a novel antitumour agent based on the endogenous aminoethane sulphonic acid, taurine. Preclinical studies with tauromustine have revealed its potent antitumour activity against several experimental tumours in vivo and in vitro [1,2] and its efficacy against nitrosourea - resistant tumours [3].

An HPLC method was described for the determination of tauromustine and its metabolites in plasma and urine [4,5]. However, this method used a cyano column, the half life of which is short [6], as it efficiency decrease with time, necessitating frequent changes of the column. Recently, a polarographic method was developed for the determination of tauromustine in tablets (7) and a fluorimetric method was described for the determination of tauromustine in formulations and biological fluids (8).

Although, tauromustine is easily decomposed in the dry state, nothing was published concerning its determination in presence of its degradation products. It is thus evident that, there is still a need for a more reliable method for the determination of tauromustine in presence of its degradation products as well as its metabolites.

Experimental

Apparatus:

HPLC pump : LKB 2150 provided by LKB-produkter AB, S-16126 BROMMA, Sweden.

Column : 5 μm Lichrosorb RP 18 (250 X 4 mm)
Injector loop : 50 μL.
Detector : LKB 2151 variable wavelength monitor.
Recording integrator : LKB 2220
Filter : Gamma 16 (5 μm)

Reagents and Materials:

Tauromustine, tablets and metabolites were kindly provided by Pharmacia, LEO, AB, Helsingborg, Sweden. Stock Solutions; aqueous solutions containing 1 mg/ml of TM was prepared, this solution was further diluted with water to give the final concentration required for preparation of calibration graph.

The mobile phase was an isocratic mixture of acetonitrile - water - acetate buffer (9) pH 5 (40 : 55 : 5), degassed befor use.

HPLC Analysis of Standared Solution of TM.

Standared solution containing various concentration of TM were chromatographed and the response peak heights were measured. The concentration of TM was calculated from the calibration graph or from the regression equation.

HPLC Analysis of TM Tablets:

Twenty tablets (20 and 50 mg) were weighed and pulverized. An accurately weighed amount of the powder equivalent to 100 mg of TM was extracted with 5X15 ml water, filtered and completed to 100 ml with water. Working solutions were prepared by dilution with water to contain 0.2 - 4 μg/ml.

HPLC Analysis of TM and its Metabolites in Biological Fluids:

The plasma or urin samples (5ml) were acidified with 2 M hydrochloric acid [10 μl per ml of plasma or urin], aqueous solutions of 1mg/ml of tauromustine (TM), demethyl tauromustine (DMTM) and didemethyl tauromustine (DDMTM) were added and the solution was thoroughly mixed in a vortex mixer for 15 min and diluted to 25 ml in a measuring flask with phosphate buffer (pH 6). The mixture was transformed to 50 ml separating funnel and extracted with 3X10 ml of chloroform, the organic phase was filtered through dry sodium sulphate then evaporated under a gentle stream of nitrogen. The residue was dissolved in bidistilled water. The solutions were then diluted with water to the working calibration region.

Effect of heat on degradation rate of TM in raw material.

TM standard solution was stored at ambient temperature (25± 0.2^{0}C) and 50 ^{0}C. Zero-time sample measurements were carried out

when the study began only on samples from flasks to be stored under ambient conditions. All original flaskes samples stored under accelerated conditions. The flasks were re-closed tightly by hand between sampling. For all storage conditions, the entire study was performed on duplicate flaskes of TM raw material. For aqueous solutions, samples equivalent to 100 mg of TM raw material were dissolved in 100 ml water, samples were diluted 1: 100 using water and poured into 10 ml flaskes and closed. the flaskes were placed in oven set at 50 ^{0}C and at specified time intervals individual samples were taken for analysis.

The percentage recoveries of TM in the pharmaceutical preparations were calculated either from a calibration graph obtained under the same conditions or by the external standard method.

Results and Discussion

The effect of acetonitrile content and pH on the number of theoretical plate (NTP) and phase capacity ratio (K`) for TM were studied. Adjusting the pH of 40% acetonitrile to 5 by using acetate buffer gave high resolution power of the mobile phase.

The resulting peak heights were proportional to TM concentration over the range 0.2-4 μg/ml with minimum detectability [S/N=2] of 2 ng/ml. Linear regression analysis of the plot of TM concentration over the cited concentration range and the measured peak heights gave the following regression equation:

$$C = 0.0107 \pm 0.567\ P \qquad (R^2 = 0.9997)$$

Where C is the concentration of TM in μg/ml and P (cm) is the peak height, R is the correlation coefficient.

To test the validity of the method it was applied to the determination of standard solutions of TM. The results abridged in Table I show that the proposed method is accurate and precise. The proposed method was further applied to the determination of TM in tablets, the results are in good agreement with reference method according to Table I . Tablets excipients such as talc, starch, lactose, gum, magnesium stearate did not interfere with the assay.

A typical chromatogram of TM in presence of its metabolites DMTM and DDMTM after extraction from biological fluids are shown in Fig. 1. The retention time was suitable enough to allow separation

Table I : Performance data for the determination* of tauromustine

Determination of tauromustine in	$\bar{X}$	S . D.	t**	F**
(1) Raw material by : -				
a - Proposd mthod .	100.1	0.7	0.1 (2.1)	1.1 (5.6)
b - Reference method (8)	100.1	0.6		
(2) Tablet (20 mg) by : -				
a - Proposed method .	99.8	0.7	1.1 (2.1)	1.5 (5.6)
b - Reference method (8)	99.6	0.6		
(3) Tablet (50 mg) by : -				
a - Proposed method .	99.9	0.6	1.1 (2.1)	1.6 (5.6)
b - Reference method (8)	99.3	0.7		
(4) In plasma	89.9	2.0		
with				
a - D M T M	89.6	0.6		
b - D D M T M	90.3	0.7		
(5) In urine	92.9	2.7		
with				
a - D M T M	93.3	1.5		
b - D D M T M	93.1	1.3		

* Each result is the average of four separate determination .

** Values in brackets are tabulatd -t- and -F- values (P = 0.05)

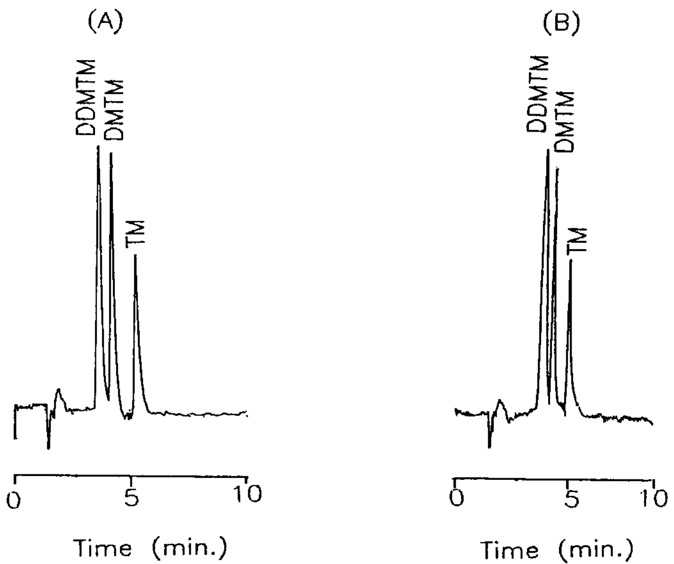

Fig. 1 :Chromatograms of urine (A) and plasma(B) spiked with 2.0 µg/ml DDMTM,DMTM and TM,the mobile phase; acetonitrile-water-acetate buffer (40:55:5) pH 4.5 for plasma and pH 6 for urine, flow rate 1.5 ml/min,detector wavelength 235 nm.

of TM from its metabolites. The method was tested for the presence of interfering peaks originating from various sources. These sources of interference could be eliminated by adjusting the pH of the mobil phase. Normally a pH value of 4.5 was chosen for plasma samples and of 6 for urine samples. The plasma or urine samples were acidified first then adjusted to pH 6 before extraction with organic solvent, this procedure minimized interference from other endogenous substance. Samples were extracted according to the procedure described above and the results are show in Tables I.

Accelerated stability study on TM was performed by heating the aqueous solutions of TM at 50 ^{0}C for different periods of time ranging from 5 - 120 min. The degraded solutions were analysed by the proposed method and the percentage remaining of undecomposed drug were calculated from the corresponding

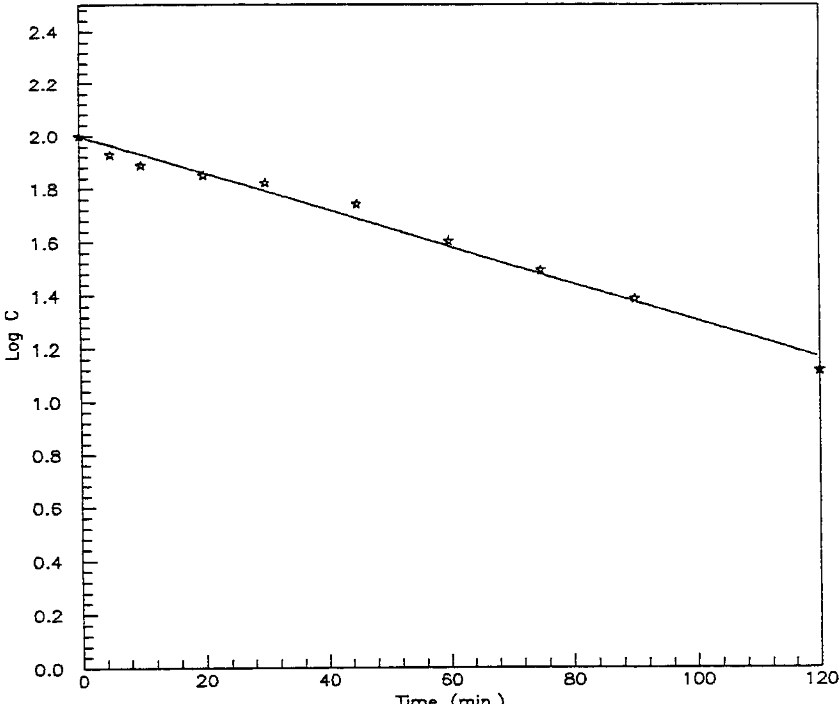

Fig. 2 : Plot of Log concentration remaining versus pH for the degradation of TM in aqueous solution at 50 C.

calibration graph . Plotting the value of the concentration remaining [Log. C.] against time indicate that TM degradation followed first-order kineties and gave a linear relationship [Fig.2.]. The presence of the degradation products of TM did not interfere in the determination of TM. Similarly, the stability of TM in alkalin medium (0.1 M sodium hydroxide) was studied. The drug was completely degraded in alkaline medium at once and could not be detected by proposed HPLC method.

On conclusion, the proposed HPLC method is rapid, sensitive, selective and accurate. The method is suitable for regular determination of TM. It is suitable for checking stability of its formulations.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) ASSAY FOR THE DETERMINATION OF CHLORHEXIDINE IN SALIVA FILM

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ABSTRACT

An HPLC assay for the determination of chlorhexidine in small samples (<1 µl) of saliva is described. A base deactivated reverse phase C-18 narrow bore column (ODS-B Exsil) was used for the analysis. Saliva samples were collected on Periopaper strips and chlorhexidine was extracted with 0.1 ml mobile phase. The optimal mobile phase comprised 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid, 7 mM sodium laurylsulphate and column temperature was maintained at 55°C. Benzethonium was included as the internal standard and a dual wavelength UV absorbance detector was used to analyse chlorhexidine at 254 nm and benzethonium at 275 nm. Triplicate standard curves were linear for the range of concentration 1 to 15 ng/sample ($R^2 > 0.99$). If 0.5 µl of saliva is collected then 2 to 30 µg/ml chlorhexidine can be measured. The intra-assay variability, determined from repeated injections of quality control standards containing 1, 3.75 and 12.5 ng chlorhexidine/tube was 12.9, 4.4 and 1.5% respectively.

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INTRODUCTION

Chlorhexidine is a bisdiguanide antiseptic discovered in 1956 [1]. It has a broad spectrum of antimicrobial activity and has been shown to be effective against plaque bacteria [2-5]. When given as a mouthrinse the drug is distributed throughout the oral cavity and its effectiveness is, in part, due to its ability to reversibly bind to the tissue surfaces in the mouth and drug can be detected in the saliva for up to 24 hours in some individuals [6-9]. Analysis of the chlorhexidine concentration usually involves collection of samples from the saliva pool and average concentrations are reported. Problems identified with the use of chlorhexidine mouthrinses include the bitter taste imparted by the high drug concentration (0.2 %w/v) and tooth discolouration that occurs with prolonged use [10, 11]. Attempts to overcome these problems have resulted in the development of delivery systems that employ smaller quantities of chlorhexidine and deliver the drug to specific sites in the mouth [12-14]. It is therefore important to monitor the chlorhexidine concentration at the desired site of action to assess the effectiveness of the delivery system.

The major problem encountered in analysis of drugs at some sites in the mouth is the small amount of fluid available for sampling. The volume and thickness of the saliva film vary throughout the mouth and an average thickness of 0.07 to 0.1 mm has been reported by Collins and Dawes [15]. In periodontal pockets the volume of fluid depends on the severity of inflammation and an average volume of 0.5 μl has been reported at diseased sites compared to 0.04 μl at healthy sites [16]. Filter paper strips are used to collect these small samples, but the drug must be extracted and diluted to provide sufficient volume for injection in HPLC analysis [17-19]. HPLC assays have been reported for the determination of chlorhexidine in pharmaceutical preparations [20, 21] or biological fluids [22-25], but these are not sensitive enough to allow analysis of chlorhexidine in the saliva film or periodontal pocket.

We report an HPLC assay for the determination of chlorhexidine using a reverse phase base deactivated C-18 narrow bore column and show that it may be used to determine the chlorhexidine concentration in the saliva film at the gingival margin after administration of a 0.2 %w/v mouthrinse.

MATERIALS AND METHODS

Chemicals and reagents

Chlorhexidine diacetate B.P. was purchased from ICI Chemicals (Wellington, New Zealand). The internal standard, benzethonium chloride was AnalaR grade purchased from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile, methanol and glacial acetic acid were HPLC grade purchased from Ajax Chemicals Pty. Ltd. (Auburn, N.S.W., Australia) and sodium laurylsulphate and triethylamine were HPLC grade purchased from BDH chemicals Ltd (Poole, England). Deionised water was produced with a Millipore Milli-Q system (Bedford, MA, U.S.A.).

Equipment

A Spectra Physics HPLC system was used comprising a SP8800/8810 ternary pump, a Spectra System UV 2000 dual wavelength detector, a SP4400 Chromjet integrator and a Rheodyne injector with a 50 µl sample loop. The stainless steel column, 10 cm x 2.1 mm i.d. was packed with 5 µm C18 ODS-B Exsil purchased from HiChrome Ltd. (Berkshire, England).

Drummond microcap tubes (2 µl) were purchased from Drummond Scientific Co. (Broommall, PA, U.S.A.). Periopaper strips were purchased from Harco Electronics Ltd. (Winnipeg, Canada). Polypropylene centrifuge tubes (Eppendorf) were purchased from Salmond-Smith Biolab (Christchurch, New Zealand). Saliva sample volumes were calculated from masses determined using a five decimal place Sartorious analytical balance and a saliva density of 1.002 - 1.012 g/ml [26].

Optimisation of the HPLC method for chlorhexidine

The mobile phase was pumped at a rate of 0.5 ml/min for all analyses. The effects of changes in the concentration of ion-pairing agent, sodium laurylsulphate

and acetonitrile on the retention and separation of chlorhexidine and benzethonium were investigated. Samples contained 1 μg/ml chlorhexidine and 10 μg/ml benzethonium in mobile phase.

Extraction of chlorhexidine from filter paper strips

A 10 μg/ml chlorhexidine solution was prepared in deionised water. Samples of about 0.5 μl were measured with Drummond microcap tubes and placed onto Periopaper strips. The exact volume of each sample was calculated from the length of liquid in the Drummond microcap tube. The Periopaper strips consist of a strip of filter paper attached to a plastic tag. The plastic tags were removed and the filter paper strips were placed in 0.6 ml polypropylene tubes (Eppendorf). 0.1 ml of the extracting solution, which comprised 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid and 7 mM sodium laurylsulphate was added to each tube. These were vortexed for one minute, sonicated for 20 minutes (Bransonic ultrasonic bath, 100 W, 55 000 Hz), then vortexed again prior to analysis (extracts). Controls were prepared by direct addition of the 0.5 μl samples into 0.1 ml of extracting solution and were treated in the same way as the extract samples. The internal standard (benzethonium) was included in the extraction solution at a concentration of 2 μg/ml and both the chlorhexidine and benzethonium peaks were analysed at 254 nm. Extraction and control experiments were performed in triplicate and the percentage extracted was calculated by comparison of the peak height ratio after correction for the sample volume. In addition, a similar dilution of the original chlorhexidine solution was performed using larger volumes i.e. 10 μl in 2 ml (standards).

Preparation of chlorhexidine standards

Standard curves were prepared, on three days, for the extraction of chlorhexidine from Periopaper strips to validate the assay procedure. For each standard curve triplicate chlorhexidine solutions (100 μg/ml in deionised water) were prepared and diluted to give standards with chlorhexidine concentrations of 2, 5, 10, 20 and 30 μg/ml in deionised water. These were diluted 1 in 200 (10 μl

in 2 ml) with mobile phase containing the internal standard (2 μg/ml benzethonium). 0.1 ml was transferred to 0.6 ml polypropylene tubes (Eppendorf) containing one piece of filter paper (cut from a Periopaper strip) per tube. The tubes were pre-rinsed with methanol. 50 μl of each standard was injected into the HPLC and chlorhexidine peaks were analysed at 254 nm while the internal standard peaks were analysed at 275 nm using a dual wavelength detector. Peak height ratios were calculated and results expressed as the amount of chlorhexidine per tube (1, 2.5, 5, 10 and 15 ng/tube chlorhexidine). Within day accuracy and precision were determined by preparation of three quality control solutions containing 2, 7 and 25 μg/ml chlorhexidine. These were processed in the same way as the standards and analysed repeatedly (n=5) in a random order throughout the first standard curve. The chlorhexidine concentrations were determined from the standard curve and the mean, coefficient of variation and percent deviation from the theoretical concentration were calculated. Between day variation was assessed by comparison of the slope and intercept of the three standard curves prepared on three separate days.

Measurement of the chlorhexidine concentration in the saliva film after administration of a 0.2 %w/v mouthrinse

The chlorhexidine concentration in the saliva film at the gingival margin was measured in two subjects after administration of a 0.2 %w/v chlorhexidine mouthrinse. The mouthrinse was prepared in deionised water and 10 ml was rinsed around the oral cavity for one minute. Samples were collected on Periopaper strips at the gingival margin on the buccal side of the upper second premolars (left = BL and right = BR) and the lingual side of the lower second premolars (left = LL and right = LR) immediately prior to application of the mouthrinse then at 0.5, 3, 6 and 8 hours post-mouthrinse.

The sample volume was measured by mass and samples were stored at 4°C until analysis. Periopaper strips were placed in 0.6 ml polypropylene tubes (Eppendorf) and weighed using a five place analytical balance (w_1). To collect a sample the Periopaper strip was removed and placed at the sampling site in the oral cavity, then the plastic tag was cut and the filter paper strip returned to the

Eppendorf tube. The plastic tag was retained and weighed again with the Eppendorf tube containing the saliva sample (w_2). The sample volume was calculated as the weight difference (w_2 - w_1) multiplied by the density of saliva, which was taken to be 1.002-1.012 g/ml (total saliva) [26].

Standards at concentrations of 1 and 15 ng/tube were prepared in triplicate as above and used to determine the slope and intercept of the standard curve at the time of sample analysis. Samples were prepared by addition of 0.1 ml of the extracting solution to the tube containing the sample on Periopaper and vortexed, then sonicated for 20 minutes (Bransonic ultrasonic bath) and vortexed again before analysis. Quality control samples of concentrations 2, 7 and 25 μg/ml were prepared as previously and analysed in a random order throughout the run.

RESULTS AND DISCUSSION

Optimisation of the HPLC method for chlorhexidine

Good peak shape was obtained for chlorhexidine when the base deactivated column (ODS-B Exsil) was used. In comparison, normal reverse phase materials such as C-18 ODS Hypersil produced poor peak shape with significant tailing. Initially, these effects were overcome with addition of a competitive amine, triethylamine, into the mobile phase [27]. However some tailing remained and the minimum quantifiable concentration of this earlier assay was 0.1 μg/ml. This would allow measurement of chlorhexidine at concentrations greater than 10 ng/tube (or 20 μg/ml in 0.5 μl saliva) which was not good enough as the minimum inhibitory concentration of chlorhexidine for some oral pathogens is 8 μg/ml [28]. Inclusion of the hydrophobic ion-pairing agent, sodium laurylsulphate into the mobile phase increased the retention of chlorhexidine and improved the peak shape. The effects of alterations in the concentration of sodium laurylsulphate is shown in Figure 1. Separation of chlorhexidine and benzethonium peaks could be obtained with a mobile phase comprising 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid and 5 mM sodium laurylsulphate. This separation could be maintained and the analysis time reduced from 8 to 4 minutes if the acetonitrile concentration was increased to 60 % v/v.

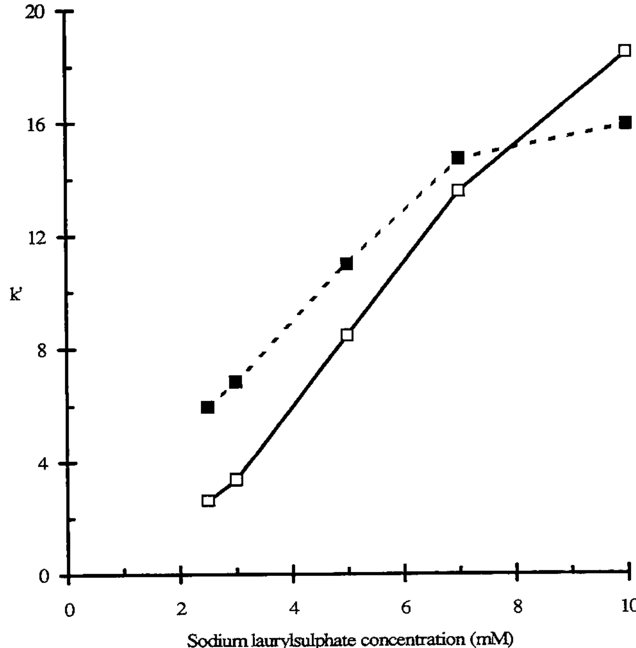

FIGURE 1

Effect of alterations in the concentration of sodium laurylsulphate on the retention of chlorhexidine —□— and benzethonium - - ■ - - . Acetonitrile concentration = 55 %v/v, glacial acetic acid concentration = 0.2 %v/v.

When dilute chlorhexidine solutions were prepared in acidic conditions (50 ng/ml chlorhexidine in 1 %v/v glacial acetic acid) a decrease in the chlorhexidine peak height was noticed. These solutions were stored in 1.5 ml polypropylene tubes and it was thought that the chlorhexidine may have adsorbed to the container surfaces. Table 1 shows the changes in chlorhexidine peak height with time for solutions in 1 %v/v glacial acetic acid. The loss of chlorhexidine was not prevented by addition of triethylamine (0.25 %v/v). It was thought that addition of a competitive amine would prevent or reduce adsorption if it bound to the same sites as chlorhexidine, but as a reduction in peak height of similar magnitude was observed to that seen in 1 %v/v glacial acetic acid it would appear

TABLE 1

Variation in chlorhexidine peak height for solutions containing 50 ng/ml chlorhexidine in 1 %v/v glacial acetic acid stored in 1.5 ml polypropylene Eppendorf tubes.

Solvent	Time (h)	Chlorhexidine peak height mean ± s.e.m. (n=2)
1 %v/v glacial acetic acid	0	606 ± 37
	3	186 ± 28
	5	ND
with 5 mM sodium laurylsulphate	0	1280 ± 60
	3	1290 ± 60
	5	1330 ± 110
with 0.25 %v/v triethylamine	0	524 ± 4
	3	88 ± 19
	5	ND

ND = not determined.

the amine groups are not responsible for this interaction. Addition of 5 mM sodium laurylsulphate to the solution prevented the chlorhexidine loss and the peak height remained stable for at least five hours. It is possible that ion-pairing of chlorhexidine and sodium laurylsulphate prevents the interaction between the polypropylene tube and the ionised chlorhexidine.

Extraction of chlorhexidine from filter paper strips

Table 2 shows the peak height ratios for chlorhexidine extracted from Periopaper strips. Although there was no significant difference between the peak

TABLE 2

Extraction efficiency for extraction of chlorhexidine from Periopaper strips

Solution	Peak height ratio mean ± s.e.m. (n=3)
extracts	2.19 ± 0.05*
controls	2.06 ± 0.11*
standards	2.10 ± 0.12*

* no significant differences $p>0.05$

height ratios when analysed using an ANOVA ($p>0.05$), the peak height ratio for the controls tended to be lower than that for extracts or standards. This may be due to the difficulty removing the sample from the Drummond microcap tube in the absence of a Periopaper strip. If the peak height ratios for extract samples is compared with the standard solutions, 100 percent extraction was achieved.

Analysis of standards and extracts

Some interference peaks were extracted from the Periopaper strips and these were separated from the chlorhexidine and internal standard peaks by adjustment of the column temperature and mobile phase composition. The increased temperature caused sharpening of peaks and decreased the retention time. Optimal conditions for analysis of chlorhexidine and benzethonium in the presence of Periopaper strips was a mobile phase comprising of 55 %v/v acetonitrile, 0.2 %v/v glacial acetic acid and 7 mM sodium laurylsulphate and a column temperature of 55°C. Standard curves were linear over the range of chlorhexidine concentrations 2 to 30 μg/ml ($R^2 > 0.99$). These gave final amounts of chlorhexidine after extraction of 1 to 15 ng per tube. The within day variability and accuracy and the between day variability are shown in Table 3.

TABLE 3

a) Within day reproducibility and accuracy

Chlorhexidine concentration (ng/tube)	Mean observed concentration	n	C.V. (%)	Accuracy (%)
1	1.13	4	12.4	113
3.75	3.67	5	4.4	97.8
12.5	12.2	5	1.5	97.7

b) Between day variability

Standard curve	R^2	Slope ± std. error	Intercept ± std. error
Day 1	0.995	0.109 ± 0.001	-0.024 ± 0.014
Day 2	0.998	0.101 ± 0.001	0.058 ± 0.009
Day 3	0.999	0.1003 ± 0.0007	0.051 ± 0.006

Measurement of the chlorhexidine concentration in the saliva film after administration of a 0.2 %w/v mouthrinse

The chlorhexidine concentration in saliva, at the gingival margin of the second pre-molars, is shown in Table 4 and typical chromatographs for saliva samples are shown in Figure 2 and Figure 3. The chlorhexidine concentration was generally higher at the gingival margin of the upper second pre-molars (BL and BR) and this was expected as these samples were taken from the buccal side, whereas samples from the lower second pre-molars were taken from the lingual side (LL and LR). It is possible that chlorhexidine was more rapidly diluted at these lower sites as they are closer to the major salivary glands and a greater mixing of the saliva by the tongue would be expected. These factors have been shown by Dawes and Weatherell [29] to be important in the distribution and clearance of fluoride in the oral cavity. A large variation was observed in the chlorhexidine concentrations

TABLE 4

Chlorhexidine concentration (μg/ml) in the saliva film at the gingival margin at four sites within the mouth after rinsing with a 0.2 %w/v chlorhexidine solution

	Subject one					Subject two				
	pre-rinse	post-rinse (h)				pre-rinse	post-rinse (h)			
Site*		0.5	3	6	8		0.5	3	6	8
BL	0	81	12	-	-	0	194	40	9	27
BR	0	ND	20	6	6	0	94	29	25	22
LL	0	19	23	-	-	0	26	28	30	10
LR	0	15	26	-	-	0	71	23	9	18

* BL = buccal gingival margin on the upper left second pre-molar.
BR = buccal gingival margin on the upper right second pre-molar.
LL = lingual gingival margin on the lower left second pre-molar.
LR = lingual gingival margin on the lower right second pre-molar.

\- = chlorhexidine less than 1 ng/tube.
ND = not determined.

achieved at different sites in each subject and between subjects. This was particularly apparent in the first post-rinse sample and may be due to differences in the degree of mixing and rates of clearance from the individual sites. Subject one ate a meal between the samples at times three and six hours and this may be responsible for the lack of chlorhexidine at six and eight hours post rinse. In contrast, subject two did not eat over the eight hour period and concentrations of between 10 and 27 μg/ml were measured eight hours after rinsing. These values are similar to those obtained when radiolabelled chlorhexidine was used [7]. The advantage of this method is that radiolabelled chlorhexidine is not required and the

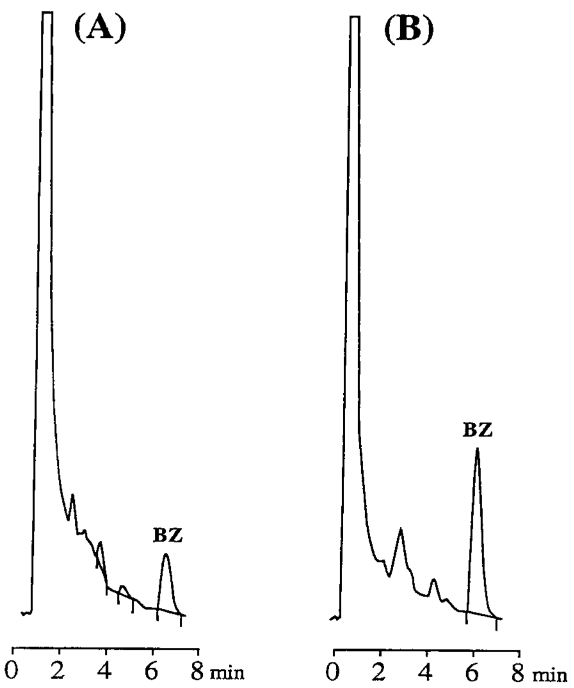

FIGURE 2

Typical chromatograph of saliva extracts. Blank saliva sample, Subject 1 (sampling time = pre-mouthrinse; sample site = buccal gingival margin on the upper right second pre-molar; saliva volume = 0.72 µl). Detector wavelengths; (A) = 254 nm and (B) = 275 nm. BZ = internal standard (2 µg/ml benzethonium).

concentration at different sites can be determined. It was interesting to note that chlorhexidine was not able to be detected in samples taken from the gingival margin in the subject who ate during the post rinse period. This may indicate that the gingival margin clearance is accelerated by eating and although chlorhexidine may be detected in the saliva pool there may be sites within the mouth where an effective concentration is not maintained. Further studies are being undertaken to confirm this and to determine the clinical significance.

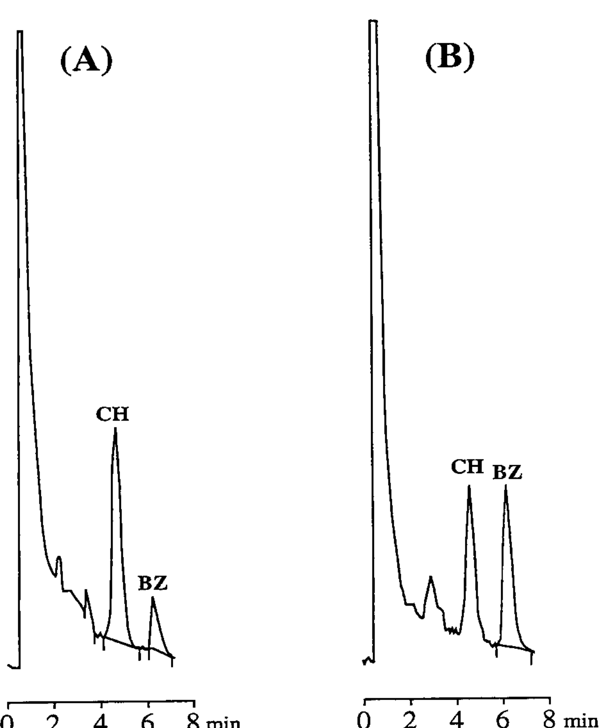

FIGURE 3
Typical chromatograph of saliva extracts. Subject 2 (sampling time = 3 hours post-mouthrinse; sample site = buccal gingival margin on the upper right second pre-molar; saliva volume = 0.46 µl). Detector wavelengths; (A) = 254 nm and (B) = 275 nm. CH = chlorhexidine; BZ = internal standard (2 µg/ml benzethonium).

CONCLUSION

This assay allows measurement of chlorhexidine concentrations greater than 2 µg/ml in 0.5 µl saliva. Since the minimum inhibitory concentration of most oral pathogens is greater than 8 µg/ml [28] it allows monitoring of individual sites within the mouth and determination of the effectiveness of chlorhexidine delivery systems in producing and maintaining effective antibacterial concentrations.

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EXTRACTION PROCEDURES FOR THE HPLC DETERMINATION OF 5-FLUOROURACIL IN BIOLOGICAL SAMPLES

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ABSTRACT

An extraction procedure was optimized for the reversed-phase high performance liquid chromatographic determination of 5-Fluorouracil in rabbit plasma, liver, kidney, lung and heart samples. The extraction conditions are adapted to each type of matrix in order to achieve high extraction yields (*ca.* 90%) and interference-free chromatographic peaks.

INTRODUCTION

5-Fluorouracil (5-Fu) is widely used as an antitumour drug, so the determination of residual concentrations of this substance in various body parts following administration is of great interest in order to optimize therapeutic doses and identify potential metabolic pathways or side effects. Most of the revised literature on this substance is concerned with clinical, pharmaco – kinetic and therapeutic aspects rather than with its analytical determination. Lately, special attention has been paid to the extraction and determination of this drug in plasma samples in preference to tissue samples, available literature on which is rather scant.

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Sample pretreatment procedures used in the determination of 5-Fu are quite varied in nature, but most involve a long string of steps, one of which is common to most: deproteination, which can be accomplished by using a precipitating agent [1–4], membrane ultrafiltration [5] and ion-exchange [6], chiefly. A second step involves extraction from the matrix with an appropriate mixed solvent (*e.g.* *n*-propanol/water [7], *n*-propanol/ether [8], chloroform/methanol [9] or chloroform/propanol [10]).

After the drug is isolated, it is normally quantified by high performance liquid chromatography (HPLC).

As a rule, the proponents of such procedures fail to mention recoveries or any differences arising from the type of matrix involved —many authors use a single, scarcely efficient procedure for every type of sample [11]. This prompted us to develop extraction/clean-up procedures providing high drug recoveries from such matrices as plasma, liver, kidney, lung and heart, using *n*-propanol/ether mixtures as extractants and optimizing the working conditions for each type of matrix —the goodness of each procedure was checked by using HPLC. All samples employed in this work were from experimental rabbits.

EXPERIMENTAL

Chemicals

5-Fluorouracil (5-Fu) was purchased from Sigma (St Louis, MO). Ammonium dihydrogen phosphate and all other chemicals used to prepare buffers were analytical-reagent grade and supplied by Merck (Darmstad, Germany). The water employed was purified by passage through a Nanopure II system from Barnstead (Newton, MA). All solvents used were HPLC-grade and obtained from SDS (Peypin, France).

Apparatus

The chromatographic set-up used consisted of a CD4000 multi-solvent partitioning pump and an SM4000 variable-wavelength UV-visible detector, both from LDC Analytical (Riviera Beach, FL), in addition to a JCL6000 Chromatography Data system from Jones Chromatography (Colorado).

An ultrasonic bath, a vibromatic stirrer, a centrifuge (all three from Selecta, Spain) and a rotavapor from Büchi (Italy) were also used.

Chromatographic conditions

The column used was 25 long × 0.46 cm ID and packed with Spherisorb 5 ODS resin of 5-μm particle size from Phenomenex (Torrance, CA). The mobile phase was 0.05 M phosphate buffer of pH 3.5 and was pumped at a flow-rate of 1 ml/min. Samples were injected by means of a Marathon autosampler from Spark Holland (Emmen, The Netherlands) furnished with a fixed-volume (20-μl) loop. Detection was performed at 254 nm.

Experimental animals

The animals used in the experiments were untreated rabbits from which plasma and tissue samples were withdrawn on sacrificing.

Plasma samples were collected in tubes containing sodium heparin that were centrifuged for 10 min and frozen until analysis. Tissue samples were ground, added physiological serum and frozen.

All experiments involving these animals were carried out in compliance with accepted norms.

Extraction/clean-up

Experiments were always performed in duplicate by using two groups of samples of the same weight for each type of matrix, to which known amounts of 5-Fu were added. One group was used to obtain the chromatogram background and the other to assess recovery.

Preliminary experiments involving ultrafiltration and solid–liquid extraction with cartridges containing various materials (C_{18}, C_8, NH_2, CN and PH functions) provided poor recoveries, so solvent extraction with various mixtures was assayed next. Thus, an amount of 0.5 g of tissue or 1 ml of plasma was added the extractant; the mixture was immersed in an ultrasonic bath for an appropriate time and then centrifuged in order

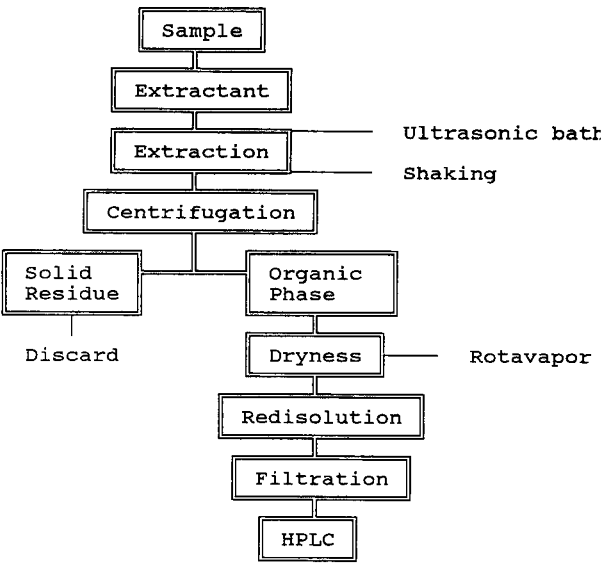

Figure 1 – Scheme of the overall procedure.

to separate the two phases. The organic phase was then evaporated to dryness in a rotavapor and the solid residue dissolved in 1 ml of 0.05 M ammonium phosphate. The resulting solution was used to inject 20-μl aliquots into the chromatograph. The process is schematically depicted in Fig. 1.

After several preliminary experiments, the best results were found to be provided by an *n*-propanol/ether mixture, consistent with previous findings in dealing with vitreous material [12]. However, the previously developed procedure was inapplicable to all the sample types studied in this work as it resulted in the simultaneous extraction of compounds that coeluted with 5-Fu or in very low extraction yields. Consequently, the influence of parameters potentially affecting the extraction process for each type of sample was studied in order to establish the optimal conditions for maximal recovery of 5-Fu with minimal extraction of potential interferents.

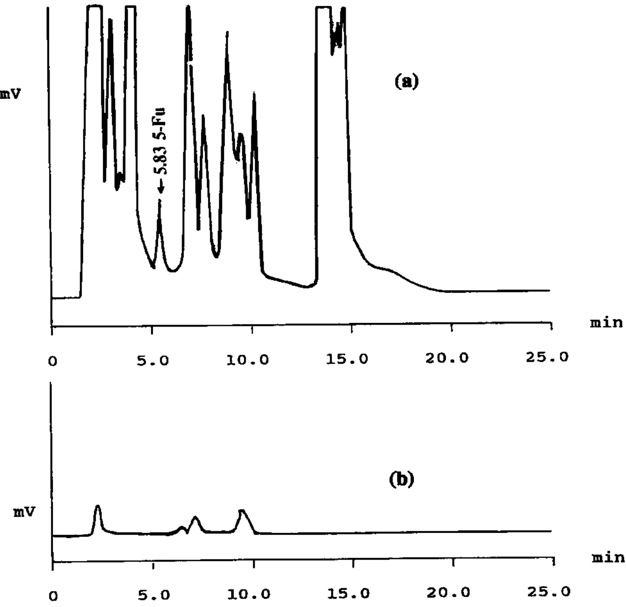

Figure 2 – Chromatograms obtained for a plasma sample on treatment with *n*-propanol-/ether mixtures of two different compositions: (*a*) 95:5, and (*b*) 5:95.

RESULTS AND DISCUSSION

The experimental variables whose effect on the extraction yield was investigated included the composition of the *n*-propanol/ether mixture, extractant volume, protein precipitant, matrix pH and ionic strength, shaking time, and pH of the redissolving solution.

Composition of the *n*-propanol/ether mixture

The various types of matrix studied were subjected to extraction with *n*-propanol-/ether mixtures of different composition, *viz.* from 5:95 to 95:5. Increased proportions of ether in the mixture resulted in neater chromatograms for all the samples, but also

TABLE 1. Optimal extractant composition.

Matrix	Composition	Recovery %
Liver	16:84	31.10 ± 0.40
Kidney	20:80	34.40 ± 0.52
Lung	88:12	45.32 ± 0.38
Heart	40:60	43.75 ± 0.47
Plasma	88:12	49.00 ± 0.32

in decreased 5-Fu recoveries, as can be seen in the chromatograms for plasma (in Fig. 2), which were similar to those obtained for the other samples.

Also, each matrix was found to give rise to different coextracted interferents, so the mixture composition must be adjusted accordingly. Table 1 gives the 5-Fu recoveries obtained by using the optimal extractant composition for each type of matrix.

Extractant volume

After the most suitable extractant composition for each type of sample was established, the effect of using different extractant volumes between 5 and 30 ml on 5-Fu recovery was studied. The results were similar for all the samples. As can be seen in Fig. 3 for heart tissue, increasing extractant volumes resulted in predictably increasing recoveries for both 5-Fu and coextracted substances, so 15 ml (*viz.* the lowest volume that resulted in an optimal 5-Fu/interferent signal ratio) was chosen.

Attempts at raising 5-Fu recovery by using successive extractions with 15 ml of mixture provided no significantly improved results in terms of analyte extraction yield; on the other hand, they gave rise to considerably increased extraction of interferents and lengthened the procedure exceedingly. Hence, a single extraction with the optimal volume was performed in all subsequent experiments.

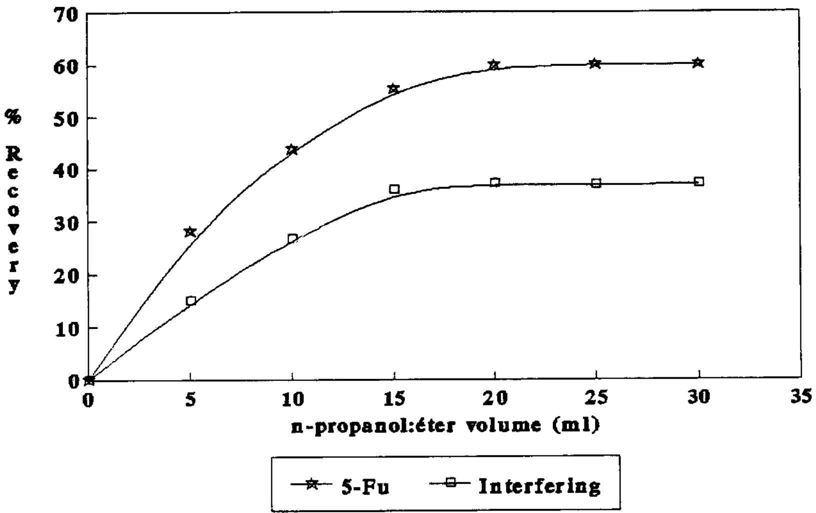

Figure 3 – Variation of 5-Fu recovery from a heart sample as a function of the extractant volume.

Protein precipitant

In order to avoid extraction of interferents giving rise to disturbing peaks in the chromatograms, samples were treated with various reagents typically used as protein precipitants, the most effective of which proved to be trichloroacetic acid and sodium acetate.

Trichloroacetic acid was tested at various concentrations (1 – 10% w/v) and in different volumes (< 1 ml). The results obtained showed its presence to be beneficial for the lung and heart samples and indifferent for the other types of sample. Figure 4 shows the variation of the analyte recovery from the lung and heart samples with the precipitant concentration and volume used. The highest recoveries were obtained by using 100 μl of 2% trichloroacetic acid.

The liver and kidney samples were treated with sodium acetate, on which they exerted a greater clearance effect than did trichloroacetic acid. The best results were

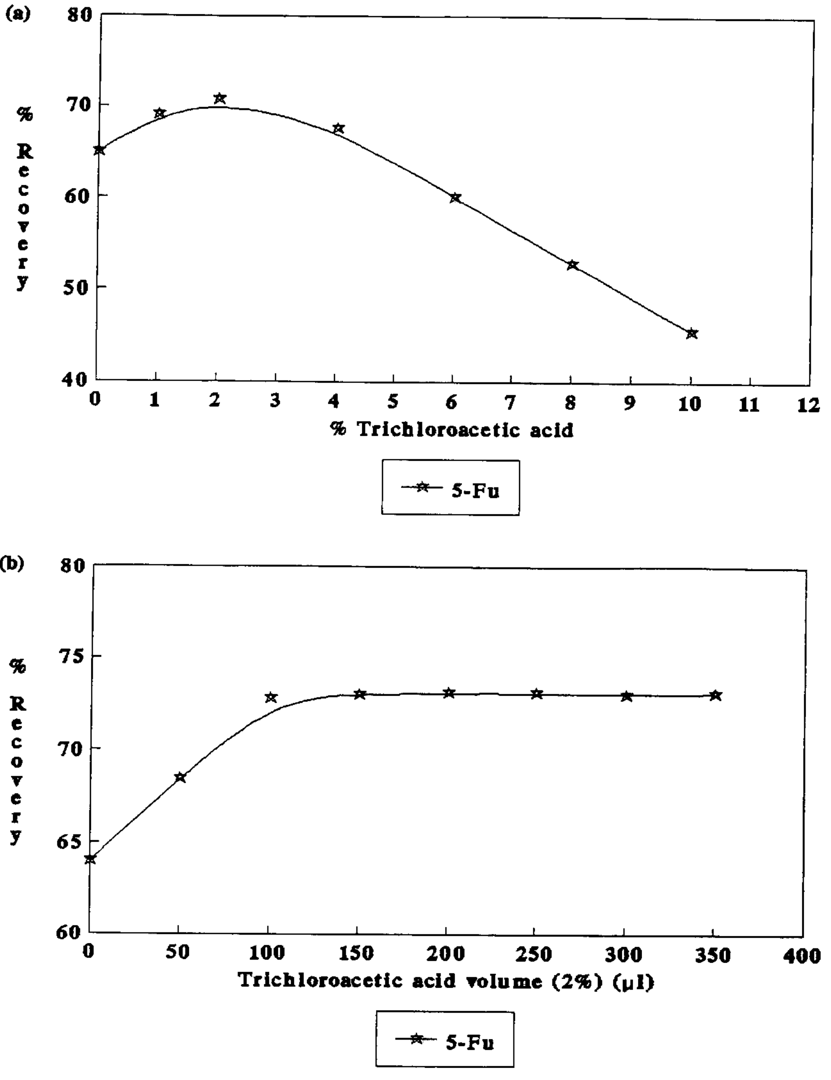

Figure 4 – Variation of 5-Fu recovery from a lung sample as a function of (*a*) the concentration and (*b*) volume of protein precipitant used.

provided by a volume of 50 μl of 1 M sodium acetate at pH 6 for liver and pH 5 for kidney, as can be seen from Fig. 5, which shows the chromatograms obtained for kidney tissue with and without the precipitant added.

Neither precipitant increased the 5-Fu recovery from plasma or resulted in a cleaner sample, though.

Matrix pH and ionic strength

The two groups of samples were added various amounts of acids, bases and salts prior to extraction with a view to improving the results. The solutions used included 2 N NaOH (V < 10 μl), 2 N H_2SO_4 (V < 10 μl), 1 M tris-hydroxymethylaminomethane (TRIS) buffer (V < 2 ml), K_2S (V < 10 μl) and saturated (4 g/20 ml) Na_2SO_4 (V < 2 ml).

The results obtained revealed the plasma, lung and heart samples to benefit from the addition of 2 N H_2SO_4, which increased 5-Fu recoveries without significantly raising coextraction of interferents (Table 2). The optimum acid concentrations for this purpose were found to be 2 μl for plasma and 5 μl for the lung and heart samples.

Also, addition of sodium sulphate —but none of the other solutions— to the liver and kidney samples was found to favour passage of the analyte into the organic phase, the effect being a function of the volume of salt solution added (Fig. 6). In fact, increasing volumes up to 0.5 ml gave rise to markedly increasing 5-Fu recoveries with no increased coextraction of interferents; however, volumes higher than 0.5 ml detracted from the extraction yield. A volume of 0.5 ml was thus chosen as optimal for the extraction of 5-Fu from liver and kidney tissues.

Shaking time

Figure 7 shows the 5-Fu recoveries obtained by using different shaking times (the results for the other four types of sample were quite similar). As can be seen, recoveries increased substantially with increase in the extraction time up to 10 min; longer times resulted in very small variations or even no changes after 20 min. A shaking time of 15 min (*viz.* the shortest providing the highest possible yield) was thus selected.

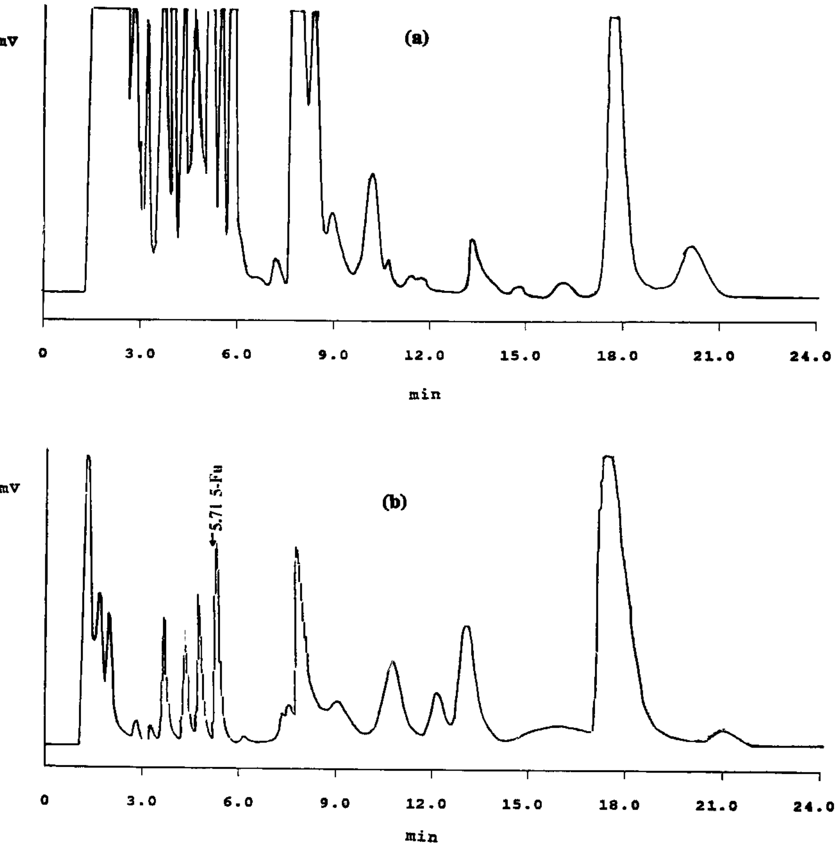

Figure 5 – Chromatograms obtained for kidney samples with (*a*) no acetate added, (*b*) 50 μl of 1 M sodium acetate (pH 6) added.

TABLE 2. Influence of H_2SO_4 volume on 5-Fu recovery %.

Matrix	0 μl	2 μl	4 μl	6 μl	8 μl	10 μl
Plasma	49.0	70.2	67.8	62.4	50.2	47.1
Lung	62.0	71.2	80.5	80.5	73.2	66.2
Heart	60.5	68.9	79.4	79.4	70.3	65.6

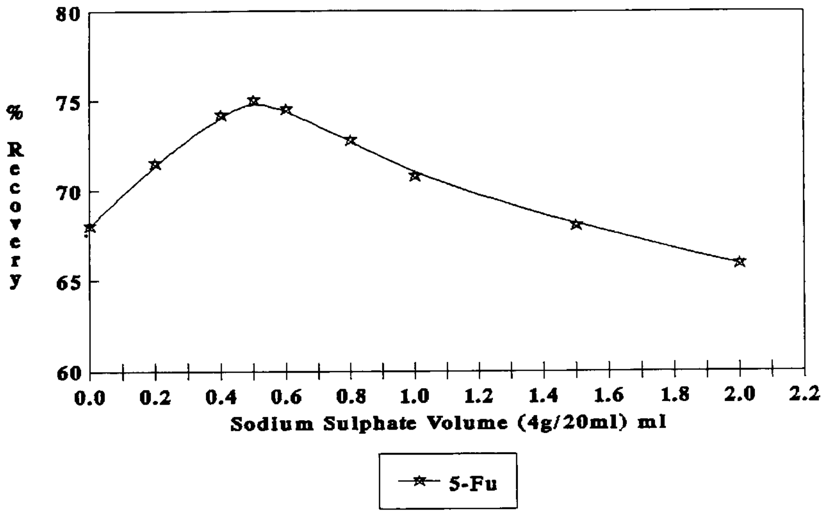

Figure 6 – Variation of 5-Fu recovery from a liver sample as a function of the volume of sodium sulphate used.

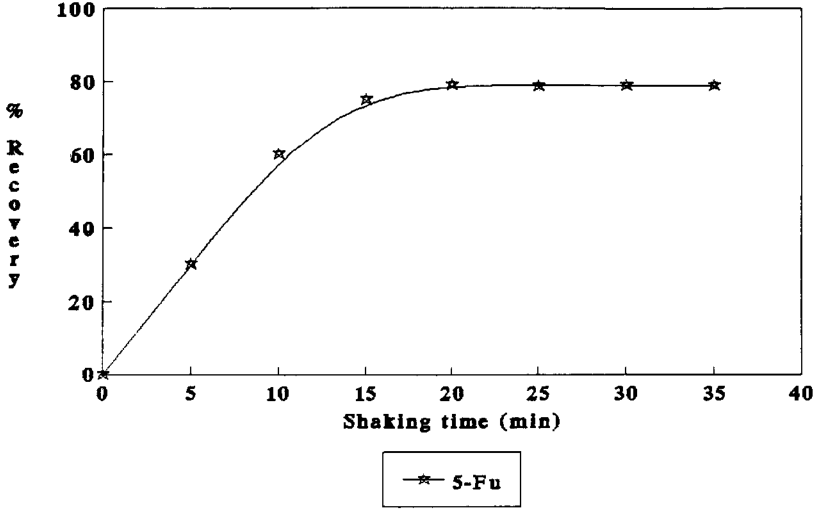

Figure 7 – Variation of 5-Fu recovery from a kidney sample as a function of the extraction time.

pH of the redissolving solution

The influence of the pH of the 0.05 M ammonium dihydrogen phosphate solution used to dissolve the analyte after evaporation of the organic phase on the analyte recovery was studied by adjusting it to values between 2 and 11 with a strong acid or base. Figure 8 shows the results obtained for the different types of matrix. Based on them, the most suitable pH for the 0.05 M ammonium dihydrogen phosphate solution, of which a volume of 1 ml was used in every case, was 2.5 for plasma and lung, 3 for kidney, 5 for heart and 11 for liver samples.

Table 3 summarizes the most suitable working conditions for each type of sample and the final 5-Fu recoveries obtained under them. As can be seen from the chromatograms for the liver sample in Fig. 9 (similar to those obtained for the other types of sample), the proposed method allows the accurate quantitation of 5-Fluorouracil.

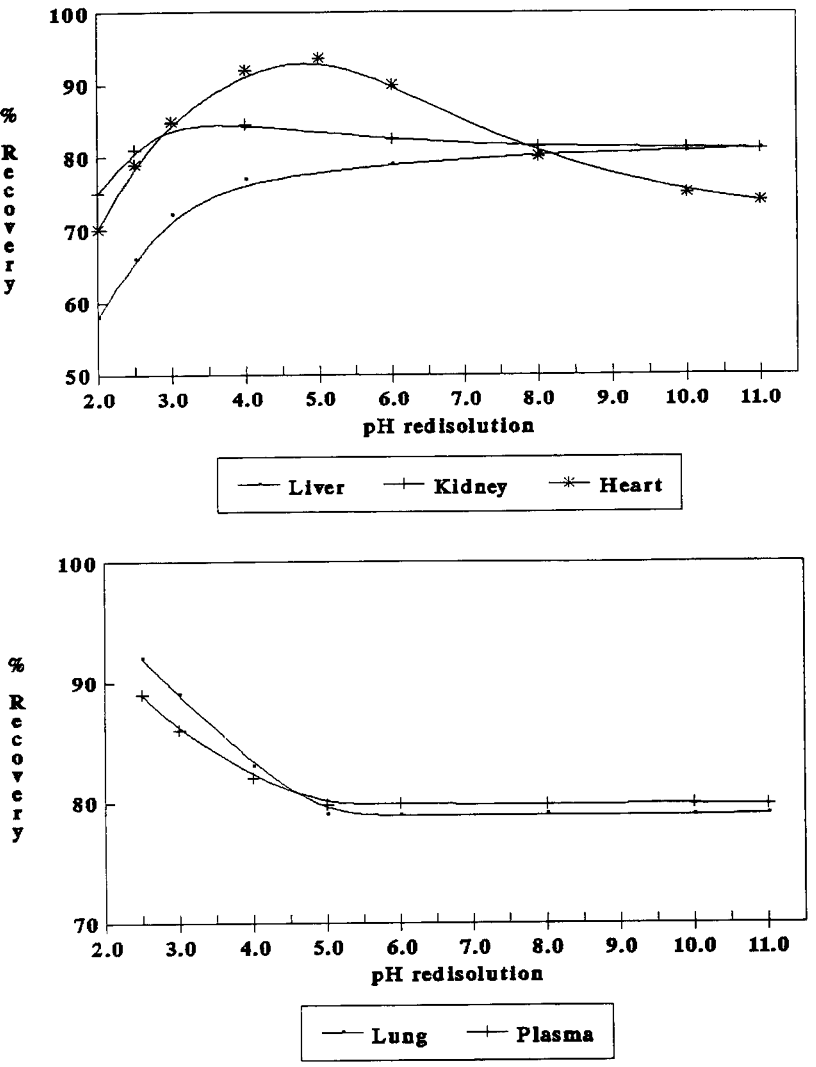

Figure 8 – Variation of 5-Fu recovery as a function of the redissolving solution pH.

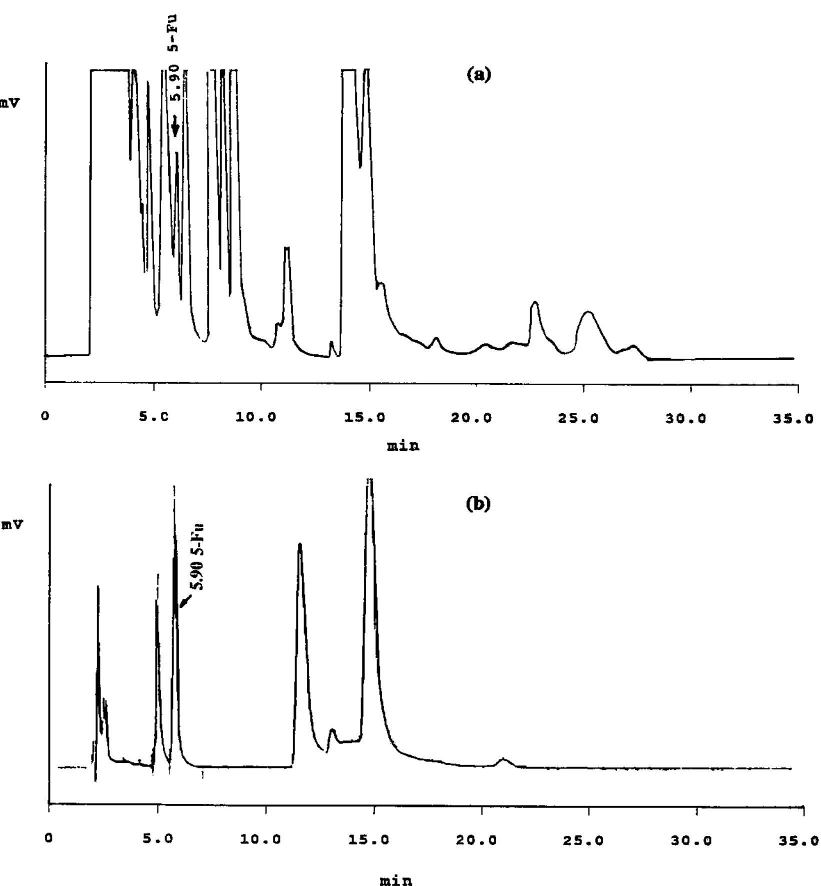

Figure 9 – Chromatograms obtained for a liver sample subjected to extraction with 15 ml of extractant (*a*) and the proposed procedure as shown in Table 3 (*b*).

TABLE 3. General procedure for sample preparation.

Matrix	Liver	Kidney	Lung	Heart	Plasma
Sample	0.5 g	0.5 g	0.5 g	0.5 g	1 ml
H_2SO_4 2 N	-	-	5 μl	5 μl	2 μl
Na_2SO_4 (4g/20ml)	0.5 ml	0.5ml	-	-	-
NaAcO 1 M	50 μl	50 μl	-	-	-
	pH 6	pH 5	-	-	-
Cl_3AcOH	-	-	2 %	2 %	-
	-	-	100 μl	100 μl	-
n-propanol/ether (15 ml)	16:84	20:80	88:12	40:60	88:12
Ultrasonic bath	30 s	30 s	30 s	30 s	30 s
Shaking	15 min	15 min	15 min	15 min	15 min
Centrifugation	15 min	15 min	15 min	15 min	15 min
pH phosphate (1 ml)	11	3	2.5	5	2.5
Recovery %	81.20	84.75	95.04	93.89	89.00

CONCLUSIONS

The results obtained in this work clearly reveal that type of matrix involved influences the final recovery of 5-Fu. Thus, plasma samples have a different effect from those of the four tissues assayed, of which liver and kidney on the one hand, and lung and heart on the other, bear some similarities.

The results also show that *n*-propanol and ether in the extracting mixture have opposing effects and that the most suitable composition for the mixture varies from sample to sample.

In contrast with the widespread use of a single extraction procedure, we believe each type of matrix should be extracted under specific, optimized conditions.

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DETERMINATION OF FUMARIC ACID, MALEIC ACID, AND PHTHALIC ACID IN GROUNDWATER AND SOIL

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ABSTRACT

When present at > 1 μg/mL, each title compound was determined in groundwater by ion-exclusion chromatography after sample acidification and filtration. For groundwater with one or all analyte concentrations of < 1 μg/mL, the acid anions were first concentrated from a 100-mL sample using a quaternary amine anion-exchange cartridge. The acids were recovered by eluting the cartridge with 1 mL of 1 N H_2SO_4 and two 2-mL deionized water washes; this solution then was examined by anion-exclusion chromatography. For soil, the acids were extracted from a 10-g sample with 20 mL of 1 N H_2SO_4 and two 15-mL water washes. This extract was filtered then analyzed by anion-exclusion chromatography. All analyses used 25-μL injections into the HPLC column which was maintained at 60°C and eluted with a 0.6 mL/min. flow of 0.02 N H_2SO_4. Analytes were monitored with a UV detector operated at 200 nm. The analysis procedures for groundwater were validated with solutions which were fortified with from 50 ng/mL to 200 μg/mL of each analyte; recoveries ranged from 90 to 110%. The soil method was validated using fortified samples which contained each acid at concentrations of from 5 to 160 μg/g. Recovery values were between 81 and 120%. For samples exhibiting minimal detector response from compounds other than the acids of interest, 100-μL injection volumes provided an estimated detection limit of 1 μg/g for soil and 10 ng/mL for groundwater.

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INTRODUCTION

For an investigation of a landfill site, procedures for the determination of fumaric acid, maleic acid, and phthalic acid in soil and groundwater samples were needed. Detection limits of 10 $\mu g/g$ for soil and 1 $\mu g/mL$ for groundwater were required; however, estimating analyte concentrations at one tenth these levels was a goal. Since many organic acids have been quantitated by gas chromatography after derivatization (1-3), similar methodology was considered. For the dicarboxylic acids of interest here, bis(trimethylsilyl) esters have been prepared and their chromatographic behavior studied (4,5). However, developing a procedure for isolating the acids in a matrix suitable for derivatization and conducting the derivatization was not felt to be the preferred route. Therefore, liquid chromatography was investigated.

Chromatography literature abounds with HPLC methods for determining aliphatic and aromatic acids with mono- and dicarboxylate functionalities. For example, physiological fluids have been analyzed for organic acids using reversed-phase, ion-exchange, and ion-exclusion techniques as discussed by Bulusu et.al.(6). Ion-exclusion chromatography (IEC) has been applied in many areas (7) using various detection systems (8). Thus, IEC has been used to quantitate organic acids in sugar cane process solutions using refractive index detection (8) and, by incorporating an anion-exchange suppressor column, conductivity detection was possible (9). For anions which exhibit UV absorption spectra, the most direct approach is single-column IEC with UV detection. Grosjean et.al. pointed out the advantages of this technique and demonstrated its application in the measurement of atmospheric carboxylic acids (10). We were able to apply a similar approach to soil and water by developing sample preparation methods that resulted in aqueous H_2SO_4 solutions for the acids of interest. These solutions were conveniently analyzed by anion-exclusion HPLC with UV

detection. This paper presents our sample preparation and chromatographic procedures along with results from validation studies.

EXPERIMENTAL

Instrument and Operating Conditions

The liquid chromatograph was a Hewlett Packard Model 1090L equipped with a PV5 solvent delivery system, column oven, an automatic liquid sampler with variable-volume injector, and a model 1040A photodiode array detector. The instrument was operated with revision 5.22 software using a 79994A ChemStation which was linked to a 9153C disk drive and a 2225A ThinkJet printer. The column was a 300 x 6.5 mm ORH-801 preceded by an ion Guard™ GC-801 guard column. These were packed with a cation-exchange resin in the hydrogen form and were purchased from Interaction Chromatography. Injection volumes were 25-μL and analyses were conducted at 60°C with a 0.6 mL/min flow of 0.02 N H_2SO_4. For quantitation, the detector signal was monitored at 200 nm (10 nm bandwidth) relative to a reference wavelength of 550 nm (100 nm bandwidth). Analysis run times usually were 40 min (extended to 55 min for samples with numerous late-eluting components); during this time the detector signal was recorded/integrated from 4.5 to 25 min.

Special Supplies

Fumaric Acid	-	Aldrich, 99+% (Cat. 24,074-5)
Maleic Acid	-	Aldrich, 99% (cat. M15-3)
Phthalic Acid	-	Aldrich, 99+% (cat. 24, 022-2)
Sulfuric Acid	-	Baker, Ultrex®, Ultrapure Reagent (Cat. 4802-05)
Water	-	Deionized, from Millipore, Milli-Q purifier system.

Extraction Cartridges	-	3-mL, Baker-10 SPE disposable columns (Cat. 7091-3). These contained 500 mg of 40 μm, 60A silica gel with a quaternary amine (N+) anion exchange functionality.
Glass Fiber Filters	-	Whatman, grade GF/B (Cat. 1821-042) and GF/D (Cat. 1823-042) with 4.25 cm diameters.
Buchner Funnels	-	Coors (Cat. 60239), 43 mm i.d.
Microparticulate Filters	-	Gleman #4187, 3-cm diameter, Acrodisc PF which has a 0.8 μm prefilter and a 0.2 μm membrane filter.
Centrifuge Tubes	-	Nalgene, 50-mL, Oak Ridge FEP (#3114-0050)

Preparation of Instrument Calibration Solutions

Stock solution A which contained 4000 μg/mL of each acid was prepared by combining and dissolving 4.000 g of each acid in 1000 mL of water. Stock solution B was prepared to contain 1000 μg/mL of each acid by diluting stock solution A with water. Calibration solutions with acid concentrations of 200 or 100 μg/mL were prepared by diluting either 20 or 10 mL of stock solution B with 20 mL of 1 N H_2SO_4 and sufficient water to give 100-mL solutions. Other calibration solutions (0.25 to 50 μg/mL) were made by appropriately diluting the 100 μg/mL solution with 0.2 N H_2SO_4.

Preparation of Fortified Samples

Deionized water was fortified with from 50 ng/mL to 50 μg/mL of each acid by serially diluting stock solution B. Groundwater was fortified with analytes (50 ng/mL to 1 μg/mL) by diluting 5 mL of fortified deionized water to 100 mL with sample water; for concentrations from 1 to 200 μg/mL, 250

μL of water was removed from a 5-mL sample aliquot and replaced with 250 μL of an appropriate fortified deionized water solution to provide the required fortification level.

For soil samples, a 10 ± 0.05 g portion (not dried) of screened (8 mesh) and mixed soil was weighed into a 50-mL beaker or 50-mL centrifuge tube. Fortification concentrations of 5, 10, 20, 40, 80 and 160 μg/g were obtained by respectively dispersing 50, 100, 200, 400, 800 and 1600 μL of stock solution B onto the soil surface. These samples were not analyzed until they had air dried for at least one hour.

Sample Preparation for Groundwater

For samples with >1 μg/mL of each analyte, a 5-mL aliquot was acidified with 25 μL of conc. H_2SO_4, then a few mL of this solution was filtered with a 5-mL syringe fitted with an Acrodisc PF membrane filter. HPLC analysis then was conducted.

The following procedure was used to determine analytes whose concentrations were <1 μg/mL. A SPE extraction cartridge was inserted into a 250-mL vacuum filtration flask which was attached to an adjustable vacuum manifold (set to 15 inches Hg). Each column was conditioned by eluting with 3 mL of methanol followed by two 3-mL deionized water washes. The wash liquids were discarded then a 125-mL feed reservoir was attached to the cartridge. To the reservoir 100 mL of sample was added after it was first filtered through a Buchner funnel with a GF/B filter. With application of vacuum a 4-5 mL/min flow of sample through the cartridge was observed. The eluted groundwater was discarded and a 16 x 125 mm collection tube was placed inside the vacuum flask. The cartridge was eluted with 1 mL of 1 N H_2SO_4, followed by two 2-mL deionized water washes. The volume of the resulting extract was adjusted to 5.0 mL with deionized water. This extract then was ready for HPLC analysis.

Sample Preparation for Soil

Into a 50-mL beaker, 10 $\pm$ 0.05 g of screened (8 mesh) and mixed soil (not dried) was weighed, then 20 mL of 1 N H_2SO_4 was added. This mixture was thoroughly blended and allowed to stand for 20 minutes. The resulting slurry was vacuum filtered using a Buchner funnel which held a GF/D filter. While the soil cake was still moist, it was washed with 15 mL of water which first was used to rinse the extraction beaker. After a second 10-mL water wash, the combined filtrate volume was adjusted to 50 mL with water. For HPLC analysis, a 3-mL aliquot of extract solution was filtered using a 5-mL syringe fitted with an Acrodisc PF membrane filter.

For soils containing high concentrations of clay, the 10-g sample was weighed into a 50-mL centrifuge tube to which 25 mL of 1.0 N H_2SO_4 was added. The mixture was blended, allowed to stand for 20 minutes, then centrifuged for 5 minutes at 5000 RPM. The clarified acid solution was decanted into a 50-mL volumetric flask. To the centrifuge tube, 25 mL of water was added and the soil cake slurried. This mixture was centrifuged at 5000 RPM for 5 minutes then the liquid layer was transferred to the 50-mL volumetric flask. After adjusting the sample volume to 50.0 mL with water, an aliquot was filtered (Acrodisc PF membrane filter) and examined by HPLC.

Calculation of Results

Area responses for analyte peaks in the analyzed solutions were compared to areas produced by analytes in calibration solutions. The following equation was used to calculate analyte concentrations in samples:

$$\frac{\text{Analyte Area from Sample Soln.}}{\text{Analyte Area from Calib. Soln.}} \times \text{Calib. Conc.} \times F = \text{Sample Conc.}$$

where F is the dilution/concentration factor. F is 1.0 for water samples with no analyte concentration step and 0.05 when the 20-fold enrichment process was used. For soil, F was 5 due to a factor of 5 dilution from sample preparation.

RESULTS AND DISCUSSION

Chromatography

Ion-exclusion chromatography provided excellent separation of the acid analytes. The retention times followed the order: maleic acid < fumaric acid < phthalic acid. Elution ordering for organic acids usually is explained by their respective acid strengths (pK_a values) and hydrophobic adsorption effects from the ion-exchange resin (7, 11-13). In general, increased compound acidity results in shorter retention time; therefore, from their acidity values (14), maleic acid (pK_a = 1.8) should elute first followed by phthalic acid (pK_a = 2.9) and then fumaric acid (pK_a = 3.0). However, the aromatic function of phthalic acid strongly interacts with the aromatic resin lattice so an extended retention time is noted. Retention is also a function of temperature and concentration of the eluting sulfuric acid. Increasing temperature causes shorter retention especially for phthalic acid (Table 1). Elution of each acid is retarded by increases in concentration of H_2SO_4 eluent (Table 2). We chose 60° to speed the analysis and to reduce column operating pressure. For rapid analyses, the concentration of H_2SO_4 in the eluent should be minimized; however, we selected 0.02 N H_2SO_4 for all analyses. This was necessary because some soil extracts exhibited a large tailing response just prior to the maleic acid peak (see Figure 1 for example). Part of that response was from the H_2SO_4 which was present in all extracts. For most soil analyses the early-eluting peak was not a problem (see Figure 2) and was never a concern for water samples. However, to assure adequate resolution for quantitation of maleic acid, the high-strength eluent was employed.

Respective UV maxima occur at 205, 210 and 200 nm for maleic acid, fumaric acid and phthalic acid. Best overall responses for all acids was obtained at a wavelength setting of 200 nm. This low value was possible since the isocratic eluent of 0.02 N H_2SO_4 produced minimal background absorbance resulting in high signal-to-noise ratios. Figure 3 is a

TABLE 1
Effect of Temperature on Retention*

Temperature (°C)	Retention Time (minutes) Maleic Acid	Fumaric Acid	Phthalic Acid
40	5.88	11.43	21.94
50	5.78	10.70	19.49
60	5.69	10.10	17.43

* Using 0.01 N H_2SO_4 eluent

TABLE 2
Effect of Eluent Strength on Retention*

H_2SO_4 (N)	Retention Time (minutes) Maleic Acid	Fumaric Acid	Phthalic Acid
0.005	5.14	9.51	15.99
0.010	5.70	10.06	17.40
0.015	6.05	10.28	17.98
0.020	6.31	10.38	18.28
0.030	6.71	10.49	18.63
0.050	7.18	10.63	19.02

* Elution temperature was 60°C

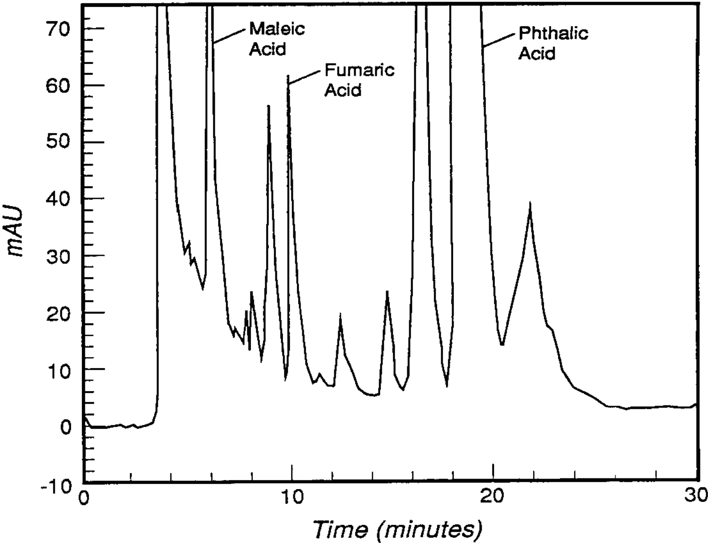

FIGURE 1. Chromatogram from 25-μL injection of extract from contaminated soil which produces a large tailing response prior to maleic acid.

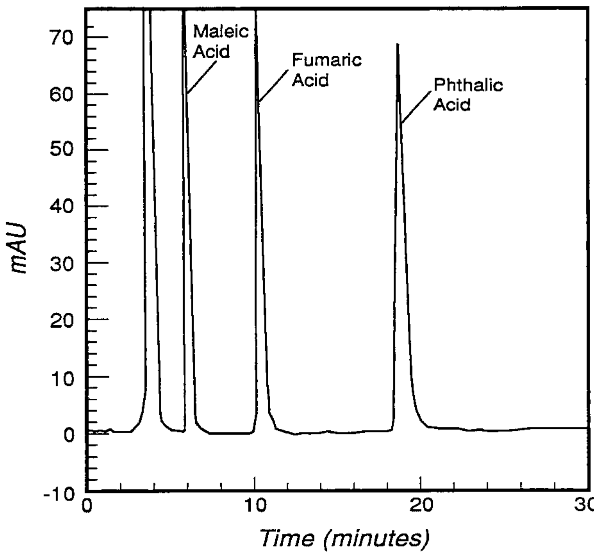

FIGURE 2. Chromatogram from 25-μL injection of extract from uncontaminated soil that had been fortified with 50 ppm of each acid.

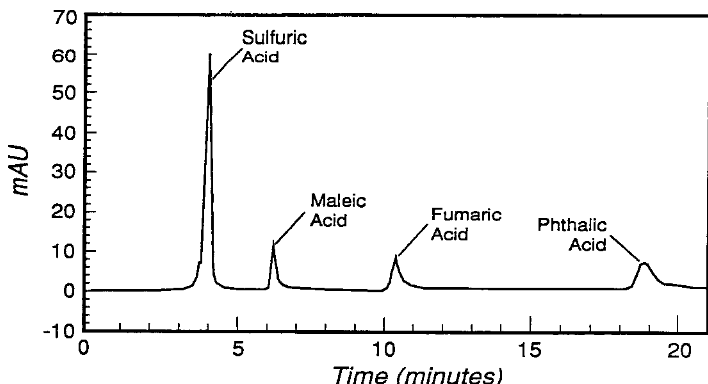

FIGURE 3. Chromatogram from 25-μL injection of a calibration solution which contained 1 ppm of each acid.

chromatographic trace for a calibration solution containing 1 μg/mL of each acid. Even with responses of only about 10 mAU, the peaks were accurately integrated by the data system. Detector linearity was maintained with up to 5 μg of each component being injected into the column. Thus, with 25-, 50- and 100-μL injection volumes, respective ranges for detector linearity were found to be 1-200 μg/mL, 0.5-100 μg/mL and 0.25-50 μg/mL. In all cases, correlation coefficients of >0.999 were calculated. Although component responses were linear with 50 and 100 - μL injections, peak broadening was noted so large injections were only used in special situations (see later discussion) to achieve detection limits below 5 μg/g with soils and below 50 ng/mL with water samples.

Typically, HPLC analyses give best results when calibration solutions are similar to sample solutions. For water analyses this criteria was met. For soils the extract acidity was about twice the concentration present in calibration solutions, but this had no observable impact on the chromatography. In fact, calibration solutions in 0 to 0.8 N H_2SO_4 were

tested. For maleic acid, retention gradually increased with higher H_2SO_4 levels; in 0.8 N H_2SO_4 only a 0.1 minute shift was seen. The other analytes exhibited no changes.

Water Analyses

For analyte concentrations >1 μg/mL, samples only need to be acidified and filtered prior to HPLC examination. Filtration was demonstrated not to cause loss of target acids by comparing analyte responses from calibration solutions (1, 25 and 50 μg/mL) before and after filtration. In all cases, the response differences were < 2%. Using a groundwater sample from a landfill well, no problems due to filtration or sample matrix were found. This was shown by recovery tests with samples fortified with analytes to 1, 10, 50 and 200 μg/mL. For these, recovery values ranged from 96 to 102% (single test per level).

To achieve lower detection limits an analyte concentration step was needed prior to HPLC analysis. Anion exchange has been used to isolate organic acids from various aqueous samples such as sugar cane process juices (8), urine samples (5,6,15), beer (16), and precipitation (17). Using a similar approach, the analyte acids were conveniently extracted from a 100-mL groundwater sample onto an anion-exchange cartridge. The acids then were recovered in a 5-mL solution (20-fold enrichment) by eluting the cartridge with H_2SO_4 and deionized water. No prior sample treatment was carried out other than filtration to remove any particulates that might have plugged the extraction cartridge. Table 3 presents method validation data from recovery studies using fortified deionized water and fortified groundwater. Concentrations tested were from 50 ng/mL to 50 μg/mL; recoveries ranged from 89 to 110%. The 50 μg/mL test demonstrates sufficient cartridge capacity to handle samples that need to be concentrated but have one or two of the acids at > 1 μg/mL.

TABLE 3

Recovery Results from Fortified Water Samples After Using Anion-Exchange Isolation/Enrichment of Analytes

A - Deionized Water B - Groundwater

Fortification Conc. (ppm)	Average % Recovery*		
	Maleic Acid	Fumaric Acid	Phthalic Acid
A-0.05	90 (1.1)	95 (2.6)	96 (2.4)
B-0.05	89	91	91
A-0.25	97 (3.6)	99 (2.6)	99 (3.6)
B-0.25	102	101	103
B-0.50	92	93	96
A-1.00	95 (6.8)	95 (5.3)	95 (7.4)
B-1.00	100	100	101
A-2.00	96 (2.3)	96 (2.6)	94 (2.5)
A-10.0	108 (3.3)	109 (3.5)	104 (4.3)
A-50.0	89 (10.4)	96 (11.7)	100 (13.3)

* Values in parentheses for A samples are % relative standard deviation for 4 tests; for 50 ppm there were eight tests. For B samples only one test done at each concentration.

TABLE 4
Recovery Results from Fortified Soil Samples

	Average % Recovery[a]		
Fortification Conc. (ppm)	Maleic Acid	Fumaric Acid	Phthalic Acid
5	92 (1.8)	99 (8.2)	88 (1.9)
10	82 (3.9)	97 (2.1)	93 (6.6)
20	87 (6.5)	95 (4.8)	101 (2.5)
40	102 (9.3)	105 (8.1)	111 (11.3)
80	90 (9.7)	92 (10.3)	98 (11.6)
160	107 (4.1)	108 (3.5)	115 (1.3)
5[b]	87	100	90
15[c]	83	90	92

a Values in parentheses are % relative standard deviation for three tests; for 5 ppm there were four tests.

b Using centrifugation procedure with sandy/clay soil (one test).

c Using centrifugation procedure with clay soil (one test).

The recovery data of Table 3 verifies a 50 ng/mL method detection limit. However, for samples exhibiting minimum background HPLC response,a detection limit estimated at 10 ng/mL can be attained with 50 or 100-μL injection volumes.

Soil Analyses

Sulfuric acid is very effective in extracting the analytes from soil. Filtration of the soil slurry usually resulted in some suspended material in

extracts so a final clarification with a membrane filter is recommended. Recovery values for a sandy/clay soil which was fortified with from 5 to 160 μg/g of each acid are given in Table 4; results from 81 to 120% were obtained. For this specific sample (local farm soil) and with many of the submitted landfill soils, the filtration process took about 20 minutes. However, a few samples were mostly clay and formed a very sticky mass that was virtually unfilterable. To handle these samples, extractions had to be conducted in centrifuge tubes. The clay slurry centrifuged so well that all the resulting clarified extract could be poured off the clay layer. Isolating the extract by this method also worked with other soil types that typically would be filtered. The centrifugation procedure takes about as long as for filtration; therefore, choice of technique is not an issue since either gives comparable results (see Table 4).

The recovery data in Table 4 show that a 5 μg/g method detection limit can be reached. An estimated detection limit of 1 μg/g is possible when the background HPLC responses are minimal and 50 or 100-μL injection volumes are used.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 17(7), 1653-1657 (1994)

BEHAVIOUR OF HUMIC ACIDS ON Fe(III)-IMPREGNATED SILICA GEL COMPARED WITH MODEL SUBSTANCES

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ABSTRACT

Behaviour of humic acids under the conditions of thin layer chromatography on Fe(III)-impregnated silica gel with tap water as developer was investigated. Compounds having similar functional groups as the humic material were compared and conclusions extended to natural conditions were drawn.

INTRODUCTION

In our previous papers we presented the study of the behaviour of some benzene derivatives related to humic material in conditions simulating the natural ones (1-3). These were: thin layer chromatography on silica gel impregnated with Fe(III)-hydroxy/oxide (4) as the support and water as the developer. The model compounds were benzene derivatives with hydroxy and/or carboxy groups representing types of structures that might occur in humic acids. The R_f-values of the compounds investigated served us as a lead for their solubility i.e. movability of the Fe(III)-complexes formed. By using this simple system we could follow the conduct of compounds with various functional groups and deduce on their abilities to detach Fe-ion from the support to the solution (5). Summarizing the results obtained with

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model substances, the differences in activities of carboxy and hydroxy groups in the benzene ring became evident.

In the present work we now examined the behaviour of commercially isolated humic acids as the natural occuring molecules under the same conditions.

EXPERIMENTAL

The chemicals and the procedures were the same as described previously (1-3). For TLC precoated plates of silica gel G_{254} (Merck) were used. Humic acids, as Na- or NH_4-salts, were commercially products purchased by: "EGA" Germany, "FLUKA" Switzerland and "GMS" Chechoslovakia.

RESULTS AND DISCUSSION

In Table 1 the behaviour of model compounds, simulating humic acid structure on Fe(III)-impregnated plates, taken from earlier publications, is presented. They are representative compounds having characteristic numbers and positions of carboxy and hydroxy groups. One can see that differences in the mobilites of the compounds exist with respect to the kind of functional groups. Looking at that one of presumed structures of humic acids (Fig. 1) it can be seen the similarities of the composition of the functional groups in the compounds tested with that of humic structure. From the behaviour of the hydroxy benzenes (Table 1: I, II, III and IV) on Fe(III)-impregnated plates it can been concluded that the related functional groups make iron and possibly also other metals under natural conditions movable by turning them to water soluble complexes. On the other hand, benzene carboxilic acids (Table 1: V, VI and IX) behave differently indicating retardation of the metal. Salicylic acid, as well as other phenolic acids (2), having the carboxilic- and hydroxy- groups, also form soluble complexes with Fe-ion and consequently moved considerably. An addditional prove would be the compounds with blocked hydroxy-groups (like: trimethoxy benzoic acid, Table 1:IX) with small Rf's.

In Table 2 the R_f values of three humic acid preparations in the salt and H^+-forms, respectively, together with their chemical characteristics, are presented. By

Table 1. R_f x 100 values of hydroxy and carboxy benzene derivatives on Fe(III)-impregnated silica gel. Developer: tap water

No	Compound	Structures	R_f x 100	Ref
I	Catechol	OH, OH	78	3
II	Resorcinol	OH, OH	91	2
III	Quinol	HO, HO	88	2
IV	Pyrogallol	HO, OH, OH	0 - 7	3
V	Benzoic acid	COOH	16	3
VI	o-Phthalic acid	COOH, COOH	23	3
VII	Salicylic acid	COOH, OH	90	1
VIII	Gallic acid	COOH, HO, OH, OH	0 - 37	3
IX	3,4,5-Trimethoxy-benzoic acid	COOH, H_3CO, OCH_3, OCH_3	6	1

Figure 1.

One of the presumed structures of humic acid

Table 2.

R_fx100 values of humic acids on Fe(III)-impregnated silica gel and their COOH content

Developer: tap water

Humic Acid	R_fx100		COOH* (meq/g)	
	Original	H+ form**	Original	H+ form**
"EGA"	0 - 88	11	1.3	4.4
"FLUKA"	0 - 88	11	0.5	4.4
"GMS"	0 - 60	3	6.5	5.8

*Determined by $Ca(OAc)_2$ method (7)

**Transformation into H+ form was performed by repeated shaking with 0.1N HCl and centrifugating followed by washing with distilled water till the negative reaction to chloride

comparing the R_f - values of the model substances with those of humic acids under the same conditions (Table 1 and 2), one can conclude that humic acids (in the original forms) as well as compounds like catechol, quinol, resorcinol and salicylic acid move considerably on Fe(III)-impregnated plates. Due to the exchange capacity of the Na^+ or NH_4^+ ion of humic acid with Fe-ion from the support, depending on the stability and solubility of the formed complexes, the tailing effect can be explained. Looking to the behaviour of humic acids in the H^+-form (Table 2) one can recognize similar small mobilities as with model carboxilic acids.

Summarizing the above results it can be presumed that during the chromatographic process complexes between Fe(III) from the support and the active functional groups from humic acids were formed, causing successive attaching and detaching of Fe(III) from the support of Fe(III)-hydroxy/oxide. The result is the moving by tailing of the so formed more or less soluble complexes. All these results so far could partially give the answer how the process of metal migration in soils and sediments is progressing.

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LIQUID CHROMATOGRAPHY CALENDAR

1994

APRIL 10 - 15: 207th ACS National Meeting, San Diego, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036, USA.

APRIL 19 - 22: Rubber Division ACS, 145th Spring Technical Meeting, Palmer House Hotel, Chicago, Illinois. Contact: C. Morrison, Rubber Division, P.O. Box 499, Akron, OH 44309-0499, USA.

MAY 8 - 13: HPLC '94: Eighteenth International Symposium on High Performance Liquid Chromatography, Minneapolis Convention Center, Minneapolis, Minnesota. Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, USA.

MAY 23 - 25: International Symposium on Polymer Analysis and Characterization (ISPAC-7), Les Diablerets, Switzerland. Contact: Howard G. Barth, ISPAC Chairman, DuPont Company, Central Research & Development, P. O. Box 80228, Wilmington, DE 19880-0228, USA or Dr. M. Rinaudo, CERMAV-CNRS, B. P. 53X, 38041 Grenoble Cedex France.

JUNE 1 - 3: Joint 26th Central Regional/27th Great Lakes Regional Meeting, ACS, Kalamazoo and Huron Valley Sections. Contact: H. Griffin, Univ of Michigan, Ann Arbor, MI 48109, USA.

JUNE 1 - 3: International Symposium on Hormone & Veterinary Drug Residue Analysis, Congress Centre Oud Sint-Jan, Brugge, Belgium. Contact: Prof. C. Van Peteghem, University of Ghent, L:aboratory of Food Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 5 - 7: VIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques & Applications in Chromatography and Capillary Electrophoresis, Congress Centre Oud Sint-Jan,

Brugge, Belgium. Contact: Prof. Dr. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Dept. of Pharmaceutical Analysis, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 12 - 15: 1994 Prep Symposium & Exhibit, sponsored by the Washington Chromatography Discussion Group, at the Georgetown University Conference Center by Marriott, Washington, DC. Contact: Janet E. Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 15: Fourth International Symposium on Field-Flow Fractionation (FFF'94), Lund, Sweden. Contact: Dr. Agneta Sjogren, The Swedish Chemical Society, Wallingatan 24, 2 tr, S-111 24 Stockholm, Sweden.

JUNE 19 - 23: 24th National Medicinal Chem. Symposium, Little America Hotel & Towers, Salt Lake City, Utah. Contact: M. A. Jensen & A. D. Broom, Dept. of Medicinal Chem, 308 Skaggs Hall, Univ of Utah, Salt Lake City, UT 84112, USA.

JUNE 19 - 24: 20th International Symposium on Chromatography, Bournemouth International Centre, Bournemouth, UK. Contact: Mrs. Jennifer A. Challis, The Chromatography Society, Suite 4, Clarendon Chambers, 32 Clarendon Street, Nottingham, NG1 5JD, UK.

JUNE 24 - 27: BOC Priestly Conference, Bucknell University, Lewisburg, PA, co-sponsored with the Royal Soc of Chem. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

JULY 31 - AUGUST 5: American Society of Pharmacognosy Annual Meeting, International Research Congress on Natural Products, joint meeting with the Assoc. Français pour l'Enseignement et la Recherche en Pharmacognosie, and the Gesellschaft fur Arzneipflanzenforschung, and the Phytochemical Society of Europe, Halifax, Nova Scotia, Canada. Contact: D. J. Slatkin, P. O. Box 13145, Pittsburgh, PA 15243, USA.

AUGUST 21 - 26: 208th ACS National Meeting, Washington, DC. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 5 - 7: 25th International Symposium on Essential Oils, Grasse, France. Contact: Assoc. des Ingenieurs et Techniques de la Parfumerie (A.I.T.P.), 48 ave. Riou-Blanquet, 06130 Grasse, France.

OCTOBER 16 - 19: 46th Southeastern Regional Meeting, ACS, Birmingham, Alabama. Contact: L. Kispert, Chem Dept, Univ of Alabama, Box 870336, Tuscaloosa, AL 35115, USA.

NOVEMBER 2 - 5: 29th Midwestern Regional Meeting, ACS, Kansas City, Kansas. Contact: M. Wickham-St. Germain, Midwest Res. Inst, 425 Volker Blvd, Kansas City, MO 64110, USA.

NOVEMBER 13 - 16: 50th Southwest Regional Meeting, ACS, Fort Worth, Texas. Contact: H. C. Kelly, Texas Christian Univ, Chem Dept, Ft. Worth, TX 76129, USA.

DECEMBER 4 - 6: IBEX'94, International Biotechnology Expo & Scientific Conference, Moscone Center, San Francisco, California. Contact: Cartlidge & Associates, Inc., 1070 Sixth Avenue, Suite 307, Belmont, CA 94002, USA.

1995

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 31 - JUNE 2: 27th Central regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 15 - 17: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 26 - MARCH 1: 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 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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.

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

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Additional Typing Instructions

1. The manuscript must be prepared on good quality **white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). The typing area of the first page, including the title and authors, should be 5½ inches wide by 7 inches high (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches high (14 cm x 21.6 cm).

2. The **title, abstract, tables and references** are typed single-spaced. All other text should be typed 1½-line spaced or double line spaced.

3. It is essential to use **dark black** typewriter or printer ribbon so that clean, clear, **solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed on separate pages, one table to a page. A table may not be longer than one page. If a table is larger than one page, it should be divided into more than one table. The word **TABLE** (capitalized and followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures (drawings, graphs, etc.)** should be professionally drawn in **black** India ink on separate sheets of **white** paper, and should be placed at the end of the text. They should not be inserted in the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). **Photographs** should be professionally prepared *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the word FIGURE and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The **reference list** should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.