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UNIFORMLY SIZED POLY(VINYLPHENOL) POROUS BEADS: A VERSATILE HPLC SEPARATION MEDIUM ALLOWING FAST SWITCHING BETWEEN SIZE-EXCLUSION, NORMAL PHASE, AND REVERSED PHASE CHROMATOGRAPHY

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ABSTRACT

Uniformly sized, 10 μm porous poly(4-*tert*-butoxycarbonyloxystyrene-co-divinylbenzene) beads with controlled porosity were prepared by polymerization of uniform size droplets consisting of small polymeric particles that were used as shape templates and enlarged with monomers and porogens. The *tert*-butoxycarbonyl groups were removed by a thermal treatment that affords the desired poly(4-hydroxystyrene-co-divinylbenzene) separation medium. The pore size of the beads does not change when immersed in THF as the beads are additionally crosslinked upon heating in the deprotection step. The beads were tested in size-exclusion, normal phase, and reversed phase chromatography. The size-exclusion calibration curve is almost linear in the range of molecular weights from 100 to about 480 000. Columns packed with the polymeric phenol beads can be switched between normal and reversed-phase chromatography simply by changing the solvent without removing from the chromatograph and their chromatographic properties do not change even after several hundred injections *in different chromatographic modes* including also multiple washing with aqueous sodium hydroxide. The retentions in separations of series of homologous alkylbenzenes, N-substituted, and ring substituted anilines were found to be linear functions of the number of carbon atoms, while interactions with the phenolic hydroxyls increased the retention times of N-alkylanilines. Separation of a protein mixture using a column packed with the beads has also been demonstrated and very high protein recoveries have been obtained.

INTRODUCTION

In spite of large number and great variety of separation media that are available for use in HPLC (1), the search for new, non-conventional packings suitable for demanding and specialized applications continues. Two broad categories of columns are the object of much development. First are the specialized packings, developed and optimized for a very specific type of separation. Among other applications, these columns are particularly well suited for routine or repetitive analyses in which a large number of similar samples are processed or for the monitoring of mixtures of defined compositions. Typical examples include columns for size-exclusion chromatography (SEC) with narrow exclusion limits, or beads with immobilized antibodies. The second type of columns are more versatile and may be used for different modes of chromatography without tedious repacking by simple adjustments in the chromatographic conditions. These packings are ideally suited for research and development laboratories where a wide variety of separations are performed frequently.

Current separation media may be divided into two large classes: inorganics and polymers. The most widely used inorganic packings are based on silica which, in its native state, contains siloxane groups with pK_a close to 7 (2). The acidity of siloxane hydroxyl groups of silica makes it similar to weak cation exchangers and limits its direct use in some chromatographic processes (1). While still less widely used that silica columns, polymeric stationary phases have attracted much interest lately, as they may offer a broader variety of chemistry and chemical stability even under extreme conditions of pH or solvents. In some modes of chromatography, such as ion-exchange chromatography (IEC) and SEC, polymeric stationary phases have assumed a dominant position.

The first textbook example of use of a macroporous polymer in HPLC-like liquid chromatography was described by Moore almost 30 years ago (3). Since their introduction in 1964, the styrene-divinylbenzene resins are still the most extensively studied polymeric phases for HPLC. However, their direct use is limited by their hydrophobic nature though this is clearly not a problem for their application to SEC in non-aqueous solvents such as tetrahydrofuran, and to reversed-phase chromatography (RPC) with acetonitrile or propanol as one of the components of the mobile phase. Overall, the styrene-divinylbenzene resins are considered to be typical nonpolar packings.

Chemical modification of styrene-divinylbenzene copolymers may be used to increase their polarity. For example, strongly acidic ion-exchangers are produced by direct sulfonation of the styrene matrix. Another route to increasing the acidity of the styrene-divinylbenzene matrix involves copolymerization with unsaturated aliphatic acids. For example, the incorporation of aliphatic carboxyls with pK_a values of about 5 results in weakly acidic ion-exchange resins. Ion- exchange chromatography is based on electrostatic interactions between the charged groups of both the dissolved compounds and the surface of the packing material.

Numerous polar packings for HPLC have been developed based on crosslinked polymers of 2-hydroxyethyl methacrylate, 2,3-dihydroxypropyl methacrylate, vinyl alcohol, hydroxylated polyethers or other monomers containing aliphatic hydroxyls. They are used directly in aqueous SEC, or, after derivatization, in the retentive modes of chromatography (1). These packings are not designed to interact in an electrostatic fashion with the compounds to be separated since their aliphatic hydroxyls have pK_a values of only approximately of 15-18.

The pK_a values of phenols being close to 10, their acidity is intermediate between organic acids and aliphatic hydroxyls. Polymeric phenols showed little potential for use as chromatographic media (4,5) until a method of polymerization of 4-*tert*-butoxycarbonyloxystyrene followed by deprotection was developed (6) and used in standard suspension polymerization to afford defined crosslinked porous beads (7). Both the pK_a of the phenol moiety and its shielding by adjacent *ortho* ring-substituents proved to be important variables for the control of the chromatographic properties of this novel medium. Its good performance in normal-phase chromatography was documented recently in a series of separations of substituted aromatic and aliphatic amines (8).

We have now vastly improved the capabilities of this new phenolic medium both with the preparation of highly efficient and uniformly sized beads of crosslinked poly(vinylphenol) and with their application in SEC, and in the direct and reversed-phase modes of chromatography of low molecular weight compounds and proteins.

EXPERIMENTAL SECTION

Preparation of Monodispersed Porous Beads

Uniformly sized shape-template latex particles 1.4 μm were prepared by emulsifier-free emulsion polymerization of styrene as described in detail elsewhere (9). The solid content in the aqueous dispersion was 29.2 wt%. To 0.80 mL of this dispersion was added 2.38 mL dibutyl phthalate emulsified by sonication in 12 mL of 0.25 wt% aqueous solution of sodium dodecyl sulfate (SDS). After all the tiny droplets of emulsified dibutyl phthalate had disappeared, a mixture of 21.7 g 4-*tert*-butoxycarbonyloxystyrene and 14.4 g divinylbenzene (80% DVB, Dow Chemical Co.)(monomers), 54 g cyclohexanol (porogenic diluent), and 0.36 g azobisisobutyronitrile (free radical polymerization initiator) emulsified in 220 mL of aqueous 0.25 wt% SDS was added. The resulting mixture was stirred slowly in a 500 mL round-bottomed glass reactor (Büchi, Uster, Switzerland) at room temperature until it contained only the swollen polymer particles. The contents of the reactor was supplemented with 90 mL of 4 wt% aqueous poly(vinyl alcohol) (Polyviol W 25/140, Wacker Chemie, Burghausen, Germany). The polymerization was then allowed to proceed under stirring with an anchor type stirrer (100 RPM) at 70 °C for 15 hours. The polymer beads were transferred to a 1000 mL beaker and washed by repeated decantation in water and methanol to remove the suspension stabilizer, surfactant, and the porogenic diluent. The original polystyrene shape-templates

were removed by extraction in toluene, the beads were washed with ethanol and finally dried in air.

Thermal Cleavage of the Protecting Groups

The deprotection of the phenol hydroxyls by removal of *tert*-butoxycarbonyl groups was done according to the method described in detail earlier (7,8). The beads were heated to 220-230°C in a flask immersed in an oil bath for 90 min. The flask was connected to a high-vacuum pump and the pressure inside the flask was kept under 25 Pa. The complete removal of the *tert*- butoxycarbonyl groups after the thermal cleavage was confirmed by the loss of the strong carbonyl band near 1760 cm^{-1} in the IR spectrum.

Chromatographic Experiments

The chromatography was carried out using an IBM LC/9560 ternary gradient liquid chromatograph equipped with a Rheodyne 7125 loop injector valve. The chromatographic bands were monitored by a HP 1050 UV detector (Hewlett-Packard, Avondale, Penn., USA) at 254 and 218 nm for low molecular weight compounds and polystyrene standards, and proteins, respectively. The beads were slurry packed with 50% aqueous acetonitrile into stainless steel columns of different sizes under constant pressure (11 MPa).

The size-exclusion chromatography was performed in 50 mm x 8 mm i.d. and 250 mm x 7 mm i.d. columns in THF with toluene and polystyrene standards with molecular weights ranging from 1250 to 2 950 000 (Polymer Laboratories). The normal phase, reversed-phase, and gradient elution chromatography experiments were carried out in a 50 mm x 8 mm i.d. column. Switching between the different chromatographic modes was done simply by changing the original mobile phase in the column to methanol followed directly by pumping the new mobile phase. The new mobile phase was pumped at 0.5 mL/min through the column for 2 hours to allow good equilibration. Detailed chromatographic conditions are described in the captions to Figures. All solvents used were of HPLC grade (Fisher Scientific). The proteins, i.e. cytochrome C (from bovine heart, MW 12 400), ribonuclease A (from bovine pancreas, MW 12 600), lysozyme (from chicken egg white, MW 13 900), carbonic anhydrase (from bovine erythrocytes, MW 29 000), chicken egg albumin (MW 44 000), and human serum albumin (MW 69 000), were purchased from Sigma. Protein recovery was calculated from the ratio between the protein peak area measured under standard chromatographic conditions and the peak area of the same amount of protein injected into the chromatographic system from which the column was removed and the inlet and outlet capillaries were connected with an empty column having a volume close to the V_i of the column tested.

Characterization of the Beads

Specific surface areas were determined from nitrogen sorption-desorption measurement and calculated according to the B.E.T. method. Pore size distributions in dry beads were calculated from mercury intrusion volumes (Combined BET Sorptometer and Mercury Porosimeter, Porous Materials, Inc., Ithaca, NY, USA). The pore size distribution and specific pore volume were also determined in the swollen state by inverse size exclusion chromatography (10) with polystyrene standards as probes.

RESULTS AND DISCUSSION

Porous copolymers of 4-vinylphenol, or its 2,5-disubstituted derivatives, with divinylbenzene have been shown to be very efficient separation media in the separation of amines by normal phase chromatography. Alkyl substitution in the positions ortho to the phenolic hydroxyl proved to be a powerful tool for the fine control of access to this phenolic hydroxyl by the compounds to be separated (8).

The first beads containing phenolic hydroxyls were prepared by standard suspension polymerization, therefore, they had a broad particle size distribution (7,8). Despite the lack of size classification, the columns packed with the novel stationary phase were very efficient except for their excessive back-pressure at linear flow rates exceeding about 10 cm/min (8). In order to improve on our earlier work we applied a preparation method which results in monodispersed beads as demonstrated in the preparation of packings based on copolymers of styrene and divinylbenzene (11-13).

The preparation method is based (14-16) on controlled swelling of small monodispersed size-templates (1.4 μm latex particles) followed by a suspension polymerization in the presence of porogenic substances (11-16) while the coalescence of the droplets is excluded by an extensive stabilization. This technique is only efficient when monomers with low water solubility, such as styrene, methyl methacrylate, glycidyl methacrylate, divinylbenzene, or ethylene dimethacrylate, are used. Since 4-*tert*-butoxycarbonyloxystyrene is a nonpolar, hydrophobic monomer that is poorly soluble in water, it is well suited for this kind of polymerization.

Physical Properties of the Packing

Monodispersed porous beads 10 μm in diameter were prepared by the method described in the experimental section. Their porous properties as checked in both the dry state and in a liquid medium are presented in Table 1. The specific surface area amounting to more than 150 m^2/g is sufficiently large for retentive modes of chromatography. The polymerization conditions, i.e. 40% of crosslinking monomer in the monomer mixture and 60% of porogenic diluent contained in the overall organic phase, were

TABLE 1
Properties of Porous Poly(Vinylphenol-co-Divinylbenzene) Beads

particle size, μm	10
specific surface area, m^2/g [a]	151
pore volume, mL/g [b]	1.45
average pore size, nm	
B.E.T.	11.1
size exclusion chromatography	13.5
mercury porosimetry	15.0
exclusion limit, MW [b]	480 000

[a] According to B.E.T. measurement. [b] According to inverse size exclusion chromatography.

chosen to obtain a chromatographic medium with moderately high exclusion limit and a large pore volume with good mechanical stability. The thermolyzed *tert*-butoxycarbonyl groups represent 33% of the original weight of monomers added in the polymerization. Their removal contributes to an increase in the pore volume which then amounts to 1.45 mL/g. This value compares favorably to standard polymeric separation media and represents a porosity of over 60%. Moreover, since the content of divinylbenzene units in the copolymer does not change in the deprotection step, its actual percentage in the final beads increases to 60 wt%.

The pore size distribution measurements performed both with the beads in THF by means of reverse size-exclusion chromatography, and in the dry state using mercury porosimetry coincide unusually well (Figure 1). This confirms the rigidity of the beads and the negligible swelling of the polymer chains within the beads when used in THF. The almost linear dependency of back pressure on flow rate in the whole range from 0 to 5 mL/min documents the pressure stability of the packing and the lack of particle deformation at high flow rates. It should be pointed out that even at a flow rate of 5 mL/min the back pressure does not exceed 14 MPa (Figure 2) and, owing to its monodispersity, the packing compares favorably to commercial styrene-divinylbenzene packings (17).

Size Exclusion Chromatography

The calibration curve measured in the determination of pore size distribution (Figure 3) already indicated that the porous polymer may be a good packing for SEC. The curve is almost linear in the entire range, from molecular weight of about 100 to 500 000. The pore volume in the 50 mm x 8 mm i.d. column (V_t = 2.5 mL) used for testing was V_i = 0.95 mL. As a low interstitial volume results from the monodispersity of the beads as well as the good packing technique (V_o = 0.76 mL, 30% of column volume),

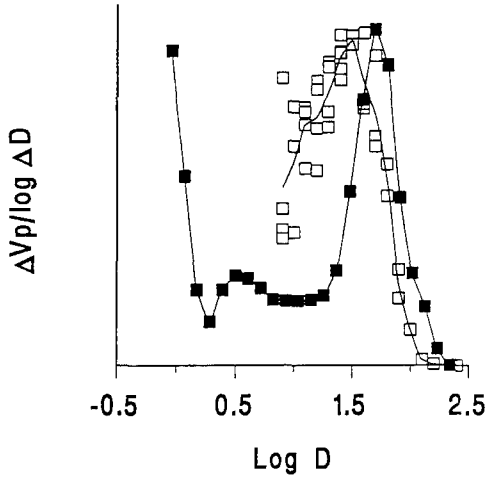

FIGURE 1. Pore size distribution curves of the beads calculated from inverse size exclusion chromatography (■) and from the mercury porosimetry (❑).

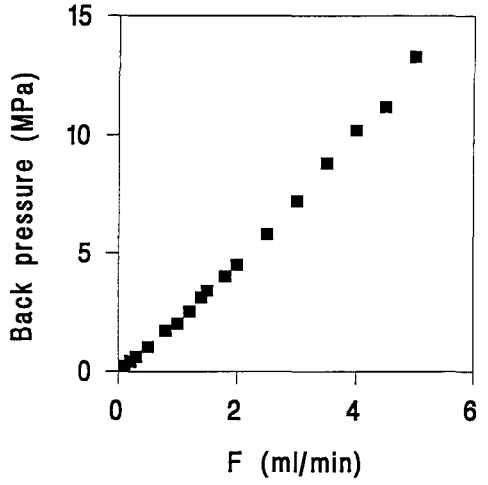

FIGURE 2. Effect of the flow rate on back-pressure. Conditions: column 50 mm x 8 mm i.d.; mobile phase, water-acetonitrile (1:1); analyte, toluene.

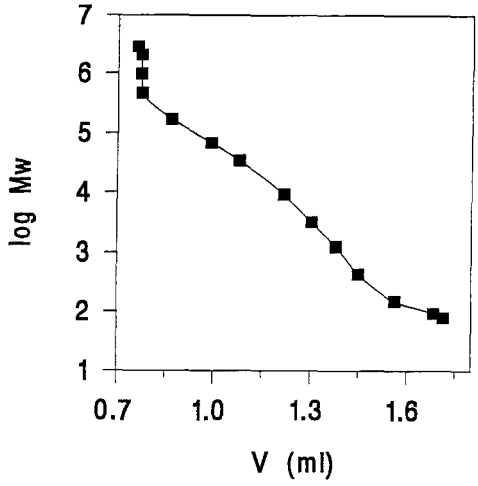

FIGURE 3. Molecular weight calibration with polystyrene standards and toluene in tetrahydrofuran with porous poly(vinylphenol-co-divinylbenzene) beads. Conditions: column 50 mm x 8 mm i.d.; flow rate 0.5 mL/min.

the phase ratio $f = V_i/V_o = 1.26$ is well within the range typical for SEC supports (1). The column efficiency measured with toluene in THF is 27 000 plates/m at a flow rate 0.5 mL/min.

The beads were repacked in a longer column (250 x 7 mm i.d.) and used in the separation of a mixture of polystyrene standards. Figure 4 shows very good resolution between the peaks of toluene and polystyrene with MW 1250, while the other peaks are also separated quite well. The characteristics of the column are similar to those of the shorter one: $V_t = 9.62$ mL, $V_o = 3.30$ mL (34%), $V_i = 3.98$ mL, $f = 1.21$. Column efficiency calculated for toluene was 19 300 plates/m at a flow rate 1 mL/min, 18 200 plates/m at 0.5 mL/min, and 12 800 plates/m at 0.2 mL/min.

Normal Phase Chromatography

The phenol groups on the surface of the beads increase the polarity of the separation medium making it useful for separations in the normal phase mode. The uniformly sized phenolic beads were tested in the separation of more complex mixtures (Figure 5) containing aniline and dimethylaniline (basic compounds), phenol (acidic compound), nitrobenzene (electron acceptor compound), and toluene (nonpolar compound), which is eluted first in hexane containing 5% of ethyl acetate, 2% methanol and 0.1% diethylamine used as the mobile phase. The peaks exhibit some fronting but the separation selectivity is good.

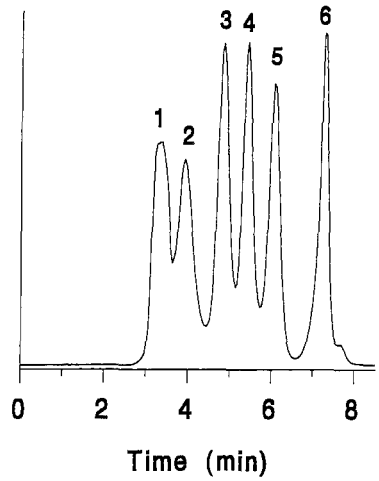

FIGURE 4. Separation of polystyrene standards with molecular weight 295 000 (1), 170 000 (2), 34 000 (3), 9 200 (4), 1 250 (5), and toluene (6). Conditions: column 250 mm x 7 mm i.d.; flow rate 1 mL/min; solvent THF.

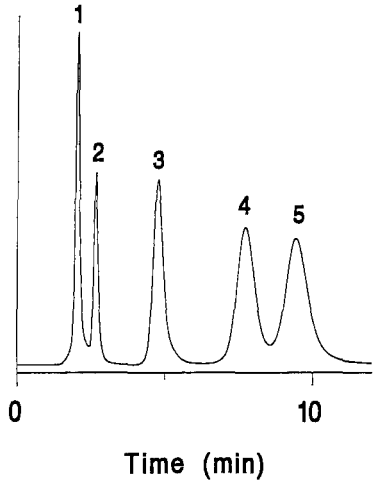

FIGURE 5. Separation of toluene (1), N,N-dimethylaniline (2), nitrobenzene (3), phenol (4), and aniline (5) by normal phase chromatography. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: hexane-ethyl acetate-methanol (93:5:2) with 0,1% diethylamine added.

TABLE 2
Capacity Factors k', Separation Selectivities α, and Asymmetry Factors A_s of Poly(Vinylphenol- co-Divinylbenzene) Column as Measured with Chemical Homologs and Other Test Compounds

Compound	k'	α	A_s
benzene	2.74		1.13
		1.33	
toluene	3.64		1.39
		1.32	
ethylbenzene	4.80		1.52
		1.37	
propylbenzene	6.58		1.59
		1.40	
butylbenzene	9.25		1.62
		1.38	
pentylbenzene	12.72		1.78
aniline	1.58		1.61
		1.40	
4-methylaniline	2.21		1.64
		1.39	
3,5-dimethylaniline	3.08		1.75
N,N-dimethylaniline	5.42		1.39
		1.33	
N-ethyl-N-methylaniline	7.23		1.36
		1.39	
N,N-diethylaniline	9.78		1.35
acetone	0.23		1.50
		2.65	
phenol	0.60		1.50
		1.69	
2-methylphenol	1.01		1.34
		1.49	
pyridine	1.50		1.43
		2.03	
nitrobenzene	3.05		1.63

Conditions: column 50 mm x 8 mm i.d.; mobile phase, acetonitrile-water 1:1; flow rate 1 mL/min.

Reversed Phase Chromatography

Since reversed phase HPLC is the most widely used mode of chromatography, it was useful to determine the capabilities of the packing in this chromatographic mode. The hexane based solvents in the column used in the previous tests were simply replaced by methanol, then by a mixture of water-acetonitrile without removing the column from the chromatograph. Several separations of mixtures, each containing very similar compounds, were made in an isocratic mode to confirm the usefulness of the packing medium in reversed phase chromatography. Table 2 summarizes the retention data, selectivities and peak symmetry factors obtained in some model separations. The peak symmetry factor of all separated compounds ranges from 1.13 to 1.78 and is generally good. An excellent peak symmetry ranging from 1.35 to 1.39 is characteristic of the separations of N-substituted anilines.

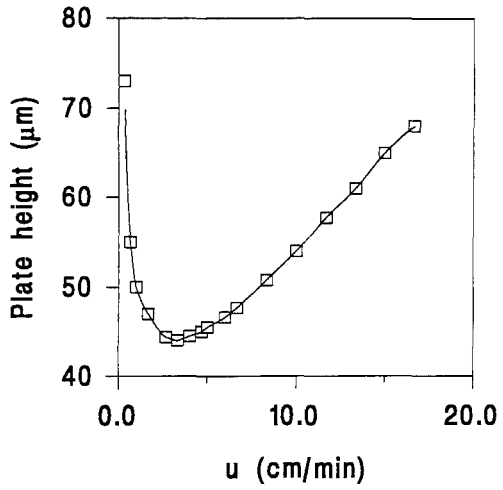

FIGURE 6. Effect of the linear flow velocity on the efficiency of poly(vinylphenol-co-divinylbenzene) column. Conditions: column 50 mm x 8 mm i.d.; mobile phase, water acetonitrile (1:1); analyte toluene.

A plot of plate height *vs.* linear flow velocity (Figure 6) obtained for the monodisperse 10 μm poly(vinylphenol) beads from a measurement in 1:1 v/v acetonitrile-water, shows that the plate height achieved with the beads is comparable to that for poly(styrene-co-divinylbenzene) stationary phases (17,18) with the optimum linear velocity at about 1 ml/min.

The separation of a mixture similar to that used in normal phase chromatography is shown in Figure 7. Obviously, toluene is now eluted last as it is the most non-polar compound in the mixture, while phenol, which is more polar, is located close to the front of the chromatogram. The inverse elution sequence is typical for the change from normal to reversed phase chromatography.

The non-polar alkylbenzenes are baseline separated within 20 minutes at a flow rate of 1 mL/min (Figure 8). The standard linear relationship between the logarithm of number of carbon atoms in alkylbenzenes in the series from benzene to pentylbenzene (Figure 9) has a slope of 0.134 which is close to that found for styrene-divinylbenzene packings (19).

The separation of ring and N-alkyl substituted anilines may be driven by both polarity and hydrogen bonding between the solutes and the stationary phase. The poly(vinylphenol) packing separates substituted anilines very well (Figure 10a). Figure 10b shows an example of separation of benzylamine and N-substituted anilines.

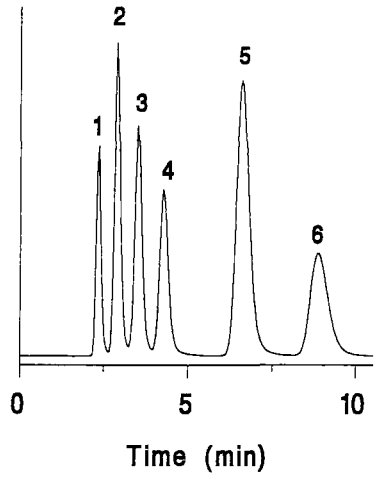

FIGURE 7. Separation of acetone (1), phenol (2), 2-methylphenol (3), pyridine (4), nitrobenzene (5), and toluene (6) by reversed-phase chromatography. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: acetonitrile-water (1:1).

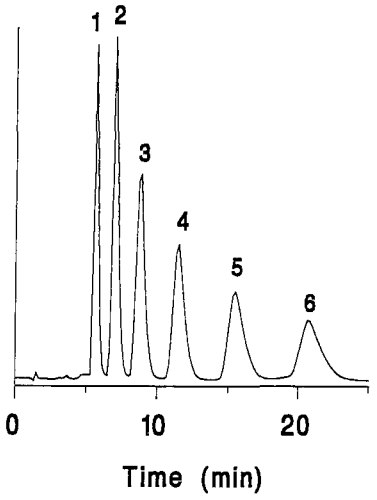

FIGURE 8. Separation of alkylbenzenes by reversed-phase chromatography. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: acetonitrile-water (1:1). 1- benzene, 2-toluene, 3-ethylbenzene, 4-propylbenzene, 5-butylbenzene, 6-pentylbenzene.

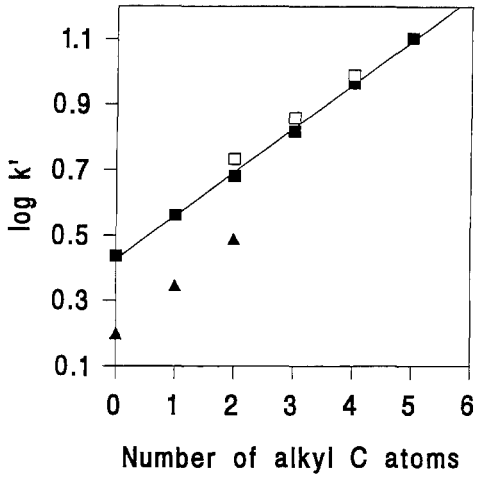

FIGURE 9. Variation of log k' with the number of carbon atoms for compounds in homologous series. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: acetonitrile-water (1:1). Alkylbenzenes (■), N-alkylanilines (❑), ring alkyl substituted anilines (▲).

The values of log k' appear to be a linear function of the carbon atom number with a slope similar to that of other separations (Figure 9). In contrast to styrenic phases (19), the retention of N-alkylanilines exceeds the retention of alkylbenzenes. This may be assigned to the stronger interaction of the N-alkylaniline with the phenolic hydroxyls of the packing. The retention of ring-substituted anilines is lower. Benzene and aniline behave as the first members of the alkylbenzenes and ring substituted anilines homologous series, respectively, while aniline does not fit as the parent compound in terms of its retention behavior within a series of N-substituted anilines.

The pore size of the beads and their good efficiency in isocratic reversed-phase chromatography of small molecules also suggests the use of this chromatographic medium for the separation of proteins. The gradient elution separation of a model mixture containing 6 proteins is depicted in Figure 11. The proteins are well separated at the flow rate of 1 mL/min in a 20 min gradient (Figure 11a). Increased flow rate and decreased gradient time make the chromatography faster but at the expense of lower resolution (Figure 11b). Despite the presence of phenolic hydroxyl groups on the surface of the polymer matrix, there is no irreversible protein adsorption in the beads. Table 3 documents the very high recovery (close to 100 %) for all individual proteins tested under reversed phase conditions.

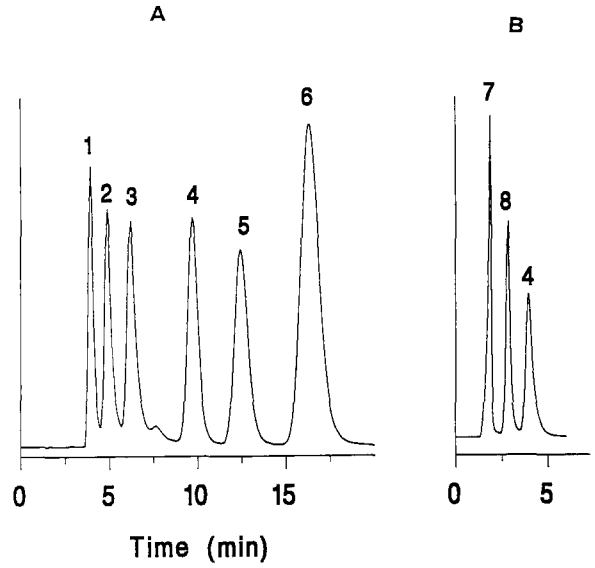

FIGURE 10. Separation of aniline derivatives by reversed-phase chromatography. Conditions : column 50 mm x 8 mm i.d.; flow rate 1 mL/min; (a) mobile phase, acetonitrile-water 1:1. 1-aniline, 2- 4-methylaniline, 3- 3,5-dimethylaniline, 4- N,N-dimethylaniline, 5- N-ethyl-N-methylaniline, 6- N,N-diethylaniline; (b) mobile phase, methanol -water-THF (70:25:5) with 0.6% acetic acid; 7- benzylamine, 8- N-methylaniline.

TABLE 3
Protein Recovery from a Poly(Vinylphenol-co-Divinylbenzene) Column

Protein	Recovery, %
Ribonuclease	98
Cytochrome C	104
Lysozyme	99
Human serum albumin	107
Carbonic anhydrase	94

Conditions: column 50 mm x 8 mm i.d.; mobile phase 0.1% v/v aqueous trifluoroacetic acid/acetonitrile (1:1 v/v); flow rate 1 mL/min; protein concentrations 10 mg/mL; injected volume 20 μL.

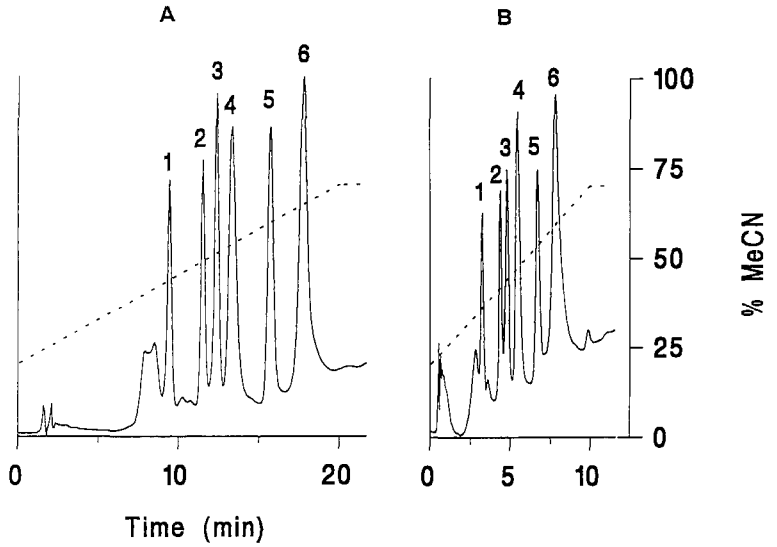

FIGURE 11. Separation of protein mixture by reversed-phase chromatography. Conditions: column 50 mm x 8 mm i.d.; mobile phase, 0.1% trifluoroacetic acid in water containing 20% acetonitrile (eluent A) or 70% acetonitrile (eluent B). 1- ribonuclease A, 2- cytochrome C, 3- lysozyme, 4- human serum albumin, 5- carbonic anhydrase, 6- chicken egg albumin. a) flow rate 1 mL/min, gradient time (from 100% eluent A to 100% eluent B) 20 min; b) flow rate 3 mL/min, gradient time 10 min.

Column Stability

As most of the measurements described above were done on a single column, the chromatographic mode had to be changed from reversed phase to normal phase and back several times. Since these changes did not appear to have any effect on the efficiency, k' values, and on the back pressure a systematic test of reproducibility of chromatographic properties was initiated. This test involved 300 injections of toluene or a mixture of benzene, toluene and ethylbenzene in acetonitrile-water (1:1 v/v) interspersed with over 100 injections in other modes (e.g. normal phase and gradient elution) within a three months period. A typical protocol for this long-term test is as follows:

(a) 25 Injections of hydrocarbon mixture, each tenth injection being toluene alone.

(b) 20 Protein injections in a gradient mode.

(c) Repeat hydrocarbon injections as in (a), each tenth injection being toluene alone until a total of 100 hydrocarbon injections (10 toluene data points).

(d) Wash the column with aqueous sodium hydroxide (1 mole/L), then acidify.

(e) 50 Hydrocarbon injections as above.

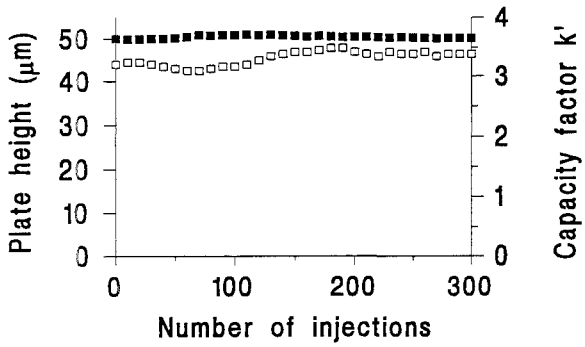

FIGURE 12. Stability of poly(vinylphenol-co-divinylbenzene) column as measured from changes of plate height (❑) and toluene capacity factor k' (■) upon multiple injections. Conditions: column 50 mm x 8 mm i.d.; mobile phase, water-acetonitrile (1:1 v/v); flow rate, 1 mL/min; analyte, toluene.

(f) Switch to normal phase and perform 50 injections of a mixture of toluene, aniline, and N,N-dimethylaniline under the conditions of Figure 5.

(g) 50 Hydrocarbon injections as above.

(h) Repeat washing as in (d).

(i) Repeat (e), (f), (g), (h).

Figure 12 documents the hydrocarbon injections in reverse phase and shows that the column efficiency only decreases very slowly with the number of injections while the capacity factor does not change. It should be emphasized that Figure 12 shows only the injections of toluene which were interspersed with other injections as noted above.

No changes in selectivity was observed during the series of 300 injections. Column washing was carried out using 50 volumes of 1 mol/L aqueous sodium hydroxide to determine whether or not phenol group ionization had an effect on the reproducibility of the chromatographic separation. The original column properties were restored within 10 hours of equilibration in a flow of mobile phase containing water, acetonitrile and trifluoroacetic acid. The back pressure *vs.* flow rate dependency did not differ from that shown in Figure 2 even after using the same column for a total of more than 500 injections. These results clearly document the stability of the column properties even after numerous chromatographic mode changes and washing with sodium hydroxide. This makes the column superior to the silica based media as no silica phases can withstand the washing with NaOH used in the testing protokol of this medium.

CONCLUSION

Poly(4-hydroxystyrene-co-divinylbenzene particles prepared by a swelling of polystyrene shape template particles followed by a suspension polymerization affords uniformly sized spherical beads are highly chemically stable and show the rigidity and durability necessary to withstand rapid changes from highly polar to non polar solvents. The beads are versatile and can be used efficiently for size-exclusion, normal phase and reversed-phase chromatography of low molecular weight compounds as well as proteins. In contrast to the preparation of typical polymeric separation media, the preparation method used also includes a deprotection step at a relatively high temperature and results in a polymeric separation medium with increased rigidity and minimized swelling ability. The phenolic moieties in the beads contribute greatly to the separation process through their interaction with polar compounds. In comparison with the water compatible modified polystyrene stationary phase (20), the poly(vinylphenol) phase do not require any additional chemical modification procedure and is more flexible for solvent changes.

ACKNOWLEDGEMENT

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USE OF DERIVATIVE SPECTROPHOTOMETRY IN THE RESOLUTION OF OVERLAPPED PEAKS IN LIQUID CHROMATOGRAPHY AND ITS APPLICATION IN THE ANALYSIS OF ACTIVE COMPONENTS IN INSECTICIDE FORMULATIONS

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A procedure for the determination of active components in insecticide formulations showing overlapped peaks in liquid chromatography has been devised. The procedure is based in the use of derivative spectra of the components obtained by a diode-array spectrophotometer around the maxima signal of the chromatographic peak, and has been applied to the analysis of mixtures of piperonyl butoxide, neopynamine and fenitrothion with satisfactory results.

One of the most serious problems with which chromatography workers are confronted is the occurrence of only partially resolved peaks arising from coelution of solutes in the sample or from similarities between their retention times. Traditionally, this type of

problem was addressed by modifying the experimental conditions by trial and error until the aforesaid errors were minimized. Thus, different mobile or stationary phases (columns) or even working techniques (e.g., isocratic or gradient elution) were tested, which was time-consuming and involved consumption of expensive solvents.

The advent of multi-dimensional detection systems and the affordability of personal computers provides with software that allows storage and subsequent processing of chromatographic data has fostered developments of new experimental procedure for the characterization of unresolved peaks. There are three basic alternatives to computer-assisted resolution of chromatographic peaks, namely:

(a) Fitting the chromatogram peak to known functions. There are some precedents to the use of this alternative in gas chromatography ranging from the use of Gaussian and non-Gaussian models, to convoluted Gaussian curves with exponential decay and fast Fourier transform techniques. Solutions involving comparison of logarithmic spectra [1] or the use of recent chemometric methods [2-6] have been tested in liquid chromatography as implemented with diode-array detectors.

(b) Integration by tracing a line perpendicular to the baseline from the valley between two peaks or one joining the valley and the end of the second peak (skimming) by computing the area of each separately from the two zones thus established.

(c) Use of derivative techniques. As far as practical applications, the information supplied by derivative spectroscopy used as detection system in liquid chromatography has been exploited in two different

ways, namely: i) By using the first derivative of the elution profile obtained at the wavelength of the absorption maximum. In theory, the derivative should be zero at this point and therefore the dissapearance of the main peak may reveals the presence of other constituents with different absorption features. This procedure is called "null spectral derivative technique" [7]; ii) By using the complete derivative obtained by recording the elution profile. This procedure, known as "spectral derivative mapping technique" was studied theoretically by Grant et al. [8], who discussed specific cases where the spectral curves of potential impurities lay within the spectral band of the major components. This procedure has been applied in the resolution of diverse mixtures [9,10]

Other procedures have been described also. Thus, Hayashi et al. [11] proposed a one-dimensional Kalman filter, run in real time, to resolve partially overlapped chromatographic peaks using a one-dimensional empirical model based on prior measurements of peak shape and location. A Kalmen filter, based on repetitive filtering of diode-array spectra obtained accross a chromatogram, has been developed recently [12]. Campins [13] developed a procedure, named "H-point standard additions method" in which the analytical signal absorbance values (peak-heights) registered at the retention time of the analyte are used.

In the present work, a new procedure has been developed by using the second derivative spectra of the components obtained around the maxima signal of the overlapped chromatographic peaks. A diode-array spectrophotometer is used with this purpose. This procedure, which has been applied to the analysis of active components in

insecticide formulations, make possible the easy transformation of a chromatographic problem in a spectrophotometric problem.

EXPERIMENTAL SECTION

Chemicals

All the reagents were of analytical-reagent grade and acetonitrile (mobile phase) were of LC grade. Standard solutions of insecticides were prepared by dissolving in acetonitrile pure piperonyl butoxide, neopynamine and fenitrothion (Chem Service Inc.) at different concentrations.

Apparatus

Spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer linked to a data system (Lab Calc from Galactic Ind. Co.) for data acquisition and storage. The system was coupled to a quaternary pump (Hewlett-Packard, 1050 series). The analytical column was a 10μm Lichrosorb RP-8 (25 cm x 4.6 mm i.d.) from Merck.

Procedure

The solvent flow-rate was set at 2 ml min^{-1} and 20 μl of each sample were injected. The detector was set to collect a spectrum every 1 s (over the range 190-310 nm). Spectrophotometric measurements were performed between the maximum (peak) and minimum (valley) of the second-derivative spectrum, viz. between 226-230, 230-234, 234-238 and 238-242 nm for neopynamine, 206-214 nm for piperonyl butoxide and between 200-210 nm for fenitrothion. A calibration graph was

previously constructed for each component from solutions of known concentrations of each. The concentration of neopynamine should be between 2.8-5.2 x 10^{-5} M, while that of piperonyl butoxide should lie in the range 1.4-2.6 x 10^{-4} M and the fenitrothion one between 6-14 x 10^{-5} M.

RESULTS AND DISCUSSION

The mixtures of fenitrothion, neopynamine and piperonyl butoxide show peaks with a great overlapping in liquid chromatography under the experimental conditions habitually used. The use of the second-order derivative spectra obtained around the maximum signal of the chromatographic peak make possible the discrimination of the spectral bands of each component of the mixture. Zero-order uv-visible spectrophotometry cannot be used with this purpose owing to extensive spectral overlap of the absorption bands of these compounds, but the second-derivative spectra show differents peaks (Fig. 1) which make possible the resolution of the mixtures.

The second-order derivative spectra show different peaks which allow to realize measures to various wavelengths, whereas that piperonyl butoxide and fenitrothion show a peak only, both to lower wavelengths. In all cases, the measurements of the derivative signal is made between a maximum (peak) and a minimum (valley), the intensity of which is dependent on the concentration of the corresponding compound. Table 1 list the figures of merit of the calibration graphs run for the three substances assayed.

The procedure was compared with other methods: conventional absorption spectrophotometry (without chromatographic separation) (1),

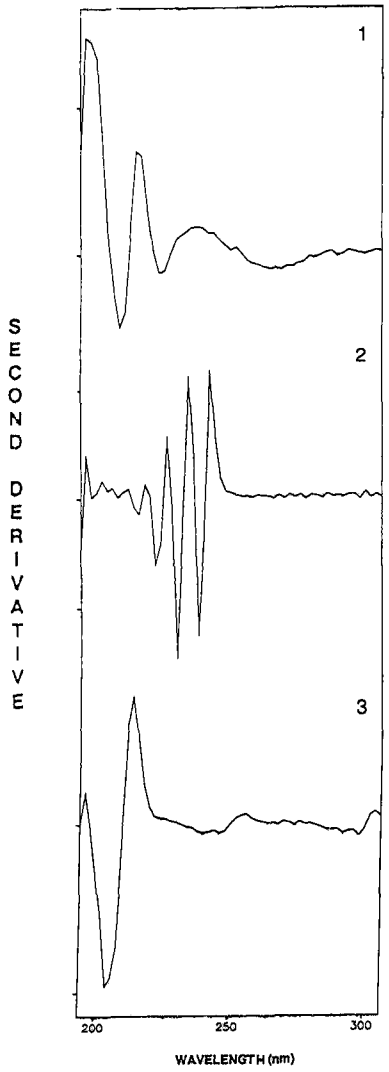

Figure 1. Second-order derivative spectra of piperonyl butoxide (1), neopynamine (2) and fenitrothion (3).

Table 1. Figures of merit of the calibration graphs.

Insecticide	Calibration equation	Wavelength range (nm)	Coef. of correlation	%RSD (n=6)
Neopynamine	h = 0.0661 C + 0.0008	226 - 230	0.997	0.97
	h = 0.0834 C + 0.0063	230 - 234	0.994	0.86
	h = 0.0785 C + 0.0050	234 - 238	0.990	0.82
	h = 0.0760 C + 0.0125	238 - 242	0.990	1.29
Piperonyl Butoxide	h = 0.0853 C + 0.1953	206 - 214	0.994	2.11
Fenitrothion	h = 0.0257 C - 0.0143	200 - 210	0.998	2.50

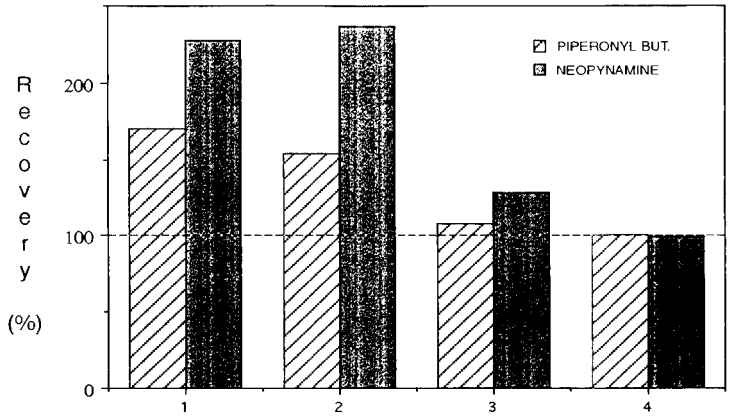

Figure 2. Recovery values obtained for the determination of neopynamine and piperonyl butoxide in commercial formulation.
METHODS: 1, conventional absorption spectrophotometry; 2, liquid chromatography; 3, liquid chromatography-zero order spectrophotometry; 4, liquid chromatography-second derivative spectrophotometry. Results are the average of six determinations.

Table 2. Analysis of commercial formulations by liquid chromatography-second derivative spectrophotometry.[a]

Formulation No	Piperonyl butoxide		Neopynamine		Fenitrothion	
	Stated concentration (10^{-5} M)	Found (10^{-5} M)	Stated concentration (10^{-5} M)	Found (10^{-5} M)	Stated concentration (10^{-5} M)	Found (10^{-5} M)
1	20.3	20.7	4.0	3.9	-	-
2	20.0	20.8	-	-	10.0	10.1
3	-	-	5.0	5.4	8.0	7.9

[a] The values are based on repetitive measurements on seven samples.

liquid chromatography (2), and liquid chromatography using the conventional spectra of the peak (3) and second-order derivative spectra (4). Results obtained with the binary mixture neopynamine-piperonyl butoxide are represented (Fig. 2). Procedure 4 (proposed in this work) offers the more precise recovery. Similar results are obtained with the other binary mixtures.

The procedure devised was applied to the resolution of three binary mixtures of the insecticides assayed. These mixtures were prepared artificially with a composition similar to commercially available formulations. Results are summarized in Table 2.

This method make possible the analysis of compound showing appretiable overlapping in their chromatographic peak without modification of the experimental variables used. Analysis is possible if the compounds showed different derivative spectra.

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COMPETITIVE BINDING OF CATECHOLAMINES, INDOLEAMINES, ACETYLCHOLINE, AND RELATED METABOLITES TO VARIOUS GLASSY CARBON MATERIALS

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ABSTRACT

We have previously demonstrated the utility of a pre-column containing glassy carbon particles in the determination of acetylcholine and choline by liquid chromatography with electrochemical detection (Ikarashi et al., 1992). Such a pre-column adsorbs neurochemically related catechol and indole derivatives and, thus, prevents interference in the chromatogram for acetylcholine and choline. In the current studies, we report the results of examining three important and related characteristics of this adsorption process. First, the source materials and conditions used in the preparation of the glassy carbon particles substantially affect adsorption of the 'interfering' catecholamines, indoleamines, and related metabolites. Second, the primary adsorption sites appear to have a substantial amount of graphite-like,

rather than diamond-like, character. Third, the Langmuirian adsorption of the catechol and indole compounds is at least partly competitive, indicating the probable involvement of common adsorption sites.

INTRODUCTION

In the development of methodology for the determination of catecholamines, indoleamines, acetylcholine, and related metabolites in rat and mouse brain tissues using liquid chromatography with electrochemical detection (LCEC), we previously attempted to directly utilize the supernatant of a perchloric acid homogenate to avoid the additional isolation steps normally required for acetylcholine (ACh) and choline (Ch) (1). While the determination of catecholamines, indoleamines, and related metabolites were quite amenable to the direct injection of this homogenate (2), direct injection of the homogenate into the separate LCEC setup normally employed for ACh and Ch, as basically described by Potter et al. (3-4), exhibited both an undesirable large solvent front response and other, unidentified peaks. The unidentified peaks were shown to be catecholamine related species (1). Quite by accident, we discovered that glassy carbon particles (IRICA Type CP-2250) were capable of adsorbing these interfering substances in the LCEC analysis of ACh and Ch, while showing virtually no adsorption of the targeted quaternary amines. A pre-column packed with the glassy carbon particles not only eliminated the interfering peaks, but also substantially decreased the size of the solvent front. Subsequent examination of adsorption by these same particles exhibited a Langmuir type adsorption for virtually all the catecholamines, indoleamines, and related metabolites while not adsorbing any of the quaternary amines; adsorption constants and the number of available adsorption sites were determined for each of the species of concern (6). In general, the adsorption favored the non-ionic form of the compounds of concern over their ionic congeners. Adsorption of the more hydrophobic species was preferred over that of the more hydrophilic species; thus, the indolic compounds were preferentially adsorbed over the catechol species.

Glassy carbon is typically formed in two separable steps (7). In the first, a polymeric precursor is heated with pressure to 300-400°C, under which conditions the material experiences an endothermic thermal reforming. Subsequently, the material is slowly heated to a final curing temperature of 1000-3000°C, with coalescence of stabilized polymer chains occurring between 400 and 1000°C. Between 1000 and 2700°C, local distortions due to internal defects and bonds with

adjacent ribbons are essentially eliminated. During this entire carbonization process, elements other than carbon are expelled from the precursor, and the carbon atoms in materials heated above 2700°C are virtually all in the sp^2, or graphite-like, form as opposed to the sp^3, or diamond-like, form. However, the particular properties of a given glassy carbon material may vary considerably depending on both the polymeric precursor and the exact method of manufacture. Thus, we decided to investigate alternative glassy carbon materials to see if they possessed the same desirable adsorption properties we had previously observed with the IRICA Type CP-2250 (1,6).

Our previous studies also left unanswered two other pertinent questions. These concern the nature of the adsorption site on the glassy carbon and the possible competition between the various neurochemicals studied for a limited total number of available adsorption sites. These issues are also addressed in the current report.

MATERIALS AND METHODS

Reagents

The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO: acetylcholine (ACh) chloride, choline (Ch) chloride, 5-hydroxytryptamine (5-HT) creatinine sulfate monohydrate, 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA) hydrobromide, norepinephrine (NE) hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). Ethylhomocholine (N,N-dimethyl-N-ethyl-3-amino-1-propanol or, simply, EHC) was prepared as described previously (3-4). Chemicals used in the LCEC eluting solvents were obtained from various manufacturers in the highest purity available.

Glassy carbon particles, 100-200 mesh from both the Mitsubishi Pencil Co., Ltd. (Gunma, Japan) and the Tokai Carbon Co., Ltd. (Tokyo, Japan) and derived from phenolic resin fibers, were heated to final curing temperatures of 1000, 1400, 2000, and 3000°C for the initial studies (c.f., Fig. 2). Plastic formed carbon materials (PFC), also 100-200 mesh, were supplied by the Mitsubishi Pencil Co.; these were created by mixing 0, 50, 60, 80, and 100% pure graphite with a vinyl chloride resin and pyrolyzing to form a 'glassy carbon' at a final temperature of 1400°C (c.f., Fig. 3). The investigation of possible competition between different adsorbates employed the 100-200 mesh glassy carbon particles from Tokai Carbon Co. heated to a final temperature of 3000°C (c.f., Figs. 4-8).

LCEC for Catecholamines, Indoleamines, and Related Metabolites

The LCEC system employed for determination of the catecholamines, indoleamines, and related metabolites consisted of a PM60 pump, a CC-4 injector, a Biophase® ODS-IV column (3 μm, 4x110 mm), an LC-4B amperometric detector, a dual glassy carbon electrode (E_{app} = +0.70 volts vs. Ag/AgCl), and an LC-22A temperature controller (35°C), all from Bioanalytical Systems, Inc. (Tokyo, Japan). An SCI Chromatocorder from Yokogawa Co., Ltd. (Tokyo, Japan) was used for data processing. The eluting solvent was a 0.050 **M** citrate buffer, pH 3.20, containing 0.80 m**M** sodium 1-octanesulfonate and 0.50 m**M** disodium ethylenediaminetetraacetic acid; the typical flow rate was 0.8 mL/min. A typical chromatogram for a standard mixture of components is shown in Fig. 1A. Detection limits for the compounds of concern were ≤ 1 pmol.

LCEC for Acetylcholine and Choline

The LCEC system employed for the determination of the acetylcholine, ethylhomocholine, and choline consisted of an LC100P pump, an LC100S injector, and an LC100W/F work station for data processing from Yokogawa Co. Components obtained from Bioanalytical Systems included an LC-4A amperometric detector with dual platinum electrodes (E_{app} = +0.50 volts vs. Ag/AgCl), an LC-22A temperature controller (35°C), an Acetylcholine Separation Column (3 μm, 4x60 mm), and a post-column (4x5 mm) containing immobilized acetylcholinesterase and choline oxidase. The mobile phase was a 0.050 **M** phosphate buffer, pH 8.40, containing 1.0 m**M** disodium ethylenediaminetetraacetic acid and 0.40 m**M** sodium 1-octanesulfonate; typical flow rates were 0.80 mL/min. A chromatogram for a standard mixture of ACh, EHC, and Ch is shown in Fig. 1B. Detection limits for the compounds of concern were 2-5 pmol.

Adsorption Determinations

All determinations of amount adsorbed and percent adsorbed employed 50 mg of the glassy carbon material tested and 1.00 mL of a 0.10 **M** phosphate buffer, pH 7.0, containing various amounts of NE, DA, 5-HT, 5-HIAA, HVA, DOPAC, ACh, EHC, and/or Ch. The carbon particles and buffer solution were mixed for a few seconds on a vortex mixer, and the supernatant was collected by passing the

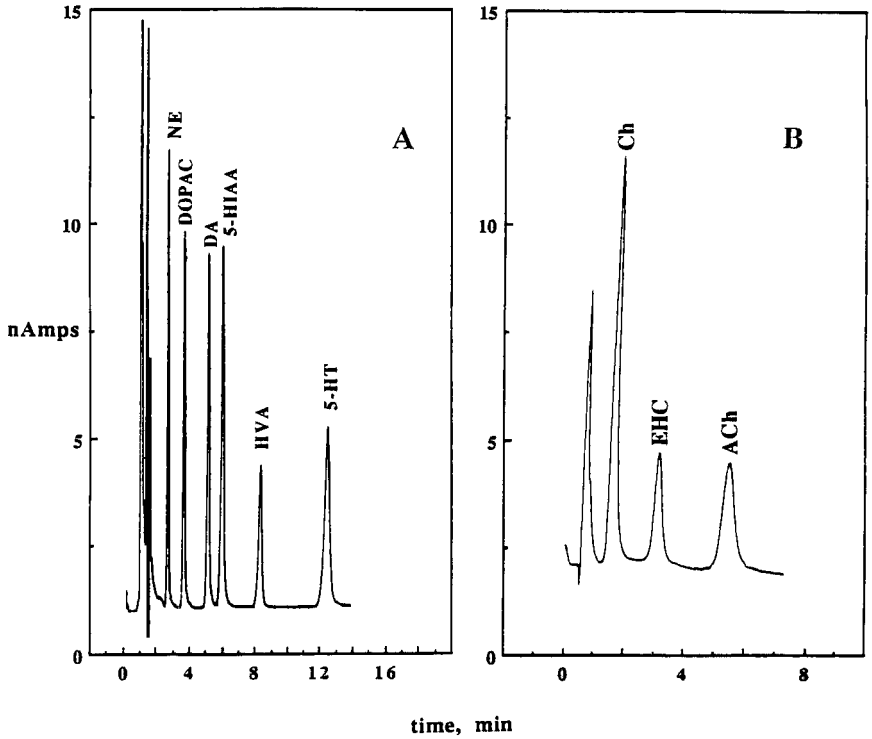

FIGURE 1. Chromatograms obtained from injection of separate mixtures of indoles/catechols and quaternary amines into the LCEC systems designed for the determination of catecholamines, indoleamines, and related metabolites (A) and for the determination of acetylcholine and choline (B). Injections were 4.0 μL aliquots of phosphate buffers containing 2.00 nmol/mL of the substances labeled in each trace.

mixture through a 0.45 μm Millipore® filter; an accurate 4.0 μL aliquot of the supernate was then injected into the LCEC for quantitation of the compound(s) of concern. The amount absorbed for a compound is given as:

$$\text{Amount Absorbed (nmol/g)} = \frac{C(PH - PH_{GC})(1.00\ \text{mL})}{0.050\ \text{g}}$$

where

C = proportionality constant to convert peak height to concentration, μM/nA or [(nmol/mL)/nA],

PH = peak height for compound using the phosphate buffer with no glassy carbon particles, nA,

PH_{GC} = peak height for compound after exposure to the glassy carbon particles, nA,

and the percent adsorbed is given as:

$$\% \text{ Adsorbed} = \frac{(PH - PH_{GC})}{PH} \times 100$$

All determinations are reported as the mean of duplicate measurements.

RESULTS

Effect of Nature of Glassy Carbon Material on Adsorption

In all these experiments, the phosphate buffer aliquots exposed to the glassy carbon materials contained 4.0 nmol of each of the nine neurochemicals listed above under reagents. The percent adsorbed for each of the neurochemicals as a function of the final curing temperature used in the preparation of the glassy carbons, each derived from phenolic resin fibers, is shown in Fig. 2. While no measurable amount of any of the compounds tested was adsorbed onto the 1000 and 1400°C glassy carbons, a small amount of 5-HT (20%) only was adsorbed onto the 2000°C material. The 3000°C material showed adsorption of all the catecholamine and indoleamine related compounds, with maximal adsorption of 5-HT (88%) and minimal adsorption of DOPAC (5%). No adsorption of ACh, EHC, or Ch was observed onto any of the glassy carbon materials tested.

Using the same nine component phosphate buffer mixtures, the effect of adding various amounts of graphite to the material used in the preparation of the glassy carbon particles was examined. In these studies, the glassy carbon precursor was composed of a vinyl chloride resin with 0, 50, 60, 80, or 100% graphite. The carbon mixture was heated to a final curing temperature of 1400°C. The results, shown in Fig. 3, clearly indicate a general trend toward increased adsorption of all of the individual compounds at increased graphite content of the carbon mixture. While the exact order of maximal to minimal adsorption for the catechols and indoles was not identical to that seen in the previous Fig. 2, the same general trend prevailed, with 5-HT and 5-HIAA showing the strongest interaction with the glassy carbon. Additionally, the quaternary amines were not immune to adsorption with this graphite-containing glassy carbon. Some of the EHC (15%)

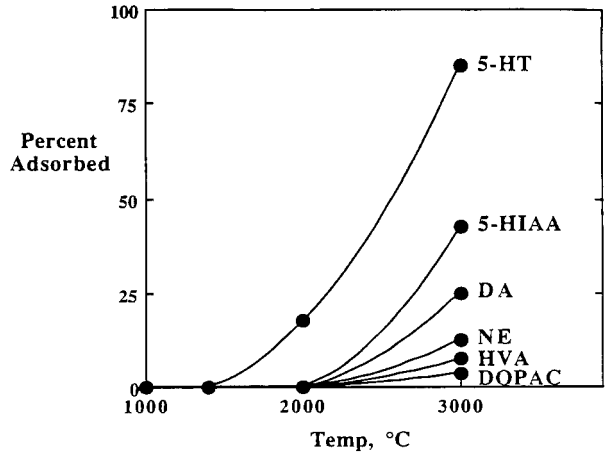

FIGURE 2. Adsorption of various neurochemicals onto glassy carbon derived from phenolic resin fibers as a function of the temperature of the final heat treatment. The glassy carbon particles were exposed to 1.00 mL of a phosphate buffer containing 4.0 nmol/mL of the chemicals shown as well as the same concentration of ACh, EHC, and Ch. No adsorption of the latter three quaternary amines was observed.

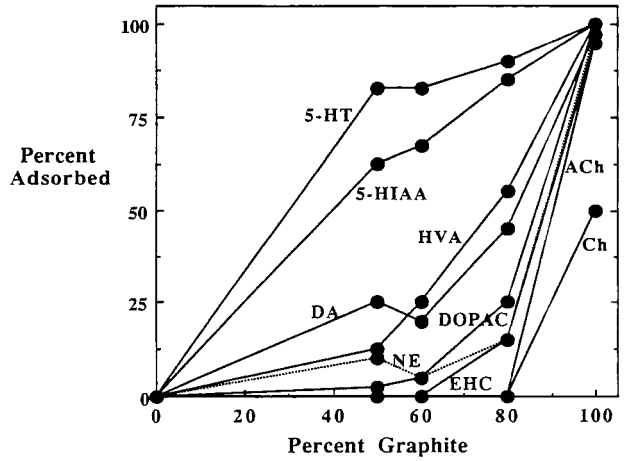

FIGURE 3. Adsorption of various neurochemicals onto glassy carbon derived from a mixture of graphite and polyvinyl chloride as a function of the original graphite content. The glassy carbon particles were exposed to 1.00 mL of a phosphate buffer containing 4.0 nmol/mL of the nine chemicals shown.

was adsorbed on the 80% graphite material, and all three quaternary amines (ACh, EHC, and Ch) were clearly and substantially adsorbed on the pure graphite heated to 1400°C.

Competitive Adsorption of Neurochemicals onto Glassy Carbon

In a preliminary investigation of possible competition between neurochemicals for a limited number of adsorption sites on the glassy carbon particles, we measured the amount of individual components adsorbed from a six component catechol/indole mixture. The individual phosphate solutions tested contained 2.0, 4.0, and 10.0 nmol/mL of each of the compounds. The glassy carbon employed in this and all subsequent competitive tests was the 3000°C material derived from the phenolic resin fibers. As seen in Fig. 4, as the amount of the individual components in the mixture increased, an increased amount of 5-HT and, to a lesser extent, 5-HIAA were adsorbed; however, the amounts of DA, NE, HVA, and DOPAC adsorbed clearly decreased as the amount of all the components increased. These results suggest competition between at least some of the compounds for shared adsorption sites on the carbon.

To further investigate this phenomena, we selected three compounds (5-HT, 5-HIAA, and DA), representing strongly, moderately, and weakly adsorbed substrates, respectively. The adsorption of each of these three species was tested at concentrations varying between 2 and 100 nmol/mL individually, i.e., with no other compounds in the buffer solution exposed to the carbon particles. As seen in Fig. 5, in the absence of other components, 5-HT is, indeed, the most strongly adsorbed; on the other hand, in the absence of other components, the adsorption of both 5-HIAA and DA are quite similar. Employing a Langmuir framework and least squares fitting routine along with the data for Fig. 5, we obtained values for the adsorption constants and the number of adsorption sites for the compounds shown. The pertinent adsorption equation is

$$K_{ads} = \frac{[SA]}{[S]\ [A]}$$

where K_{ads} is the adsorption constant (μM^{-1}) for the particular form (neutral or ionic) of the compound adsorbed, [SA] is the concentration of adsorption sites in the mixture of 1.00 mL of phosphate buffer and 50 mg of glassy carbon which are

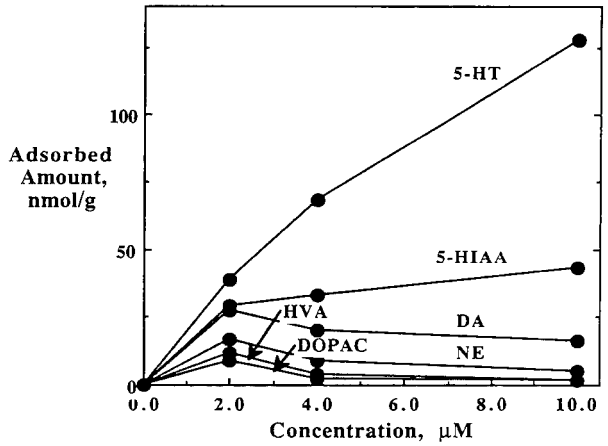

FIGURE 4. Adsorption of six catechols/indoles from a mixture containing increasing concentrations of each. The phosphate solution contained 2.0, 4.0, or 10.0 μM of each of the species shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

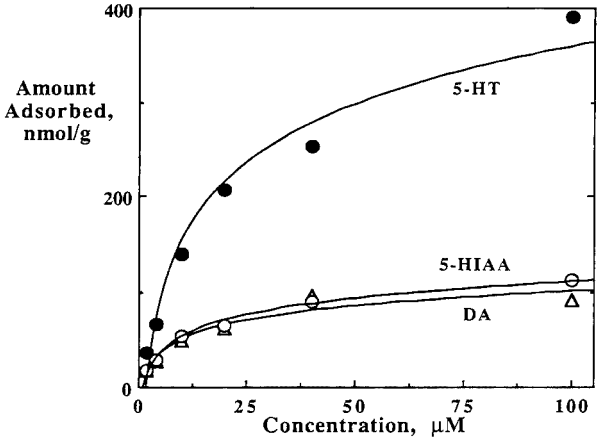

FIGURE 5. Adsorption of 5-HT, 5-HIAA, or DA alone with increasing concentrations of the substrate. The phosphate solutions used in this case contained only one of 5-HT, 5-HIAA, or DA at the concentration shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

occupied by the adsorbate, [A] is the concentration of unoccupied adsorption sites, and [S] is the concentration of the adsorbable form of the component in the phosphate solution (respectively, 5-HT free base, 5-HIAA anion, and DA free base), where all concentrations are expressed as μM or nmol/mL. The total number of available adsorption sites for a particular species, C*, is expressed on a per gram of glassy carbon basis and is defined for these experiments, each using 50 mg of glassy carbon, as

$$C^* = \frac{(\text{nmol}_{SA} + \text{nmol}_A)}{0.050\ \text{g}}$$

where

$$\text{nmol}_{SA} = [SA]\ (1.00\ \text{mL})$$

and

$$\text{nmol}_A = [A]\ (1.00\ \text{mL})$$

Using this formulation, the least squares fitted K_{ads} and C* values (± s.d.) for 5-HT, 5-HIAA, and DA, respectively, were: 781±267 and 188±37; 0.189±0.018 and 93±6; and, 21±6 and 71±11. It is also pertinent to note that the fraction of the adsorbable form of the compound relative to the total amount of free, unbound compound in solution at pH 7.0 are, respectively, 0.141%, 99.9%, and 1.30%; these values were obtained using the pK_a values of 9.85, 4.14, and 8.88, respectively, for deprotonation (8-10). The derived C* values for these three compounds were, notably, approximately the same within a factor of ca. 2; this would indicate that the number of available adsorption sites is approximately the same for all three compounds thus examined.

Employing a fixed, initial concentration of 5-HT of 10 nmol/mL, we examined the adsorption of both 5-HT and 5-HIAA while varying the initial concentration of the latter between 2 and 100 nmol/mL. As seen in Fig. 6, increasing concentrations of 5-HIAA provided increased amounts of adsorbed 5-HIAA at the expense of the adsorption of the 5-HT. In a comparable experiment using fixed concentrations of 5-HT and varying concentrations of DA, as shown in Fig. 7, a similar result was obtained; the amount of adsorbed DA increased with increasing initial concentrations of DA, while the amount of 5-HT adsorbed from the fixed concentration of 5-HT declined correspondingly.

In a final experiment, a fixed concentration of 10 nmol/mL of 5-HT was combined with concomitantly increasing concentrations of both 5-HIAA and DA. As seen in Fig. 8, the increasing concentrations of 5-HIAA and DA provided, as

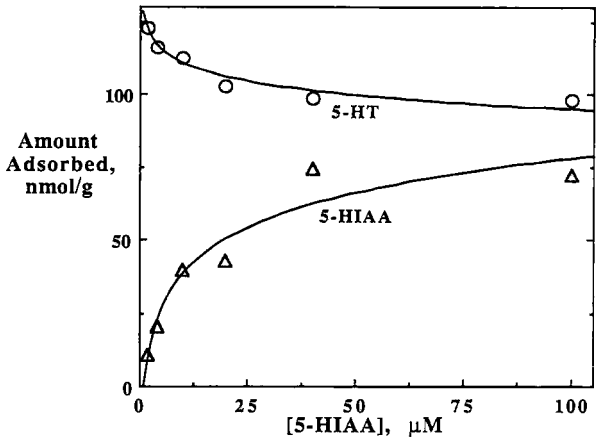

FIGURE 6. Competitive adsorption of 5-HT and 5-HIAA. The phosphate solution contained a fixed, 10 μM 5-HT, while the concentration of 5-HIAA was varied as shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

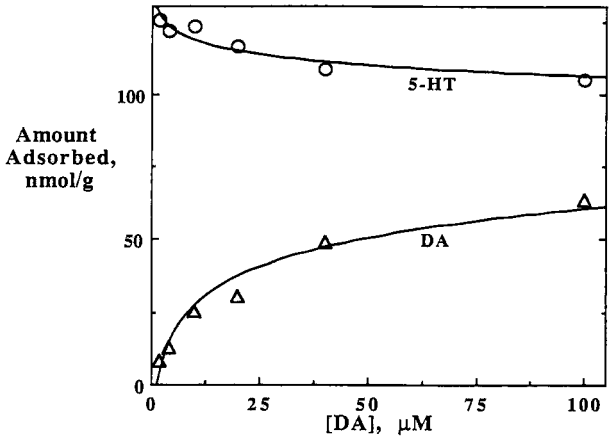

FIGURE 7. Competitive adsorption of 5-HT and DA. The phosphate solution contained a fixed, 10 μM 5-HT, while the amount of DA was varied as shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

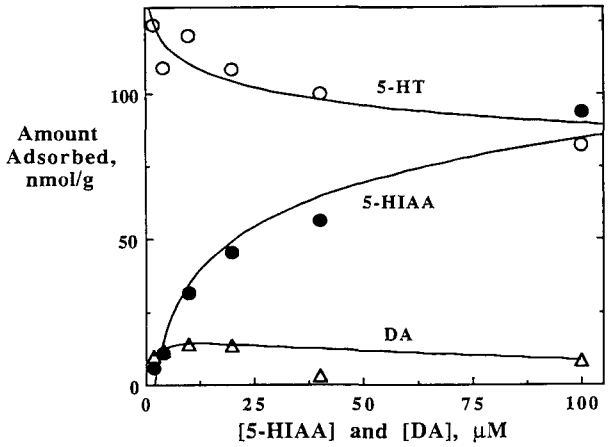

FIGURE 8. Competitive adsorption of 5-HT, 5-HIAA and DA. The phosphate solution contained a fixed, 10 μM 5-HT, while the concentrations of both 5-HIAA and DA were simultaneously varied as shown. The glassy carbon used was the 3000°C variety derived from phenolic resin fibers.

might have been expected, correspondingly increasing amounts of adsorbed 5-HIAA and decreased amounts of adsorbed 5-HT. However, the amount of adsorbed DA decreased as its concentration and that of 5-HIAA increased above 20 nmol/mL; this indicates fairly substantial competition between DA and 5-HIAA for binding sites on the glassy carbon, with DA losing the battle.

DISCUSSION

Glassy carbon is a categorization for carbon materials which includes a considerably wide range of substances. The nature of the starting material(s) used in preparation, the initial modest heat treatment with or without high pressure, the final carbonization step at high temperature, the rate(s) of heating in the treatment step(s), and possible mixing or mechanical manipulations between the treatments inevitably produces carbon materials which exhibit a variety of distinctive physical properties (7,11-16). However, the details for preparation of the many different available glassy carbons are frequently only available within a given manufacturing organization, where they are carefully maintained as trade secrets. Our previous

study (1,6) demonstrated the utility of IRICA Type CP-2250 glassy carbon, used as a pre-column in the determination of ACh and Ch, in the elimination of interfering catechols and indoles via effective adsorption processes. The current results with the glassy carbon derived from phenolic resin fibers prepared by either Mitsubishi Pencil Co. or Tokai Carbon show that these materials are also suitable for the same application. However, substantial adsorption of the interferents without concomitant adsorption of the quaternary amines for these latter materials was only achieved with the materials treated at a final carbonization temperature of 3000°C as opposed to the 2250°C of the previously employed IRICA material; thus, we can only recommend the 3000°C material from these two manufacturers for use in pre-columns in ACh and Ch determinations.

The chemical nature of the adsorption site for catechols and indoles on glassy carbon is at least partly addressed by the studies using varying amounts of graphite in conjunction with a vinyl chloride resin as the starting materials for a glassy carbon which received final heat treatment at 1400°C. Increasingly larger amounts of catechols and indoles were adsorbed on the final material as the fraction of graphite was increased, implicating graphite-like domains as the site(s) of adsorption. At the highest fractions of graphite tested (80-100%), even the quaternary amines were adsorbed; thus, too much graphite-like character in the glassy carbon could be deleterious for the utilization of such materials in the determination of ACh and Ch. The investigation discussed in the previous paragraph also supported the involvement of graphite-like domains in the adsorption process. It is well known that the graphite-like character of glassy carbons increases as the final heating temperature increases, with the carbon atoms in materials heated to $\geq$ 2700°C existing almost completely, if not exclusively, in an sp^2 hybridization. Adsorption of the catechols and indoles in this investigation did, indeed, increase as the final temperature of heat treatment was increased. Further, since the 50% graphite material in the second case exhibited roughly comparable adsorption properties for the catechols and indoles to the phenolic resin derived material heated to a final temperature of 3000°C in the first case, one might conclude that approximately one-half of the latter material is composed of graphitic domains.

Investigation of the adsorption of individual components from a six component mixture of catechols and indoles revealed possible competitive binding between these compounds. Further examination with 1, 2, and 3 component mixtures of 5-HT, 5-HIAA, and DA confirmed this competition. While 5-HT was most strongly

attracted to the adsorption sites, at least some of the adsorbed 5-HT could be displaced by either 5-HIAA or DA. In the 3 component mixture, the attraction of the 5-HT and, particularly, 5-HIAA for the binding sites was relatively so strong that adsorption of DA was suppressed. Adsorption binding constants were derived for the one component mixtures, and their magnitudes were found to fall in the order of 5-HT(free base) >> 5-HIAA(anion) ≈ DA(free base). The number of available adsorption sites for all three species was within a relatively small factor of ca. 2, supporting the concept of sharing of the same adsorption sites by the different compounds. The competitive studies for the two and three component mixtures were consistent with the derived adsorption constants, the number of available sites, and the assumption of coincident adsorption sites when one also takes into consideration the fraction of the free (unbound) compound which exists in the appropriate form (protonated or deprotonated) for adsorption.

The amount and degree of adsorption of neurochemically related catechols and indoles onto glassy carbon, thus, is highly dependent on the nature of the starting materials and processing used in the formation of the final glassy carbon. Adsorption of these compounds appears to predominantly involve the graphite-like domains, as opposed to the diamond-like domains. Among the catechols and indoles, the adsorption is a competitive process, indicating accessibility of the same binding sites to more than one of the neurochemicals simultaneously. The 3000°C glassy carbon material obtained from either Mitsubishi Pencil Co. or Tokai Carbon Co. are suitable replacements as pre-column materials for the previously employed IRICA Type CP-2250 glassy carbon in the determination of ACh and Ch by direct injection of the homogenate supernate of tissue samples.

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STUDIES ON MOLECULAR WEIGHT DETERMINATION USING REFRACTIVE INDEX AND MULTI-ANGLE LASER LIGHT SCATTERING DETECTORS IN SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

The response of differential refractive index and light scattering detectors, used in size exclusion chromatography, depends on the concentration and mass of the polymer respectively. In order to evaluate the detection capabilities of these detectors, polystyrene sample SRM 706 and three commercial polybutadiene rubber samples were analyzed. Molecular weight data obtained by using these detectors have been compared in the present work.

INTRODUCTION

Size exclusion chromatography (SEC) is a rapid and convenient means to determine molecular weight (MW) and molecular weight

distribution (MWD) of macromolecules[1,2]. A differential refractive index (DRI) detector, placed after SEC columns, is usually employed to detect the molecular species eluting according to their hydrodynamic size. A correlation between DRI detector response, which is proportional to the concentration of the eluting species, and MW of the unknown sample is required for deriving MW data from SEC. Universal calibration approach, suggested by Benoit *et al*[3], has been found to be applicable to a wide range of polymers[4-6] by using Mark-Houwink relation : $[\eta] = K M^{\alpha}$, where $[\eta]$ is the intrinsic viscosity, M is the viscosity average MW and K and α are the Mark-Houwink constants. To enable computation of MW of an unknown polymer, generally narrow MWD polystyrene (PS) standards are used on account of their easy commercial availability. But the knowledge of Mark-Houwink constants for PS and the unknown polymer for the same solvent system and operating conditions are required *a priori*. Since commercial availability of well characterized narrow dispersity polymers other than PS and values of the related Mark-Houwink constants in literature are limited, calibration of SEC columns is often complicated. A mass sensitive detector, connected to SEC system along with the conventional DRI detector, has been found to be helpful in addressing such problems[7].

In the present study, a broad MWD PS standard SRM 706 and three commercial polybutadiene rubber (PBR) samples have been evaluated for MW and MWD by SEC and SEC coupled with a multi-angle laser light scattering (SEC/MALLS) detector. A comparative study of the MW data obtained by using two different approaches has been presented in this work. MALLS detector has also been used as a stand-alone unit for determining weight-average MW of SRM 706 and the values obtained compared with those available in the literature.

EXPERIMENTAL

Materials

Narrow MWD PS calibration standards, ranging from 2K to 1800K, and SRM 706 were obtained from Pressure Chemical Co., U.S.A. and National Institute of Standards and Technology, U.S.A., respectively. PBR samples, received from three different commercial sources, consisting of > 96 % *cis*-1,4 content, were produced by using cobalt (#1 and #2) and nickel (#3) catalyst systems. HPLC grade toluene (S.D.Fine-chem, India) was used as mobile phase and solvent for polymers after filtering through 0.1 mμ filter (Alltech Associates, U.S.A.).

Sample Preparation

PS standards (0.1-0.2 % w/v, depending on MW), were dissolved in toluene and filtered through 0.45 mμ membrane filter (Millipore Corporation, U.S.A.) before injection. PBR solutions (0.3 % w/v) were filtered first through a Whatman No. 41 filter paper and later through 0.45 mμ membrane filter. The undissolved polymeric gel was discarded and a part of this solution was vacuum evaporated for calculating polymer concentration. The solution was appropriately diluted before injection.

SEC

Waters 590 solvent delivery system, U6K injector, R401 DRI detector and 745 B data module, equipped with GPC calibration module, were used for SEC measurements. A set of four Ultrastyragel columns (Waters Division of Millipore, U.S.A.) having nominal porosity 500, 10^3, 10^4 and 10^5 was used at a flow rate of 0.9 ml/min. The detector

sensitivity and data module attenuation used were (-) 32 and 16 respectively. A 250 µl Hamilton syringe was used for injection. The values of Mark-Houwink constants, K and α for PBR in toluene at ambient temperature, taken as 5.19×10^{-4} dL/gm and 0.679 respectively for the present work[8], were also calculated by the procedure suggested by Houquiang *et al*[9] employing MW data at different retention volumes from SEC/MALLS measurements. Thus intrinsic viscosity [η] of the 12 narrow dispersity PS standards was calculated by using Mark-Houwink equation ($K=0.997 \times 10^{-4}$ dL/gm, $\alpha=0.73$ for PS in toluene)[10] and then universal calibration curve equation ln [η]. M (= $K.M^{\alpha+1}$) and RV was computed, where M is the peak MW and RV the corresponding retention volume.

SEC/MALLS

For light scattering measurements in flow mode, Waters liquid chromatograph ALC/GPC 150C and DAWN-F MALLS photometric detector (Wyatt Technology Corpn., U.S.A.) were connected together, as described schematically by Jackson *et al*[11]. A pulse dampener (Model: free Flow, Alltech Associates, U.S.A.) and 25 mm high pressure filter with 0.2 mµ membrane filter (Millipore Corporation, U.S.A.) were connected after pump for reducing its pulsations and on-line filtration of mobile phase. The column set and other chromatographic conditions were same as used for SEC measurements, except the DRI detector sensitivity and scale factor used were (-)64 and 10 respectively. The data manipulation and calculations were done using ASTRA software (supplied by Wyatt Technology Corpn., U.S.A.) with the help of an IBM AT computer and 80387-SX math co-processor, connected to a printer.

The calibration constant of MALLS was calculated by ASTRA while DRI detector constant,(dn/dV), was determined by sequentially injecting 200 µl of four solutions of 233K narrow MWD PS standard having different concentrations at a flow rate of 0.1 ml/min. As the PS solution passed through RI detector cell, the maximum voltage for each concentration was noted. DRI difference (dn) was calculated as concentration (gms/ml) times DRI increment for PS (taken as 0.11)[12]. The DRI detector calibration constant was given by the various values of slope (dn) versus voltage least-squares fit, obtained with the help of a computer programme. The DRI increment (dn/dc) for PBR in toluene at 632.8 nm and MW data were calculated using ASTRA[13].

MALLS

Weight-average MW of PS and PBR samples was determined by DAWN-F87 software (Wyatt Technology Corpn.) after disconnecting it from 150 C and replacing the flow cell with sample vials. The calibration was done by measuring the scattering intensity, $I(\theta)$, of toluene at 90°, given by the equation:

$$I(\theta) = \frac{V_{90} - V_{90,dark}}{V_L - V_{L,dark}} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

where V_{90} and V_L are the measured voltages for toluene at 90° and the laser monitor respectively while the subscript 'dark' indicates the corresponding voltage when the incident radiation source is turned off. The calibration constant A_{inst} is calculated as :

$$A_{inst} = \frac{R(\theta)}{2 \cdot I(\theta)} \quad \ldots\ldots\ldots\ldots\ldots (2)$$

where $R(\theta)$ is the Rayleigh ratio[7] for toluene (taken as 1.406×10^{-5} for the present work)[13] at 632.8 nm, the wavelength of incident radiation. The 15 photo detectors of MALLS, at different fixed angles ranging from 26.57° to 128.66°, were normalized relative to 90° by measuring the scattering intensity of a 2 % solution of 25K narrow MWD PS standard in toluene, known to act as an isotropic scatterer for the incident radiation wavelength. The normalization coefficient of the ith detector, N_i, was calculated by DAWN-F87 according to the equation given below:

$$R(\theta)_i = N_i \cdot I(\theta)_i \qquad \ldots\ldots\ldots\ldots (3)$$

where $R(\theta)_i$ and $I(\theta)_i$ are the normalized excess Rayleigh ratio and the scattering intensity respectively for the ith detector at angle θ; the normalization coefficient for 90° detector taken as unity.

The weight-average MW for a given sample was determined by measuring the scattering intensities of four solutions at different concentrations using DAWN-F87. Data manipulation and calculations were done using SKOR-F87 and AURORA (Wyatt Technology Corpn.) software using Zimm plot technique[14].

RESULTS AND DISCUSSION

The MW-retention time (RT) relationship from SEC measurement using 12 narrow MWD PS standards ranging from 2K to 1800K was found to be : log MW = 10.755 - 0.164 RT. The standard error of estimate and correlation coefficient were calculated as 0.056 and 0.998 respectively. The value of DRI constant for use in ASTRA was found to be 4.195×10^{-4} RIU per volt while the calibration constant A_{inst} for batch mode measurements was calculated as 2.14×10^{-5}. The MW averages for SRM 706 determined from SEC, MALLS and SEC/ MALLS

alongwith literature data[15-21] are given in Table 1. The weight average MW obtained in the present study using different setups, and those available from literature are in excellent agreement, except that of Mori[15] who applied a concentration correction method to the results obtained by using 0.4% polymer solution. A comparative study of the cumulative MWD of SRM 706 by SEC and SEC/MALLS (Fig. 1) indicates that the low MW tail, consisting of about 12 % polymer, has a poor detector response from MALLS while rest of the data are slightly over estimated probably due to higher sensitivity of the light scattering detector. The smoothened data chromatograms obtained from DRI and at 9th detector of MALLS, which corresponds to 68.2° fixed angle and 66.34° scattering angle, are shown in Fig. 2. The MALLS response, being MW dependent, is different from concentration dependent DRI detector signal. Consequently, the low MW peak appearing at 30 ml elution volume in DRI chromatogram is not detected by MALLS.

The average value of DRI increment for the three samples, calculated by injecting known amounts of PBR using ASTRA[13], was found to be 0.040 which is close to the value obtained by direct measurement[22]. The SEC/MALLS chromatogram of a typical sample (light scattering signal at 90° fixed angle) is shown in Fig. 3. The SEC response is smooth while MALLS chromatogram shows similar multiple peaks in all the three samples, probably due to the presence of solute aggregates which remain unnoticed by DRI. The MW averages of these samples obtained by SEC and SEC/MALLS are given in Table 2. It is observed from the data that MW averages for PBR, obtained by mass detection technique, are higher as compared to SEC. Number-average and z-average MW values calculated from measured values of weight-

TABLE 1

Molecular Wight Data of Polystyrene Standard SRM 706

Method	$\bar{M}_w \times 10^{-5}$	$\bar{M}_n \times 10^{-5}$	$\bar{M}_z \times 10^{-5}$	$\bar{M}_w/\bar{M}_n$	Ref.
SEC	2.57	1.25	3.91	2.05	*
	2.85	1.37	—	2.08	15
	2.56	1.21	—	2.12	10
LALLS	2.60	—	—	—	16
	2.59	—	—	—	17
	2.58	—	—	2.10	NIST
MALLS	2.60	—	—	—	*
SEC/VIS	2.61	1.03	—	2.53	18
SEC/ LALLS	2.76	1.59	3.97	1.73	19
	2.63	1.49	4.17	1.77	20
SEC/ MALLS	2.63	1.72	4.04	1.53	21
	2.61	1.30	4.04	2.01	*

SEC = Size exclusion chromatography, LALLS = Low angle laser light scattering, MALLS = Multi-angle laser light scattering, SEC/LALLS = SEC coupled with LALLS detec tor, SEC/MALLS = SEC coupled with MALLS detector, SEC/VIS = SEC coupled with a viscometric detector, NIST = National Institute of Standards and Technology, U.S.A., $\bar{M}_w$ = Weight-average molecular weight, $\bar{M}_n$ = Number-average molecular weight, $\bar{M}_z$ = z-averge molecular weight, * = Present work

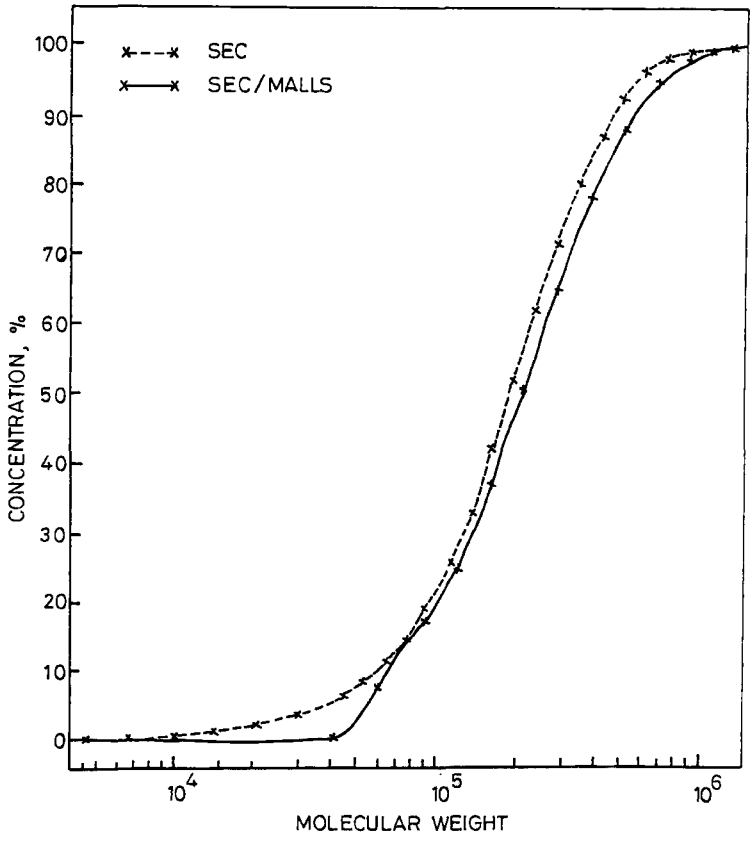

FIGURE 1 Cumulative molecular weight distribution of SRM 706

average MW, which is believed to be over estimated due to aggregation, are also higher. Since the MW averages for PS and PBR in the present study were determined using the same configuration, connecting tubes and operational parameters and the agreement for PS data obtained by SEC and SEC/MALLS being excellent, reasons for variation in PBR data were studied further. Thus the Mark-Houwink constants K and α for PBR used in the present study[8], were determined by the method suggested

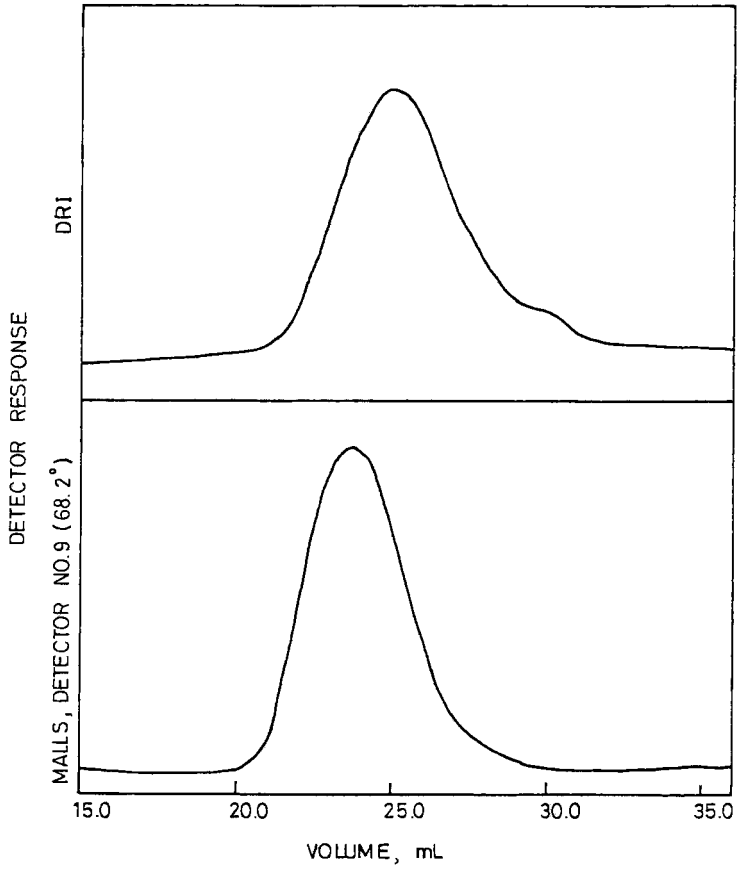

FIGURE 2 Smoothened data chromatograms from DRI and MALLS detectors

by Houquiang *et al*[9] using the weight-average MW data obtained by MALLS. The computed universal calibration curve was : ln $[\eta].M$ = 34.828 - 0.728 RV, from which the values of intrinsic viscosity $[\eta]_i$ for the ith slice corresponding to the retention volume RV_i for a particular sample were calculated. The average values of K and α, calculated from

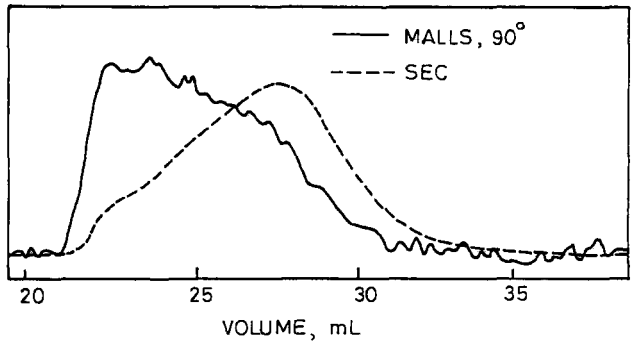

FIGURE 3 SEC/MALLS chromatogram for a typical PBR sample

TABLE 2

Molecular Weight Data on Polybutadiene Rubber Samples

Sample	#1		#2		#3	
Method	SEC	SEC/ MALLS	SEC	SEC/ MALLS	SEC	SEC/ MALLS
MW	$\times 10^{-5}$	$\times 10^{-5}$	$\times 10^{-5}$	$\times 10^{-5}$	$\times 10^{-5}$	$\times 10^{-5}$
$\bar{M}_w$	2.56	2.66	2.40	2.59	2.53	2.68
$\bar{M}_n$	0.88	1.07	0.76	1.10	0.80	1.00
$\bar{M}_z$	5.43	6.75	5.09	6.65	5.61	6.67

$\bar{M}_w$ = Weight-average molecular weight, $\bar{M}_n$ = Number-average molecular weight, $\bar{M}_z$ = z-average molecular weight

the MW data of SEC/MALLS for the three samples, were found to be 4.96×10^{-4} dL/gm and 0.69 respectively which are close to the values used in the present work. The values of constants adopted for PS[10] in the present study were in the middle range of literature values covering a range of 2K to 900K. The values of these constants used for the present work were, therefore, found to be comparable and utility of SEC/MALLS in determining these values for unknown systems established. Ambler[8] has reported that nickel based catalysts produce predominantly linear PBR, cobalt based catalyst was considered to show similar behavior. The higher values of MW averages are, therefore, considered to be due to the presence of aggregates present in the solution.

CONCLUSIONS

The SEC/MALLS data for PS and PBR are found to be comparable with the results obtained by SEC and the setup provides a rapid means for determining the Mark-Houwink constants for the polymeric systems that follow the principle of universal calibration. It has also been found useful in calculating differential refractive index incremant for unknown polymeric systems, required for light scattering studies, from the mass of solute present in the peak.

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A MECHANISTIC EVALUATION FOR THE RESOLUTION OF ENANTIOMERS OF α-ARYLPROPIONIC ACID DERIVATIVES ON π-BASIC CHIRAL STATIONARY PHASES

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ABSTRACT

A chiral recognition mechanism involving face to edge π-π interaction for the separation of the two enantiomers of the 3,5-dinitroanilide derivatives of nonsteroidal anti-inflammatory drugs(NSAIDs) on chiral stationary phase(CSP) **1** has been proposed. The inverse chromatographic resolution trends observed in resolving the two enantiomers of the 3,5-dinitroanilide derivatives of α-phenylalkanoic acids and α-(p-alkylphenyl)propionic acids on CSP **1** and **2** may support the postulated chiral recognition mechanism.

INTRODUCTION

Enantiomers of chiral drugs often show different metabolic behavior and pharmacological activity. Among the nonsteroidal anti-inflammatory drugs (NSAIDs), those related to α-arylpropionic acids contain many examples of this

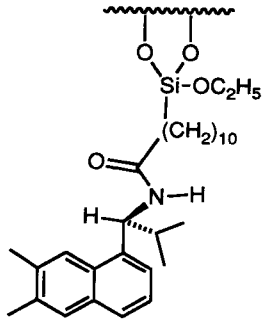
contrasting behavior. For example, (S)-(+)-ibuprofen is biologically more active than its (R)-(-)-enantiomer and the difference in activity is compensated by metabolic inversion of the (R)-(-)-enantiomer *in vivo*.[1,2] The decreased rate of metabolism and excretion of benoxaprofen in elderly patients caused by the inversion of (R)-(-)-benoxaprofen to its (S)-(+)-enantiomer leads to hepatotoxicity and, consequently, the drug has been withdrawn from the market.[3] Among the commercial NSAIDs related to α-arylpropionic acids, only naproxen is sold as a single enantiomer.

Because of the biological significance of the two enantiomers of NSAIDs, accurate and convenient means of measuring the optical purity of these substances has been sought.[4,5] In addition, recent FDA guidelines for marketing of chiral drugs require the establishment of techniques for the development and quality control of new chiral drugs.[6] Liquid chromatographic separation of enantiomers on CSPs should be the method of choice. The enantiomers of NSAIDs related to α-arylpropionic acids have been separated by liquid chromatography as their π-basic amide derivatives on a π-acidic CSP derived from (R)-N-(3,5-dinitrobenzoyl)phenylglycine[7-10] or without derivatization on a CSP based on protein or cellulose.[11-13] Recently, based on the reciprocity conception of chiral recognition, improved CSPs have been developed for the stereoselective separation of underivatized NSAIDs.[14,15]

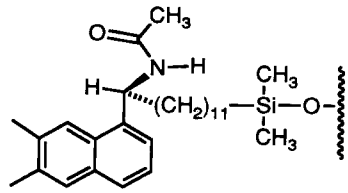
Recently, we reported that CSP **1** which has been widely used for the resolution of variety of racemates[16-18] can be used for the separation of commercial racemic NSAIDs as their 3,5-dinitroanilide derivatives **3**.[19] At that time, we proposed a chiral recognition mechanism utilizing the edge to face π-π interaction between the α-aryl group of analytes and the 6,7-dimethylnaphthyl group of CSP **1**. In this paper, we wish to provide additional experimental observations which support this chiral recognition mechanism.

EXPERIMENTAL

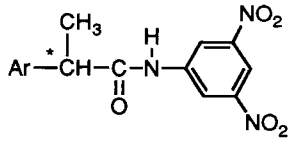
Chromatographic analysis was performed with an instrument consisting of a Waters model 510 pump, a Rheodine model 7125 injector with a 20 μl sample



CSP 1



CSP 2



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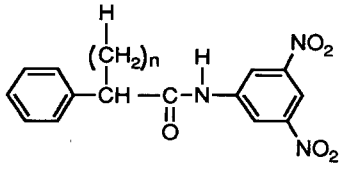

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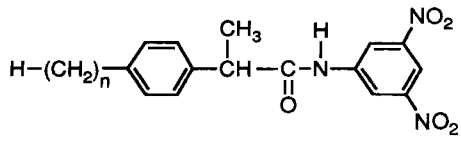

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loop, a Youngin model 710 absorbance detector with a 254 nm UV filter and a Youngin D520B computing integrator. All chromatographic data were obtained by using a 250 mm x 4.6 mm I. D. stainless-steel column packed with CSP **1** or **2**. CSP **2** used in this study was prepared according to the known procedure except for the use of dimethylchlorosilane instead of trichlorosilane in the hydrosilylation step.[16] All chromatographic separations were carried out at

a flow rate of 2.00 ml/min with a mobile phase of 10 % 2-propanol in n-hexane. Column void volume was measured by injecting 1,3,5-tri-*t*-butylbenzene.[20]

The α-phenylalkanoic acids used in this study were prepared from phenylacetic acid by the known procedures.[21] A series of α-(p-alkylphenyl)propionic acids was prepared from alkylbenzenes. Alkylbenzenes purchased from Aldrich were treated with acetyl chloride in the presence of anhydrous aluminum chloride in dichloromethane at 0 °C to afford the p-alkylphenyl methyl ketones. These methyl ketones were converted to the corresponding thiomorpholides following the Kindler-Willgerodt procedure[22] and then to p-alkylphenylacetic acids by use of acidic hydrolysis(50 % H_2SO_4 for p-alkyl = methyl, propyl : acetic acid/c-HCl (3/1, v/v) for p-alkyl = hexyl, octyl, decyl).[23] The p-phenylacetic acids prepared in this study were treated with LDA (lithium diisopropylamide, 2.3 eq.) in dry THF at -78 °C followed by methyl iodide (1.3 eq.) to afford the α-(p-alkylphenyl)propionic acids.

The α-phenylalkanoic acids and α-(p-alkylphenyl)propionic acids were converted to their 3,5-dinitroanilide derivatives **4** and **5** by treatment of the corresponding acid chlorides with 3,5-dinitroaniline and triethylamine in dry dichloromethane at room temperature as described previously.[24] All compounds prepared in this study were fully characterized by ^{1}H NMR and IR.

The elution orders for the two enantiomers of 3,5-dinitroanilide derivatives **4** and **5** on CSP **1** and **2** were determined by the TRAC (tracking of absolute configuration) technique[16] based on the absolute elution order of the two enantiomers of the 3,5-dinitroanilide derivative of α-phenylpropionic acid, the optically active form of which is commercially available.

RESULTS AND DISCUSSION

In our previous report, we proposed the chiral recognition model shown in Figure **1** for the resolution of the two enantiomers of N-3,5-dinitrobenzoyl derivatives **3** of NSAIDs on CSP **1** based on their elution orders and on a consideration of space-filling molecular models.[19] In this model, both the CSP and analyte are presumed to interact in their lowest energy conformations.[16,19]

To briefly review this chiral recognition model, CSP **1** and the analyte are proposed to interact through the π-π complexation between their respective 6,7-dimethylnaphthyl and 3,5-dinitroanilide groups and through the hydrogen bonding between the carbonyl oxygen of the CSP and the amide N-H hydrogen of the analyte. In this event, the face of the α-aryl substituent of the (R)-analyte is positioned at the edge of the 6,7-dimethylnaphthyl ring of the CSP, invoking the face to edge π-π interaction which has received increased attention as an associative force between aromatic rings in the recent studies.[25-27] On the contrary, the α-aryl substituent of the (S)-analyte is directed to the acyl connecting arm of the CSP and, hence, it experiences some degree of steric hindrance. Consequently, the diastereomeric (R,S)-complex shown in Figure 1 is energetically more favorable than the (S,S)-complex. This chiral recognition model is quite similar to that recently proposed for the resolution of N-(3,5-dinitrobenzoyl) derivatives of α-arylalkylamines on CSP **1**.[28]

In the chiral recognition model shown in Figure 1, the methyl substituent at the chiral center of the (R)-analyte is oriented alongside the acyl connecting arm of the CSP. Therefore, it is expected that the long alkyl substituent at the chiral center of the (R)-analyte may intercalate between the connecting arms of the CSP and make the diastereomeric (R,S)-complex increasingly more unfavorable as the alkyl chain length increases. To test this postulate, 3,5-dinitroanilide derivatives **3** of a series of α-phenylalkanoic acids were prepared and resolved on CSP **1**. Figure 2a shows the dependence of the retention of the two enantiomers on the length of the alkyl substituent at the chiral center of the analyte.

The more rapid decrease of the retention of the (R)-analyte than that of the (S)-analyte observed can be rationalized by considering the intercalation of the alkyl substituent at the chiral center of the (R)-analyte between the connecting arms of the CSP. As a result, the enantioselectivity decreases continuously as the length of the alkyl substituent at the chiral center of the analyte increases (Figure 2b). The maximum in the retention of the (R)-analyte and in the enantioselectivity at n=2 noted in Figure 2 may be a consequence of conformational factors. As the alkyl substituent at the chiral center of the analyte changes from methyl to ethyl, there is a significant change in steric bulk

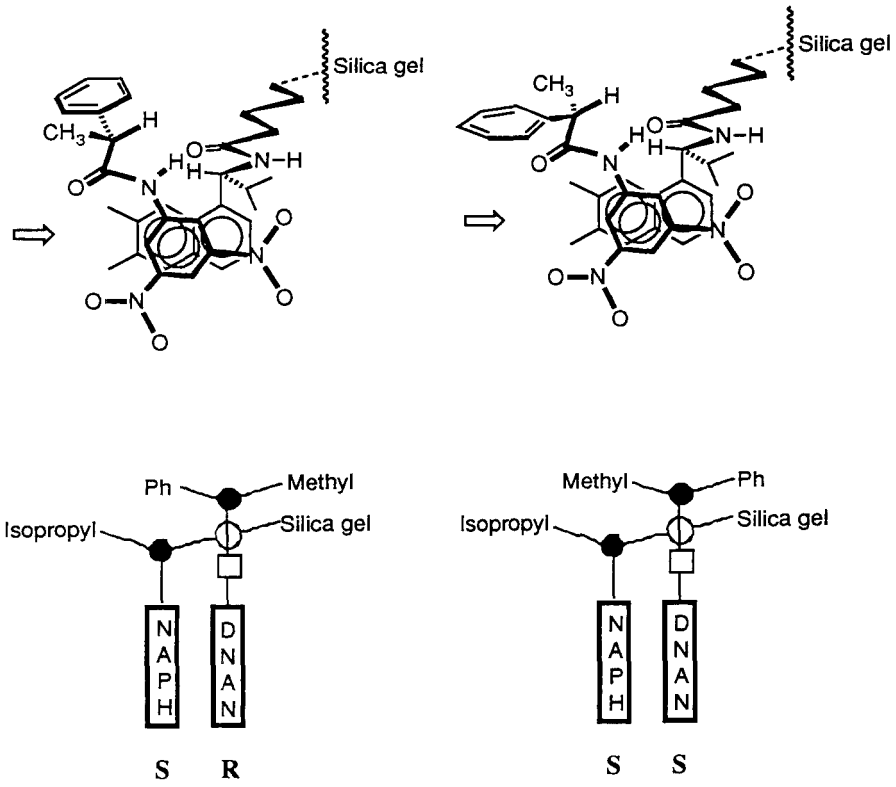

Figure 1. The proposed chiral recognition model. Above : the two diastereomeric (R,S)- and (S,S)-complexes between the 3,5-dinitroanilide derivative of racemic α-phenylpropionic acid and (S)-CSP **1**. Below : schematic representation of the above diastereomeric complexes viewed from the arrow direction. solid circle : methine hydrogen oriented toward the viewer in the CSP and away from the viewer in the analyte. empty circle : carbonyl oxygen oriented toward the viewer in the CSP. empty square : N-H hydrogen oriented away from the viewer in the analyte.

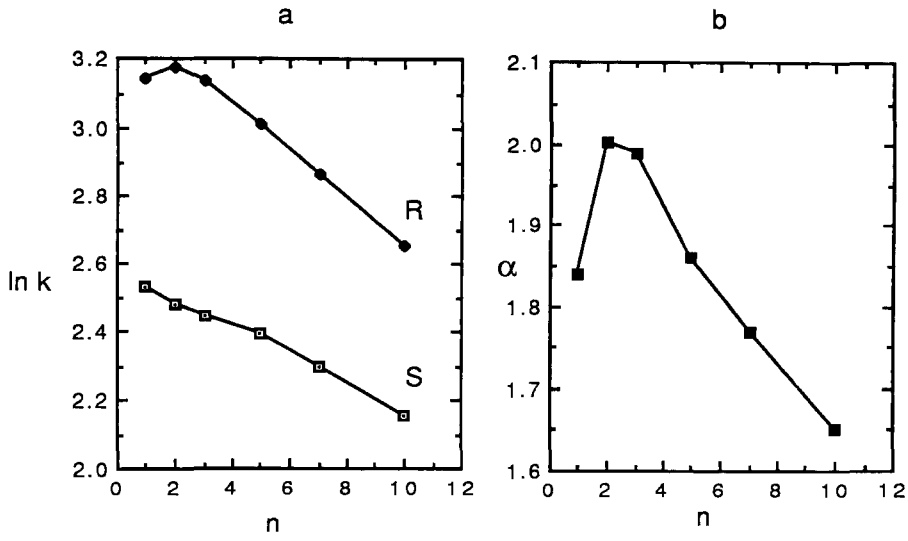

Figure 2. (a) Retention and (b) enantioselectivity trends of the two enantiomers of **4** on CSP **1**. Chromatographic conditions are given in the Experimental.

and, consequently, the conformational preferences should be altered. However, the conformational preferences may not be altered by further lengthening of this alkyl chain because the changes in the structure occur at sites remote from the stereogenic center.

From the chiral recognition model shown in Figure 1, it is also expected that the trends for resolution of N-3,5-dinitrobenzoyl derivatives **4** of a series of α-phenylalkanoic acids on CSP **2**, the connecting arm of which is oriented in the opposite way to that of CSP **1**, should be the reverse to those observed on CSP **1**. On CSP **2**, the alkyl substituent at the chiral center of the less retained (S)-enantiomer of **4** should intercalate between the connecting arms of the CSP as shown in Figure 3. Consequently, as the alkyl chain length increases, the retention time of the (S)-enantiomer should decrease more rapidly than that of the (R)-enantiomer. All of these expectations are consistent with the experimental observations as shown in Figure 4a and 4b. The discrepancy in the continuous increase of enantioselectivity shown in Figure 4b may be a result of the conformational factors discussed above.

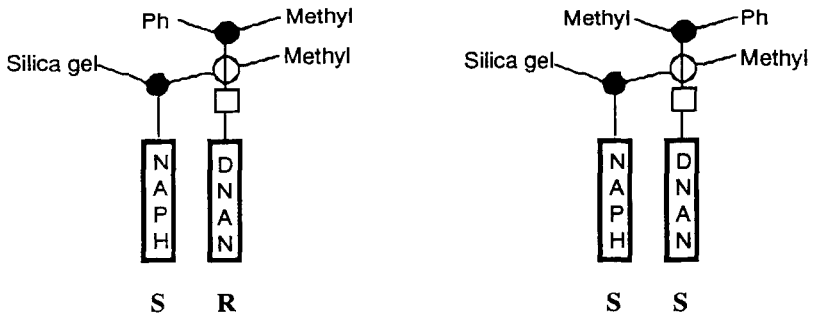

Figure 3. Schematic representation for the two diastereomeric (R,S)- and (S,S)-complexes between the 3,5-dinitroanilide derivative of racemic α-phenylpropionic acid and (S)-CSP **2**. See the caption of Figure 1 for the meaning of solid circle, empty circle and empty square symbols.

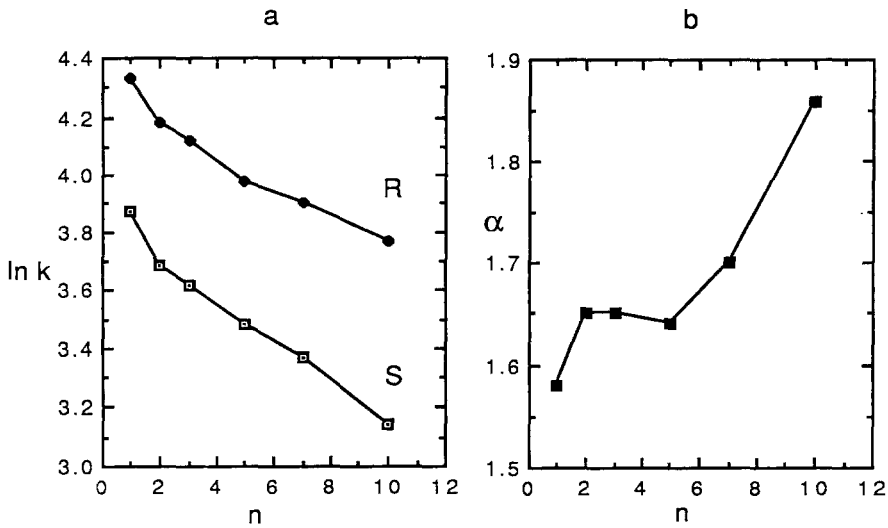

Figure 4. (a) Retention and (b) enantioselectivity trends of the two enantiomers of **4** on CSP **2**. Chromatographic conditions are given in the Experimental.

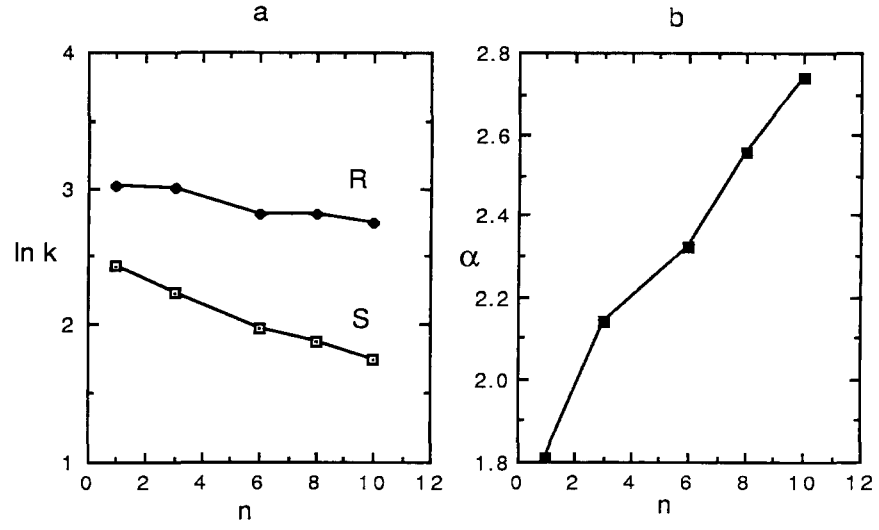

Figure 5. (a) Retention and (b) enantioselectivity trends of the two enantiomers of **5** on CSP **1**. Chromatographic conditions are given in the Experimental.

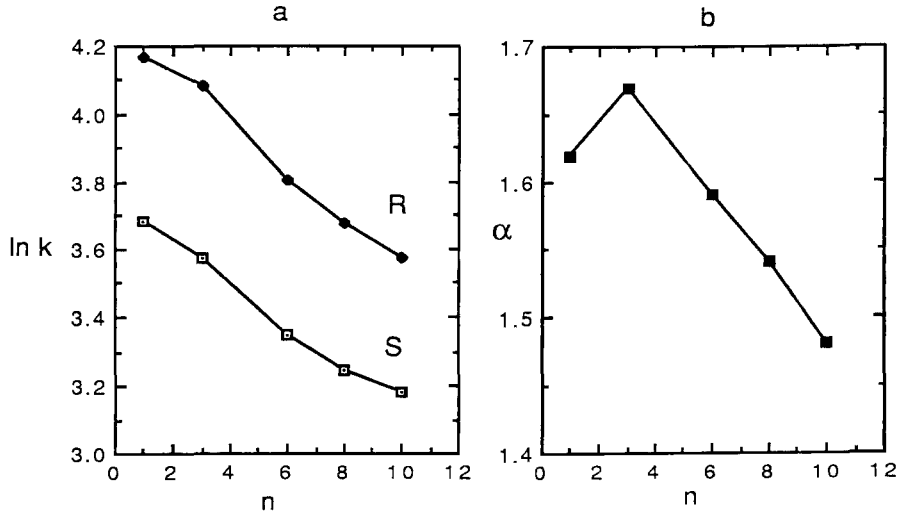

Figure 6. (a) Retention and (b) enantioselectivity trends of the two enantiomers of **5** on CSP **2**. Chromatographic conditions are given in the Experimental.

Based on the chiral recognition model shown in Figures 1 and 3, the trends for the resolution of 3,5-dinitroanilide derivatives **5** of α-(p-alkylphenyl)propionic acids are expected to be opposite to those for the resolution of 3,5-dinitroanilide derivatives **4** of α-phenylalkanoic acids. The p-alkyl substituent of the less retained (S)-enantiomer is anticipated to intercalate between the connecting arms of CSP **1**. Hence, the retention of the (S)-enantiomer should decrease more rapidly than that of the (R)-enantiomer and the enantioselectivity should increases as the p-alkyl substituent increases in length. On CSP **2**, the p-alkyl substituent of the more retained (R)-enantiomer can intercalate between the connecting arms of the CSP as expected from the model shown in Figure 3. This should lead to a decrease in the stability difference between the two diastereomeric complexes shown in Figure 3 and consequently to a decrease in enantioselectivity. Figures 5 and 6 show data obtained for trends in the resolution of 3,5-dinitroanilide derivatives **5** of α-(p-alkylphenyl)propionic acids on CSP **1** and **2**. The experimental results are fully compatible with the expectations based on the chiral recognition mechanism discussed above.

In conclusion, the trends we have noted in studies of the resolution of 3,5-dinitroanilide derivatives **4** and **5** on CSP **1** and **2** support the proposed chiral recognition model which emphasizes the importance of face to edge π-π interaction in addition to the hydrogen bonding and face to face π-π interaction between the 3,5-dinitroanilide derivatives of NSAIDs and the CSP. As shown in this and previous studies,[28] face to edge π-π interactions now seem to play an important role as an attractive force in chiral recognition by a CSP. To provide more solid evidence for the role of the face to edge π-π interaction, spectroscopic and/or crystallographic data are required. Efforts are underway in our laboratory to prove these issues.

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ASSAY OF RP 69698, A NOVEL LTB4-ANTAGONIST, IN PLASMA BY HPLC AND ITS APPLICATION FOR CHARACTERIZING PHARMACOKINETICS OF THE DRUG IN DOGS

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ABSTRACT

To support the preclinical development of RP 69698, a novel antagonist of LTB4, a high-performance liquid chromatographic (HPLC) procedure was developed for its determination in plasma. The method involves extraction of RP 69698 from deproteinized plasma with hexane:ethyl acetate under acidic conditions followed by reversed-phase HPLC with detection at 258 nm. The method is rapid, simple, and is applicable to dog, rat, and monkey plasma. Validation studies using dog plasma showed that the values obtained for parameters of linearity, precision, and accuracy were within acceptable limits. Based on analysis of 0.5 ml of plasma, the lower limit of quantitation was determined to be 25 ng/ml. The method has been successfully applied to determine the pharmacokinetic parameters of RP 69698 in the dog following intravenous and intragastric administration. The results of the dog study indicated rapid clearance of the drug from plasma (0.4 L/hr/kg) and limited distribution (volume of distribution at steady state =0.24 L/kg). When given intragastrically as a solution in PEG 400, absolute bioavailability of the drug was 59% but when given as an aqueous suspension in 0.5% methylcellulose, the drug was poorly absorbed with absolute bioavailability estimated to be about 6%.

INTRODUCTION

Leukotriene B4 (LTB4) has been shown to play a critical role in the onset of late-phase asthma by promoting the migration of eosinophils into respiratory tissues (1). RP 69698, 2-[[5-methyl-5-(1H-tetrazol-5-yl)hexyl]oxy]-4,6-diphenylpyridine, is a specific inhibitor of LTB4 binding to polymorphonuclear leukocytes (PMNs) with IC50 ranging from 2.2 nM to 14.5 nM depending upon the species from which the PMNs were obtained (2). RP 69698 is undergoing evaluation as a potential therapeutic agent for the treatment of asthma. To support preclinical development of the drug, a method was developed to analyze RP 69698 in the plasma. This report describes the method and its validation using dog plasma. In addition, applicability of the method is shown in a pharmacokinetic study conducted in dogs following intravenous and intragastric administration of the drug.

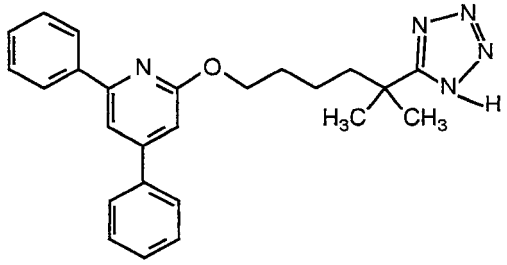
MATERIALS AND METHODS

Materials

The test compound, RP 69698, was synthesized at Rhône-Poulenc Rorer (Collegeville, PA). RP 69698 has a molecular weight of 413.5. The internal standard (I.S.) used was 5,7-dihydro-5,5,7,7-tetramethyl-3-(3-nitrophenyl)-furo[3,4,-e]-1,2,4-triazine, and was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. The structures of RP 69698 and I.S. are shown in Figure 1.

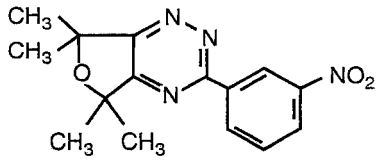
HPLC grade ethyl acetate, hexane, acetonitrile and sodium acetate were obtained from Fisher Scientific Co. (Pittsburgh, PA).

RP 69698



2-[[5-methyl-5-(1H-tetrazol-5-yl)hexyl]oxy]-4,6-diphenylpyridine

INTERNAL STANDARD



5,7-Dihydro-5,5,7,7-tetramethyl-3-(3-nitrophenyl)furo[3,4-*e*]-1,2,4-triazine

FIGURE 1. Structures of RP 69698 and internal standard (I.S.).

Acetic acid (analytical grade) and polyethylene glycol (PEG) 400 (certified grade) were also obtained from Fisher Scientific Co. Methylcellulose (viscosity of a 2% solution at 25°C: 400 cps) was obtained from Sigma Chemical Co. (St. Louis, MO). Deionized water was produced by a Milli-Q reagent water system (Millipore Corp., Bedford, MA). Control dog plasma (heparinized) was obtained from

either in-house supply or from Lampire Biological Laboratories (Pipersville, PA).

Preparation of Standards

Stock solutions of RP 69698 were prepared in acetonitrile at concentrations of 2.5 mg/ml or 1.0 mg/ml. The I.S. stock solution was prepared in acetonitrile at a concentration of 1.0 mg/ml. The working solutions of RP 69698 and I.S. were prepared by diluting stock solutions with HPLC mobile phase. Stock and working solutions were stored at room temperature in amber-colored volumetric flasks. Plasma standards, in the range of 25 ng/ml to 50 μg/ml, were prepared by aliquoting appropriate volumes of a stock or working solution of RP 69698 and adjusting to 5 ml with blank (drug free) plasma. Reference control concentrations (75, 250, and 750 ng/ml and 2.5 and 12.5 μg/ml) were similarly prepared for monitoring the performance of the assay during each run. Plasma standards and controls were stored at -20°C until used or up to a maximum of 1 month.

Buffer Solutions

Two sodium acetate buffer solutions, one adjusted to pH 5.0 and the other adjusted to pH 6.1, were used during the analytical procedure. The pH 5.0, 0.1M sodium acetate buffer was prepared by mixing 14.8 ml of 0.2M acetic acid and 352 ml of 0.2M sodium acetate and then bringing the volume to 1 liter with deionized water. The pH 6.1, 0.1M sodium acetate buffer was prepared by combining 15 ml of 0.2M acetic acid with 485 ml of 0.2M sodium acetate and then bringing the volume to 1 liter with deionized water. The buffer solutions were stored at room temperature for up to a week.

HPLC Instrumentation and Operating Conditions

The chromatographic analyses were performed using a Waters (Milford, MA) HPLC system consisting of a model 510 pump, model 484 UV detector and a model 712 autosampler (WISP). The system was controlled by a 840 data control station. The HPLC peaks were identified by retention time with reference to standard compounds and quantitated using peak area ratio of RP 69698 to I.S. Analog data were collected, digitized and stored using chromatography software (version 4.1) obtained from Perkin Elmer Nelson systems (Cupertino, CA). The chromatographic conditions were as follows:

Column:	Partisil 10 (particle size 10μm), ODS-3 (25cm x 4.5mm I.D.) analytical column (Whatman, Clifton, N.J.)
Guard Column:	C18 RCSS Guard Pak-C18 precolumn module (Waters Assoc., Milford, MA.)
Temperature:	30°C
Mobile Phase:	48% Acetonitrile and 52% 0.1M sodium acetate (pH 6.1) buffer
Flow Rate:	2 ml/min
Run Time:	35 min
Detection:	Absorbance at 258 nm
Retention Time:	RP 69698 - approximately 10 min I.S. - approximately 14.5 min

Extraction Procedure

For sample preparation, frozen plasma was thawed at room temperature and a 0.5 ml aliquot was transferred to a 2.2 ml

polypropylene tube. To this, 50 µl of I.S. solution of appropriate conentration (2 µg/ml for low concentration range curve and 50 µg/ml for the high concentration range curve) was added and mixed. The plasma was deproteinized by adding 0.5 ml of acetonitrile followed by centrifugation at 6000 g for 10 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY). The supernatant was transferred to a 16 x 150 mm disposable glass tube and acidified by adding 1 ml of 0.1M sodium acetate buffer adjusted to a pH of 5.0. RP 69698 and I.S. were extracted from the buffered matrix by mixing with 4 ml of hexane/ethyl acetate (3/1;v/v) and vortexing the mixture for 30 s. The organic (upper) layer was aspirated and transferred to a 15 ml conical centrifuge tube. The extract was evaporated to dryness under a stream of nitrogen at room temperature and the residue was reconstituted in 200 µl of HPLC mobile phase. After mixing, either 20 µl (high concentration range curve) or 100 µl (low concentration range curve) of the reconstituted extract was injected onto the HPLC column.

Validation Studies

The recoveries of RP 69698 and I.S. were estimated in the range of 50 ng/ml to 5 µg/ml by comparing detector response to pure authentic standards with the response obtained from equivalent amounts added to and recovered from plasma with correction made for the fraction of reconstituted sample injected onto the column. Since a broad range of concentrations was expected to be analyzed by this method, to achieve better performance, standard curves were prepared in two concentration

ranges. A low concentration standard curve ranged from 25 ng/ml to 1000 ng/ml and a high concentration curve ranged from 1 μg/ml to 50 μg/ml. The linearity of the method was based on evaluation of correlation coefficient and variability of slope for within-day and day-to-day analyses. The precision of the method was based on determination of the coefficient of variation (CV) for within-day and day-to-day analyses. The accuracy of the method was determined from percentage difference between extrapolated values and nominal (spiked) values for reference controls. The lower limit of quantitation (LOQ) was defined as the concentration which could be quantified with acceptable accuracy (difference <15%) and precision (CV <15%). The stability of RP 69698 in plasma was determined under the conditions of storage (-20°C).

Dog Pharmacokinetic Study

Six male beagle dogs, weighing about 8 to 10 kg, were obtained from White Eagle Laboratories (Doylestown, PA). After 2 weeks of acclimatization period, the dogs were given each of the following three treatments according to a 3-way crossover design during the three-week study period. Treatment 1 and 2 consisted of intravenous (I.V.) and intragastric (I.G.) administration, respectively, of RP 69698 given as a solution in PEG 400 at a dose of 2.5 mg/kg. Treatment 3 consisted of I.G. administration of the drug given as a suspension in 0.5% methylcellulose suspension. Blood samples were withdrawn from the cephalic vein into heparinized syringes (Sarstedt, Princeton, NJ) at 0 (pre-dose), 2 (I.V. only), 5, 10, 20, 30, and 45 min and at 1, 1.5, 2, 4, 6, 8, and 24 hr following each treatment. The samples were centrifuged

at 600 g for 10 min and plasma was separated and stored at -20°C until analyzed.

Sample Analysis

On the day of analysis, plasma samples were thawed, and an aliquot (0.5 ml) was removed. Based on the results of exploratory studies, the sample was analyzed by using either low concentration range curve (50 μl of 2 μg/ml I.S.) or high concentration range curve (50 μl of 50 μg/ml I.S.). When using the low concentration range curve, if the extrapolated value exceeded the upper limit of the curve (1 μg/ml), the sample was reanalyzed using the high concentration range curve. Similarly, when utilizing the high concentration range curve for extrapolation, if the sample concentration was below 1 μg/ml, the sample was reanalyzed using the low concentration curve.

Data Analysis

The plasma concentration data were analyzed using the SIPHAR computer program (SIMED, Cedex-France) to provide the following pharmacokinetic parameters whenever appropriate: maximum observed plasma concentration (Cmax), time when Cmax was observed (tmax), the area under the curve from time zero to the time when the concentration was last quantifiable (AUC_{0-t}), the area from time zero to infinity ($AUC_{0-\infty}$), systemic clearance (CL), the volume of distribution at steady state (Vdss), and elimination half-life ($t_{\frac{1}{2}}$) values for the α- and β-phases. Absolute bioavailability (F) was determined from the ratio of dose normalized AUCs obtained for I.G. vs. I.V. administered drug.

RESULTS AND DISCUSSION

Chromatography

Initial development of the method indicated that, by using acetonitrile and 0.01M sodium acetate buffer as mobile phase, RP 69698 eluted as a symmetrical peak using a C18 column. The optimal mobile phase for separation of RP 69698 and I.S. was established by varying the pH of the buffer, to which the elution of the drug but not of the I.S., was found to be highly susceptible. Under the HPLC operating conditions described in the Materials and Methods section, the retention times of RP 69698 and I.S. were found to be 10 ($\pm$0.5) and 14.5 ($\pm$0.5) min, respectively. The seemingly large variability in retention times was mainly due to slight differences in the pH of the mobile phase. With any given mobile phase, however, the retention times remained unchanged during the course of an analytical run.

Preliminary studies established that RP 69698, which has an octanol/water partition coefficient of 3045 (log P= 3.48), could be extracted from deproteinized plasma under slightly acidic conditions with a relatively non-polar solvent. Further studies led to the selection of hexane/ethyl acetate (75/25; v/v) as the most suitable solvent for extraction. The choice of the I.S. was based on matching of physico-chemical properties and chromatographic behavior of RP 69698 with materials available in our library of compounds.

Blank plasma obtained from several species including dog, rat, monkey, and humans were tested and found to show no significant endogenous peak that would interfere with the analysis of RP 69698. Although the method has also been used for sample

analysis obtained from studies in rats and monkeys, the validation studies reported here were performed with dog plasma. Typical chromatograms of blank dog plasma and dog plasma spiked with RP 69698 and an appropriate amount of I.S. are shown in Figure 2.

Assay Validation

The recoveries of RP 69698 and I.S. were determined for three representative concentrations of 50, 500, and 5000 ng/ml. As shown in Table 1, the mean recoveries were $\geq$79% at all concentrations for both compounds. The recoveries were quite reproducible (CV<10%) and there was no evidence for concentration-dependence within the tested range of 50 ng/ml to 50 μg/ml. By using non-weighted least square regression analysis, the standard curves were found to be linear in the range of 25 ng/ml to 1000 ng/ml and 1 μg/ml to 50 μg/ml. The correlation coefficients were >0.992 for all curves. The slopes were quite reproducible with CV values determined to be <2% for both within-day and day-to-day analysis. The results of within-day and day-to-day precision for both the low and high concentration range curves are shown in Table 2. The CV was <10% at all concentrations except at 1 μg/ml, where the day-to-day precision was about 15.8%. The accuracy of the assay appeared to be quite good as determined from % difference of extrapolated values from the nominal (spiked) concentrations (Table 3); at all levels the mean difference was <15%. A concentration of 25 ng/ml was established as the LOQ based on acceptable estimates obtained for precision (CV<15%) and accuracy (%difference <15%) at this level. Based on reanalysis of spiked standards stored at -20^{o}C, it was found that the drug was stable in dog plasma for at least three months.

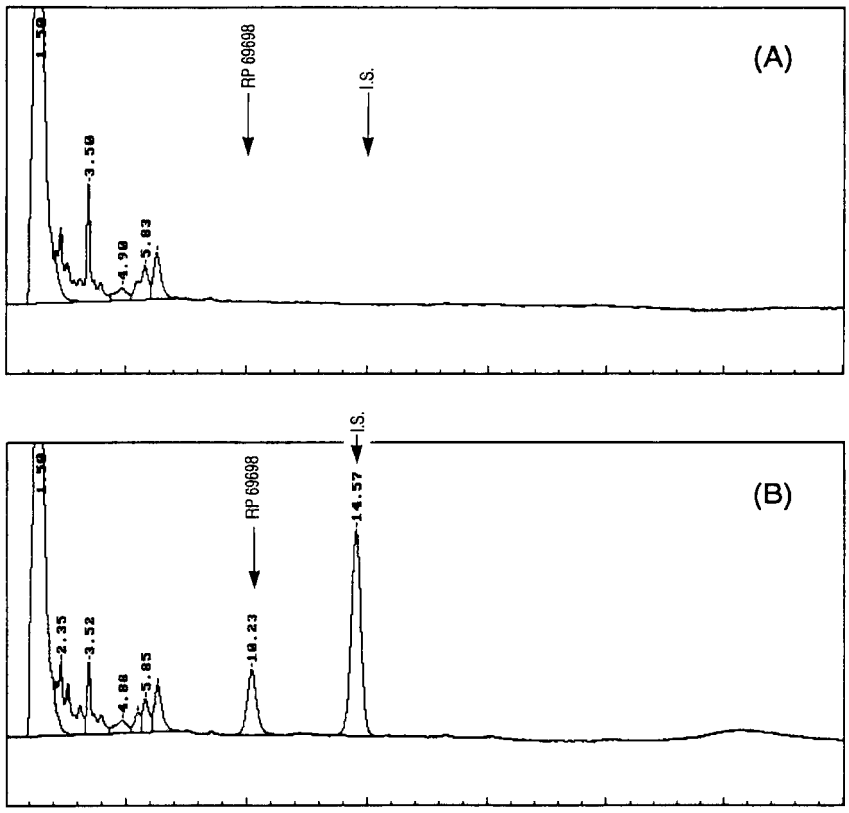


FIGURE 2. Typical chromatograms obtained following analysis of blank dog plasma (A) and plasma spiked with 100 ng/ml RP 69698 and 200 ng/ml of I.S. (B).

Pharmacokinetic Characteristics of RP 69698 in the Dog

The plasma concentration data obtained following various treatments of RP 69698 are summarized in Table 4 and the results of pharmacokinetic analysis of these data are shown in Table 5. The plasma concentrations of RP 69698 in samples taken at 6, 8 and 24 hr post-treatment were generally below LOQ.

TABLE 1

Extraction Recoveries of RP 69698 and I.S. from Dog Plasma

Compound	Concentration (ng/ml)	% Recovery (Mean ± S.D.)(n=3)
RP 69698	50	89±6
	500	79±2
	5000	80±3
I.S.	50	82±1
	500	86±2
	5000	83±3

TABLE 2

Within-day and Day-to-Day Precision of the Analytical Procedure

RP 69698 Spiked Conc.	Within-Day Analysis (Mean±S.D.)(n=3) Conc. Measured	Day-to-Day Analysis (Mean±S.D.)(n=3) Conc. Measured
Low Concentration Range Curve		
(ng/ml)	(ng/ml)	(ng/ml)
25	22.4±1.7	26.7±1.8
50	44.7±3.2	50.9±1.8
100	100.6±2.7	103.2±4.6
200	193.5±1.3	198.0±5.8
400	420.5±5.0	393.9±4.2
1000	993.4±1.9	1002.5±1.3
High Concentration Range Curve		
(μg/ml)	(μg/ml)	(μg/ml)
1	1.1±0.1	1.2±0.2
5	5.1±0.0	5.3±0.3
10	10.1±0.1	10.3±0.2
25	24.1±0.2	23.4±1.3
50	50.4±0.1	50.7±0.6

TABLE 3

Within-day and Day-to-Day Accuracy of the Analytical Procedure

RP 69698 Nominal Conc.	Within-Day Analysis (Mean±S.D.)(n=3) Conc. Measured	% Diff.	Day-to-Day Analysis (Mean±S.D.)(n=3) Conc. Measured	% Diff.
Low Concentration Range Curve				
(ng/ml)	(ng/ml)		(ng/ml)	
75	76.9±2.0	-2.5±2.6	78.8±4.2	-5.0±5.6
250	251.6±8.9	-0.6±3.5	254.7±12.0	2.0±5.9
750	749.0±7.3	0.1±1.0	759.0±5.8	1.2±2.9
High Concentration Range Curve				
(μg/ml)	(μg/ml)		(μg/ml)	
2.5	2.7±0.1	-2.7±10.1	2.4±0.4	6.7±6.1
12.5	12.2±0.0	2.7±0.5	10.1±2.1	10.0±14.9

TABLE 4

Plasma Concentrations of the Unchanged Drug in Dogs Following Treatment with RP 69698 Administered Either as a Solution in polyethylene glycol (PEG) 400 or as a Suspension in 0.5% Methylcellulose (MC).

		RP 69698 Plasma Conc. (Mean±S.D.)(n=6) (μg/ml)		
Time Post-dose (min)	Route Vehicle Dose	I.V. PEG 400 2.5 mg/kg	I.G. PEG 400 2.5 mg/kg	I.G. 0.5% MC 5.0 mg/kg
2		15.51±2.04	NS	NS
5		10.20±2.01	0.02±0.03	BQL
10		8.08±1.75	0.54±0.39	BQL
20		5.62±1.45	1.74±0.91	BQL
30		4.07±1.34	2.02±1.09	BQL
45		2.43±1.10	2.12±0.82	0.04±0.03
60		1.28±0.82	1.74±0.89	0.07±0.07
90		0.91±0.99	1.26±1.12	0.13±0.17
120		0.28±0.16	0.58±0.22	0.30±0.22
240		0.03±0.03	0.07±0.05	0.14±0.11
360		BQL	0.03±0.06	BQL
480		BQL	BQL	BQL

NS = No sample taken at this sampling time
BQL = Below Quantitation Limit (0.025 μg/ml)

TABLE 5

A Summary of the Pharmacokinetic Parameters of RP 69698 Determined in Dogs Following Intravenous and Intragastric Administration of the Drug

TREATMENT			PHARMACOKINETIC PARAMETER*							
ROUTE	VEHICLE	DOSE (mg/kg)	Cmax (μg/ml)	tmax (hr)	Abs. Rate ($t_{\frac{1}{2}}$; hr)	$t_{\frac{1}{2}}\alpha$ (hr)	$t_{\frac{1}{2}}\beta$ (hr)	CL (L/hr/kg)	Vdss (L/kg)	F (%)
I.V.	PEG 400	2.50	15.51[1] $\pm$2.04	0.03[2]	-	0.19 $\pm$0.12	0.53 $\pm$0.13	0.39 $\pm$0.09	0.24 0.07	-
I.G.	PEG 400	2.50	2.32 $\pm$0.84	0.67 $\pm$0.13	0.25 $\pm$0.08	NC	0.54 $\pm$0.16	-	-	59.03 $\pm$33.06
I.G.	0.5% Methyl-cellulose	5.00	0.31 $\pm$0.21	2.67 $\pm$1.03	NC	NC	NC	-	-	5.68 $\pm$4.58

* Values given are Mean$\pm$S.D. for n=6

1 Mean concentration at the earliest sampling time

2 Earliest sampling time (2 min)

NC = Not calculated due to inadequate data

Following an I.V. dose, CL of the drug occurred relatively rapidly at a rate of 0.39 L/hr/kg. The Vdss was estimated to be about 0.24 L/kg, suggesting limited distribution and providing evidence for the lack of extensive tissue binding. The plasma concentration data was best described by a biexponential curve. A typical best-fit of the data is shown in Fig. 3. The average $t_{\frac{1}{2}}$ value for the α-phase, which most likely represents the distribution phase, was estimated to be about 0.19 hr. The average $t_{\frac{1}{2}}$ for the β-phase was about 0.59 hr which seems to indicate that the drug is rapidly eliminated from the central compartment.

When administered I.G. as a solution in PEG 400 at a dose of 2.5 mg/kg, Cmax averaged about 2.32 μg/ml. In the individual dogs, the tmax was either 30 min or 45 min with an average value estimated to be about 0.67 hr. The plasma concentration data was best described by either a biexponential (Fig. 3) or a triexponential curve. The first exponent described the absorption phase, whereas the other exponent(s) described the elimination phases(s). Due to a somewhat slower absorption rate ($t_{\frac{1}{2}}$=0.25 hr), the α-phase was not discernible. The average $t_{\frac{1}{2}}$ for the β-phase was estimated to be about 0.54 hr, which is similar to that determined after I.V. administration. In two of the six dogs, an additional elimination phase with $t_{\frac{1}{2}}$ of about 1.16 hr and 3.85 hr was also apparent.

When RP 69698 was given as a suspension in 0.5% methylcellulose at a dose of 5 mg/kg, the concentrations attained in plasma were quite low. The average value for Cmax was

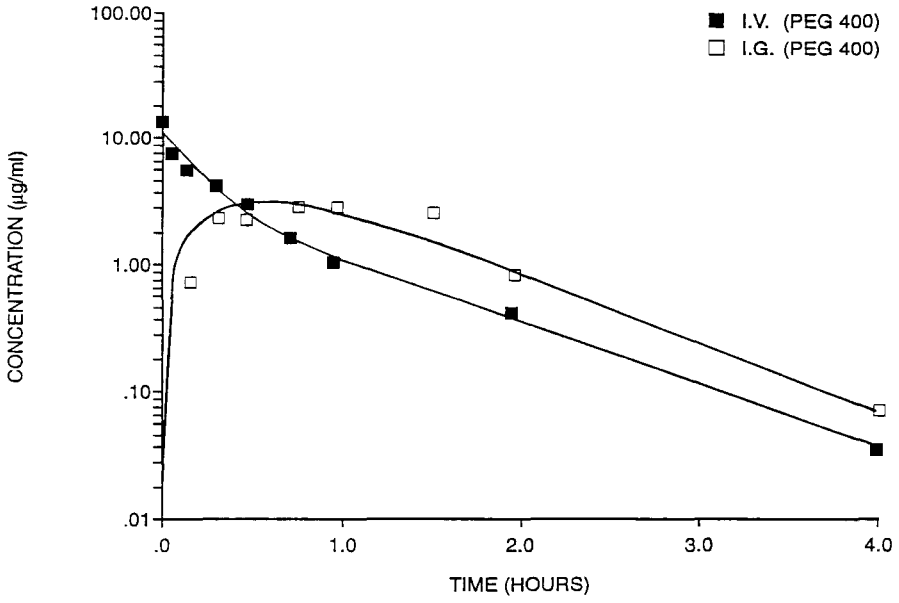


FIGURE 3. A representation of the best fit of the plasma concentration data obtained in a dog following I.V. and I.G. treatment with 2.5 mg/kg RP 69698 given as a solution in PEG 400.

estimated to be 0.31 µg/ml which was attained at about 2.67 hr (tmax). A plot of the data indicated no clear pattern of decline and, therefore, no effort was made to calculate the elimination $t_{\frac{1}{2}}$(s).

Based on ratio of AUCs obtained following I.G. vs. I.V. administered drug, the bioavailability of RP 69698 was estimated to be about 59% when given as a solution but only 6% when given as a suspension in 0.5% methylcellulose. These data indicate that solubility of the drug apparently becomes the limiting factor for absorption of the drug when given as a suspension.

CONCLUSIONS

The method described in this report for the assay of RP 69698 in plasma was developed to obtain pharmacokinetic characteristics of the drug and to perform toxicokinetic studies. The method is simple, specific and adaptable to plasma from several species. Validation studies, performed with dog plasma as the matrix, showed that by utilizing low and high concentration range standard curves, concentrations of 25 ng/ml or higher can be quantitated with satisfactory precision and accuracy. RP 69698 was found to be stable in dog plasma when stored at -20^{o}C up to about 3 months. The suitability of the method was demonstrated by analyzing plasma samples from a pharmacokinetic study of the drug in dogs. The results of this study indicated rapid clearance of the drug from plasma and limited distribution. The plasma elimination of the drug was characterized as biphasic with t½s of about 11 min and 35 min for the distribution (α) and elimination (β) phases respectively. Also, the study showed poor bioavailability of the drug from suspension relative to the solution, thus, indicating that the dissolution of the drug in gastrointestinal tract limits the absorption of the drug. Availability of the analytical method helped in identifying the critical issues related to the development of the drug.

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HPLC ANALYSIS OF TAURINE IN HUMAN PLASMA SAMPLE USING THE DABS-Cl REAGENT WITH SENSITIVITY AT PICOMOLE LEVEL

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ABSTRACT

A high-performance liquid chromatographic method for the evaluation of taurine in human plasma samples is presented. Two different extraction procedures have been employed: perchloric acid extraction and filtration using the CF-50 Amicon membrane, obtaining comparable results. After extraction the samples were derivatized with the 4-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS-Cl) reagent and the DABS-derivatives, included DABS-taurine, analyzed by reversed-phase HPLC obtaining a sensitivity at picomole level. However, the plasma filtration using the CF-50 Amicon membranes is a quicker procedure (10 min) also allowing the simultaneous evaluation of acid-labile amino acids such as asparagine, glutamine and tryptophan and a higher recovery of taurine (>96%). The taurine levels in human plasma samples of ten normal adults (mean age, 20 years) using the perchloric extraction and the plasma filtration were 38±5 and 42±4 nmol/ml of plasma respectively.

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INTRODUCTION

Taurine (2-aminoethanesulfonic acid) is an amino acid containing sulphur widley distributed in vertebrate tissues. It is present in relatively high concentrations in several vital organs where it could regulate some of their physilogical functions (1). The role of this amino acid in the enteroheptatic circulation of bile salts, in the renal transport and in the binding of calcium ions in the myocardial tissue is known (2). In the central nervous system taurine is important as a neurotrasmitter and a neuromodulator (3-6). Human, nonhuman primate species and cats present a poor ability to synthesize taurine so they are largely dependent on an exogenous source. The symptoms of a taurine depletion in these species are eye defects and growth retardation (7). Some authors have shown a possible relationship between a lower level of taurine in the plasma and a depressive illness (5) as well as differences between plasma levels of this metabolite in epileptic and non-epileptic children (8). Stephan et al. (9) using human hepatoblastoma cell line (Hep G2) have shown that taurine enhances the LDL receptor activity suggesting an interesting role of this metabolite on hepatic cholesterol catabolism. Taurine is present in tissues and physiological fluids in a very low concentration, ranging approximately from 1-50 μM. For this reason a method with high sensitivity is requirred for its evaluation. In the last

few years high-performance liquid chromatography (HPLC) analysis has become the most successful method for taurine determination. Many authors have reported different HPLC methods for the evaluation of this amino acid. Reversed phase HPLC determination either a pre-column derivatization with o-phthalaldeyde (OPA) (10,11), o-phthalaldeyde + 2-mercaptoethanol (12,13), dansyl chloride (14,15), phenylisothiocyanate (PITC) (16) or a post-column derivatization (17) have been described; other authors have proposed ion-exchange HPLC chromatography (18-21).

In this paper we report a procedure (extraction and RP-HPLC analysis) which allows to evaluate the concentration of this metabolite in human plasma samples with a sensitivity at picomole level. Furthermore this procedure allows the simultaneous analysis of the standard amino acids and some of their derivatives (22). The procedure proposed can also be applied to evaluate the distribution of this compound in other physiological fluids such as amniotic liquid or in the supernatant of cell cultures as well as in food samples.

MATERIALS AND METHODS

Chemical. DABS-Cl were purchased from Fluka (Buchs,Switzerland). Free L-taurine for the preparation of DABS-taurine

calibration mixture was obtained from Sigma (Sigma Chemical Co., St. Louis, MO). Acetonitrile, ethanol, isopropanol of HPLC grade and potassium dihydrogen phosphate and the other chemicals were purchased from Carlo Erba (Milan, Italy). CF50 Amicon membranes were obtained from Amicon (Lexington, MA). Triple-distilled water was prepared in the laboratory and used for the preparation of buffers. The buffers used for the HPLC analysis were filtered through a 0.22-μm Millipore filter (Millipore, Bedford, MA).

HPLC analysis. A Gold liquid chromatographic system from Beckman (Beckman, Berkeley, CA) was used throughout this work. This system consisted of two Model 126 pumps, a PC-8300 solvent programmer, a Model 210 sample injection valve, and a Model 166 variable-wavelength uv-visible range detector, equipped with a 12-μl flow cell. Integration of peak areas was obtained by means of a Shimadzu C-R6A Chromatopac electronic integrator (Shimadzu Corp., Kyoto, Japan). The separation of DABS-amino acids was performed using a 3-μm Supelcosil LC-18 T column (15 cm x 4.6 mm i.d.) protected with a guard column 5-μm Supelcosil LC-18 T (2 cm x 4.6 mm i.d.) (Supelco, Bellefonte, PA). The mobile phases used were: 25 mM potassium dihydrogen phosphate, pH 6.8 (solvent A), and acetonitrile-isopropanol (80:20) (solvent B). The gradient program was:

1 min at 20% of solvent B, 4 min at up to 23% of solvent B, 7 min at 23% of solvent B, 11 min at up to 27% of solvent B, 7 min at 30% of solvent B, 9 min at up to 60% of solvent B, 1 min at up to 70% of solvent B, 5 min at 70% of solvent B. The gradient was then returned in a minute to 20% of solvent B and the initial condition restored in 6 minutes. The flow rate was 1.5 ml/min and the detection was performed at 436 nm. The separation of free amino acids present in human plasma, as DABS-derivatives, reported in Fig. 1, was obtained using the analytic conditions described. A calibration curve was performed injecting different amounts of DABS-taurine ranging from 1 to 50 pmoles. For more details concerning the HPLC separation of DABS-amino acids see *Stocchi et.al.* (22).

Blood Collection. The human blood samples used for the experiments were obtained from 10 normal adult subjects with a mean age of 20 years. The blood was collected in heparin and immediately centrifuged at 3000 rpm x 10 minutes, at 4°C. The plasma was removed and used for the following extraction with perchloric acid or filtration through a CF-50 Amicon membrane.

Extraction with Perchloric Acid. To one milliliter of plasma 0.5 ml of 5% (v/v) perchloric acid solution was added. The sample was

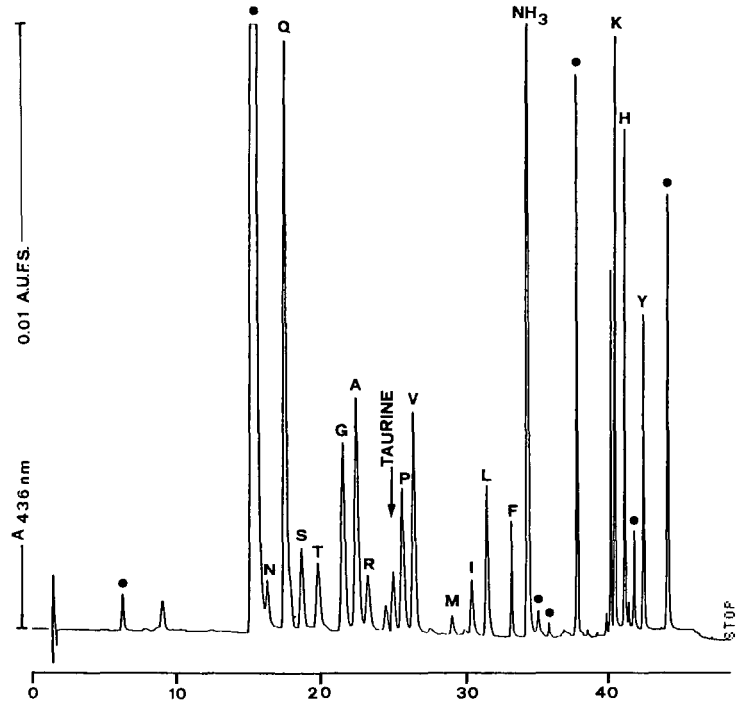

Fig. 1 Determination of DABS-taurine in human plasma by reversed-phase high-performance liquid chromatography using a Supelcosil LC-18 T (15 cm x 4.6 mm i.d.), 3-µm particles, column. The experimental conditions to obtain the separation are described in detail under "Materials and Methods". Solvent A, 25 mM potassium dihydrogen phosphate, pH 6.8; solvent B, acetonitrile-2-propanolo (80:20). Flow rate, 1.5 ml/min; room temperature. 5 µl of sample were injected for the HPLC analysis. ●, By-products originating from the excess of reagent. The chromatogram was obtained using a variable-wavelength detector , Model 166, from Beckman equipped with a 12 µl flow cell.

centrifuged at 3000 rpm x 10 min, the supernatant removed and neutralized with K_2CO_3 3M. The solution was again centrifuged at 3000 rpm x 5 minutes and the supernatant was removed and brought to a final volume of 2 ml. 10 µl were used for the reaction with DABS-Cl as reported below.

Filtration on CF-50 Amicon membrane. 4 milliliters of plasma were placed on a CF-50 Amicon membrane and centrifuged at 2500 rpm x 10 minutes. 10 µl of the filtrate were used for the dabsylation procedure reported below.

Derivatization with DABS-Cl. 10 microliters of plasma sample either perchloric acid extract or filtrate were derivatizated in an Eppendorf tube adding 10 µl of 0.1 M sodium hydrogen carbonate, pH 9.0 and 40 µl of DABS-Cl solution (4 nmol/ µl acetonitrile, freshly prepared). The reaction was performed at 70°C x 10 min and the volume was brought to 500 µl with 70% (v/v) ethanol solution. The sample was centrifuged at 14000 rpm x 3 min and 5 µl used directly for the HPLC analysis as previously described (22). Sample after perchloric extraction has half the concentration compared with the sample obtained after filtration on CF-50 Amicon membrane.

RESULTS AND DISCUSSION

The DABS-Cl reagent used for precolumn derivatization of primary and secondary amino acids can also be used for evaluation of L-taurine. For this metabolite a possible role has been proposed as neuromodulator and neurotransmitter also involved in many other biological processes (4-5). Furtermore, this metabolite is usually present in physiological fluids in very low concentrations (μMolar range) and therefore a method with high sensitivity for its evaluation is required. For this purpose the precolumn derivatization using the DABS-Cl reagent seems appropriate giving a sensitivity at picomole level.

In Fig.1 reports the complete RP-HPLC separation of amino acids present in human plasma (included L-taurine) as DABS-derivatives, using the method reported in this paper. The method shows a high reproducibility and a sensitivity at picomole level. Table I reports the plasma concentration of taurine of 10 normal adult human subjects. These data have been compared with those obtained by other authors (4-5) using different conditions of extraction and analysis (4-5). In our case we used a perchloric acid extraction and filtration of plasma with CF 50 Amicon membranes, obtaining the same results. However, plasma filtration is a more rapid procedure which also allows the simultaneous evaluation of plasma acid-labile amino acids such as

TABLE I

TAURINE LEVEL IN HUMAN PLASMA OF NORMAL ADULTS

Reference	Taurine (nmol/ml)
Tachiki (3)	45±4
Perry (2)	56±2
Present study (perchloric extraction)	38±5
(plasma filtration)	42±4

Data are expressed as nmoles per milliliter of plasma and are the mean value (± standard deviation) of 10 blood samples, from normal adult subjects (mean age, 20 years), centrifuged immediately after collection.

asparagine, glutamine and tryptophan. The procedure proposed (extraction and HPLC analysis) could also be used for the evaluation of this metabolite in other physiological fluids.

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MICROBORE HPLC DETERMINATION OF POLYETHER ANTIBIOTICS USING POSTCOLUMN DERIVATIZATION WITH BENZALDEHYDE REAGENTS

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ABSTRACT

Monensin, narasin, and salinomycin have been separated by HPLC on microbore column packed with octadecyl-bonded silica gel and detected via postcolumn color reaction with derivative of benzaldehyde in packed-bed reactor. Various benzaldehyde reagents have been tested in batch and 4-dimethylaminobenzaldehyde (DMABA) and vanillin selected as most promising ones. Requirements and characteristics of postcolumn reaction with the two reagents have been investigated and optimized and DMABA has been shown as superior over currently used vanillin reagent. The applicability of postcolumn derivatization with DMABA has been demonstrated by monensin assay in premixes and feeds.

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INTRODUCTION

Monensin, narasin, and salinomycin are fermentation-derived polyether monocarboxylic antibiotics used mainly as feed additives in the prevention of coccidiosis in poultry and as feed efficiency enhancers in cattle. The antibiotic monensin is a mixture consisting of two or more components, i.e. monensin Factor A as the main derivative and monensin Factors B and, if any, C and D.

Typical matrices for the analytical determination of the three antibiotics are fermentation broths, premixes, feeds, and environmental samples. The antibiotics are routinely analyzed by bioassay methods which lack speed and specificity.

Golab et al. (1) introduced a photometric method based upon the reaction of monensin with vanillin in sulfuric acid solution. The method uses Komarowski reaction (2) which is known as a color reaction of higher alcohols with aromatic aldehydes. The color produced is due to aldol condensation products. The reaction of a secondary alcohol (or a similar compound) with benzaldehyde which proceeds in strong acid media of sulfuric acid can be schematically described by the two consecutive steps (3)

$$C_6H_5CHO + R_2CHOH \rightleftarrows C_6H_5CH_2OH + R_2C=O \qquad (A)$$

$$R_2C=O + C_6H_5CHO \rightleftarrows \text{condensation products} \qquad (B)$$

Later on, the reaction with vanillin has been applied to postcolumn derivatization of salinomycin after normal phase HPLC separation (4) and to that of the three antibiotics separated by reversed phase HPLC method (5). Reaction detection with vanillin has become the most used detection means in HPLC of monensin,

narasin, and salinomycin. Recently, high speed liquid chromatographic determination of these antibiotics in feeds using postcolumn reaction with vanillin (6) has been described and the correlation between HPLC determination of monensin and its microbiological assay has been investigated (7).

In this work monensin, narasin, and salinomycin have been separated on a microbore column and derivatized in packed-bed reactor instead in generally used (5-8), up to 9 m long, reaction capillary. Applicability of various benzaldehyde reagents to derivatization reaction has been evaluated and parameters of postcolumn derivatization have been studied.

EXPERIMENTAL

Reagents and Solutions

Stock solutions (0.1 mg/ml) of monensin (960 μg/mg purity), narasin (934 μg/mg; both from Lilly Research Center, USA), and salinomycin (960 μg/mg; Höchst, Germany) were prepared in methanol from their sodium salts and stored under refrigeration.

Vanillin, (4-hydroxy-3-methoxybenzaldehyde, anal. grade, Loba Chemie, Germany), salicylaldehyde (2-hydroxybenzaldehyde, pure, Reachim, USSR), 4-nitrobenzaldehyde, 4-dimethylamino-benzaldehyde (both pure), 3,5-dibrom-4-hydroxybenzaldehyde, and 3,4-dimethoxybenzaldehyde (both recrystallized, all from Lachema, Czechoslovakia) were employed as derivatization agents.

Distilled methanol and water redistilled in a quartz still were used. Other reagents were of analytical grade purity (Lachema, Czechoslovakia).

Mobile phase was prepared as described previously (7, 8) and consisted of methanol, water and glacial acetic acid at a ratio of 940:59:1.

To prepare a derivatization reagent solution, concentrated sulfuric acid was slowly mixed with methanol under stirring and, after cooling to room temperature, weighted amount of benzaldehyde reagent was added. The solution was freshly prepared daily.

In batch experiments, methanolic solutions contained 5 μg/ml (ca. 7 μmol/l) monensin, benzaldehyde reagent at concentrations from 15 to 20 mmol/l, and 20 mmol/l sulfuric acid. These solutions were heated for one minute in a water bath at 60 °C, cooled to room temperature and their absorbance was measured at the wavelengths of absorption maxima.

Solid samples of premixes (DBBR2; 0.5 g) and medicated feeds (KSBR2; 5 g; all from Institute for State Control of Veterinary Biopreparates and Medicaments, Brno, Czech Republic) were extracted with 15 ml of methanol by mixing 2 h on a shaker and filtered. Extracts of feed additives were transferred into 50-ml volume flasks and diluted with methanol. Feed extracts were evaporated to the volume of approx. 3 ml and diluted in a volume flask with methanol to 10 ml. Volumes of 3 μl of final solutions were injected onto chromatographic column and assayed for monensin using DMABA as derivatization reagent.

Apparatus

Two syringe pumps, model MHPP 20, were used to deliver the mobile phase and reagent solution. Samples were injected using a model LCI 30, six-port injection valve with 3-μl sample loop (all Laboratory Instruments Prague, Czechoslovakia), onto a CGC glass column 150x1 mm i.d. packed with Separon SGX C18 (5 μm) silica gel with octadecyl groups (Tessek Prague, Czechoslovakia). Separations were performed at an ambient temperature.

Effluent and reagent flow were joined in a low dead volume T-piece (Swagelok, USA) and mixed in a home-made, glass, 150x1 mm i.d., packed-bed reactor filled with acid-washed glass beads, 40-70 μm in diameter. The reactor was placed in a thermostated water-bath jacket. A model HP 1050 multiple wavelength detector, equipped with a 1-μl cell, 5 mm in path length (Hewlett-Packard, Japan), was used for detection and data was evaluated by using a model CI 100 integrator and a TZ 4620 recorder (Laboratory Instruments Prague, Czechoslovakia).

Spectrophotometric investigations were done on a model Specord M 40 spectrophotometer (Carl Zeiss Jena, Germany), however, absorption curves of derivatization products were measured on a chromatographic detector introduced above.

Acidity was measured with a model OP-208 pH-meter equipped with an OP-0808-P glass-Ag/AgCl combined electrode using phthalate and phosphate standard buffers (all Radelkis, Hungary).

RESULTS AND DISCUSSION

Batch Experiments

In preliminary experiments, absorption curves of products of derivatization reactions with vanillin, 4-dimethylaminobenzaldehyde (DMABA), 3,4-dimethoxybenzaldehyde (DMOBA), and salicylaldehyde (SA) were measured to determine their absorption maxima listed in Table 1. Reagents with electron-accepting substituents (i.e. 4-nitrobenzaldehyde and 3,5-dibrom-4-hydroxybenzaldehyde) did not react with the antibiotics.

Absorbance of reaction products was rapidly increasing with a reaction time up to ca. 40 s while practically constant absorbance values were observed after 1-min or longer reaction. This was not valid for DMABA, where absorbance values were increasing during a period of 2 min. Absorbance was also increasing with benzaldehyde reagent and sulfuric acid concentrations and becomes independent of them if these were higher than 20 mmol/l. Different behavior was observed by the use of DMABA, where absorbance reached a maximum values at 15 mmol/l DMABA, and then was sharply decreasing up to 40 mmol/l, and at higher DMABA concentrations remained at low levels.

Calibration curves of individual antibiotics measured at conditions used in previous batch experiments were linear in the range from 1 to 15 μg/ml. Values of y-intercept were low, ranging from -34 to 13 mAU. The slope values of calibration curves were decreasing from DMABA, vanillin, and DMOBA to salicylaldehyde and, thus, DMABA was shown as a reagent more sensitive than

TABLE 1

Conditional Molar Absorption Coefficients Determined from Calibration Curves at Wavelengths of Absorption Maxima.

Concentration of antibiotics 1-15 μg/ml, 15 mmol/l vanillin, 17 mmol/l DMABA, 16 mmol/l DMOBA, and 20 mmol/l salicylaldehyde (SA); 20 mmol/l sulfuric acid. Reaction time 1 min at temperature of 60 °C.

	Vanillin	DMABA	DMOBA	SA
Monensin [a]	3.77 (520)	5.39 (585)	3.28 (511)	2.15 (480)
Narasin	3.72 (525)	4.08 (598)	3.31 (512)	1.61 (498)
Salinomycin	3.85 (521)	5.12 (597)	3.35 (513)	1.93 (502)

Values in 10^{-4} l mol^{-1} cm^{-1} (nm). [a] Based on molecular weight of monensin Factor A.

vanillin in spite of proceeding the reaction for only 1 min. The values of conditional molar absorption coefficients determined from the slopes are introduced in Table 1. For postcolumn derivatization, DMABA and vanillin were chosen as the most promising reagents.

Postcolumn Derivatization

The effect of mobile phase and derivatization reagent flow rates and, thus, also the effect of column efficiency and that of reaction time on detector response were studied using a reagent solutions containing 0.34 mol/l DMABA or 0.33 mol/l vanillin and

0.75 mol/l sulfuric acid. The derivatization reaction was performed at 75 °C. Injected samples contained 0.1 mg/ml of each antibiotic. Products of derivatization were monitored at 592 (DMABA) or 520 nm (vanillin). The total flow rate was varied from 15 to 75 μl/min at the ratio of mobile phase/reagent flow rate equal to 2:1. At higher total flow rates, corresponding to a hold-up time between column outlet and detector from 50 to ca. 85 s, peak heights were increasing linearly and a sharper increase was observed for DMABA than for vanillin reagent. Further, the increase of peak heights was lower and, finally, at hold-up time from ca. 2 to 3 min, the peak heights of individual antibiotics practically did not change. As an optimum, a reaction time of 110 s, i.e. a total flow rate of 35 μl/min was chosen. A total dead volume in the T-piece, packed-bed reactor, and connecting tubing was calculated as 64 μl.

At the total flow rate of 35 μl/min the ratio of mobile phase/reagent flow rate was varied from 4:1 to 1:4. The best detector response was found at flow rates of 20 and 15 μl/min of mobile phase and reagent solution, respectively.

The increase of peak heights of individual antibiotics with increasing reaction temperature is demonstrated in Fig. 1. Formation of bubbles in a reactor was observed at temperatures higher than 80 °C. A temperature of 75 °C was chosen as optimum for both derivatization reactions.

The effect of DMABA and vanillin concentrations on the detection of antibiotics is shown in Fig. 2. In the concentration range studied, the peak heights were increasing linearly with vanillin concentration while by DMABA application practically

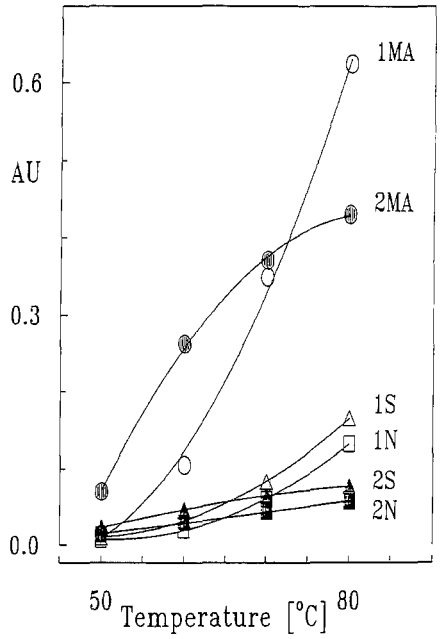

FIGURE 1 Peak heights vs. reactor temperature. Acidity of postcolumn reagent, 0.75 mol/l H_2SO_4; reaction time, 110 s; injected sample, 0.1 mg/ml each antibiotic; detected at 592 or 520 nm after derivatization with DMABA (open symbols) or vanillin (closed symbols). Curves: 1MA and 2MA - monensin Factor A (0.5 mol/l DMABA and 0.66 mol/l vanillin in the reagent solution), 1S and 2S - salinomycin, 1N and 2N - narasin (0.24 mol/l DMABA or vanillin).

constant values were obtained at c(DMABA) > 0.6 mol/l. If the concentration of benzaldehyde reagent was higher than 0.7 mol/l, the baseline was noisier and, therefore, concentrations of 0.5 mol/l DMABA and 0.66 mol/l vanillin were chosen as a good compromise between signal and noise level. Concentration of sulfuric acid in reagent solutions was varied from 0.15 to 2.0 mol/l. Peak heights

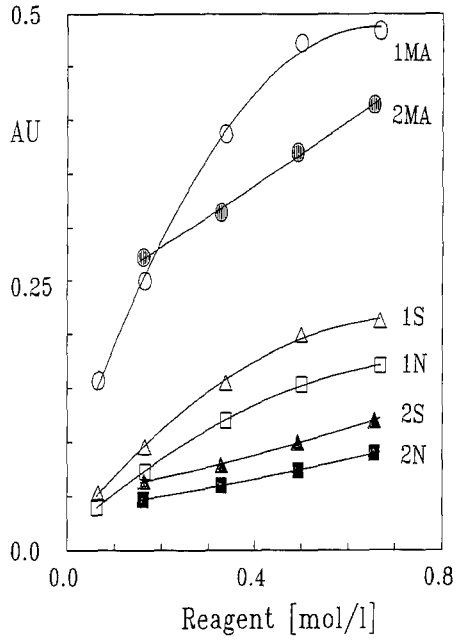

FIGURE 2 Peak heights vs. derivatization reagent concentration. Reactor temperature 75 °C, other conditions given at Fig. 1. Open symbols: derivatization with DMABA - curves 1MA (monensin Factor A), 1N (narasin), and 1S (salinomycin); closed symbols: with vanillin - curves 2MA, 2N, and 2S.

increased with increasing acidity, however, at $c(H_2SO_4) > 1.1$ mol/l the increase was already low and, simultaneously, higher blank absorbance of vanillin reagent was observed. Sulfuric acid concentration of 1.2 mol/l was taken as optimum in both reagent solutions.

Calibration curves, peak height [AU] vs. antibiotic concentration [μg/ml], measured under optimized conditions were linear up to 1 mg/ml. Some parameters of calibration curves are

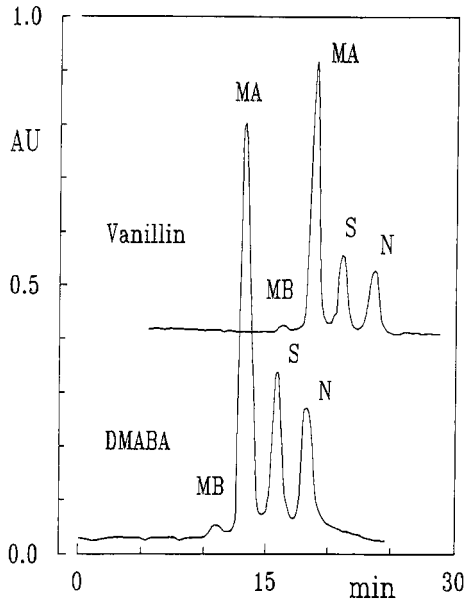

FIGURE 3 Chromatograms of monensin Factor A (MA), monensin Factor B (MB), narasin (N), and salinomycin (S) detected via postcolumn reaction with DMABA or vanillin. Injected sample, 0.1 mg/ml each antibiotic (monensin A and B as total monensin). Other conditions as in Table 2.

introduced in Table 2. The chromatograms presented in Figure 3 illustrate the separation of monensin, narasin, and salinomycin onto microbore column and differences in their detection via postcolumn reaction with DMABA or with vanillin. The detection method based on the reaction with DMABA was evaluated for the determination of monensin in premixes and medicated feeds. Recoveries which are listed in Table 3 ranged from 88.4% to 102.6% across the different sample types and lower values were found for feed samples.

TABLE 2

Slope Values of Calibration Curves and Sensitivities for Individual Antibiotics.

Eluent, 94% v/v methanol containing 0.1% v/v acetic acid, flow 20 μl/min; postcolumn reagent, methanolic solution of 0.50 mol/l DMABA or 0.66 mol/l vanillin, 0.75 mol/l sulfuric acid, flow 15 μl/min; derivatization, 110 s at 75 °C; sample, 3 μl, 20-100 μg/ml of each antibiotic; detection, 592 (DMABA) or 520 nm (vanillin).

Antibiotic	DMABA		Vanillin	
	ϵ [a]	S [b]	ϵ [a]	S [b]
Monensin A	7 780	1.7	5 190	2.0
Narasin	2 700	2.3	1 320	3.9
Salinomycin	3 610	2.2	1 600	3.3

Values of y-intercepts were between -1.9 and 9.8 mAU (DMABA) or -0.1 and 15.2 mAU (vanillin). [a] Slope values as conditional molar absorption coefficients [l mol^{-1} cm^{-1}]; [b] Sensitivities at a signal-to-noise ratio of 2 [μg/ml].

TABLE 3

Determination of Monensin A by Microbore HPLC Using Derivatization with DMABA.

Sample [a]	Monensin A [μg/g]	Recovery [d] [%]
1 [b]	70.3 ± 4.6	93.7
2 [b]	49.5 ± 3.7	88.4
3 [b]	9 063 ± 181	102.6
4 [c]	10 061 ± 408	100.6

[a] Feeds (1, 4) and premixes (5, 6); [b] 3 weighted amounts, 2 injections; [c] 3 weighted amounts, 5 injections; [d] Based on bioassay results. Other conditions as in Table 2.

Higher concentrations of sulfuric acid and benzaldehyde reagent, longer reaction time and, on the other hand, lower reaction temperature than recommended previously (6-8) have been employed in detection methods described. However, by using the microcolumn HPLC system, reagent consumption and, thus, analysis costs have been substantially lower. Linear working range has been found to be one order of magnitude broader than reported earlier (6). Applying DMABA to derivatization, higher sensitivities, particularly for narasin and salinomycin, than with vanillin, which has been widely used to this purpose, have been attained. Postcolumn reaction with DMABA offers a viable method for efficient and sensitive detection of monensin, narasin, and salinomycin.

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DETERMINATION OF TEMAZEPAM AND ITS ACTIVE METABOLITE, OXAZEPAM IN PLASMA, URINE AND DIALYSATE USING SOLID-PHASE EXTRACTION FOLLOWED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive and rapid assay of temazepam and its active metabolite, oxazepam in plasma, urine and dialysate was developed. This assay involves solid phase extraction followed by HPLC analysis. Mean recoveries were 97 % for temazepam and 70 % for oxazepam. The limit of detection was 5 ng/ml of biological fluids for each compound. One advantage of this method lies in the solid phase extraction which is more rapid and convenient than liquid liquid extraction.

This assay maybe useful for monitoring concentrations of temazepam and oxazepam in plasma, urine and dialysate of patients under continuous ambulatory peritoneal dialysis (CAPD).

INTRODUCTION

Temazepam (1 chloro 2,3 dihydro 3-hydroxy-1-methyl-5-phenyl-1H-1,4 benzodiazepin-2-one) is a benzodiazepin used in the short term treatment of insomnia. It is prescribed for to uraemic patients being hemodialysed or under CAPD. We are investigating the behaviour of temazepam and its entry through the peritoneum in uraemic patients under CAPD.

Temazepam is metabolised to an inactive glucuroconjugated compound (1) but also to oxazepam, an active metabolite (2). We developed an assay method for both temazepam and oxazepam in various fluids (plasma, urine and dialysate). These compounds were separated by gas chromatography (3,4,5) or high performance liquid chromatography (6,7,8,9,10) after extraction from biological fluids.

We have developed a method of separation by HPLC after solid phase extraction. The two compouds were extracted from plasma, urine and dialysate and then analysed using the same procedure.

MATERIALS AND METHODS

Reagents

Compounds and reagents were of analytical grade (>99% purity).

Temazepam was generously given by Wyeth France (Paris, France), and climazolam by Hoffman Laroche (Basel, Switzerland). Oxazepam was purchased from Sigma Chemicals (St Louis, USA). Other reagents methanol (Carlo Erba, Milan, Italy) and acetonitrile (Prolabo, Paris, France).

Potassium phosphate buffer (0.01M, pH 5.6) was obtained by diluting 10 ml of 1M potassium phosphate buffer (pH 5.6) to 1 l with distilled water. The 1M buffer was prepared by mixing 94.8 ml of 1M potassium dihydrogenphosphate solution (KH_2PO_4, Merck, Darmstadt, Germany) with 5.2 ml of 1M dipotassium hydrogen phosphate solution (K_2HPO_4, Merck, Darmstadt, Germany).

Reagents used for solid-phase extraction were methanol, distilled water, acetonitrile-distilled water (30:70 v/v) and acetonitrile-distilled water (20:80 v/v).

Stock solutions (1 mg/ml) were prepared by dissolving 10 mg of temazepam, oxazepam or climazolam in 10 ml of methanol. Standard solutions containing a mixture of temazepam, oxazepam and climazolam were prepared as detailed in Table I (climazolam as internal standard was at a set concentration of 400 ng/ml).

TABLE I : Preparation of standard solutions using stock solutions (1 mg/ml) of temazepam, oxazepam and climazolam

	1	2	3	4	5
OXAZEPAM	10 µl (20 ng/ml)	25 µl (50 ng/ml)	50 µl (100 ng/ml)	100 µl (200 ng/ml)	250 µl (500 ng/ml)
TEMAZEPAM	10 µl (20 ng/ml)	25 µl (50 ng/ml)	50 µl (100 ng/ml)	100 µl (200 ng/ml)	250 µl (500 ng/ml)
CLIMAZOLAM	200 µl (400 ng/ml)	200 µl (400 ng/ml)	200 µl (400 ng/ml)	200 µl (400 ng/ml)	200 µl (400 ng/ml)

HPLC procedure

The HPLC system consisted of a L5000 LC Controller module equipped with a pump (655 A-11 liquid chromatography), a L4250 UV-Visible detector and a D2000 chromato-integrator (all from Merck-Hitachi, Darmstadt, Germany).

The analysis was performed on a Lichrocart 125-4 Lichrospher 100 RP 18 endcapped 5 micron analytical column (Merck, Darmstadt, Germany). A Lichrocart 4mm X 4 mm ID Lichrosorb RP 18-5 micron precolumn was used (Merck, Darmstadt, Germany)). The mobile phase was acetonitrile-potassium phosphate buffer (0,01 M, pH 5.6) (40-60 v/v). The analysis was performed in isocratic mode. The flow rate was 1.6 ml/min. The UV detector wavelength was 254 nm.

The injection volume was obtained with a 20 microlitre injection loop.

The column was flushed daily with methanol-water (50:50 v/v)

Solid-phase extraction

The solid-phase extraction was performed using the Vac-Elut system (Analytichem International, Harbor City, USA) and C18 100 mg Bond Elut cartridges (Analytichem International, Harbor City, USA).

The extraction procedure comprised four steps:

* Sample preparation ,deproteination: 1 ml of acetonitrile-distilled water (30:70 v/v) was added to 1 ml of plasma or 1 ml of urine or 10 ml of dialysate. After homogenisation (vortex mixing for 10 s), the samples were centrifuged for 5 min at 4000g.

* Conditioning of the cartridges : 2 ml of methanol and then 2ml of distilled water were run through the cartridges.

The sample (first prepared) was then run through the conditioned cartridges.

* Washing : 2 ml of acetonitrile-distileled water (20:80 v/v) was run through the cartridges. This was followed by drying for 3 or 4 min.

* Elution : the cartridges were eluted with four 200 microlitre volumes of methanol.

The eluate was then evaporated under nitrogen and the residue taken up in 100 microlitres of methanol which 20 microlitres were injected.

Quantification

The internal standard was climazolam. Calibration curves were obtained by plotting the peak area ratios (peak area of temazepam or oxazepam over peak area of

climazolam) as a fonction of concentration of temazepam or oxazepam per ml of plasma, urine or dialysate. Concentration of temazepam or oxazepam for a patient was calculated by interpolation from the calibration curve.

RESULTS AND DISCUSSION

Chromatography

With our chromatographic system, we can separate the two compounds (oxazepam and temazepam) and the internal standard as shown in figure 1. Retention times are stated on the chromatogram.

Quantitative analysis : validation of the method

* Linearity : good linearity was observed for temazepam and oxazepam within the concentration ranges studied (20–500 ng/ml) for each biological fluid was (plasma, urine, dialysate). Slopes and correlation coefficient are given in table II.

* Precision : this was evaluated by intra–assay and inter–assay variabilities. Results are given in tables III and IV.

* Limit of quantification : 5 ng/ml for each compound in all three biological fluids.

* Recoveries : these were calculated at two different concentrations : 50 and 200 ng/ml. Values for recoveries are given in table V.

Figure 2 shows a chromatogram of a blank plasma extract and plasma sample containing 100 ng/ml of oxazepam and temazepam.

Figure 3 shows a chromatogram of a blank urine extract and urine sample containing 100 ng/ml of oxazepam and temazepam.

Figure 4 shows a chromatogram of a blank dialysate extract and dialysate sample containing 100 ng/ml of oxazepam and temazepam.

Clinical application

This assay was used to estimate the entry of temazepam and its active metabolite, oxazepam through the peritoneum during CAPD in uraemic patients.

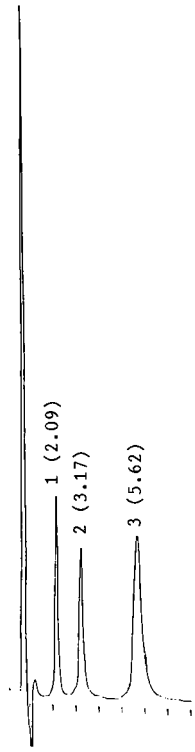

Figure 1 : Chromatographic separation of mixture containing oxazepam 100 ng/ml (peak 1), temazepam 100 ng/ml (2) and climazolam 400 ng/ml (3).

TABLE II : Linearity of the method : values of slopes and correlation coefficients.

		Slope	Correlation coefficient
Temazepam	**Plasma**	0,00486	0,999
	Urine	0,00520	0,999
	Dialysate	0,00501	0,999
Oxazepam	**Plasma**	0,00322	0,999
	Urine	0,00370	0,999
	Dialysate	0,00441	0,999

TABLE III: Precision of the method; intra-day assay variability

		TEMAZEPAM		OXAZEPAM	
BIOLOGICAL FLUID	**CONCENTRATION ADDED (ng/ml)**	**CONCENTRATION FOUND (ng/ml)***	**COEFFICIENT OF VARIATION (%)**	**CONCENTRATION FOUND (ng/ml)***	**COEFFICIENT OF VARIATION (%)**
Plasma	50	49.1+/- 3.7	7.5	52.6 +/- 5.1	9.7
n = 10	200	202.7 +/- 12.2	6.0	211.4 +/- 12.8	6.1
Urine	50	48.9 +/- 2.4	4.9	52.2 +/- 3.8	7.3
n = 10	200	203.3 +/- 5.8	2.9	207.0 +/- 5.8	2.8
Dialysate	50	48.0 +/- 4.4	9.2	48.3 +/- 6.1	12.6
n = 10	200	202.0 +/- 10.6	5.2	200.1 +/- 11.6	5.8

*** mean +/- standard deviation**

TABLE IV: Precision of the method; inter-day assay variability

		TEMAZEPAM		OXAZEPAM	
BIOLOGICAL FLUID	**CONCENTRATION ADDED (ng/ml)**	**CONCENTRATION FOUND (ng/ml)***	**COEFFICIENT OF VARIATION (%)**	**CONCENTRATION FOUND (ng/ml)***	**COEFFICIENT OF VARIATION (%)**
Plasma	50	48.7 +/- 4.1	8.4	49.0 +/- 5.1	10.2
n = 10	200	206.3 +/- 9.6	4.7	210.0 +/- 11.6	5.5
Urine	50	47.1 +/- 2.4	5.1	48.0 +/- 4.6	9.6
n = 10	200	199.1 +/- 5.8	2.9	203.4 +/- 5.7	2.8
Dialysate	50	44.9 +/- 5.4	12.0	52.7 +/- 5.7	10.8
n = 10	200	211.9 +/- 13.3	6.3	209.7 +/- 8.9	4.2

*** mean +/- standard deviation**

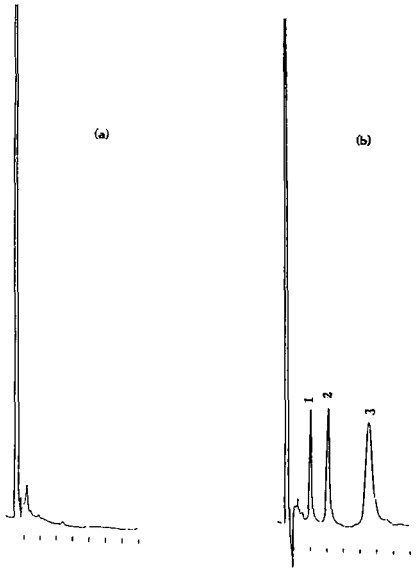

Figure 2: Chromatograms of blank plasma extract (a) and plasma sample (b) containing 100 ng/ml of oxazepam (1) and temazepam (2) and 400 ng/ml of climazolam (3).

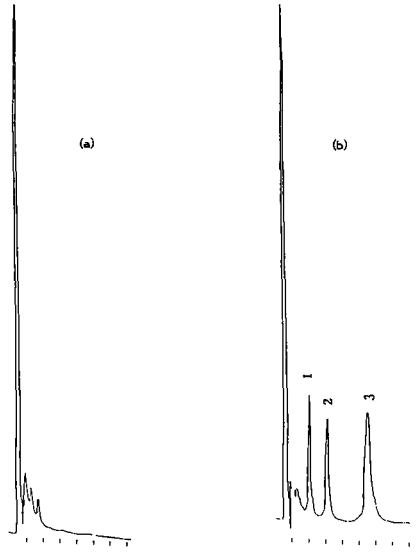

Figure 3 : Chromatograms of blank urine extract (a) and urine sample (b) containing 100 ng/ml of oxazepam (1) and temazepam (2) and 400 ng/ml of climazolam (3)

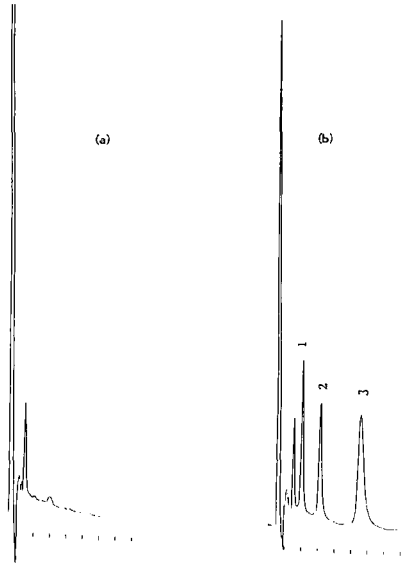

Figure 4 : Chromatograms of blank dialysate extract (a) and dialysate sample containing 100 ng/ml of oxazepam (1) and temazepam (2) and 400 ng/ml of climazolam (3).

TABLE V: Recoveries of temazepam and oxazepam in plasma, urine and dialysate

COMPOUND	BIOLOGICAL FLUID	CONCENTRATION (ng/ml)	RECOVERY (%)	COEFFICIENT OF VARIATION (%)
Temazepam	Plasma	50	96.3	13.8
		200	97.1	9.1
	Urine	50	99.0	9.6
		200	99.8	8.6
	Dialysate	50	96.2	12.5
		200	94.4	9.9
Oxazepam	Plasma	50	60.1	9.5
		200	74.0	9.4
	Urine	50	60.2	13.5
		200	74.5	13.8
	Dialysate	50	69.6	11.8
		200	85.3	14.2

In conclusion, this rapid and convenient assay method involving solid phase extraction followed by HPLC analysis is suitable for routine clinical monitoring of temazepam and oxazepam in plasma, urine and dialysate.

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DETERMINATION OF CHLORAMPHENICOL IN CHICKEN MUSCLE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND UV-DIODE ARRAY DETECTION

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ABSTRACT

A rapid and sensitive method for the determination of chloramphenicol in chicken muscle tissue is described. The method consists of mixing of the ground sample with anhydrous sodium sulphate and acetonitrile, followed by centrifugation and liquid-liquid partition of the supernatant with n-hexane. The acetonitrile extract is evaporated to dryness and the residue dissolved in dichloromethane. Purification was achieved by solid phase extraction silica cartridge and the chloramphenicol is eluated with a acetonitrile/water mixture (20%). Analysis is performed by high-performance liquid chromatography and Diode-Array detection where the peak's identity can be confirmed by comparing retention time and UV-spectra with external standard.

Linearity was studied up to 30 ng injected amount. Mean recoveries from spiked chicken muscle samples were 78.3% with a variation coefficient of 5.77%. The detection limit was 0.01 mg/kg. All the examined samples (50) proceeding from Spanish slaughterhouses turned out to be negative in chloramphenicol.

INTRODUCTION

Antibiotics have been widely used to treat human and animal diseases causing a lot of problems for the health authorities. These problems may have a toxic or allergic character caused by the accumulation of the residue in food for human consumption, but may also produce microbiological selection of resistent bacterial strains.

Chloramphenicol (CAP) has a broad spectrum activity and therefore has been used, not only in veterinary therapeutics, but also as feed additives. Since its introduction in 1949, adverse reactions and side effects of CAP have been reported over the last 30 years. The most known side effect is aplastic anemia, unrelated to dosage and irreversible. Others are blood dycrasias and bone marrow depression. To protect the consumer, zero tolerance levels have been proposed for CAP in edible tissues and milk (1) as the use of chloramphenicol in food producing animals is forbidden in various countries. In others, limits on CAP residues have been fixed. For example, the policy in several European Community countries is to reduce its use in food producing animals by setting limits on CAP residues in edible tissues. Countries, such as the Netherlands, have recently forbidden the suministration of CAP in leghen.

In order to monitor effectively the occurrence of residues, specific and sensitive analytical methods are

required. The microbiological methods have not the sensitivity necessary for the detection of these amounts. Radioimmnoassay has been used to identify and quantify chloramphenicol residues in food (2,3), which offers great sensitivity and may be used for screening purposes when a great number of samples has to be analyzed.

In the last years, various chemical detection methods for CAP in food have been published, generally based on chromatographic technics such as gas chromatography (4), liquid chromatography (5,6,7,8,9,10,11,12) and a colorimetric method coupled with thin layer chromatography (13). The most sensitive methods use gas chromatography with electron capture detection and tandem MS-MS (14) for the CAP residue determination in tissues, milk and eggs. However, gas chromatography involves derivatization of the sample extract, which may cause losses and lower recoveries.

Thin layer chromatography can be useful as a preliminary screening in the range between 10 and 1000 ppb (13). In the literature, several liquid chromatographic methods (5,12) are proposed based on reversed phase separation and UV and electrochemical detection. In previous work (15) we used the method proposed by Ellen and coworkers (16), but this method failed in the determination of CAP residues in fatty chicken tissues.

Recently new innovations in the purification step were introduced by Haagsma and coworkers (17, 18) using monoclonal antibodies in the clean up procedure.

In this work, we describe a HPLC method for the determination of CAP in fatty chicken tissue, based on the extraction of the tissue with acetonitrile, a medium polar organic solvent, followed by a solid purification step with a silica cartridge and finally separation and detection with reversed phase chromatography and Diode Array detection.

MATERIALS

Reagents

Water was purified by demineralization (Mili Q, Millipore). CAP was obtained from Fluka (Büchs, Switzerland).

Anhydrous sodium sulfate, sodium chloride and sodium acetate from Panreac (Montplet and Esteban, Barcelona, Spain). Ethylacetate, acetonitrile methanol, acetic acid (HPLC grade) and dichloromethane (for residue analysis) from Merck (Darmstadt, F.R.G.). Sep-Pak silica cartridges of Waters (Milford, USA) were used.

A CAP standard solution was prepared by dissolving 100 mg of CAP in 100 ml of methanol. Working standards for HPLC were prepared in the range of 100-600 ng/ml by diluting the standard solution with CH_3CN/H_2O (30/70).

The mobile phase solvent for HPLC was acetonitrile/diluted sodium acetate buffer (30:70 v.v). The diluted sodium acetate buffer was prepared by diluting sodium acetate buffer (1M pH 4.8) 1:100 with demineralized water. The mobile phase was filtered through 0.45 mμ Millipore filter membranes.

Apparatus

The instruments used were a Sorvall Omni-mixer (Duponts Instruments), an ultrasonic bath (Selecta, Madrid, Spain), a sample concentrator under a nitrogen stream (Techne Ltd, Oxford, Cambridge U.K.), a Macrotonic centrifuge (Selecta, Madrid, Spain), a rotatory evaporator (Büchi, Flawil, Switzerland) and a Moulinex homogenizer (Moulinex, Bilbao, Spain). The HPLC equipment consisted of a Waters pump (Mod. 6000 A), an injector (Mod. U6K), and UV absorption was monitoried at 278 nm with a Diode-Array detector (Waters 990). The HPLC column used throughout this work was a Nova-Pak 4 μ, C_{18} column in a Radial compression module of Waters (Milford, USA).

Flow rate: 1 ml/min., chart speed: 0.5 cm/min.

METHODS

Extraction and clean up of muscle tissue samples

Muscle tissue was prepared removing visible fat as far as possible and homogeneizing in a Sorvall Omni-mixer.

A 10 g sample of homogeneized muscle tissue was weighed into a centrifuge tube of 100 ml and thoroughly mixed with 30 g of anhydrous sodium sulfate and 30 ml of acetonitrile during 1 min in an Omni-mixer. The mixture was centrifuged during 10 min at 4000 r.p.m. and the upper organic layer transferred to a 250 ml round-bottom flask. The remaining aqueous phase was extracted two more times each with 30 ml acetonitrile. The combined acetonitrile phases were transferred to a separation funnel and extracted twice, shaking vigourously during 30 seconds, with two portions of 60 ml n-hexane. The hexane layers were discarded and the acetonitrile extract was transferred to a round-bottom flask and evaporated till dryness under a stream of nitrogen at 50°C.

Clean-up procedure

A silica Sep-Pak Cartridge (Waters) was washed respectively with, 5 ml of acetonitrile in water (20%), 5 ml of acetonitrile, 5 ml of dichloromethane and then dried by forcing during 30 minutes a gentle stream of nitrogen through the cartridge.

The sample extract was gently pressed through the cartridge with a disposable syringe, and the cartridge was washed with two 5 ml portions of dichloromethane.

After drying with a stream of nitrogen (about 30 min.), CAP was eluted from the cartridge with 5 ml

acetonitrile in water (20%). Then 1 ml of ethylacetate was added to the eluate and the mixture was shaken. The upper layer was transferred to a clean tube and the extraction with ethylacetate was repeated twice. The combined organic phases were evaporated till dryness in a sample concentrator at 50°C under a gentle stream of nitrogen. The dry residue was dissolved in 1 ml of the HPLC eluent.

Spiked samples

For the spiking studies, homogenized muscle tissue was used.

Ground tissue samples were spiked at levels of 10, 30 and 60 μg/kg at least 15 min before extraction according to the procedure described below.

Detection and Identification by HPLC

Different amounts of CAP were injected to check the linear response of the detector. Recovery experiments were carried out on spiked samples by injection of 50 μl of the sample extract, obtained under the conditions of extraction and purification as described above.

RESULTS AND DISCUSSION

Almost all the described methods for the HPLC determination of CAP residues use ethylacetate as the

extraction solvent. However, we observed an important co-extraction of fat using this solvent, causing a lot of troubles in the purification procedure. Therefore, we changed the extraction procedure of our previously described method (15) choosing a more polar solvent as acetonitrile, which has the same extraction efficiency for CAP, but is less active in the extraction of fat.

The fatty compounds were removed from the acetonitrile extract by liquid-liquid partition with n-hexane before the clean-up step with the silica cartridge.

Linearity of response, using peak height at 278 nm vs quantity injected, was studied by sucessive injections (n=2) of 50 μl alicuots of working solutions: 100, 300, 600 ng/ml corresponding to 5, 15 and 30 ng injected CAP respectively. The calibration graph was calculated using the method of least squares and can be expressed as:

$$y = 0.44\ x + 0.10$$

where y = CAP peak height in mm at 278 nm and x = the amount of injected CAP expressed in ng. The linearity was excellent with a correlation factor r = 1.000.

A typical chromatogram of 15 ng of CAP injected in the described conditions, is shown in Fig.1.

To check the validity of the proposed extraction and clean up procedure, various chicken tissue samples were homogenized and spiked with 100, 300 and 600 μg/kg of CAP respectively and the extracts analysed.

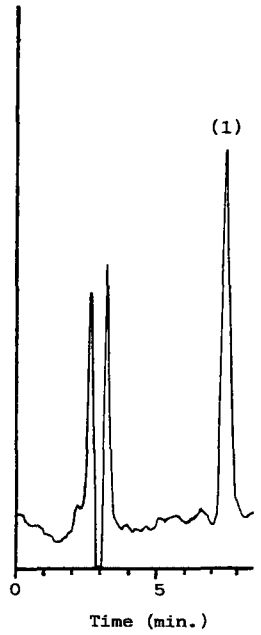

Figure 1 : Chromatogram of (1) 15 ng injected CAP in described conditions. (UV detection at 278 nm 0.005 a.u.f.s.).

Reagent blanks were extracted and analysed following the described method. Blanks are important as the influence of the surfaces of glassware may produce interfering peaks. Therefore, it is necessary to clean the used glassware by hand with a few drops of a cleansing fluid in water and rinse extensively. Alkaline soaps of the automatic dishwasher may cause undesired activation of the glass surface.

Fig. 2 shows a comparison of chromatograms of spiked (with 30 ppb CAP) and unspiked extracts of chicken muscle tissue.

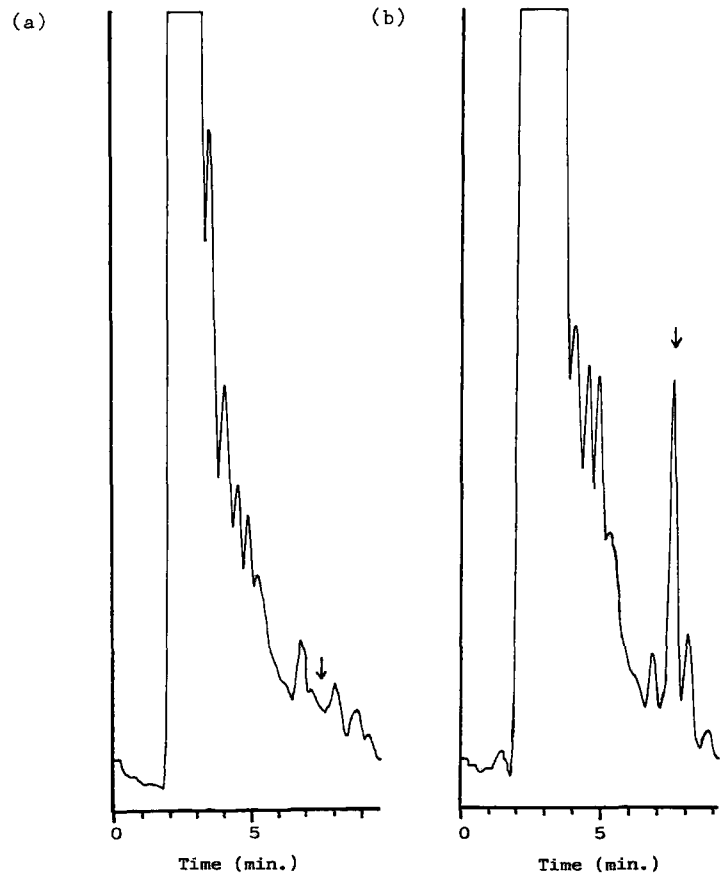

Figure 2 : Chromatograms of (a) unspiked and (b) spiked sample (30 ppb) of chicken muscle tissue (UV detection at 278 nm 0.005 a.u.f.s.).

TABLE I

Recovery of CAP in chicken muscle tissue

CAP added ($\mu g.kg^{-1}$)	% Recovery (n=4) mean	CV %
10	81.4	4.94
30	78.7	4.94
60	74.7	3.7

OVERALL = 78.3 %

Recovery results are shown in Table I. As can be seen, the recoveries are adequate in the range of the injected amounts with an overall recovery of 78.3% and CV of 5.77%.

A lot of 50 samples of chicken muscle tissue proceeding from different Spanish slaughterhouses was analysed with the proposed method.

Generally, peaks are identified by comparing the retention time with that of a standard. However, when working at trace levels, other compounds from the sample might have the same or similar retention times as the residues of interest. Therefore, an additional method for confirmation is required.

The Diode Array detector allows the possibility of getting this information about a compound as chromatographic and spectral data are both acquired and put into memory during the time of the run. These spectral data of an unknown peak can be used to confirm the identity of this peak.

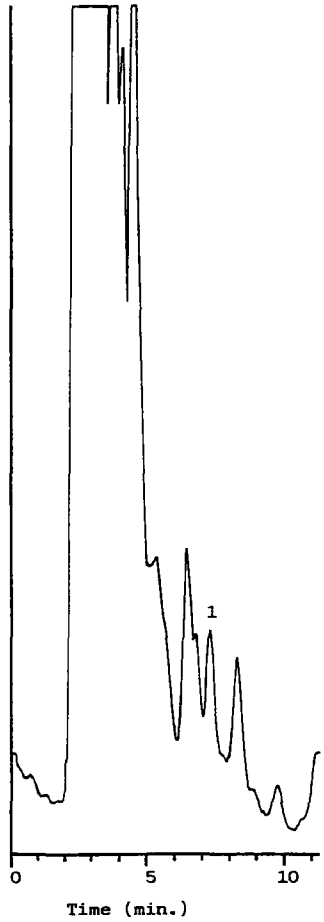

Figure 3 : Chromatogram of chicken muscle sample C 211 with a peak (1) at the retention of chloramphenicol.

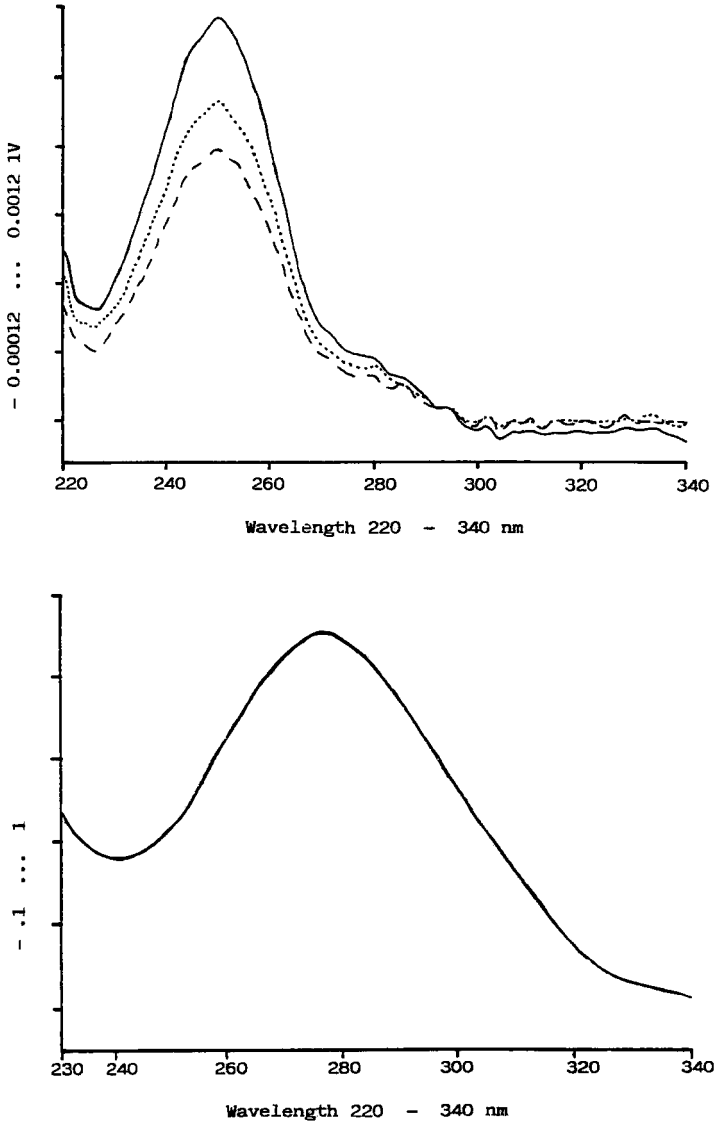

Figure 4 : Comparison of the spectra at different retention times of (a) peak (1) of chicken muscle sample C 211 and (b) external standard.

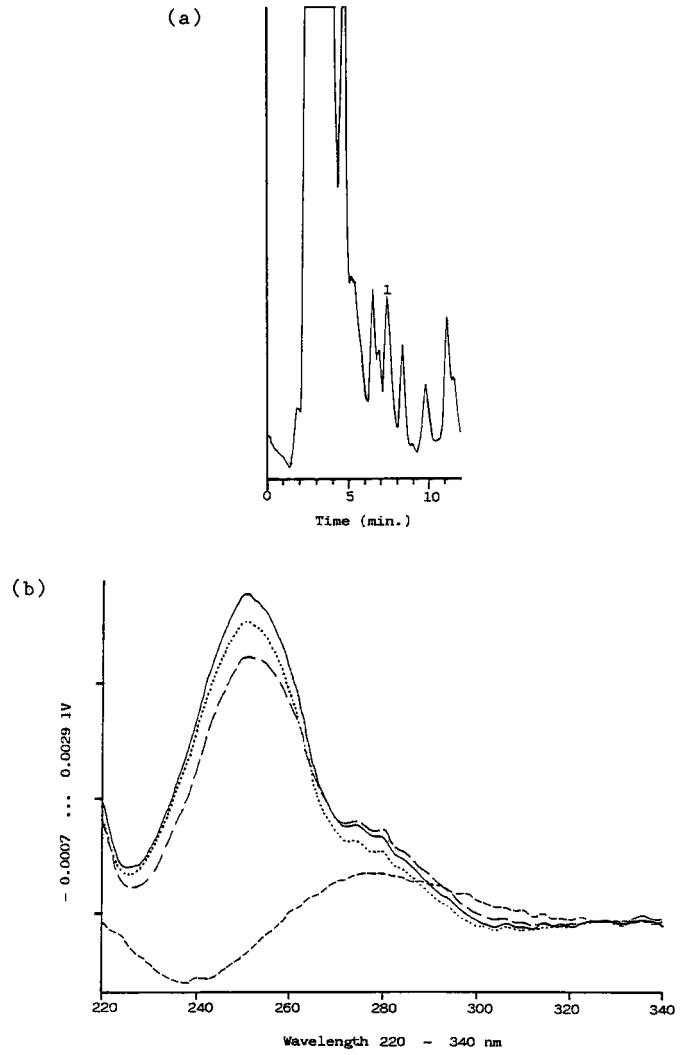

Figure 5 : a) Chromatogram of chicken muscle sample C 211 spiked with 5 ng chloramphenicol (UV detection at 278 nm 0.005 a.u.f.s.).

b) Spectra at different retention times of peak (1) of the spiked muscle sample C 211.

In neither of the analysed chicken samples could any detectable residues of CAP be detected. The detection limit was about 10 ppb; this concentration still gives well defined spectra. Moreover, the Diode-Array detector permits the "on-line" confirmation of suspicious peaks. For example, the chromatogram of sample C211 presents a peak at retention time 7.38 min, similar to that of CAP (Fig. 3). This unknown compound appeared to be a pure peak with a spectrum quite different to that of an external standard of CAP (Fig. 4a and 4b). Adding an amount of 5 ng of CAP to the sample, resulted in the appearance of the CAP spectrum in the downslope of the increased peak (1) as is presented in Fig. 5a and 5b.

Sometimes these interfering peaks were produced when samples underwent repeated thawing and freezing. So only once frozen samples must be used and thawed just before analysis.

In conclusion, the analysis of CAP by the reversed-phase clean up to separate the drug from the biological sample, is sensitive, specific and reproducible, as is demonstrated by the above mentioned results.

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DETERMINATION OF ECHINOMYCIN (NSC-526417) IN HUMAN PLASMA: COMPARISON OF CONVENTIONAL HPLC TO A CAPILLARY HPLC, ELECTROSPRAY IONIZATION AND MASS SPECTROMETRY SYSTEM

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ABSTRACT

We have compared the use of conventional HPLC to that of capillary HPLC used together with electrospray ionization and mass spectrometry (capHPLC/ESPI/MS) as a means of achieving picogram sensitivity for the analyses of a potent model anticancer compound, echinomycin. Using conventional HPLC, a lower limit of quantitation (LLQ) of 10 ng/ml plasma was obtained but capHPLC/ESPI/MS permitted a LLQ of 100 pg/ml. The latter method was found to be accurate and reproducible and provided a broad range of drug detection capability (0.1 ng/ml to 1 μg/ml). Comparison of analytical assay parameters using the capHPLC/ESPI/MS methodology to that of conventional HPLC are provided and discussed.

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INTRODUCTION

The development of potent anticancer compounds in conjunction with their administration in the clinic setting by prolonged infusion schedules has made analyses of these drugs in biological fluids by conventional HPLC or GC extremely difficult. While liquid chromatography (especially HPLC) combined with thermospray/mass spectrometry has been an essential analytical tool for the pharmaceutical industry for drug metabolism studies, it has rarely been applied to the routine determination of experimental drug levels. The recent development of extremely potent anticancer compounds, however, has brought forth a need for analytical equipment with detection capabilities down to at least the picogram/ml level. The introduction of capillary electrophoresis and capillary HPLC columns and related equipment (e.g. flow splitters and microinjectors) has provided an opportunity to combine these types of chromatography with efficient, nondestructive methods of sample introduction (i.e. electrospray ionization) into the mass spectrometer to achieve extremely sensitive and specific drug detection and assay capabilities.

In this study we have compared an analytical assay procedure for echinomycin (Figure 1) using conventional HPLC with UV detection to that using capHPLC together with electrospray ionization and mass spectrometry (Figure 2). Echinomycin (NSC 526417, quinomycin A) is one of a family of quinoxaline antibiotics originally isolated from Streptomyces echinatus (1,2). It is thought to act as a bifunctional intercalator in DNA thereby resulting in inhibition of DNA-directed RNA synthesis. The drug's specific interaction with DNA results

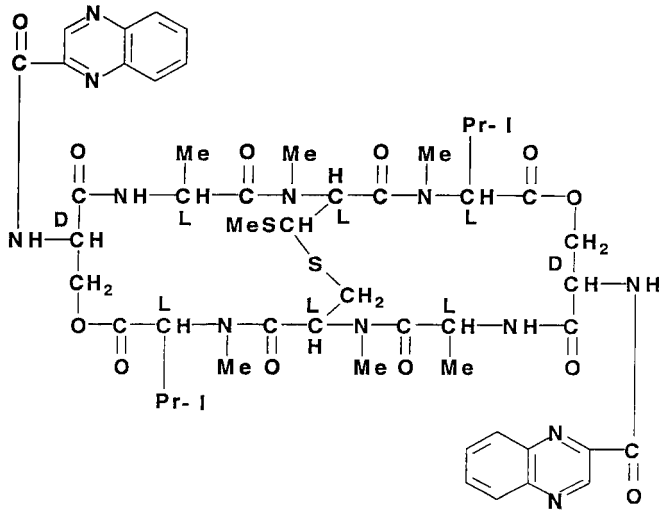

Figure 1. Structure of Echinomycin (Quinomycin A; NSC-526417).

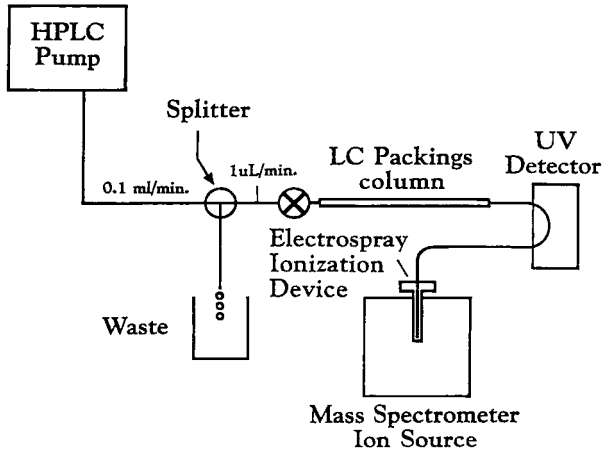

Figure 2. Schematic diagram of the capillary HPLC/electrospray ionization/mass spectrometry system.

in both inter- and intramolecular crosslinking (3,4). Although a number of Phase II studies have been completed with this drug, attempts to develop a sensitive and specific assay have been unsuccessful and, hence, there are no published reports on the clinical pharmacology of this potent anticancer compound.

In the present study we report that capHPLC with ESPI/MS can be used in an assay method which extends the limit of quantitation down to pg/ml levels with excellent reproducibility and accuracy. It is suggested that this combination of techniques may be appropriate for a wide variety of pharmaceutical compounds and their metabolites where detection limits are important and cannot be met by conventional HPLC instrumentation or methodology and where, as opposed to methods such as RIA or ELISA, qualitative knowledge of a compound's identity (via mass weight) is considered important.

MATERIALS AND METHODS

Conventional HPLC

The instrumentation consisted of a Waters Assoc. (Milford, MA) Model 6000A pump, Model U6K injector and a Model 990 photodiode array detector. The separations were performed on a Waters μBondapak C18 column (3.9 x 300 mm; Waters Assoc., Milford MA). A μBondapak C18 guard column was routinely used to protect the analytical column.

CapHPLC/ESPI/MS

The capillary HPLC equipment consisted of a fused 320 um x 150 mm C18 capillary column and an Acurate flow splitter (LC Packings, Zurich,

Switzerland), a Valco microinjector valve (Valco, Houston, TX), an electrospray interface (Analytica Inc., Branfort, CT) and a Nermag R-30-10 triple quadrapole mass spectrometer (Paris, France).

Reagents and Materials

Methanol and acetonitrile were of HPLC grade (Curtis Matheson, Scientific Inc., Houston, TX). Phosphoric acid was obtained from Fisher Scientific (Fairlawn, NJ). Echinomycin was obtained from the National Cancer Institute (Bethesda, MD). Taxol was purchased from Sigma Chemical Co. (St. Louis, MO).

Drug Extraction

BondElut C2 minicolumns (Varian Associates, Palo Alto, CA) were activated by rinsing with methanol and water as per the manufacturer's instructions. Using a low vacuum (approximately 7 psi), 1 ml of plasma containing echinomycin and the internal standard, taxol, was added to the BondElut columns. They were then rinsed with 2 ml of water followed by 2 ml of 10% acetonitrile in water. These eluants were discarded and the drug was eluted using 1 ml of 100% acetonitrile which was then dried under nitrogen. Samples were reconstituted in either 250 μl acetonitrile: water (60:40, v/v) for conventional HPLC analyses or 20 μl methanol for analyses by capHPLC/ESPI/MS.

Analytical Assay (capHPLC/ESPI/MS)

Reconstituted drug (1 μl) was injected into the capillary HPLC system. As seen in Figure 2, mobile phase (methanol) from the pumps goes through the splitter and then onto the capillary column's injector. This splitting brings the

flow rate into the 2-3 μl/min range which is the optimum flow rate for 0.32 mm (ID) capillary columns. In addition, these flow rates are also needed for optimum electrospray ionization sensitivity. Higher flow rates reduce electrospray ionization efficiency and, therefore, sensitivity. Rention times for echinomycin and taxol on the capillary HPLC column under the conditions described were 11.8 min and 12.3 min, respectively (Fig. 3).

Analytical Assay (Conventional HPLC)

Reconstituted drug (50 μl) was injected onto the HPLC column using a mobile phase of 48% acetonitrile, 6% methanol and 46% 0.1 M H_3PO_4 (pH 7.1). Flow rate was 1.5 ml/min and the detector was set at 243 nm and 0.001 AUFS. Under these conditions, echinomycin eluted at approximately 6.5 min and taxol eluted at 7.8 min (Fig. 4).

RESULTS

Extraction efficiency from human plasma was examined over a wide range of drug concentrations. Concentrations below 25 ng/ml produced recoveries which were lower (64% to 70%) than those at concentrations exceeding this value; concentrations above 25 ng/ml, however, yielded recoveries which were consistently greater than 87%. Increasing either the volume or strength of the 10% acetonitrile minicolumn wash lowered the recovery of echinomycin substantially; however, reducing the volume of the 10% acetonitrile wash resulted in retention of UV absorbing material on the C2 minicolumn which was subsequently eluted with echinomycin. While the extent of recovery from plasma was felt to be adequate, the concentration-dependent variability in recovery made

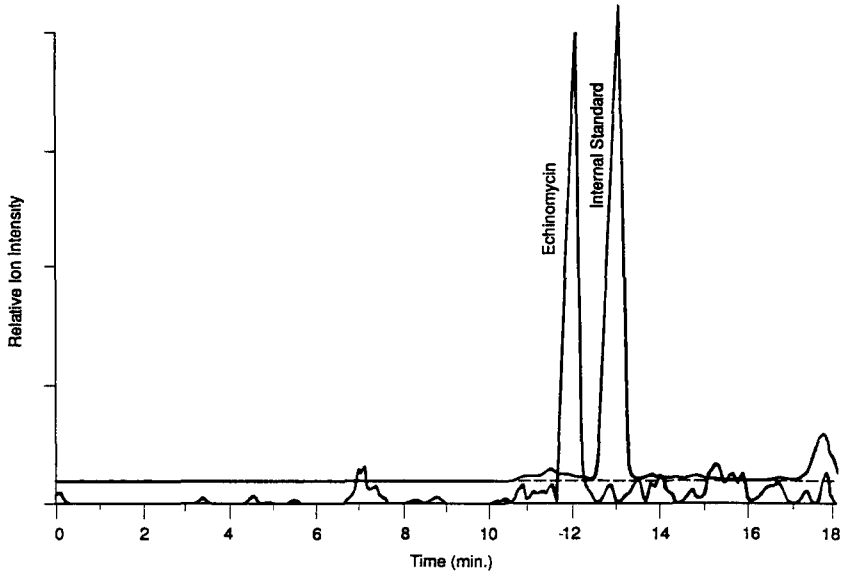

Figure 3. Mass spectrometry analyses of echinomycin (*m/z* 110) and taxol, the internal standard (*m/z* 854). The figure depicts relative ion intensities for 2 ng (on column) echinomycin and 50ng (on column) taxol.

the use of an internal standard necessary. Taxol, a structurally unrelated compound, was determined to be a useful internal standard based on its similar retention characteristics on C2 minicolumns and elution time on a conventional reverse phase C18 analytical column.

Excellent separations of internal standard and echinomycin were achieved on both the C18 analytical column (conventional HPLC) and on the capillary HPLC column. As shown in Figure 4, taxol is well resolved from echinomycin with no interfering peaks. Using taxol as an internal standard, standard curves were prepared over a wide concentration range. The lower limit of detection

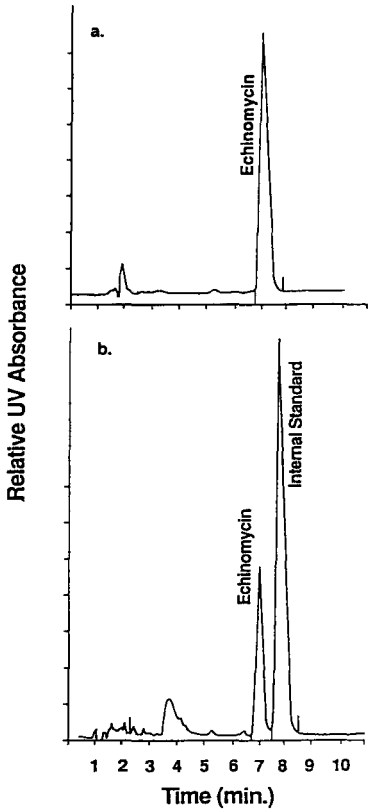

Figure 4. Representative conventional HPLC chromatogram of a) echinomycin (200 ng on column) extracted from human plasma, and b) echinomycin (200 ng) and the internal standard, taxol (500 ng), extracted from human plasma.

using the conventional HPLC approach (UV detection) was approximately 10 ng/ml. However, 50 ng/ml was determined to be the actual lower limit of quantitation. Excellent linearity of standards was obtained up to a concentration of 500 ng/ml (Figure 5).

The standard curves for echinomycin obtained using capHPLC/ESPI/MS are shown in Figures 6 and 7. While this analytical method could determine drug

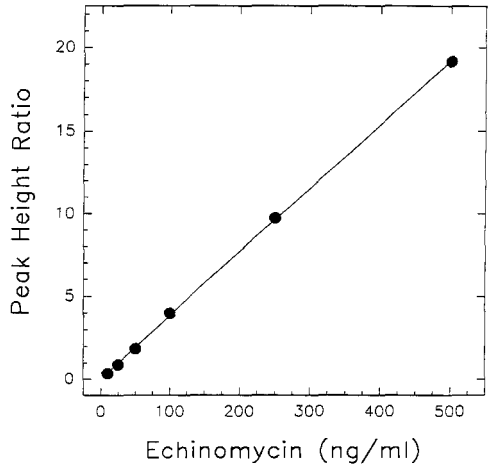

Figure 5. Standard curve for echinomycin obtained using conventional HPLC and the peak height ration method. Data are representative of curves run in triplicate on each of three different days; $r^2 = 0.98$.

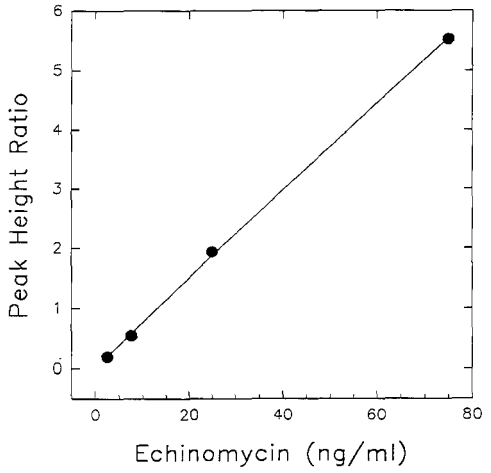

Figure 6. Standard curve (total assay range) for echinomycin obtained using capHPLC/electrospray ionization/MS. Data are presented as the average of duplicate determinations per point; $r^2 = 0.99$.

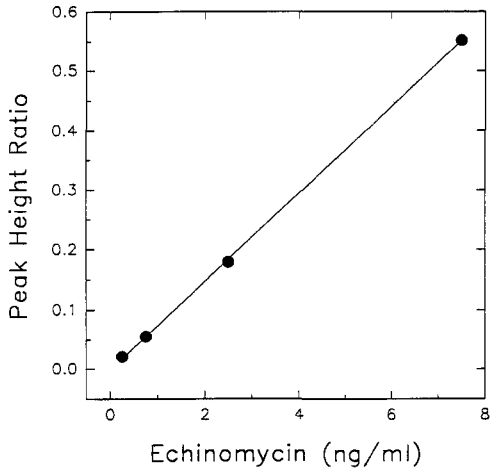

Figure 7. Standard curve (working range) for echinomycin obtained using capHPLC/electrospray ionization/MS. Data are presented as the average of duplicate determinations per point; $r^2 = 0.99$.

in plasma extracts over a wide range (2.5 to 75 ng/ml; Figure 6), the lower range of 0.25 to 7.5 ng/ml was chosen as the more useful working analytical range (Figure 7). This is also the most likely range of values which can be expected from administration of low doses of echinomycin to patients.

Assay precision and accuracy were examined for both analytical methods. Within day precision for the conventional HPLC assay was within 8% of the mean value for both concentration levels examined while precision for the capHPLC/ESPI/MS was also excellent with all relative standard deviations less than 10.5% (Table 1). Both analytical assays also showed excellent accuracy as all concentrations assayed by either method were within 4% of their respective nominal values. Between day accuracy and precision for both methods was also

TABLE 1

Within-day Accuracy and Precision

Method	N	Concentration (ng/ml) Nominal	Observed	%RSD	% Deviation from Nominal
HPLC	6	25.0	25.6	7.1	2.4
	6	250	254	4.4	1.6
capHPLC/ESPI/MS	6	0.25	0.26	10.2	3.2
	6	7.5	7.4	5.9	1.7
	6	75.0	74.0	7.0	1.3

considered as acceptable; data are presented in Table 2. The %RSD and % deviation of observed values from nominal values were all less than 12% for both analytical methods.

DISCUSSION

Echinomycin (Quinomycin A; NSC-526417) is a fermentation product derived from Streptomyces echinatus. It is a large (mol. wgt. = 1101) and extremely potent natural product which has been administered to humans with cancer at low $\mu g/m^2$ doses making it nearly undetectable by conventional chromatography methods. Echinomycin is, in fact, representative of a number of natural products from several sources including plants, marine organisms and bacteria, which have been identified, isolated and purified for potential use as anticancer drugs. Their

TABLE 2

Between-day Accuracy and Precision

Method	Day	Concentration (ng/ml) Nominal	Observed	%RSD	% Deviation from Nominal
HPLC	1	25.0	25.5	9.4	2.0
		250	257	3.1	2.8
	2	25.0	22.6	10.6	9.6
		250	255	2.9	2.0
	3	25.0	23.7	8.2	5.2
		250	251	3.0	0.4
capHPLC/ESPI/MS	1	0.25	0.26	11.2	3.5
		25.0	26.8	2.2	7.2
	2	0.25	0.24	2.9	2.0
		25.0	25.0	3.8	0.2
	3	0.25	0.26	11.5	3.2
		25.0	26.1	4.4	4.5

N = 3/day

potency presents an analytical problem especially when these agents are combined with prolonged infusion schedules. Anticipated blood levels are often far below the usual detection limit for conventional HPLC with UV or even fluorescence detection capabilities. To overcome this problem we have evaluated an analytical system which combines capillary HPLC with electrospray and mass spectrometry. As shown in the present series of studies, the capHPLC/ESPI/MS system has been found to provide detection capabilities down to at least 100 pg/ml with excellent accuracy and precision. We believe that even lower levels of compound could be easily determined if extracts derived from larger initial plasma volumes had been used.

The combination of capillary HPLC and ESP ionization has rapidly become the techniques of choice for the quantitation of "difficult to do" pharmaceutical compounds. Compounds presenting analytical challenges include those which exhibit one or more of the following characteristics: high potency with respect to pharmacodynamic action(s), poor volatility, high polarity, thermal lability, and poor UV absorbance or fluorescence capabilities.

ESP ionization has the advantage of ionizing molecules and driving them into a gas phase thorough an electrical repulsion mechanism rather than through the use of heat. This results in large currents of ions of only one mass (i.e. the molecular ion adduct). With respect to echinomycin, the molecular species *m/z* 1102^{+1} carries all the current. In addition to great enhancement of sensitivity, specificity of detection is significantly improved.

The low solvent flow (1-10 μl/min) typically used with capillary HPLC is ideally suited to ESP ionization procedures which works optimally at these low flow rates. Capillary columns also provide a very high theoretical plates (typically 15,000 to 30,000) and permit short retention times which are typically under 10 min. The resulting narrow peaks provide greater sensitivities. On theoretical grounds, the gain in sensitivity using a 0.32 mm column, instead of a 4.6 mm column is about 200 fold. From our experience, it also appears that currently available capillary columns are durable and stand up well to repetitive injections of biological extracts. The principal disadvantage to the use of capillary HPLC columns, however, is the limited injection volume which must be used. In our system a maximum of 1 μl can be injected without loss of retention characteristics and peak homogeneity. A second disadvantage is that for most applications using capillary HPLC, gradient elution programs are required to adequately separate the eluant of choice from other contaminating species. Coeluting compounds can interfere with the ESP ionization process resulting in lower sensitivities than expected. Better separation and/or cleaner sample preparations are therefore often required for capillary HPLC analyses than for conventional HPLC. Given these disadvantages, however, the present work demonstrates that capHPLC/ESP/MS is a useful analytical approach for the quantitation of pharmaceutical compounds in biological matrices which must be determined at extremely low levels.

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SIMPLE, RAPID AND SPECIFIC IDENTIFICATION AND QUANTIFICATION OF A METABOLITE OF ALPIDEM, A NEW IMIDAZOPYRIDINE ANXIOLYTIC, IN HUMAN URINE, BY DIRECT INJECTION INTO HPLC COLUMN WITH FLUORESCENCE DETECTION

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ABSTRACT

For the investigations of alpidem overdose cases in humans, an HPLC method with fluorescence detection was developed. The method was based on the identification and quantification of an alpidem metabolite (the product of oxidation of the propyl group) in urine, since no unchanged alpidem was found in urine of subjects dosed with the drug. The urinary sample, after deconjugation with β–glucuronidase/ arylsulfatase enzyme and addition of internal standard, was simply diluted (1/1) with pure water and injected onto the chromatographic system connected to spectrofluorimetric detector ($\lambda_1 = 255$ nm, $\lambda_2 = 423$ nm). The analytical column was C_8 type material specially deactivated for basic compounds, the eluent mixture was constituted by a phosphate solution 0.025M (pH=4.6), acetonitrile, methanol, 45/30/15 (v/v/v), supplied at 1.5 $ml.min^{-1}$ flow rate. The determination limit of the method, in human urine, was 20 $ng.ml^{-1}$; the method was linear in the

range 20–1000 ng.ml^{-1} in urine. The method showed a good selectivity in respect to endogenous compounds and to commonly prescribed psychotropic drugs. The method, validated over a 4 day period, by two analysts, was demonstrated to be precise and accurate.

INTRODUCTION

Alpidem is a new imidazopyridine which has an interesting anxiolytic profile at both the animal and clinical level; in several animal species, alpidem shows anxiolytic–like activities and anticonvulsant properties associated with very weak or no myorelaxant and sedative effects (1,2). In animal models, the drug seems to exert its pharmacological effect by interacting with ω_1 receptors within the GABA supramolecular receptor complex (3). The drug undergoes a first pass elimination, after oral administration to humans (4), it is actively metabolized and three circulating metabolites have been identified in plasma (5), they originate from N–dealkylation, oxidation of one N–propyl group or a combination of both processes (Fig.1).

Alpidem metabolites are mainly eliminated by biliary excretion in the feces, small quantities are eliminated in urine where alpidem unchanged is not found (6).

Since the compound has been available on the French pharmaceutical market for one year, we were requested to develop a simple and rapid analytical method suitable to detect the unchanged compound and/or its metabolites in urinary samples collected from subjects hospitalized after accidental or deliberate drug overdoses.

The determination of alpidem and metabolites in human plasma is performed by HPLC with fluorescence detection method based on a liquid–liquid extraction of the drug and its metabolites (5) from the biological matrix; recently a method was developed in our laboratory

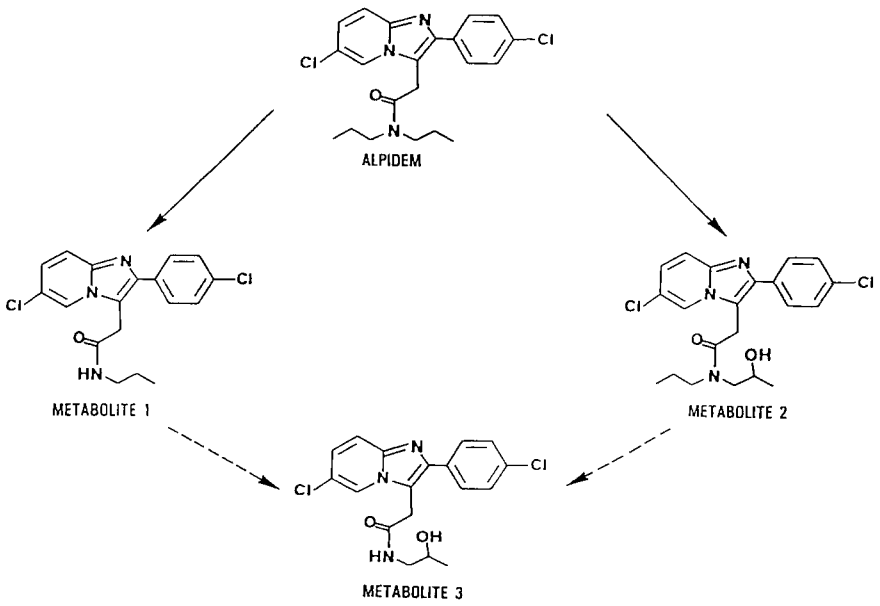

FIGURE 1: Metabolic pathway of alpidem in humans: structures of the principal metabolites

based on the column switching HPLC technique (not yet published). When both the existing methods were tried on urinary samples from subjects treated with alpidem at therapeutic dose, neither unchanged drug nor metabolites were found; however if urine was previously enzymatically deconjugated, a relevant chromatographic peak (corresponding to the retention time of the metabolite obtained from the oxidation of a N–propyl group) was discovered.

This paper describes a single and rapid method of determination of such metabolite in urinary samples by directly injecting the fluid into an HPLC column connected to a fluorescence detector.

MATERIAL AND METHOD

Chemicals

Alpidem (SL 80.0342), metabolite 1 (SL 80.0522), metabolite 2 (SL 83.0912), metabolite 3 (SL 83.0725) (Fig.1) and the internal standard (SL 80.0633) (Fig.2) were of pharmaceutical grade and provided by Synthélabo Recherche (L.E.R.S.), Chemistry Department, Paris (France). HPLC grade acetonitrile and methanol, potassium dihydrogen phosphate anhydrous (pro analysi), β–glucuronidase/ arylsulfatase (extracted from Helix pomatia, β–glucuronidase 30 U/ml, arylsulfatase 20 U/ml) were all obtained from E.Merck (Darmstadt, Germany). Pure water used for HPLC and reagent preparation was obtained from deionized water then purified through Milli Q–system (Millipore, Bedford, MA, U.S.A.).

Reagents

The phosphate buffer 0.025M (pH=4.6) used for the mobile phase was prepared from potassium dihydrogen phosphate by dissolution and dilution with pure water. The mobile phase was prepared by adding to 450 ml of phosphate buffer 0.025M, 150 ml of methanol and 300 ml of acetonitrile, then mixing and filtering on 0.22 μm filter membrane (type HV) with a clarification kit (Millipore).

Standard solutions

Stock solutions (1000 μg/ml) of metabolite 2 (SL 83.0912) and internal standard (SL 80.0633) were prepared monthly in methanol and stored at 0–5 °C conditions. The standard solutions, used for daily calibration, were prepared from stock solutions by suitable dilution with methanol in order to obtain a wide range of concentrations (Table 1).

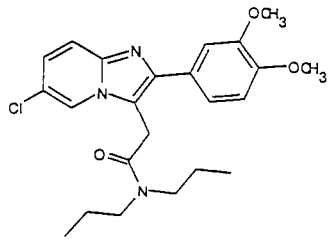

FIGURE 2: Internal standard (SL 80.0633) structure

TABLE 1

Standard solutions used for calibration

Standard solutions	Metabolite 2 concentration (ng/20 µl)	Internal Standard concentration (ng/20 µl)
1	1000	——
2	500	——
3	250	——
4	100	——
5	50	——
6	20	——
7	——	400

The standard solutions were prepared weekly and stored at 0–5 °C conditions.

Quality control samples

Quality control samples were prepared in pre–dose human urine at concentrations of about 300 and 60 ng.ml^{-1} of metabolite 2 by spiking 0.5 ml of methanolic solution of metabolite 2 (at concentration of 14.7 ng.μl^{-1} and 2.95 ng.μl^{-1} respectively) with two 25 ml aliquots of pre–dose urine. 1 ml aliquots of urinary specimens were transferred into screw–capped test tubes and deep–frozen (–20 °C) until analysis.

Chromatography

A Kontron system was used consisting of a double piston pump model 420 with a pump head for 0.01–2 ml.min^{-1} flow rate, the detector was a fluorimetric type model SFM 23 operating at 255 nm, as excitation, and at 423 nm, as emission wavelength; the automatic sample injector was a model 460 provided with a 6 port Valco valve and 100 μl loop. The chromatographic column (15 x 0.46 cm i.d.) was packed with Hypersil BDS, C_8 type material, 5 μm particle size (Shandon, Runcorn, UK); it was provided with a guard column (2 x 0.46 i.d. cm) packed with Pelliguard C_8 material, 40 μm particle size (Supelco, Bellefonte, PA, U.S.A.). The mobile phase consisted in a mixture of aqueous potassium dihydrogen phosphate (0.025M, pH=4.6), acetonitrile and methanol in the ratio 45/30/15 (v/v/v) supplied at 1.5 ml.min^{-1} flow rate (back–pressure about 85 BAR). The quantitative determinations were performed by means of a S.P. model 4290 integrator (Spectra Physics, San José, CA, U.S.A.).

Sample preparation

The frozen urine samples (pre–dose and unknowns) were thawed in a water–bath at 37 °C before pipetting at room temperature; then 1 g of pre–dose urine (for each future urinary standard) and unknown samples were weighed. To the pre–dose urine, 20 µl of the standard solutions were added for each calibration point (Table 1); 50 µl of β–glucuronidase/arylsulfatase solution were added to all samples. All the tubes containing the samples were capped and incubated in a thermostated block at 37 °C for 20 minutes, then cooled at room temperature; to all the tubes 20 µl of internal standard solution were added (Table 1) and homogenized, then the samples were diluted with 1 ml of pure water, homogenized and transferred to autosampler vials for automatic sample injection. 50 µl were injected into the HPLC system (Fig. 3, for the scheme of sample preparation).

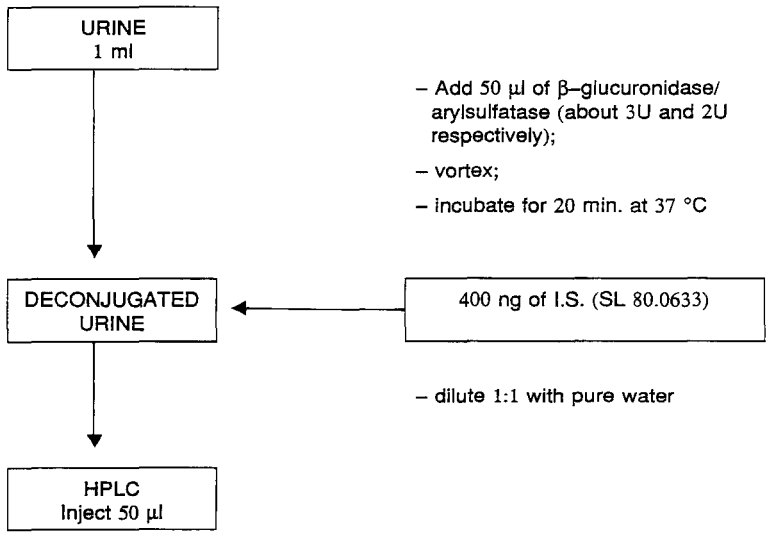

FIGURE 3: Scheme of the sample preparation for the analysis of metabolite 2 (SL 83.0912) in urine.

Quantitative determination

Peak height ratios of metabolite 2/internal standard, obtained from urinary standards, plotted versus the nominal concentration of the metabolite 2, were used to generate the linear least square regression line (calibration equation). The concentration of metabolite 2 in the unknown specimens were obtained by interpolation from the calibration equation using peak height ratios of metabolite 2/internal standard, obtained from unknown specimens. All the operations concerning quantitation and calibrations were automatically performed on an integrator SP 4290.

RESULTS AND DISCUSSION

Stability

The metabolite 2 and internal standard were stable in methanol, at least one month, if maintained at 0–5 °C conditions (stock solutions) in comparison with freshly prepared solutions. Both the compounds were stable in deconjugated and diluted human urine for at least 12 hours at room temperature (pre–injection conditions). The metabolite 2, in urine, resulted stable if submitted to 2 freezing–thawing cycles (–20 to 37 °C).

Selectivity

Several pre–dose urinary samples from different subjects were tested for the absence of interfering compounds; in no case was any chromatographic interference found at the retention time of metabolite 2 and internal standard (Fig. 4a). The possibility of interference by some commonly prescribed drugs (anxiolytics, hypnotics,

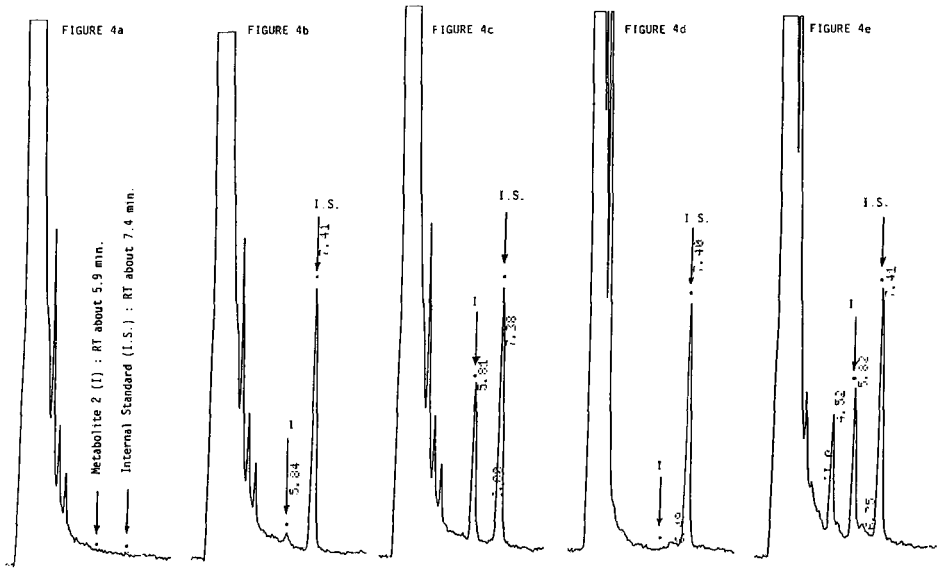

FIGURE 4a: chromatogram of pre–dose human urine after deconjugation; metabolite 2 = I, internal standard = I.S.

FIGURE 4b: chromatogram showing the limit of determination of the method in human urine deconjugated : 20 $ng.ml^{-1}$

FIGURE 4c: chromatogram of a quality control sample (QC) recovered from pre–dose human urine deconjugated at nominal concentration of 300 $ng.ml^{-1}$ of I and 400 $ng.ml^{-1}$ of I.S.

FIGURE 4d: chromatogram of a urine sample taken from a subject treated orally with 50 mg of alpidem b.i.d.; non–deconjugated sample

FIGURE 4e: chromatogram of the same sample reported in Figure 4d after being submitted to deconjugation process. The value found for metabolite 2 was 292.7 $ng.ml^{-1}$ (referred to 0–10 hours urinary collected fraction)

antidepressants, β–blockers, anti–H_2) was also checked; diazepam, nordiazepam, nitrazepam, flunitrazepam, oxazepam, lorazepam, triazolam, amitriptyline, fluoxetine, propanolol, betaxolol and ranitidine were not detectable in the described conditions. Trazodone and zolpidem and its metabolites, which possess fluorescence properties, were detectable, however these were eluted at shorter retention times than metabolite 2 and well separated from it.

Linearity

A linear correlation between peak height ratio of metabolite 2 and the internal standard versus the concentration of metabolite 2 was found in the range 20–1000 $ng.ml^{-1}$ of human urine (as shown in Fig. 5).

Limit of quantitation

The limit of quantitation was 20 $ng.ml^{-1}$ of metabolite 2 in human urine (Fig. 4b). The coefficient of variation (C.V.) of the limit of quantitation was ± 7% (n=4).

Precision and accuracy

The described method was validated by two analysts working with the same chromatographic apparatus on different days. Each analyst, after the daily calibration (performed in quadruplicate), analysed two quality control samples (low and medium concentration, performed in quintuplicate) respectively at about 300 and 60 $ng.ml^{-1}$ of metabolite 2 in human urine, over a two day period. The results of this experiment, reported in Table 2, showed good precision and accuracy. A relevant chromatogram of the experience is shown in Fig. 4c.

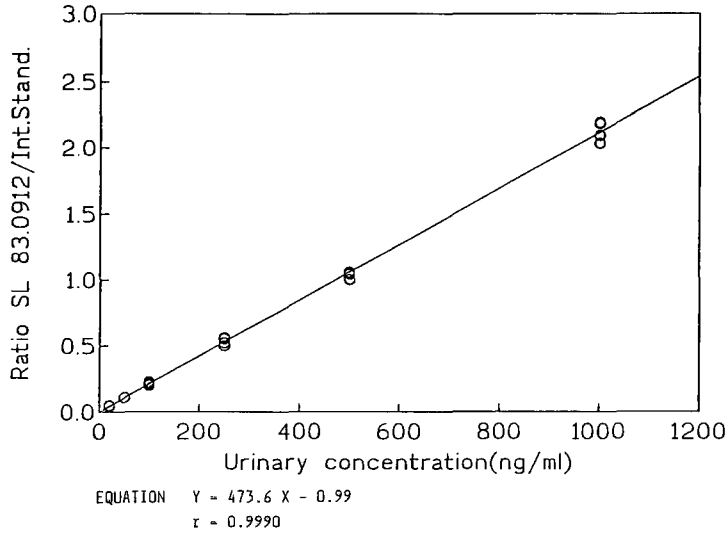

FIGURE 5: Linearity for alpidem metabolite 2(SL 83.0912) in human urine (n=24).

Application of the method to "real" urinary specimens

The method described was applied to the determination of metabolite 2 in urinary samples of volunteers administered orally with alpidem at 50 mg dose b.i.d. (therapeutic regimen); the urinary excretion values are reported in Table 3; Fig. 4d shows a chromatogram of the non–deconjugated urinary sample (fraction 0–10 hours after drug treatment) without the presence of metabolite 2, on the contrary Fig. 4e, referring to the same urinary sample submitted to deconjugation, shows the presence of metabolite 2 at concentration of 292.7 $ng.ml^{-1}$ of urine.

Conclusions

The metabolite 2 of alpidem, can be detected in human urine of subjects treated orally with alpidem only after deconjugation of the urinary samples, as a consequence, the analysis of urine samples

TABLE 2

Precision and accuracy of the method of determination of metabolite 2 (Alpidem) in human urine evaluated by analysing quality control specimens at 59.1 ng.ml^{-1} and 292.5 ng.ml^{-1} by two analysts over 4 day period.

	59.1 ng.ml^{-1}	292.5 ng.ml^{-1}
Precision C.V. intra (%) @	5.3	3.4
C.V. inter (%) #	0.0	4.3
C.V. total (%)	5.3	5.5
LS*	7.7	10.4
Accuracy **	93.2 ± 1.7	94.0 ± 4.3
n	20	20

@ C.V. intra = coefficient of variation within days
# C.V. inter = coefficient of variation between days
* 95% upper confidence limit for the relative standard deviation
** it is the recovery (expressed as per cent of the nominal concentration) with 95% as confidence limit

before and after deconjugation can be considered as an identification criterion of the intake of the anxiolytic drug. The chromatography of the deconjugated urine also showed the presence of other possible metabolites eluted in the early part of the chromatogram, not well separated from the solvent front and from other endogenous peaks. The sample preparation of the proposed method is extremely simple since it requires no extraction or clean–up steps, only incubation with

TABLE 3

Urinary excretion of metabolite 2 in two healthy volunteers administered with alpidem orally at 50 mg dose b.i.d.

Fraction	Subject B.M.		Subject V.A.	
	Collection time (hours)	Metabolite 2 (μg)	Collection time (hours)	Metabolite 2 (μg)
1	0–7	202.7	0–10	117.8
2	7–15	80.5	10–14	92.8
3	15–23	139.1	14–23	266.9
4	23–32	114.0	23–34	109.5
Cumulative excretion	0–32 (hours)	536.3 (μg)	0–34 (hours)	587.0 (μg)

the deconjugation enzyme, dilution of the sample with water and direct injection onto the chromatographic column. The selectivity of the method in regard to endogenous compounds and other eventually co–administered drugs (benzodiazepine anxiolytics, hypnotics, antidepressants, β–blockers, anti–H_2) is quite satisfactory; no interference was found. The method has to be tested on urine samples collected from subjects overdosed with alpidem, but the results on samples obtained under therapeutic treatment allow us to think that the method should be suitable in the case of overdosing. Moreover in such case the urinary levels of metabolite 2 should be higher than those found in normal dose. In conclusion the described method is proposed for the rapid identification and quantitation of the metabolite 2 of alpidem in toxicological and emergency laboratory in

case of a suspected overdose (voluntary or accidental) of alpidem; the method could also be useful for assessing drug compliance during clinical investigations (phase 3 and 4).

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PRACTICAL APPROACH FOR VALIDATING THE TLC ASSAY OF AN ACTIVE INGREDIENT IN A PHARMACEUTICAL FORMULATION

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ABSTRACT

A general approach is proposed for validating the TLC assay of an active ingredient in a dosage form. The experimental design together with the statistical tests used to evaluate the data takes into account the major aspects of the technique.

The present study is part of a work related to the experimental and statistical approach for validating an assay procedure of an active component in a dosage form. We had previously reported the possibility of using different statistical approaches on the basis of spectrophotometric data (1). This paper deals with the validation aspects of a TLC assay for the same active ingredient (diclofenac sodium) in the same tablet formulation. The proposed design and its statistical interpretation take into account the main features of the technique.

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PRELIMINARY EXPERIMENTS

There are no literature references concerning the TLC separation of diclofenac sodium (D) and its related decomposition products (Fig.1). The solvent mixture of dicloromethane-methanol in the ratio 92:8 (v/v) was found to be able to separate D from these impurities and to give an appropriate peak shape (Fig.2) and Rf value for quantitation.

Methanol was used as extraction solvent because it gave a satisfactory repeatability of loadings with the spotting device employed.

The on-plate maximum absorbance wavelength of D, 280 nm, was selected for the densitometric measurements.

EXPERIMENTAL

Apparatus, materials and chemicals.

A band applicator (Camag Linomat IV), HPTLC precoated silica gel plates 20x10 cm (Merck 60 F254) and a twin-trough chamber (Camag) were used for chromatographic development. Quantitative measurements were carried out with a chromatogram densitometer (Camag TLC Scanner II) coupled with an integrator (Merck-Hitachi D-2000).

All the chemicals were of analytical grade; D, decomposition products and commercial Voltarene L.P. tablets (100 mg of D for a tablet weight of 299 mg) were gifts from Ciba-Geigy laboratories (Basel, Switzerland).

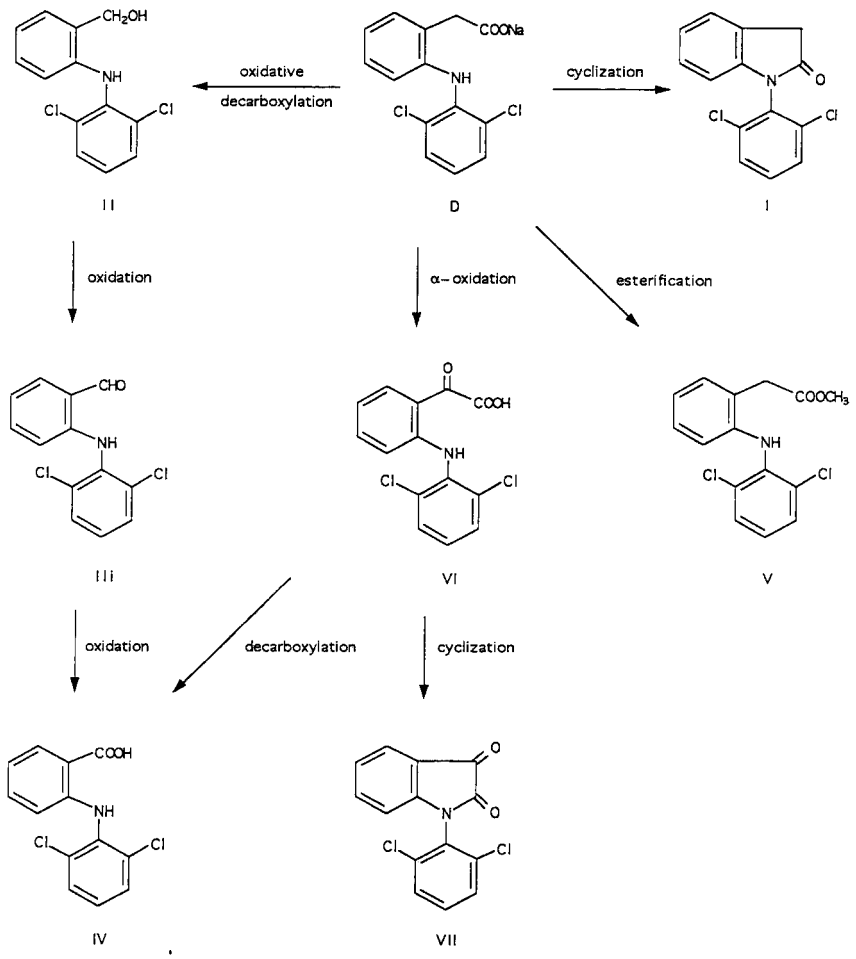

FIGURE 1. Decomposition products of diclofenac sodium.
D: diclofenac sodium
I: 1-(2,6-dichlorophenyl)oxindole
II: 2-[(2,6-dichlorophenyl)amino]benzyl alcohol
III: N-(2,6-dichlorophenyl)anthranililaldehyde
IV: N-(2,6-dichlorophenyl)anthranilic acid
V: 2-[(2,6-dichlorophenyl)amino]phenylacetic acid methyl ester
VI: {2-[(2,6-dichlorophenyl)amino]phenyl}glyoxylic acid
VII: 1-(2,6-dichlorophenyl)isatin

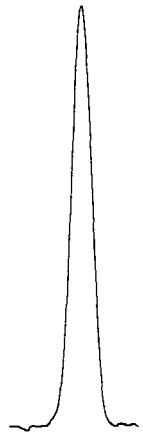

FIGURE 2. Chromatogram of a diclofenac sodiumstandard solution : 200 ng loading.

Test procedure.

-Solution for resolution test.

Prepare a stock solution (50 mgl^{-1}) of IV as follows: in a 100 ml volumetric flask, weigh accurately 5 mg of IV and add about 90 ml of methanol; dissolve by sonication and add methanol to the mark.

Prepare the solution for resolution test as follows: in a 50 ml volumetric flask, weigh accurately 50 mg of D and add 5 ml of the stock solution of IV and about 40 ml of methanol; dissolve by sonication and complete with methanol to the mark.

Prepare in triplicate the following solutions:

-Test solution.

In a 100 ml volumetric flask, place one tablet with about 90 ml of methanol, sonicate for 10 min. Allow to cool and add methanol to the mark. Centrifuge the suspension at 4000 rev/min for 30 min. Dilute 1/25 an aliquot of the supernatant with methanol to give a theoretical concentration of 40 mgl^{-1} of D.

-Standard solution.

In a 100 ml volumetric flask, weigh an accurate amount of 100 mg of D; add about 90 ml of methanol; dissolve by sonication for 5 min. Allow to cool and add methanol to the mark. Dilute 1/25 an aliquot of this solution with methanol to give a theoretical concentration of 40 mgl^{-1}.

-Sample application.

Pre-wash the plates by immersion for 1 h in methanol, dry for 30 min at 80°C. Allow to cool in a dessicator before use. Apply in triplicate 5 μl of standard and test solutions onto the plate using a bandwise applicator, at a delivery speed of 1 μl/10 s and as bandlengths of 5 mm. The space between bands and the distance from the side edge are determined by the number of samples to be analyzed.

-Chromatography.

Pre-equilibrate the plate in a twin-trough chamber (20x20 cm) with the vapors of the mobile phase dichloromethane-methanol (50 ml) in the proportion 92:8 (v/v) for 1 h. After this time, start the development and allow the mobile phase to migrate to a distance of about 60 mm which corresponds to a migration time of about 10 min. The spots can be visualized under UV light at 254 nm.

-Densitometry.

Perform quantitative measurements at 280 nm in the reflectance mode using a scanning densitometer with the parameters set as follows : monochromator bandwidth 10 nm, slit dimension 0.4x3 mm, scanning speed 0.3 mm s^{-1}. By using peak area measurements, calculate the percentage of D per tablet.

The results can be considered as valid if the performances of the chromatographic system meet the following

requirements : the Rf values for D should be about 0.27. The resolution between D and compound IV should not be lower than 0.70. The repeatability between three loadings should not be higher than 3%.

Test procedure validation.

-On-plate stability and solution stability.

A standard solution (40 mgl^{-1}) of D was applied on a plate in triplicate. After 1 h, the same solution was applied in triplicate on the same plate and the plate was immediately developed . The responses were compared.

The solution stability was assessed by loading a volume of 5 μl of a 24 h aged (at ambient temperature) and a freshly prepared standard solutions on the same plate. The response factors were compared.

-Specificity.

The interference of the related compounds and excipients of the formulation was assessed by applying 5 μl of a methanolic solution (40 mgl^{-1}) of each compound and of solutions resulting from the treatment of an analytical placebo and a placebo stressed in thermal conditions (60°C, for 7 h).

-Linearity and accuracy.

The linearity of the response of standard solutions was assessed by applying volumes of 3, 4, 5, 6 and 7 μl of the standard solution of D in triplicate on a same plate. These volumes correspond to a span of 60-140% of the theoretical content of D.

The linearity and accuracy of the test procedure were assessed by spiking five analytical placebos with D in solid form (60%, 80%, 100%, 120%, and 140% of the theoretical content). A single application of 5 μl of each test solution

was performed on each of three plates. The calibration line used to calculate the recoveries was obtained from two standard solutions (40 mgl^{-1}), each applied in volumes of 3, 4, 5, 6 and 7 μl on each plate. The average area of each pair was used to establish the regression equation.

-Repeatability.

The repeatability of the chromatographic system was assessed by applying onto the same plate six replicates (5 μl) of the standard solution of D. The RSD of peak area was calculated.

The repeatability of the test procedure was assessed by applying six times the procedure on real tablets and placebos spiked with 100% of the theoretical content. The amount of D was determined by reference to two standard solutions (40 mgl^{-1}), each applied in volumes of 3, 4, 5, 6 and 7 μl on the same plate.

RESULTS AND DISCUSSION

-On-plate stability and solution stability.

These tests should be carried out at the beginning of the validation process since they condition the validity of the other tests.

The on-plate stability should be assessed since on-plate degradation has been reported for some compounds (see e.g. ref.2). No significant difference ($p = 0.05$) was found between the responses obtained at one hour interval after the loading and no artefacts were formed within the limit of detectability of the method. D is stable on the plate for at

least one hour which is largely sufficient to cover the time necessary for band application. In addition, no significant difference ($p = 0.05$) was found in the densitometric measurements carried out at 90 min interval which allowed to cover a possible instrumental delay.

The comparison on a same plate between the response factors of solutions 24 hour aged at ambient temperature and freshly prepared were found unchanged ($p = 0.05$). This allows the solutions to be used within this delay without the results being affected.

-Specificity.

The solvent system dichloromethane-methanol 92:8 (v/v) allows the separation of D from compounds I-VII with respective Rf values of 0.27 and 0.38 for D and IV; compound VI does not migrate and other compounds are eluted near the solvent front.

-Linearity and accuracy.

* The linearity of the calibration curve (peak area *vs* applied amount) was first plotted on a graphic paper. A straight line not going through the origin was obtained (Fig.3). The regression line, calculated from the least-squares method was :

Peak area = (827.66 ± 38.35) applied amount (ng) + (58640 ± 8330) with the confidence intervals calculated at $p = 0.05$.

The coefficient of correlation was 0.997 and the coefficient of determination was 0.994. The variance analysis confirmed the linearity of the regression with a F_{cal} of regression = 2703 ($p << 0.01$) and F_{cal} of non-linearity = 2.06 ($p > 0.05$). The t-test showed that the regression line did not go through the origin ($t_{cal} = 15.21$), which should make

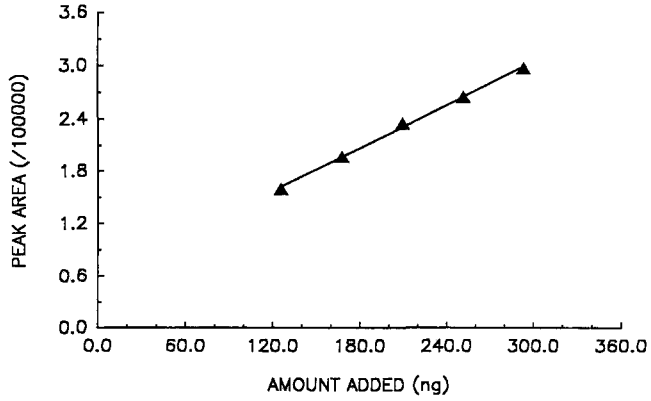

FIGURE 3. Calibration line for diclofenac sodium.

necessary to use multiple level calibration each time the procedure is carried out. However, since the test solution is at a target level, a single level calibration is possible; a dedicated software (3) may be of interest for the routine analysis in dosage forms.

*The linearity and accuracy of the procedure were investigated by repeating the chromatographic process on three plates (A, B and C) using the same standard and test solutions. This experimental approach takes into account plate-to-plate differences as well as other possible variations from one run to another (spotting, saturation, etc.). It gives an idea of the reproducibility (random errors) of the whole chromatographic system, in addition to accuracy (systematic errors).

The data were analyzed by using the three y-values (A, B and C) for each of the 5 x-concentration levels (TABLE 1) to construct the graph, after having assessed by one-way ANOVA on recoveries that there was no significant influence due to the plate.

The linear regression equation obtained was:

Amount found = (1.00 ± 0.04) amount added - (1.48 ± 7.51) at p = 0.05. The correlation coefficient was 0.998. ANOVA confirmed the linearity of the graph with F_{cal} of regression = 3092 ($p << 0.01$) and F_{cal} of non-linearity = 0.26 ($p >> 0.05$).

The resulting graph (amount found y - amount added x) should have a slope of 1 and go through the origin if the procedure is accurate and linear.

The t-test showed that the slope and intercept of the graph were not significantly different from 1 and 0 respectively ($p >> 0.05$). Therefore, the procedure proposed can be considered as linear and accurate in the range investigated. The general bias of the procedure given by the intercept corresponds to 0.73% of the value of y when x is at the 100% level. A bias of ± 2% can be accepted for the determination of an active ingredient in a pharmaceutical form (4). The experimental bias on each point (TABLE 1) is lower than 4% which is an acceptable value (5).

From the three experiments (A, B and C) the overall repeatability of recovery could be calculated (RSD = 1.87%, n = 15). The inter-plate reproducibility was found to be the same (RSD = 1.87%, n = 15).

- Repeatability.

The repeatability of the chromatographic system at the 100% level was 1.47% (6 replicate loadings on the same plate).

The repeatability of the test procedure was evaluated on real tablet samples and spiked placebos (100%) (n = 6 procedures). The diclofenac sodium content was calculated both by reference to the calibration line and the 100% calibration point (the results of which are given below in brackets).

TABLE 1. Linearity and accuracy of the test procedure.

Amount added (ng)	Amount found (ng)	Recovery (%)	Bias (%)
	123.9 (A)	101.06	1.06
122.6	118.0 (B)	96.25	-3.75
	118.9 (C)	96.98	-3.02
	165.2 (A)	99.76	-0.24
165.6	161.7 (B)	97.64	-2.36
	167.6 (C)	101.21	1.21
	204.3 (A)	98.79	-1.21
206.8	206.1 (B)	99.66	-0.34
	210.6 (C)	101.84	1.84
	243.0 (A)	98.94	-1.06
245.6	247.7 (B)	100.86	0.86
	242.8 (C)	98.86	-1.14
	272.5 (A)	96.70	-3.30
281.8	283.0 (B)	100.43	0.43
	283.9 (C)	100.75	0.75

For tablet samples, since the tablet weight is subject to small variations, each tablet was weighed before analysis and the percent of analyte recovered per tablet was also calculated with respect to the tablet theoretical weight. The diclofenac sodium content was found to be 99.05% (99.54%) for a tablet weight of 299 mg with a RSD of 2.93% (2.61%) which showed the repeatability of the procedure. The confidence interval on the assay result was 99.05% ± 3.05% (99.54% ± 2.73%). The amount of D per tablet was found to be 99.34% (99.83%) of the label claim with a RSD of 3.10% (2.77%). This result considers the variations of weight between tablets as well as the repeatability of the assay.

The recovery from spiked placebos was 98.89% (98.65%) with a RSD of 1.70% (1.55%). The confidence interval on the recovery was 98.89% ± 1.76% (98.65% ± 1.61%).

The results obtained by using either the calibration line or only a 100% calibration point do not give a significant

difference, which allows to use a single level calibration (at the targeted concentration) in routine, since the analyte is present in known amount.

CONCLUSION

It is often stated that the repeatability of TLC makes it more suited to impurity tests which are less demanding. However the results obtained in this study show that with optimized chromatographic conditions and an automated spotting device, the repeatability of the chromatographic system (RSD = 1.5%, n = 6) complies with the requirements for an active component assay in a formulation. It should be born in mind that a RSD of 2% (n = 5 or 6 injections) is generally admitted in the System Suitability Tests for HPLC (6).

The experimental design carried out to assess linearity, accuracy, reproducibility between plates and developments, as well as the statistical tests used to evaluate the data, shows that TLC is a reliable method for the determination of diclofenac in tablets. It can be used as an alternative method to HPLC for the assay of diclofenac sodium (7,8), especially for the analysis of a small number of samples.

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HIGH PERFORMANCE ION CHROMATOGRAPHY DETERMINATION OF TOTAL SULFITES IN FOODSTUFFS

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ABSTRACT

An alternative chromatographic method based upon a combination of a modified Monnier-Williams procedure and an Ion Chromatography separation and quantitation of sulfite, have been developed. The collected gaseous SO_2 is oxidized to sulfate with hydrogen peroxide. IC determination was carried out with an anion-exchange polymetacrylate column, borate/gluconate as eluent and conductivity detection. The combined method shows a good detection limit as well as a high chromatographic resolution. It is also applicable to the analysis of several foodstuffs.

INTRODUCTION

Sulfites in various forms have been added to foods for centuries. Only recently has the widespread use of sulfites in foods become an issue of health concern. Certain individuals have been found to exhibit adverse reactions (mainly asthmia) to sulfite residues in foods (1,2).

The AOAC Monnier-Williams method (3) for sulfite analysis has been the method of choice for many years, although its application has several drawbacks that have been recognised and cited in the literature (4,5).

A number of alternative methods has been developed. Ion chromatographic techniques have been reported for sulfite analysis involving, in some cases, a process of previous distillation (6,7,8) whereas in others distillation is replaced for extraction (9,10,11,12). Those techniques permit a rapid, precise and reliable sulfites determination.

In view of the fact that amongst the existing difficulties in analytical sulfite ion measurement are volatility and inestability to air oxidation, which could be overcome by the indirect detection of stable sulfate of the distilled gaseous SO_2, IC techniques can monitor the sulfite levels free of interferences from other volatile anions often present in foods as well as from the reagents employed during the extraction and oxidation of sulfite (13).

Once the above-mentioned chromatographic procedures were evaluated, we chose as working method an anion-exchange with conductivity detection and borate/gluconate as eluent to determinate sulfites by the indirect detection of sulfates.

MATERIAL AND METHOD

Reagents

All the reagents used were of analytic grade; organic solvents of high purity grade for HPLC; water was Milli-Q (Millipore Corp. Bedford M.A. 01730, U.S.A.) deionized. Standard reagents of sodium sulfite and sulfate were purchased from Merck (D-6100 Darmstadt, Germany).

Equipment

Ion-Chromatographic system (Millipore-Waters, Milford M.A, U.S.A.) ILC-1 composed of a manual injector with a 100 ul loop,

conductivity detector (430), Programmable Solvent Delivery module (590); Data Module Integrator (745) and anionic column IC-PAK™.

Distilling Unit: Kjeltec System 1026 (Tecator AB, S-26301 Höganäs, Sweden).

Samples

Analyzed samples were purchased from food stores after a sampling carrier out by The Food Health Department in different Regional Communities.

Method

EXTRACTION: Samples were extracted by acid distillation according to a modification of the original Monnier-Williams method developed in our laboratory (14).

The sample was first minced in a homogenizer (a domestic blender). Approximately 20 g of the homogenate was distilled for 10 minutes in 70 ml water containing 3 ml HCl 37%. The liberated sulfite was trapped in a solution of 0.5 ml 1N sodium hydroxide plus 4.5 ml water, and oxidized to sulfate with of about 0.3% hydrogen peroxide.

Purification was carried out by filtering 2-3 ml of extract through an 0.45 μm membrane filter (MIllex HV, Millipore). This solution was applied to a Sep-Pak C_{18} cartridge (Millipore-Waters) which was pretreated with 5 ml of methanol and 5 ml of water. Eluates aliquots of 100 μl were injected into the chromatograph.

IC DETERMINATION: High Performance Ion Chromatography was carried out under the following conditions: conductivity detection; eluent sodium borate/gluconate pH 8.5 (300 μS Conductivity); flow rate, 1 ml/min.

Linearity and sensitivity of the detector were calculated from a series of standard solutions from 1 to 100 μg/ml, prepared from sodium sulfite oxidized to sulfate with 0.3% hydrogen peroxide.

RESULTS AND DISCUSSION

As the determination of sulfite is quite difficult due to rapid oxidation of sulfite to sulfate by dissolved oxygen, and as IC with conductivity detection gives one of the most accurate and precise values for sulfate, we decided to choose this technique as working method.

The first step was to check out that oxidation of sulfite to sulfate with hydrogen peroxide was quantitative. For this purpose standard solutions with an equal concentration of sodium sulfate and oxidized sodium sulfite (with 0.3% hydrogen peroxide) were prepared. It was observed chromatographic peaks of both anions and their RT coincided, and, concentration being equal, the areas were similar.

Figure 1 shows the chromatogram for sodium sulfate and sodium sulfite oxidized under the chromatographic conditions previously described in the method. It can be seen a peak that corresponds to the excess of hydrogen peroxide used in the oxidation. To minimize it, was necessary to find the optimum hydrogen peroxide concentration that would produce complete sulfite oxidation and would not interfere with the sulfite peak. This concentration was established at 1 ml of 0.3% hydrogen peroxide each 10 ppm of sulfite to be oxidized.

Then it was considered necessary to change the distillation conditions established in our laboratory in order to determine sulfite by Iodimetric tritation, since there were chromatographic interferences with the chlorides. Therefore, different quantities and concentrations of HCl were tested to liberate standard sulfite, and they were collected in different alcaline solutions. Table 1 shows the recoveries obtained as the HCl and NaOH quantity or concentration was modified, as well as the influence from the oxidation time.

As Table 1 shows, the best recoveries were obtained when 3 ml of HCl 37% were used, and the destillate was collected in 0.5 ml of NaOH 1N. A minimum of between 2 and 3 hours being necessary to obtain a good oxidation.

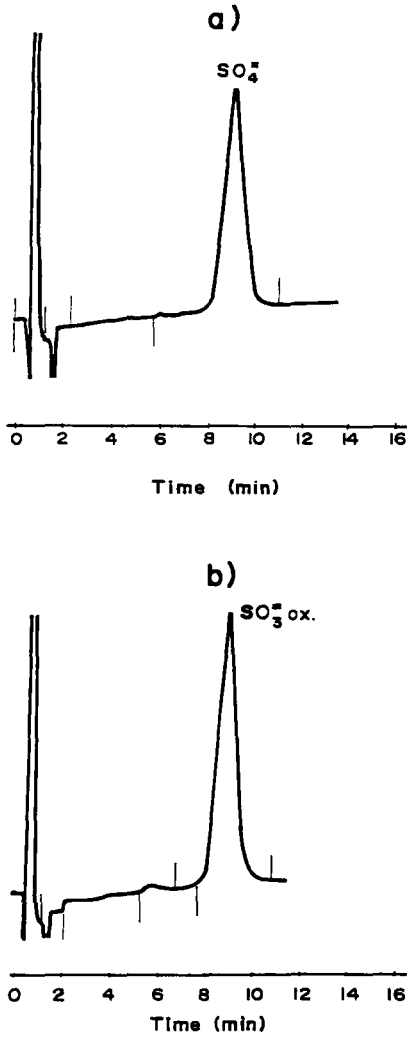

FIGURE 1.- Chromatograms of (a) sodium sulfate and (b) sodium sulfite oxidized (10 μg/ml respectively). Conditions: Waters IC-Pak anion column with Sodium Borate/Gluconate pH 8.5 eluent; conductivity detection; flow-rate: 1.2 ml/min; injection volume: 100 μl.

TABLE 1

Percentage Recovery of Sulfite (a) from standards after acid distillation.

Sulphite (ppm)	HCl (ml)	OHNa 1N (ml)	Oxidation time(hours)	Recovery (%)
20	7(HCl 37%)	5	1/2-1	*
20	1(HCl 37%)	5	1/2-1	<10
20	3(HCl 37%)	5	1/2-1	58
20	3(HCl 37%)	0.5	1/2-1	67
20	3(HCl 37%)	0.5	2-3	91
20	3(HCl 37%)	0.5	12	92

* chloride peak masked the sulfate peak
(a) average of four determinations

A linear regression analysis of the relationship between peak area versus amounts of standards was carried out within the range 0.5-100 μg/ml. The regression line obtained was y= 0.72x+0.08, with a correlation coefficient of 0.999.

The detection limit was 0.5 μg/ml under the chromatographic conditions described, although it could be lower when operating at higher sensitivity.

The mean recoveries of standards in the range 1-100 μg/ml was 90.4% (σ=17,04; n=14).

Recovery studies were performed on canned vegetable samples by adding known quantities of sodium sulfite to the sample solution prior to the distillation step. The results are given in Table 2, which indicates that satisfactory recoveries were achieved for the samples tested.

Some typical chromatograms obtained with samples of sausages (a), shrimp (b) and asparragus (c), are presented in Figure 2.

To establish whether the method is applicable to determine sulfite levels in foods, a number of samples representing a variety of foodstuffs were analyzed and compared with the modified Monier-Williams distillation technique developed in our laboratory. Table 3 shows data generated using both, the Ion-chromatographic and Monnier-Williams procedures.

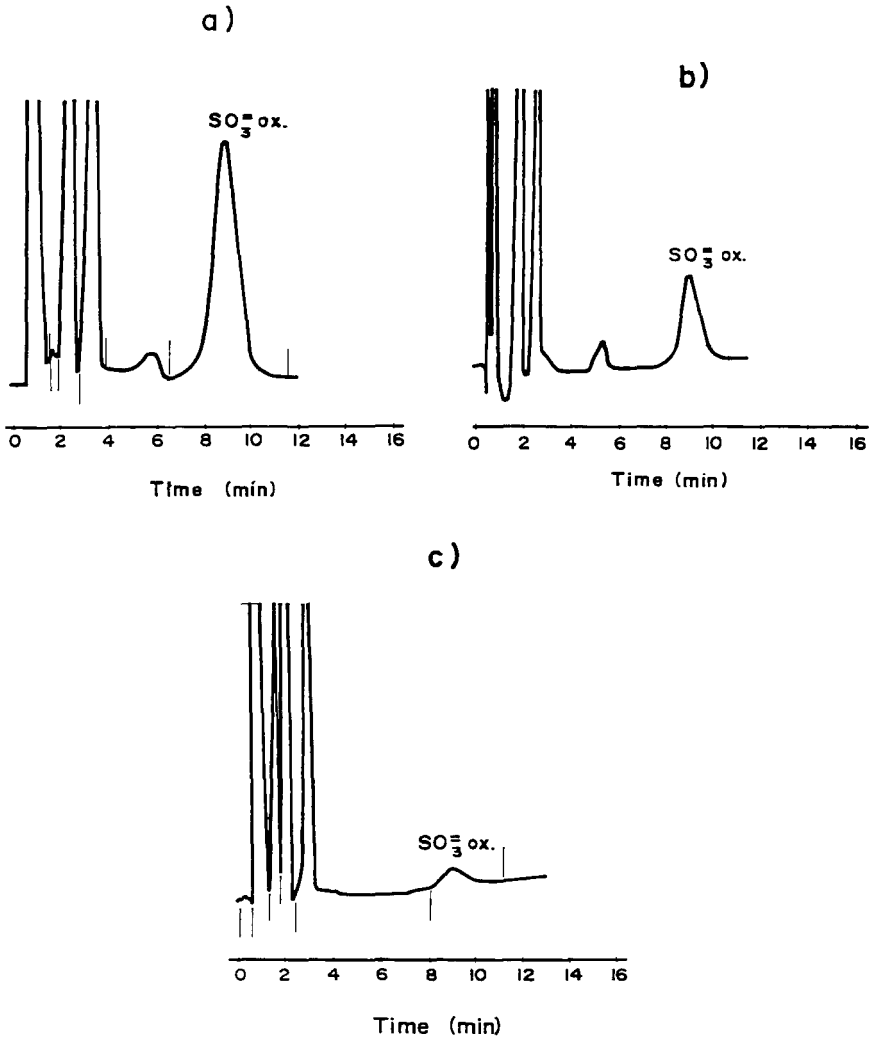

FIGURE 2.- Chromatograms obtained with: a) sausages (136 μg/g); b) shrimp (70 μg/g); c) asparragus (7 μg/g), using the proposed chromatographic method.

TABLE 2

Percentage recovery of sulfite from canned vegetable foods after acid distillation and oxidation to sulfate by IC analysis.

FOOD	Sulfite present in food (μg/g)	Sulfite added as sod. sul. (μg/g)	Sulfite found (μg/g)	Recovery (%)
Tomato	<1	50	38	76
id.	<1	100	83	83
Asparragus	16	50	49	74
id.	16	100	81	70
Artichoke	<1	20	14	70.5
id.	<1	50	42	84
id.	<1	100	87	87

Mean= 77.6%; n= 14; S.D= 6.65%

TABLE 3

Comparison of Ion Chromatography and Monier-Williams method for determining sulfite in foods.

	Sulfite, ppm (as SO_2) (a)	
FOOD	IC	M-W
Sausage 1	254	302
id. 2	313	383
id. 3	209	242
Juice	6	20
Canned mushroom	22	39
Shrimp	47	57
Canned artichokes	12	18
Tomato sauce	9	25

(a) Average of three determinations

The resulting concentrations of the method were comparable to those obtained by the modified Monnier-Williams method. The results, however, under the modified M-W method are considerably higher than those under the IC method. The fact that the acid-base tritation in the M-W method is nonspecific could be one reason for these results; any volatile acid may produce a positive interference depending on the nature of the samples.

To sum it up, our improved IC procedure combines the chemical approach of the Monnier-Williams technique with the superior detection system available through Ion Chromatography. The method permits quantitation at low parts-per-million and is not subject to the interferences associated with the Monnier-Williams procedure.

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RAPID DETERMINATION OF NITROFURANTOIN, FURAZOLIDONE AND FURALTADONE IN FORMULATIONS, FEED AND MILK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A new and rapid method is described to determine nitrofurantoin, furazolidone and furaltadone in formulations, feed and milk by reversed phase high performance liquid chromatography. A high-speed C_{18} 3x3 column is used and a mobile phase of HAc/NaAc 0.1 M (pH 3.2):acetonitrile, 90:10, was found to be the optimum to carry out the analytical separation. A photometric detection at 360 nm was selected.

INTRODUCTION

Nitrofuran derivatives are highly effective chemotherapeutic drugs well known as antibacterial agents from Dodd and Stillman studies (1). Some

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of them are widely used to fight common infections to humans and animals or characteristic infections of domestic animals or poultry. Hence, among these compounds, nitrofurantoin (1-[[5-nitro-2-furanyl]methylene]amino-2,4-imidazolidinedione), furazolidone (3-[5-nitrofurfurylideneamino]-2-oxazolidinone) and furaltadone (5-morpholinomethyl-3-[5-nitrofurfurylideneamino]-2-oxazolidinone) are formulated together in our country (Tribactina Premix from Esteve Lab.) (2). The formulation is of great utility in the control of the bacterian origin infectious processes most frequent in the intensive stock exploitation, and is administered together with feeds.

In the bibliography, there are numerous references about the analytical determination of these compounds by high performance liquid chromatography. However, nitrofurantoin and furazolidone are the subject of a higher number of studies than furaltadone. About the determination of this compound by HPLC only four references have been found, in which its determination, together with nitrofurantoin, furazolidone and other nitrofurans, is described. In two of these, columns of ciano have been used (3, 4) and reversed phase chromatography has been used in the others (5, 6). The methods are applied to the determination of these compounds in milk, meat, eggs or biological fluids (urine or plasma).

In this paper, we describe a simple and rapid method to determine any of these compounds or their mixture in its formulations, feed and milk by using high speed liquid chromatography. For this purpose, a C_{18} 3x3 column, with aqueous acetonitrile as mobile phase, has been used, and the chromatographic separation is attained in less than four minutes.

MATERIALS

HPLC system

A Perkin Elmer Series. 4 high performance liquid chromatograph, equipped with a constant flow pump, together with a Diode-Array detector (LC-235) was used. The detector was interfaced with an Olivetti PCS-286

personal computer, and an Epson LQ-570 printer was used for the graphical representation of chromatographic data. The analogue signal was taken from the output of the detector and the interface uses a 12-bits AD/DA card from Sapphire (Diamond System Corp. V.1.1), for the acquisition and digitization of the chromatographic data (7).

A program was written in BASIC that allows the representation of the chromatogram on the VDU screen, storage on diskettes and post-run manipulation, evaluation and graphical representation of the data. A convert program has been also developed with the object of transferring the files obtained with the acquisition data program to an ASCII XY format which allows the manipulation of these files with the "Data Leader" Software package from Beckman.

The chromatographic separation was performed on a Perkin-Elmer Pecosphere 3x3 CR C_{18} column (0.46 cm ID x 3.3 cm). The mobile phase was HAc/NaAc 0.1 M (pH 3.2):acetonitrile 90:10. The solvents were degassed and filtered, through a 0.45 μm cellulose acetate or PTFE membrane filter, daily before use. Samples were filtered through a Millipore swinney syringe adapter, containing a 0.45 μm cellulose regenerated membrane filter, before injection. Samples of 10 μl were injected through a Rheodyne 7125 injector with the solvent flow-rate maintained at 2 $ml \cdot min^{-1}$. Photometric detection was performed at 360 nm.

Chemicals and supplies

Nitrofurantoin, furazolidone and furaltadone obtained from Sigma Chemicals Co. were used. Standard solutions of these compounds were prepared by dissolving the appropriate amounts, in acetonitrile or dimethylformamide (DMF). Acetate buffer (0.1 M, pH 3.2) was prepared from anhydrous sodium acetate (Panreac) and glacial acetic acid (Normalsolv) in Millipore Milli-Q purified water (HPLC-grade water). All other chemicals were of analytical-reagent grade or better.

METHODS

Procedure for the determination of nitrofurantoin, furazolidone and furaltadone in formulations

A suitable aliquot of the formulation (powdered, solution or emulsion) is accurately weighed or pipetted, dissolved in DMF (filtering and washing with DMF when necessary) and diluted to a known volume. Suitable aliquots of the obtained solutions are used to prepare appropriate samples in acetonitrile, and portions for analysis are filtered before injection (10 μl) into the HPLC system.

Procedure for the determination of nitrofurantoin, furazolidone and furaltadone in feeds.

Amounts of about 10 g of finely ground feeds are accurately weighed and carefully stirred with 40 ml of DMF, during 30 minutes. The extracts are centrifuged and filtered and the residues are washed with DMF diluting to a final volume of 50 ml. Suitable aliquots of these solutions are used to proceed with the analysis.

Procedure for the determination of nitrofurantoin, furazolidone and furaltadone in milk

Aliquots of 200 ml of milk are pipetted, liophylized and washed with acetonitrile (about 75 ml) during 15 minutes. The residue is extracted with 15 ml of DMF stirring during 30 minutes, and subsequently washed with a mixture of 25 ml of acetonitrile and 5 ml of DMF. The different solutions are joined and evaporated to dryness in a rotavapor under diminished pressure. The residue is treated with DMF, filtered and diluted into a 25

ml volumetric flask with DMF. Portions for analysis are filtered before injection (10 μl) into the HPLC system.

RESULTS AND DISCUSSION

The analytical separation of nitrofurantoin, furazolidone and furaltadone in a 3x3 reversed phase C_{18} column has been investigated. Aqueous methanol or aqueous acetonitrile have been initially tested as mobile phases to perform the separation. In the Table 1 the retention times, capacity factors (k') and resolution values (R_S) for these compounds, obtained with different proportions of organic component in the mobile phase, have been summarized. It can be observed that nitrofurantoin and furazolidone are easily eluted by aqueous methanol or aqueous acetonitrile with just a 10% of organic modifier. Furaltadone, however, is strongly retained and it is necessary to use mobile phases with at less 30% of methanol or 70% of acetonitrile to obtain capacity factors lower than 10 for this compound. To improve these results, acetonitrile:water or methanol:water mobiles phases, in which the pH of the aqueous portion was modified by using acetate buffer solutions of different pH values, were assayed. The obtained results have been summarized in the Table 2. It can be appreciated that the behavior of nitrofurantoin and furazolidone do not significantly changes in the different mobile phases tested, whereas the furaltadone retention do it drastically. In the Table 3 the capacity factor values obtained for furaltadone with the various mobile phases are compared.

As can be observed, the furaltadone retention diminishes drastically when the mobile phase contains buffer solution and is generally lower when acetonitrile is used as organic modifier.

It is also interesting to denote that the retention-elution behavior of furaltadone is also strongly influenced by the ionic strength of the mobile phase, as has been proved by using different 0.05 M HAc-$NaClO_4$:acetonitrile, 90:10, mobile phases, in which the $NaClO_4$ concentration is changed, to elute the furaltadone. This fact could

TABLE 1.

Influence of the Organic Modifier Proportion on the Retention of Nitrofurantoin (NF), Furazolidone (FZ) and Furaltadone (FD)

a) Aqueous acetonitrile mobile phase

% AcCN	NF		FZ		FD		$R_{s_{1,2}}$
	t_R	k'	t_R	k'	t_R	k'	
5	3.67	14.29	4.09	16.04	---	---	0.840
10	1.49	5.21	1.74	6.25	---	---	0.930
20	0.58	1.42	0.74	2.08	19.20	79.00	0.533
30	0.36	0.50	0.43	0.79	9.39	38.13	
40					6.39	25.63	
50					4.82	19.08	
60					4.84	19.17	
70					4.14	16.25	

b) Aqueous methanol mobile phase

% MeOH	NF		FZ		FD		$R_{s_{1,2}}$
	t_R	k'	t_R	k'	t_R	k'	
10	3.05		3.14		---	---	0.180
15					13.12	61.48	
20					6.60	30.43	
25					3.71	16.67	
30					2.03	8.67	
35					1.47	6.00	
40					1.06	4.05	
50					0.51	1.43	

TABLE 2 a.

Influence of % Acetonitrile on the Mobile Phases HAc/NaAc 0.1 M:Acetonitrile, on the Retention of Nitrofurantoin (NF), Furazolidone (FZ) and Furaltadone (FD).

Buffer solution pH = 2.8

% AcCN	NF		FZ		FD		$R_{s_{1,2}}$
	t_R	k'	t_R	k'	t_R	k'	
5	4.40	17.3	4.74	18.75	7.40	29.83	----
10	1.55	5.46	1.90	6.92	4.25	16.71	0.88
15	0.81	2.38	1.04	3.33	2.72	10.33	0.74
20	0.55	1.29	0.70	1.92	2.29	8.54	0.56
25	0.40	0.67	0.50	1.08	2.08	7.67	0.36

Buffer solution pH = 3.2

% AcCN	NF		FZ		FD		$R_{s_{1,2}}$
	t_R	k'	t_R	k'	t_R	k'	
5	3.58	13.9	4.12	16.17	4.26	16.75	0.80
10	1.36	4.67	1.77	6.38	2.40	9.00	0.87
15	0.76	2.17	0.98	3.08	1.55	5.46	0.60
20	0.52	1.17	0.66	1.75	1.20	4.00	0.55

Buffer solution pH = 3.6

% AcCN	NF		FZ		FD	
	t_R	k'	t_R	k'	t_R	k'
5	3.89	15.2	4.44	17.5	3.51	13.6
10	1.51	5.29	1.88	6.83	1.55	5.46
15	0.81	2.38	1.04	3.33	1.02	3.25
20	0.56	1.33	0.70	1.92	0.74	2.08

Buffer solution pH = 4.1

% AcCN	NF		FZ		FD	
	t_R	k'	t_R	k'	t_R	k'
5	3.77	14.7	4.00	15.7	4.25	16.7
10	1.43	4.96	1.72	6.17	1.50	5.25
15	0.77	2.21	0.99	3.13	0.86	2.58
20	0.54	1.25	0.69	1.88	0.60	1.50

TABLE 2 b.

Influence of % Methanol on the Mobile Phases HAc/NaAc 0.1 M: Methanol on the Retention of Nitrofurantoin (NF), Furazolidone (FZ) and Furaltadone (FD)

Buffer solution pH = 2.84

% MeOH	NF		FZ		FD		$R_{S_{1,2}}$
	t_R	k'	t_R	k'	t_R	k'	
5	7.11	32.9	7.45	34.5	7.80	36.1	0.39
10	2.89	12.8	3.06	13.6	3.52	15.8	0.41
15	1.37	5.52	1.52	6.24	2.18	9.38	0.54
20	0.80	2.81	0.89	3.24	1.60	6.62	0.45

Buffer solution pH = 3.19

% MeOH	NF		FZ		FD	
	t_R	k'	t_R	k'	t_R	k'
5	6.99	32.3	8.17	37.9	7.22	33.4
10	2.84	12.5	3.16	14.1	2.92	12.9
15	1.29	5.14	1.51	6.19	1.53	6.29
20	0.76	2.62	0.87	3.14	1.07	4.10

Buffer solution pH = 3.60

% MeOH	NF		FZ		FD		
	t_R	k'	t_R	k'	t_R	k'	
5	8.24	38	.2	8.82	41.0	7.83	36.3
10	3.07	13.6	3.36	15.0	3.01	13.3	·
15	1.40	5.67	1.60	6.62	1.49	6.10	
20	0.82	2.90	0.94	3.48	0.97	3.62	

Buffer solution pH = 4.1

% MeOH	NF		FZ		FD	
	t_R	k'	t_R	k'	t_R	k'
5	8.07	37.4	8.80	40.9	13.12	61.48
10	3.02	13.4	3.29	14.7	4.26	19.29
5	1.42	5.70	1.62	6.71	1.90	8.05
20	0.83	2.95	0.95	3.52	1.09	4.19

TABLE 3.

Capacity Factor (k') of Furaltadone in Several Aqueous Methanol or aqueous Acetonitrile Mobile Phases

Aqueous portion composition	5% AcCN	5% MeOH	10% AcCN	10% MeOH	15% AcCN	15% MeOH	20% AcCN	20% MeOH
HAc/NaAc 0,1 M pH 2.84	29.8	36.1	16.7	15.8	10.3	9.38	8.54	6.62
HAc/NaAc 0,1 M pH 3,19	16.8	33.4	9.00	12.9	5.46	6.29	4.00	4.10
HAc/NaAc 0,1 M pH 3,60	13.6	36.3	5.46	13.3	3.25	6.10	2.08	3.62
HAc/NaAc 0,1 M pH 4,20	16.7	61.5	5.25	19.3	2.58	8.05	1.50	4.19
Water						61.50	79.0	30.43

indicate the participation of some kind of polar interaction between the stationary phase and the furaltadone.

According with these results it has been chosen, as optimum, a mobile phase of 0.1 M HAc/NaAc (pH 3.2):acetonitrile, 90:10, due to the acceptable values obtained for the capacity factors (k'_{NF} = 4,7; k'_{FZ} = 6,4 and k'_{FD} = 9,0) and the resolution next to the unity, for the nitrofurantoin and furazolidone peaks.

The mobile phase caudal, in the interval of values assayed (1.0-2.3 ml/min), do not significantly affect to peak width neither to analytical signals (peak area or peak height). Only an slight diminution on k' values is observed. A 2 ml/min caudal has been selected in order to reduce the analysis time.

With the mobile phase composition and caudal chosen, the resolution between peaks of nitrofurantoin and furazolidone has a value lower than the unity.

We have tried to improve these results by time-domain differentiation of the chromatograms. Derivative techniques have been increasing in popularity for improving the resolution of overlapping spectra in other analytical methodologies such as spectrophotometry and spectrofluorimetry, and, recently, the fundamental properties, limitations, and some applications of time-domain derivatives in liquid chromatography have been evaluated (7-10). Derivative peaks are characterized by reduced peak widths in comparison with the original, which implies a potential improvement in resolution. In figure 1 the conventional chromatogram and the first and second derivative of the two peaks of nitrofurantoin and furazolidone are shown. These have been obtained by means of a Basic program which allows storage on diskettes and post-run manipulation of the data. A CONVER program has been used to translate the initial format files to ASCII format files which can be manipulated by the "Data Leader" Software package from Beckman. The smoothing and differentiation of the chromatographic peaks with respect to time is done according to the Savitzky and Golay method (11, 12). Due to the low sensititivity obtained in the second derivative chromatograms, we have selected as analytical signals those obtained in the first maximum of the first derivative chromatogram for nitrofurantoin, $^1D_{máx}$, and in the last minimum for furazolidone, $^1D_{min}$. These signals correspond to the inflexion points of the ascending and descending lengths of nitrofurantoin and furazolidone peaks, respectively. At these points, the one to other contribution of these two peaks is lower than in the peak maxima. The optimization of smoothing and band widths used for the calculation of the derivative chromatograms has been made according with their influence on the signal to noise ratio. Smoothing through 13 experimental points and a band width of 13 experimental points have been selected.

Calibration graphs for the three compounds have been established, in the chromatographic conditions mentioned and for the different analytical signals (area or height of chromatographic peaks or $^1D_{máx}$ for nitrofurantoin and $^1D_{min}$ for furazolidone). The obtained results are summarized in Table 4.

Subsequently, we have prepared synthetic mixtures of nitrofurantoin/furazolidone (weight to weight ratio between 0.1 and 7.5) to

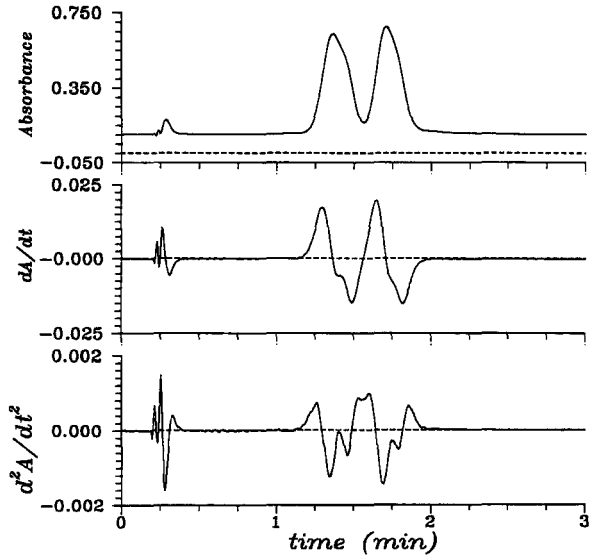

Figure 1. Conventiona chromatogram, and first and second derivative of it, corresponding to a mixture of a 500 ng of nitrofurantoin and 500 ng of furazolidone.

compare the obtained recoveries by using the selected analytical signal on the first derivative chromatogram with those obtained by integration of the conventional chromatogram with diverse criteria (baseline drawn from valley to valley; baseline drawn horizontally until a base point or baseline draw between base points).

We have found similar results by differentiation of the chromatograms or by integration of the conventional chromatograms with a baseline drawn between base points, and these results are better than those obtained with the other integration criteria. On the other hand, differentiation only slightly improve the recoveries obtained by integration of the furazolidone peaks in presence of a great excess of nitrofurantoin. Because of this, the integration to the baseline between base points have been selected. The precision of the assay was checked, by performing eleven replicate runs of the three substances. In Table 5, the results obtained by measuring the peak area or the peak height are compared. It can be appreciated that

Table 4.

Calibration Graphs for the Chromatographic Determination of Nitrofurantoin, Furazolidone and Furaltadone, as well as for Nitrofurantoin and Furazolidone Determination after Differentiation of the Chromatograms.

	Nitrofurantoin (20-900 ng)		Furazolidone (20-900 ng)		Furaltadone (20-250 ng)	
Analytical signal (y)	Straight line equation	r	Straight line equation	r	Straight line equation	r
Peak area	$y=13{,}7\times10^{3}\ X+6.30\times10^{5}$	0.9993	$y=14.2\times10^{3}\ X+5.87\times10^{5}$	0.9991	$y=10.7\times10^{3}\ X+8.05\times10^{5}$	0.9981
Peak height	$y=1.11\ X-3.70$	0.9994	$y=1.09\ X+2.99$	0.9997	$y=0.725\ X+4.44$	0.9992
$^{1}D_{max}$	$y=0.344\times10^{-4}\ X-7.70\times10^{-4}$	0.9982				
$^{1}D_{min}$			$y=0.281\times10^{-4}\ X-5.12\times10^{-6}$	0.9998		

X: analyte amounts (ng); r: linear regression coefficient

TABLE 5.

Precision of the Methods of Determination of Nitrofuratoin, Furazolidone and Furaltadone.

Analytical signal	Nitrofurantoin Area $x10^{-4}$	Height	Furazolidone Area $x10^{-4}$	Height	Furaltadone Area $x10^{-4}$	Height
$\bar{X}$	692.3	492.2	746.7	543.7	52.31	81.21
S	$2.94x10^{5}$	9.53	$2.81x10^{5}$	9.77	3.73	1.95
S_m	$8.85x10^{4}$	2.88	$8.87x10^{4}$	2.95	1.18	0.58
% error (95% confidence level)	2.85	1.31	2.69	1.21	5.10	1.61

better results are obtained when the peak height is the analytical signal. In this instance, the detection and determination limits for the three compounds, calculated on the basis of analyte response at low concentration (13) are, respectively: 4.7 ng and 15.8 ng for nitrofurantoin, 4.2 ng and 13.8 ng for furazolidone and 8.2 ng and 27.3 ng for furaltadone.

Applications

Under the optimum conditions already established, the proposed method has been applied to the determination of nitrofurantoin, furazolidone and furaltadone in several formulations, pig feed and milk, according to the above mentioned procedures. Due to the great solubility of these compounds in DMF (>50 g/l), considerably higher than in other solvents (14), this solvent has been used to extract nitrofurantoin, furazolidone and furaltadone from their formulations as well as from feed. The analysed formulations contain the three mentioned compounds (Tribactina Premix), two of them (Trionic) or one of the nitrofuran mixed with other kind of drugs.

The proposed procedure to analyse feeds has been applied to the determination of the three compounds in pig feed samples that have been

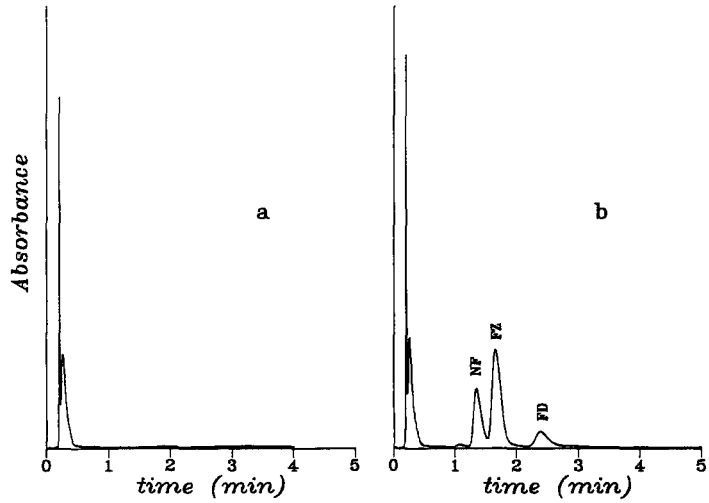

Figure 2. Chromatograms of feed samples contaminated with 0.1% Tribactine premix (b) and without Tribactine premix (a).

previously mixed with 0.1% of Tribactina Premix. This is the lowest level of dosage in which this formulation is used.

The obtained results are summarized in Tables 6 and 7 and in figure 2 the chromatograms corresponding to the analysis of a feed sample contaminated with Tribactina Premix and of a feed sample without these compounds are shown.

To execute the analysis of milk samples, previously contaminated with these compounds, different procedures have been assayed. At first, extraction of milk samples, deproteinized in acid medium, with ethylacetate has been tried. This solvent was chosen according to the solubility data (14) and the bibliographic references about the extraction of these compounds (15, 16). However, negative results were found. Because of this, the liophylization of milk samples previous to the solution of the analyte in a suitable solvent, as other alternative procedure to get preconcentration of analytes, has been assayed. As a previous experience, we have performed the analysis of powdered milk, contaminated with the nitrofurans, by using ethylacetate or acetonitrile as solvent to extract

TABLE 6.

Determination of Nitrofurantoin, Furazolidone and Furaltadone in Formulations.

a) Determination of nitrofurantoin

Formulation	Composition	Nitrofurantoin claimed (mg/g)	Nitrofurantoin found* (mg/g)
Tribactina Premix Polvo	(per 100 mg) Nitrofurantoin 60 g. Furazolidone 120 g. Furaltadone 60 g. (2,3-dihydroxibutane-dioate)	60	60.43±0.80
Furantoin comp.	(per tablet) Nitrofurantoin 50 mg.	158.8	165.09±0.24

b) Determination of furazolidone

Formulation	Composition	Furazolidone claimed (mg/g)	Furazolidone found* (mg/g)
Tribactina Premix polvo		120	124.00±0.89
Saleton	(per tablet) Furazolidone 50 mg Difenoxilate hydrochloride 2.5 mg Neomycin sulfate 3.5 mg Atropine sulfate 0.025 mg	272	270.84±0.39
Enteromicina	(per tablet) Furazolidone 250 mg Neomycin sulfate 250 mg Electrolytes Cl^- 474 mg Na^+ 346 mg K^+ 52 mg Ca^{2+} 18 mg Mg^{2+} 15 mg	84.6	84.46±0.44

(continued)

TABLE 6 *(continued)*

c) Determination of furaltadone

Formulation	Composition	Furaltadone claimed (mg/g or mg/ml)	Furaltadone found* (mg/g or mg/ml)
Tribactina Premix polvo		41	37.34±0.34
Trionic	(per gram) Chloroamphenicol 25 mg Neomycin base 40 mg Furazolidone 40 mg Furaltadone 40 mg Methylescopolamine bromine 0,2 mg Nicontinamide 20 mg	20	19.98±0.15
Panotile sol.	(per ml) Furaltadone hydrochloride 4.5 mg Polymixin B sulfate 10,000 U.I. Neomycin sulfate 3.5 mg Fluorocortisone acetate 1.0 mg Didocaine hydrochloride 40.0 mg	4.5	4.40±0.04
Altabactina	(per 200 ml) Furaltadone 4.16 g. Chloroamphenicol 4.16 g. Noemycine sulfate 10.0 g. Valeramide sulfate 41.50 g. Magnesium chloride 16.66 g.	19.6	18.40±0.74

* Each value is mean of three determinations.

TABLE 7.

Determination of Nitrofurantoin, Furazolidone and Furaltadone in Pig Feed.

Compound	Added (mg/g)	Found (mg/g)	% Recovery
Nitrofurantoin	0.0604	0.0579	95.9
		0.0591	97.8
		0.0589	97.5
	0.121	0.118	97.5
		0.121	100.0
		0.111	91.7
Furazolidone	0.124	0.119	96.0
		0.119	96.0
		0.122	98.4
	0.248	0.242	97.6
		0.245	98.8
		0.234	94.4
Furaltadone	0.0373	0.0303	81.2
		0.0326	87.4
		0.0319	85.5
	0.0746	0.0721	96.6
		0.0721	96.6
		0.0705	94.5

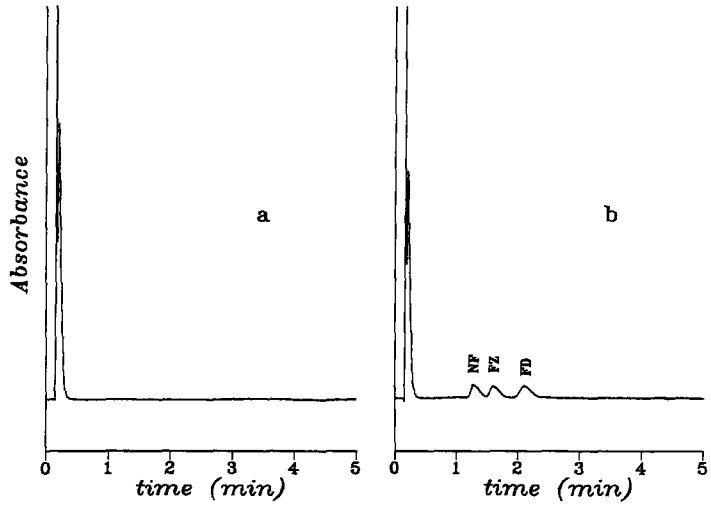

Figure 3. Chromatograms of milk samples contaminated with nitrofurantoin (0.25 ppm), furazolidone (0.25 ppm) and furaltadone (0.5 ppm) (b) and without addition of these compounds (a).

TABLE 8.

Determination of Nitrofurantoin, Furazolidone and Furaltadone in Milk

Compound	Added (μg/g)	Found* (μg/g)	% Recovery
Nitrofurantoin	0.25	0.198	79.2
		0.202	80.8
		0.211	84.4
Furazolidone	0.25	0.210	84.0
		0.214	85.6
		0.219	87.6
Furaltadone	0.50	0.405	81.0
		0.409	81.8
		0.413	82.6

these compound. Very good results were obtained with acetonitrile as extractant (recoveries greater than 95% for nitrofurantoin and furazolidone and greater than 90% for furaltadone). However, these results are not reproduced when natural milk samples, contaminated with these substances, are analysed. Because of this we have assayed a mixture of DMF-acetonitrile as the extractant according to the described procedure. The obtained results are shown in Table 8. In figure 3 the chromatograms of milk samples with and without the nitrofurans are shown.

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LIQUID CHROMATOGRAPHY CALENDAR

1994

JANUARY 10 - 14: Supercritical Fluid Chromatography and Extraction, Hyatt Regency Hotel on the Inner Harbor, Baltimore, Maryland. Contact: Larry T. Taylor, Dept of Chemistry, Virginia Polytechnic Institute & State University, Blacksburg, VA 24061, USA.

JANUARY 16 - 20: 19th IUPAC Symposium on the Chemistry of Natural Products, Karachi, Pakistan. Contact: Prof. Atta-Ur-Rahman, Director H. E. J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

FEBRUARY 2 - 3: AOAC Southeast Section Meeting, Ramada Hotel & Convention Center, Atlanta, GA. Contact: Doug Hite, Technical Services, P. O. Box 40627, Melrose Station, Nashville, TN 37204, USA.

FEBRUARY 28 - MARCH 4: PittCon'94: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd, Pittsburgh, PA 15235-9962, USA

MARCH 22 - 24: PrepTech '94, A New Conference on Industrial Bioseparations, Meadowlands Hilton Hotel, S3ecaucus, New Jersey. Contact: Symposium Manager, PrepTech '94, ISC, Inc., 30 Controls Drive, Shelton, CT 06484, USA.

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.

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 & Vetrinary 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.

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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.

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

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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.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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

1998

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

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

1999

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

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

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

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

2001

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

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

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

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