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PHOTOIONIZATION DETECTION IN PACKED-CAPILLARY LIQUID AND SUPERCRITICAL-FLUID CHROMATOGRAPHY

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ABSTRACT

A photoionisation detector (PID) was coupled to packed-capillary liquid and supercritical-fluid chromatography to study its performance. Several mobile phases were tested to evaluate the potential of liquid chromatography with photoionisation detection, LC-PID. The behaviour of the PID was not as good as in gas chromatography (GC), due to the absorption of photons by the mobile phase vapour. Therefore, the minimum detection limits (MDLs) were high compared to those in GC-PID, being at the low nanogram level for, e.g., ketones, aldehydes and amides. Coupling of the PID with supercritical-fluid chromatography (SFC) using modified carbon dioxide gave more satisfactory results. For aromatic compounds like phenanthrene and pyrene MDLs were found to

be 12 and 20 pg, respectively. These values were almost the same as found in GC-PID. Separation and detection of aliphatic C_4-C_{13} carboxylic acids and some organosulphur/phosphorus pesticides (disulfoton, ethion and sulfotepp) showed the possibility to detect several classes of compounds at the low nanogram level using methanol-modified carbon dioxide.

INTRODUCTION

The photoionisation detector (PID) is a commonly used detector in gas chromatography [1], but so far it is hardly applied in column liquid (LC) and supercritical-fluid (SFC) chromatography. However, since LC is the most versatile chromatographic separation technique for polar to medium polar compounds, combination of this technique with the PID is of interest. After the introduction of miniaturised LC, i.e. the introduction of columns with internal diameters of ≤ 1.0 mm, it became possible to use LC on-line with most gas chromatographic detectors. An excellent review on this topic is given by Kientz *et al.* [2]. The use of the PID in SFC is especially of interest when modified carbon dioxide is used as the mobile phase, since using a flame-ionisation detector (FID) then is impossible.

Reactions that can take place inside the ionisation chamber of a PID are schematically shown in Fig. 1. In Step 1 the photoionisable analyte (AB) absorbs UV light (wavelength is determined by the lamp choice). Ionisation (Step 4) of the excited analyte (AB*) occurs if the ionisation potential is equal to or smaller than the energy of the photons ($h\nu$) used. After ionisation AB^+ is directed by an electric field, present in the ionisation chamber, to the collector electrode (cathode) and the generated current, i, is measured (Step 9) [1]. There are however, several side reactions limiting the efficiency of these processes. The mobile phase (E) and the make-up gas (G) can also absorb UV light (Steps 2 and 3). The

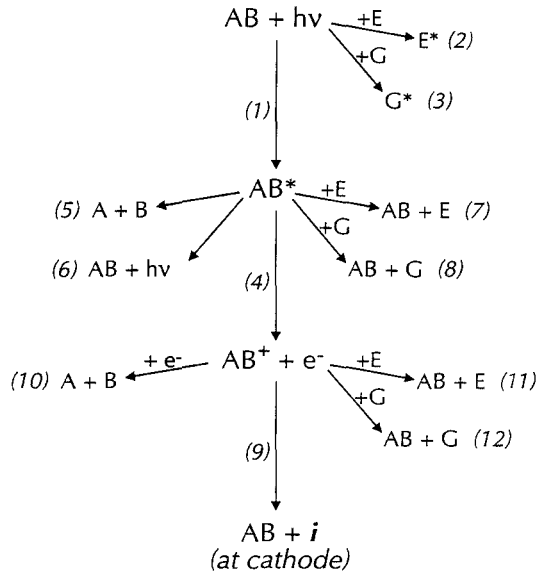

Figure 1. Reaction scheme for photoionisation detection. Abbreviations: AB, analyte; E, mobile phase and G, make-up gas.

excited AB* can, in addition to Step 4, dissociate to A and B (Step 5), emit fluorescence (Step 6), and be quenched by the mobile phase (Step 7) or by the make-up gas (Step 8) to AB. Furthermore, AB^+ can recombine with an electron (Step 10), with the mobile phase (Step 11), or wih the make-up gas (Step 12) to AB, instead of being directed to the collector electrode [5,8].

In the literature helium is frequently recommended as make-up gas because of its high ionisation potential (24.6 eV), which means that it can not be ionised under the conditions conventionally used. Moreover, it hardly absorbs at 10.2 eV (121 nm), the energy of the light source most frequently used, so Step 3 is almost negligible. The collisional quenching efficiency (Step 8) and the electron-capture efficiency (Step 12) are both

rather low and can be neglected also [8]. Another important aspect is the nature of the mobile phase. In the vapour state, normal-phase solvents like hexane and toluene can not be used with a 10.2 eV lamp because their ionisation potentials are 10.2 and 6.7 eV, respectively. Reversed-phase solvents like methanol, water and acetonitrile have, in the vapour state, ionisation potentials above 10.3 eV, but from literature it is known that they absorb rather strongly at 121 nm [9-11]. For carbon dioxide it is known that it hardly absorbs at 121 nm [12].

In principle, there are two possibilities to couple a chromatographic system with a PID. One is to use an interface to evapourate both the analytes and the mobile phase before their introduction into the detector. The other possibility is to allow direct introduction of the mobile phase containing the analytes into the detector, without evapouration (LC) or decompression (SFC) of the solvent.

Using the interface approach, Schmermund et al. [3] designed the first LC-PID system in 1975 and in 1984 Driscoll et al. [4] evaluated the applicability of LC-PID for several classes of analytes, reporting minimum detection limits (MDLs) of 3 - 700 ng. The best analyte detectability was found for halobenzenes. These studies were all performed using conventional-bore LC with flow splitting before detection. In 1987 De Wit and Jorgenson reported coupling of the PID with open-tubular LC columns (I.D. 5-10 μm). The MDL for toluene was 10 fmole and linearity was observed over three orders of magnitude [5].

The second approach was used by Locke et al. [7]. In this case only normal-phase LC can be used as the ionisation potential of water (liquid state) is very low (6.05 eV). Detection limits for polycyclic aromatic hydrocarbons (PAHs) varied between 4 and 250 pg, while the analyte detectability for substituted benzenes was 100-fold worse. Linearity was found over more than six decades. No response was found for phenols and chlorinated compounds.

The first report of using a PID for both packed-column and open-tubular SFC discussed three detectors designed for use with pressurized

mobile phases was rather disappointing [13]. With carbon dioxide the sensitivity for benzene was 10^6 times lower than when using an FID. Both argon and carbon dioxide were used as mobile phase; the detection limits found were 1000-fold lower when using argon instead of carbon dioxide. The dramatic difference was explained by different degrees of light absorption and different quenching effects.

The use of the PID under atmospheric pressure conditions in SFC seems promising. Sim et al. compared PID with FID detection for packed-column SFC and found that pressure programming had less effect on PID than on FID as the flame was quenched with the latter detector [14]. Detection limits for polyaromatic hydrocarbons were ca. 200 pg and linearity was observed over three orders of magnitude.

In the present paper the use of the PID is studied for both LC and SFC, and the results are compared with those obtainable in GC-PID. In both cases, packed-capillary columns (I.D. = 0.32 mm) were used without flow splitting and detection took place in the vapour phase. For LC-PID, this required the use of a simple interface to evapourate the LC effluent. Reversed-phase LC was performed because this mode allows the use of high-energy lamps. For SFC the use of packed columns meant that it was necessary to modify the mobile phase (carbon dioxide) with methanol and formic acid. In both cases the performance of the system was first tested with three polyaromatic hydrocarbons. Next, the potential of the system was evaluated by separating and detecting compounds without chromophoric groups.

EXPERIMENTAL

LC-PID Instrumentation

A Phoenix-20 syringe pump (Carlo Erba Strumentazione, Milan, Italy) was used to deliver the mobile phase. Samples were injected

manually using a 60 nl home-made injection valve (Free University, Amsterdam, The Netherlands). Fused-silica capillaries of different lengths (I.D. 0.32 mm; SGE, Ringwood, Australia and Chrompack, Middelburg, The Netherlands) were slurry packed with 5 μm LiChrosorb RP-18 (Merck, Darmstadt, Germany) or 5 μm RoSil C-8 (RSL, Eke, Belgium) and used as the analytical column. For detection a 52-02A PID (HNU systems, Newton, MA, USA) with a 9.5 or 10.2 eV krypton lamp was used. Signals were recorded on a Kipp & Zonen BD40 recorder (Delft, The Netherlands).

Fig. 2 shows the detector set-up. Via a low-dead volume union (Valco, Schenkon, Switzerland) the column was connected to a fused-silica capillary (SGE; I.D. 0.050 mm) which entered the glass-lined inlet (length 14.85 cm; I.D. 0.70 mm) of the detector through a capillary column adaptor. The fused-silica capillary was tightened in the capillary column adaptor by a vespel ferrule (SGE), while the other end of the adaptor was connected with a helium line from which the helium flows between the fused-silica capillary and the inner wall of the glass-lined inlet. The fused-silica capillary ended just beneath the top of the glass-lined inlet. Helium swept the mobile phase vapour into the ionisation chamber of the detector via the two holes present at the top of the glass-lined inlet and left the chamber via the glass-lined exhaust.

SFC-PID Instrumentation

A μLC-500 Micro Flow-syringe pump (ISCO, Lincoln, NE, USA) was used for mobile phase delivery and pressure control. All samples were introduced manually through a 60 nl Valco injection valve (Type CI4W) positioned on a PU 4500 gas chromatograph (Pye Unicam, Philips, The Netherlands). Fused-silica columns of different length (0.32 mm I.D.) were slurry packed with Rosil CN (5 μm; RSL), or LiChrosorb RP-18 or RP-8 (both 5 μm; Merck). The column temperature was controlled at 50°

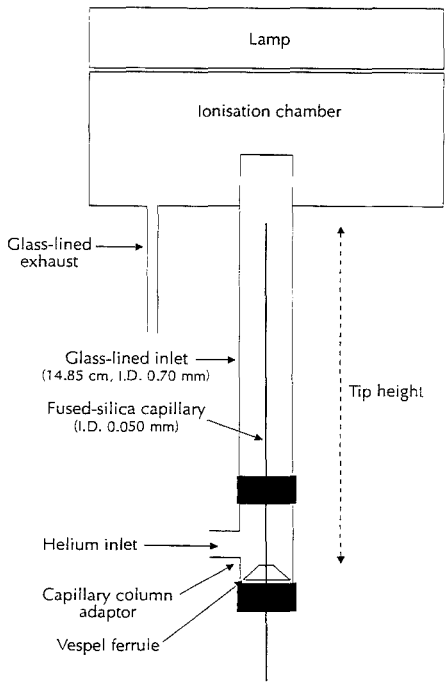

Figure 2. Design of LC-PID interface.

C by the GC oven. The PID was mounted in one of the detector positions of the GC, temperature being controlled by both the detector oven of the GC and the heater of the PID. Signals were recorded on a Kipp & Zonen BD40 recorder. Fused-silica capillaries (10 μm I.D.; Polymicro Technologies, Phoenix, AZ, USA) of different length were used for pressure restrictor or a 100 μm frit restrictor (Dionex/Lee Scientific, Salt Lake City, UT, USA) was shortened to give a flow rate of 5 μl min^{-1} at 150 bar. The restrictor was mounted in the same way as the fused-silica capillary in the LC-PID system described above. It was necessary to cool the syringe of the ISCO pump during the filling procedure in order to

obtain a maximum filling percentage. This was done by slightly releasing a nut at the top of the syringe and allowing the carbon dioxide to expand adiabatically. To obtain modified carbon dioxide a known volume of modifier was added to the syringe. The resulting percentages (expressed as % mol mol^{-1}) were calculated with interpolated carbon dioxide densities at various temperatures, taken from the tabulated data of Angus et al. [15], and the known densities of the modifiers used.

Chemicals

Helium (99.999%) and carbon dioxide (99.97%) were both obtained from Hoek Loos (Schiedam, The Netherlands). Hexane, water and methanol were of HPLC-grade and were obtained from J.T. Baker (Deventer, The Netherlands). 2-Propanol (99%), phenol (99%), acetonitrile (>99%), chloroform (>99%), N,N-dimethylformamide (>99%) and formic acid (98%) also came from J.T. Baker. 1-Butanol (99.7%), N-methylacetamide (98.5%), butanoic acid (>99%), hexanoic acid (>99%), heptanoic acid (>99%), octanoic acid (>99%), decanoic acid (>99%), dodecanoic acid (>99%), tridecanoic acid (>99%), naphthalene (>99%) and pyrene (>99%) were purchased from Merck. 2-Butanone (99%), 2-hexanone (99%), 2-heptanone (99%), acetamide (99%) and N,N-dimethylaniline (99%) came from Aldrich (Brussels, Belgium). N,N-Diethylformamide (>99%) was supplied by Lamers & Indemans ('s-Hertogenbosch, The Netherlands) and phenanthrene (99%) by Eastman Kodak (Rochester, NY, USA). 2-Pentanone (99%) came from Huls (Marl-Kreis - Recklinghausen, Germany). Propionaldehyde (97%), butyraldehyde (>99%), capronaldehyde (>98%) and valeraldehyde (98%) were purchased from Fluka Chemie (Buchs, Switzerland). Disulfoton, ethion and sulfotepp were gifts from the Governmental Food Inspection Service (Alkmaar, The Netherlands).

RESULTS AND DISCUSSION

LC-PID

The LC-PID system was optimised to obtain the best signal-to-noise ratio by varying parameters such as the detector temperature, tip height (i.e. length of the fused-silica capillary in the glass-lined inlet) and lamp intensity in the flow-injection mode. The injection valve was coupled directly to the PID via the interface capillary. Phenanthrene (11.6 ng/60 nl methanol) was chosen as test compound because of its high boiling point (340°C) and good PID response. With a tip height of 11.6 cm, a methanol flow of 3 μl min^{-1} and a helium flow of 26 ml min^{-1}, the detector temperature was varied over the range 180-300°C. It was found that the temperature should be kept at 200-250°C, i.e. significantly above the boiling point of the mobile phase, in order to prevent condensation, but it was not necessary to go up to 340°C (the boiling point of phenanthrene) because the analyte was swept into the ionisation chamber by the mobile phase vapour and the helium.

Next the influence of the tip height was investigated. When the tip position is not high enough less volatile compounds will not evapourate instanteneously at the tip end, and consequently band broadening will occur. On the other hand, when the tip position is too high, analytes can condense inside the fused-silica capillary and clog the capillary after evapouration of the mobile phase. For a relatively non-volatile compound like phenanthrene no peak broadening was observed when the tip was positioned above 8 cm. Clogging problems started to occur at tip heights of over 12.2 cm. For volatile compounds it was not necessary to optimise the tip height as no clogging was observed even at maximum tip height (13.5 cm).

Fig. 3 shows the effect of the lamp intensity on the signal-to-noise ratio (test compound, phenanthrene). The best ratio was obtained at the

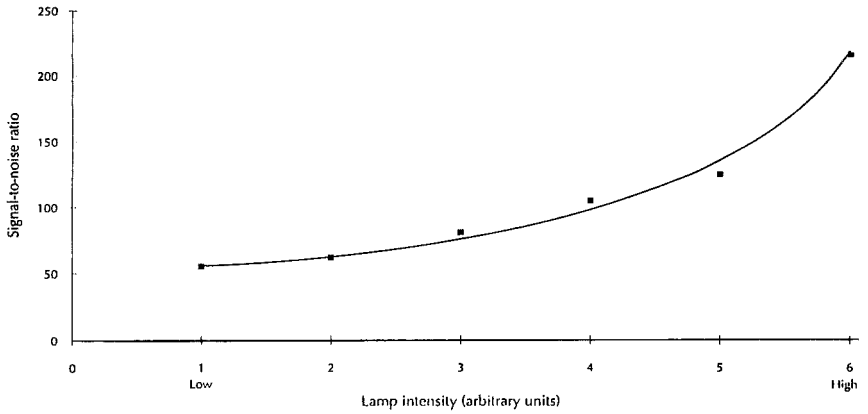

Figure 3. Signal-to-noise ratio as function of the lamp intensity in LC-PID. Conditions: injection volume, 60 nl; sample, phenanthrene, 11.6 ng; mobile phase, methanol 3 μl min^{-1} ; helium flow rate, 26 ml min^{-1}; lamp energy, 10.2 eV; detector temperature, 250°C; tip height, 11.6 cm.

highest lamp intensity, which illustrates the importance of a high photon flux because most of the photons are absorbed by mobile phase molecules.

The influence of the helium flow rate was studied in the LC mode. In the interface helium flows between the capillary and the glass-lined inlet and forces the mobile phase into the ionisation chamber. It is also used to cool both the lamp and the mobile phase in the capillary; the mobile phase evapourates only at the end of the capillary, just before the ionisation chamber. Fig. 4 shows the effect of varying the helium flow rate from 21 to 49 ml min^{-1} on the signal-to-noise ratio of a phenanthrene solution (25 ng/60 nl). The optimum helium flow rate was ca. 40 ml min^{-1}. For higher helium flow rates two opposite effects were observed. First of all, the helium dilutes the mobile phase; the background current reduction is therefore less pronounced (cf. below) and the signal-to-noise

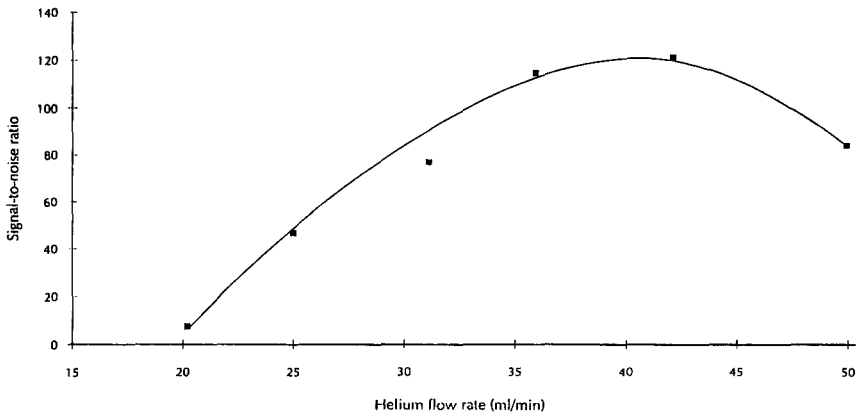

Figure 4. Signal-to-noise ratio as function of the helium flow rate in LC-PID. Conditions: packed-capillary column, 80 x 0.32 mm I.D., packed with 5 μm LiChrosorb RP-18; injection volume, 60 nl; sample, phenanthrene, 25 ng; mobile phase, methanol 3 μl min^{-1} ; lamp energy, 10.2 eV; detector temperature, 240°C; tip height, 8.3 cm.

ratio increases. Secondly, the analyte is in the detector for a shorter period of time which will decrease the signal-to-noise ratio.

Background current in LC-PID

The background current of some frequently used mobile phases was measured to study the influence on the PID response. This was done at mobile phase flow rates of 0, 1 and 3 μl min^{-1} using lamp energies of 9.5 and 10.2 eV. The helium flow rate was varied between 6 and 58 ml min^{-1}.

The background current is caused by the gold used to coat the ionisation chamber. The ionisation potential of gold is 4.71 eV, so both lamps are able to ionise the gold. The mobile phase vapour present in the

ionisation chamber can reduce the background current by absorbing the lamp radiation. This can be seen as a process competing with photoionisation of the analytes. The level of the background current is influenced by the efficiency of Steps 2 ($E + h\nu \rightarrow E^*$), 7 ($AB^* + E \rightarrow AB + E$) and 11 ($AB^+ + E + e^- \rightarrow AB + E$) of Fig. 1. Obviously these efficiencies should be as low as possible.

The background currents of the mobile phases at different mobile phase flow rates and lamp energies are listed in Table I. The background current with no mobile phase vapour present in the ionisation chamber (mobile phase flow rate, 0 μl min^{-1}) is 1.3 nA for the 9.5 eV lamp and 0.31 nA for the 10.2 eV lamp. The data in Table I show that at a mobile phase flow rate of 3 μl min^{-1}, the background current is reduced more than at 1 μl min^{-1}. This is due to the fact that at higher flow rates more mobile phase flows into the ionisation chamber and diminishes the ionisation of the gold. The only exception was found for pure water when using the 9.5 eV lamp. When the 10.2 eV lamp was used, some of the mobile phases were ionised. These are n-hexane, 1-butanol and 2-propanol with ionisation potentials of 10.18, 10.04 and 10.16 eV, respectively [8]. As a result these mobile phases yield a high background current. But even with these mobile phases the background current is lower at 3 than at 1 μl min^{-1}.

The effect of the helium flow rate on the ionisation efficiency of the 9.5 eV lamp is shown in Fig. 5. If there is only helium present in the ionisation chamber (mobile phase flow rate, 0 μl min^{-1}) there is no effect on the background current when changing the helium flow rate. When hexane was introduced as mobile phase the background current decreased, the decrease becoming larger at a higher flow rate. An increase of the helium flow rate led to higher background currents (cf. Fig. 5), because helium dilutes the mobile phase vapour.

In summary one can conclude that with flow rates of around 3 μl min^{-1} generally used in packed-capillary LC, the decrease of the

TABLE I

BACKGROUND CURRENTS FOR SEVERAL MOBILE PHASES AT DIFFERENT MOBILE PHASE FLOW RATES AND LAMP ENERGIES*.

Mobile phase	Background current (nA) at			
	lamp energy: 9.5 eV and mobile phase flow rate of:		lamp energy:10.2 eV and mobile phase flow rate of:	
	1 μl min^{-1}	3 μl min^{-1}	1 μl min^{-1}	3 μl min^{-1}
n-Hexane	1.2	0.7	2.1	1.2
Methanol	1.0	0.4	0.1	0.01
Methanol-water (70:30)	1.2	0.7	0.1	0.01
Methanol-water (30:70)	1.6	1.2	0.1	0.05
Water	1.8	2.0	0.1	0.02
Acetonitrile	1.1	0.8	0.02	0.01
Chloroform	1.0	0.7	0.1	0.03
1-Butanol-methanol-water (5:30:65)	1.6	1.3	0.5	0.4
1-Butanol	1.1	1.0	3.0	2.5
2-Propanol	1.4	1.2	1.5	0.9

* Conditions: LC column 8.0 cm x 0.32 mm I.D., packed with 5 μm LiChrosorb RP-18; detector temperature, 240°C; helium flow rate, 57 ml min^{-1}; tip height, 8.1 cm; n = 3.

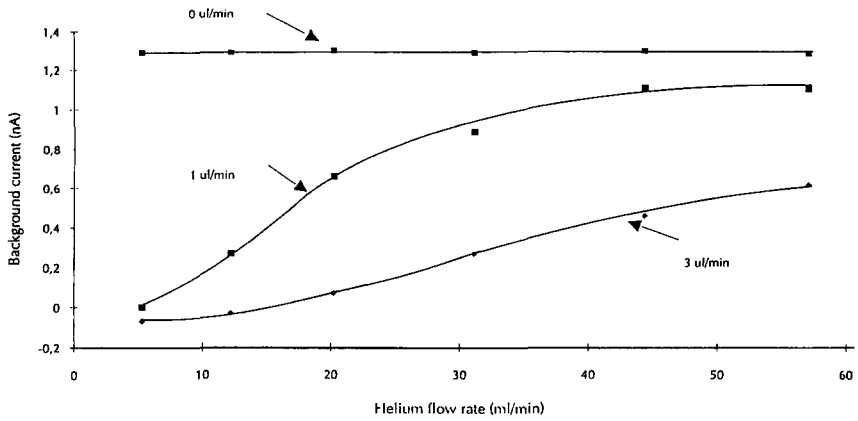

Figure 5. Background current of n-hexane versus different helium flow rates in LC-PID. Conditions: packed-capillary column, 80 x 0.32 mm I.D., packed with 5 μm LiChrosorb RP-18; lamp energy, 9.5 eV; detector temperature, 240°C; tip height, 8.1 cm.

background current will be such that a serious loss of detector sensitivity must be expected.

Analytical data

Calibration curves were constructed for several groups of compounds by injecting 60 nl samples into the system and MDLs (signal-to-noise ratio of 3) were determined. The helium flow rate was 40 ml min^{-1} and the flow rate of the mobile phase 3 μl min^{-1}. The detector temperature was set at 210°C. A 10.2 eV lamp was used because of its highest photon flux. Data concerning the calibration curves and the MDL values are given in Table II. The concentration range of the calibration curves is between 5 and 1200 ng per 60 nl injection. Each calibration curve consisted of 12 data points measured in duplicate, except the curves

TABLE II

MINIMUM DETECTION LIMITS (MDL) OF THE LC-PID SYSTEM*.

Compounds	MDL (ng)	Calibration curve $y = a (\sigma_a) x + b (\sigma_b)$	R^2
2-Butanone[I]	0.5	y = 3.45 (0.01) x + 0.4 (0.4)	0.999
2-Pentanone[I]	1.5	y = 2.25 (0.01) x + 0.7 (0.5)	0.999
2-Hexanone[I]	0.2	y = 1.77 (0.01) x + 0.6 (0.4)	0.999
2-Heptanone[I]	2.5	y = 1.18 (0.01) x + 0.3 (0.3)	0.999
Propionaldehyde[II]	1.0	y = 7.51 (0.09) x + 2.2 (0.9)	0.999
Butyraldehyde[II]	1.5	y = 5.46 (0.09) x + 1.8 (1.1)	0.998
Capronaldehyde[II]	2.0	y = 4.55 (0.15) x + 3.5 (2.1)	0.994
Valeraldehyde[II]	2.5	y = 1.31 (0.03) x + 1.1 (0.6)	0.997
Acetamide[III]	2.5	y = 3.18 (0.02) x + 2.6 (0.9)	0.999
N-Methylacetamide[III]	1.5	y = 3.76 (0.06) x + 8.0 (2.6)	0.997
N,N-Dimethylformamide[III]	1.5	y = 4.09 (0.04) x + 3.7 (1.7)	0.999
N,N-Diethylformamide[IV]	3.0	y = 8.16 (0.09) x + 4.4 (4.5)	0.999
Phenanthrene[V]	1.0	y = 1.81 (0.02) x - 0.2 (0.1)	0.999
Phenol[VI]	4.5	y = 1.63 (0.01) x - 0.5 (0.2)	0.999
N,N-Dimethyl-aniline[VII]	2.5	y = 8.95 (0.05) x + 5.7 (2.4)	0.999

* Conditions: injection volume, 60 nl; mobile phase flow rate, 3 μl min^{-1}; helium flow rate, 40 ml min^{-1}; lamp energy, 10.2 eV; detector temperature, 210°C unless otherwise stated.

I: conditions as in Fig. 6A. II: Conditions: LiChrosorb RP-18 column; mobile phase, acetonitrile-water (1:1); tip height, 10.1 cm; III: conditions as in Fig. 6B; IV: Conditions: RP-18 column; mobile phase, methanol; tip height, 8.1 cm; V: conditions: RP-18 column; mobile phase, methanol; tip height, 9.5 cm; detector temperature, 240°C; VI: conditions: RP-18 column; mobile phase, methanol; tip height, 8.0 cm; detector temperature, 240°C; VII: conditions: RP-18 column; mobile phase, methanol; tip height, 8.1 cm.

for the aldehydes which consisted of 10 data points measured in duplicate. Figs. 6A and 6B show LC-PID chromatograms for the separation of several ketones and amides, respectively.

In literature the MDLs of N,N-dimethylformamide and N,N-diethylformamide for LC-PID are 500 and 600 ng, respectively [3]. In other words, the data of Table II show values which are more than 100-fold improved. In the same paper the MDLs reported for 3-hexanone and anthracene are 700 and 75 ng, respectively. For similar compounds like 2-hexanone and phenanthrene the MDL values found with the present system are 4600 and 80 times lower. These rather remarkable gains in analyte detectability can not be explained by the fact that there is less mobile phase introduced into the detector because for phenol and N,N-dimethylaniline the MDL values found are the same as those published before [3]. It was also observed that better MDLs were invariably obtained using the 10.2 eV lamp, which is in contrast with the literature where the 9.5 eV lamp gave the best results for N,N-dimethylaniline and anthracene [3].

Although most of the compounds in Table II can also be determined by GC which provides more sensitive detection, separation by LC can be helpful because of a different selectivity of this technique [10]. On an average, the loss of sensitivity of LC-PID compared with GC-PID is about two orders of magnitude. In essence this means that LC-PID should be combined with on-line trace enrichment in order to perform trace analysis for compounds which can not be separated by GC and have no chromophoric group.

SFC-PID

Optimisation of the SFC-PID system

The composition of the mobile phase is the most interesting parameter when using PID in SFC. The 10.2 eV lamp was used

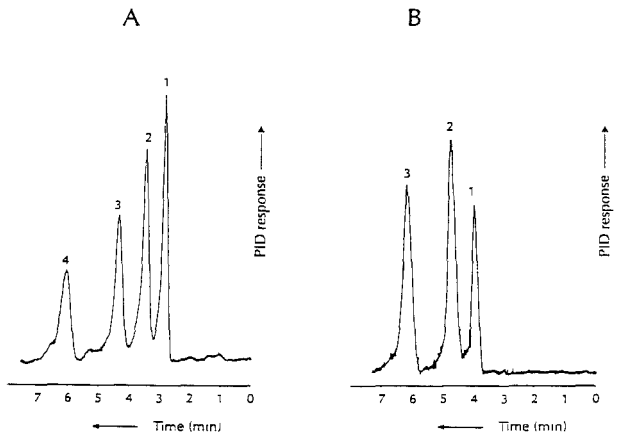

Figure 6. LC-PID of ketones (6A) and amides (6B). Conditions: helium flow rate, 26 ml min^{-1}; lamp energy, 10.2 eV; detector temperature, 240°C. (A) Packed-capillary column, 100 x 0.32 mm I.D., packed with 5 µm LiChrosorb RP-18; mobile phase, acetonitrile-water (55:45), 3 µl min^{-1} ; tip height, 11.4 cm; injection volume, 60 nl; sample, (1) 2-butanone, 85 ng; (2) 2-pentanone, 94 ng; (3) 2-hexanone, 82 ng; (4) 2- heptanone, 91 ng. (B) Packed-capillary column, 150 x 0.32 mm I.D. , packed with 5 µm Rosil C-8; mobile phase, acetonitrile-0.1mM formic acid (5:95), 3 µl min^{-1} ; tip height, 11.7 cm; injection volume, 60 nl; sample, (1) acetamide, 50 ng; (2) N-methylacetamide, 49 ng; (3) N,N-dimethylformamide, 47 ng.

throughout because of the results presented above for LC-PID. First of all the influence of the mobile phase composition on the backgound current was studied. In LC reduction of the background current caused by the mobile phases used was responsible for the low sensitivity of the detector. As expected from the literature [12], the introduction of carbon dioxide in the detector had no influence on the background current. The use of modified carbon dioxide reduced the background current of the 10.2 eV lamp with maximally 10% (ca. 4.7 mol% methanol in carbon dioxide, 5 µl min^{-1}; helium flow rate, 5 ml min^{-1}). Therefore, it was not necessary to

use high helium flow rates because helium only serves to lower the dead volume of the detector. Because, moreover, low mol% (< 2.5 mol%) of modifier are normally used in SFC, one may expect that the performance of the detector will not seriously be affected.

A problem in SFC is that the injection medium can not be the same as the mobile phase. Especially with the PID this problem is not easily solved. Common injection solvents like acetone and hexane can not be used because they cause a large PID response. Therefore, more polar solvents like ethanol, methanol or acetonitrile had to be used as injection medium. From among these solvents there is a slight preference for ethanol because it has least influence on the separation efficiency. However, even these solvents disturb the PID signal due to a solvent peak, because they do absorb light and this causes a decrease of the background current. As a consequence optimisation of the SFC-PID system could not be carried out using flow-injection.

Phenanthrene was chosen as test compound for optimisation of the system. When studying the influence of the detector temperature on the signal-to-noise ratio, it was found that an increase of the temperature from 200 to 300°C reduced this ratio 2-fold. It is therefore recommended to use a detector temperature which is as low as possible. The limitation in going to a lower detection temperature self-evidently is the nature of the analyte (e.g., volatility). The ensuing problem was partly circumvented by using the detector oven of the GC on which the PID was mounted, to heat up the glass-lined inlet of the PID in which the restrictor was situated.

Another parameter is the lamp intensity of the detector. The result was virtually identical with that observed for LC-PID shown in Fig. 3. That is, the highest lamp intensity should be chosen in order to measure under optimum conditions. One should add that working with high lamp intensities will reduce the lifetime of the lamp.

In Fig. 7 the influence of the helium flow on the signal of phenanthrene is shown for four different modified carbon dioxide phases.

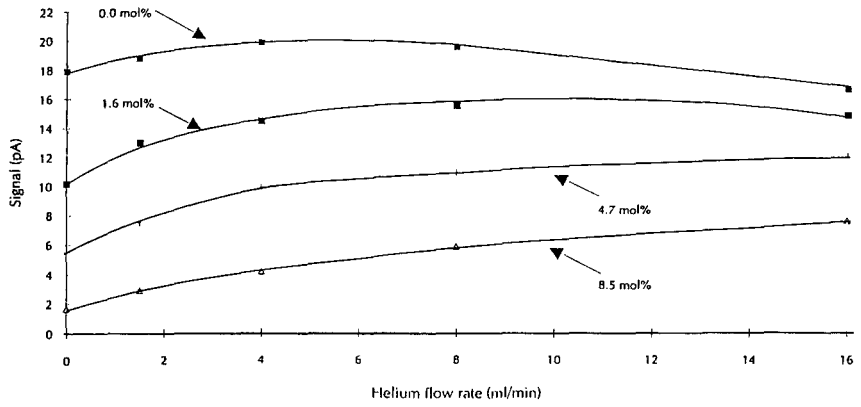

Figure 7. Influence of the helium flow rate on the signal of phenanthrene in SFC-PID for different modified mobile phases. Conditions: packed-capillary column, 150 x 0.32 mm I.D., packed with 5 µm LiChrosorb RP-18; column temperature, 54°C; restrictor length, 250 mm; pressure, 152 bar; injection volume, 60 nl; sample, phenanthrene, 450 pg in ethanol; detector temperature, 200°C; lamp energy, 10.2 eV; lamp intensity, high.

The influence of helium is rather limited when there is no methanol in the mobile phase. However, with an increasing flow rate of helium the residence time of the analyte in the detector is reduced and a somewhat lower signal is observed. If methanol is added to the mobile phase, a much stronger decrease of the sensitivity is observed which may be explained by increasing competition of side reactions such as Steps 2, 7 and 11 (cf. Fig. 1). In these instances, increasing the helium flow rate has no negative effect on the analyte signal intensity because it helps to reduce the amount of methanol in the detector. However, as Fig. 8 shows the real situation, expressed by the signal-to-noise ratio, is somewhat different: the increase in the flow rate obviously causes a noticeable increase of the noise level. Consequently, an optimum is obtained at about 2 ml min^{-1}.

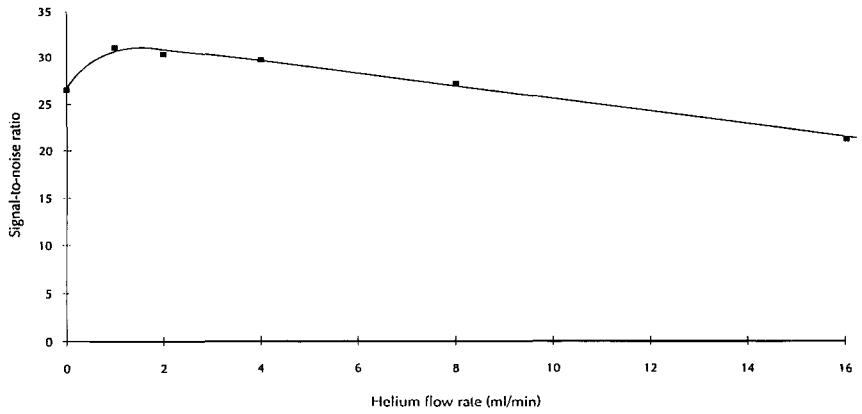

Figure 8. Signal-to-noise ratio as function of the helium flow rate in SFC-PID. Conditions: packed-capillary column, 150 x 0.32 mm I.D., packed with 5 µm LiChrosorb RP-18; column temperature, 54°C; restrictor length, 250 mm; pressure, 152 bar; mobile phase, 1.5 mol% methanol; injection volume, 60 nl; sample, phenanthrene, 130 pg in ethanol; detector temperature, 200°C; lamp energy, 10.2 eV; lamp intensity, high.

The influence of the mobile phase flow on the signal-to-noise ratio was studied using another model compound, butanoic acid. The data of Fig. 9, which was constructed at the optimal helium flow rate of 2 ml min^{-1}, show the influence at three different pressures. The highest signal-to-noise ratio is reached at the highest chromatographic pressure, which is of course due to the fact that peak broadening is larger at low mobile phase densities. As is to be expected, as regards the influence of the restrictor length, at a low mobile phase flow (long restrictor) signal-to-noise ratios are highest.

Analytical data

In order to test the analytical performance of the SFC-PID system using 1.5 mol% methanol in carbon dioxide as mobile phase, a mixture of

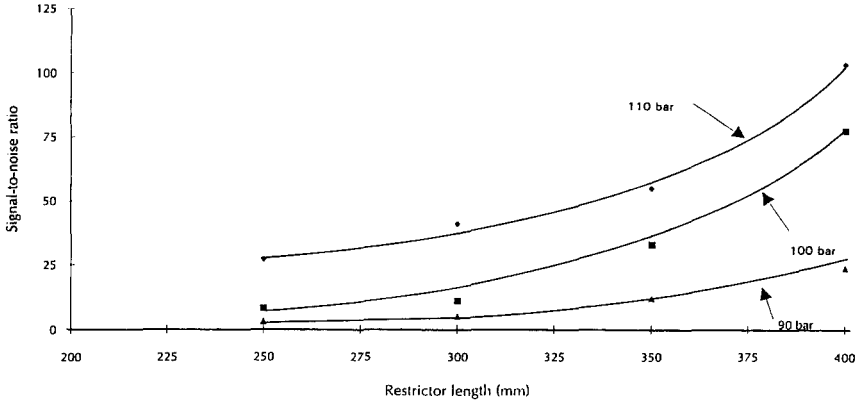

Figure 9. Signal-to-noise ratio of butanoic acid as function of the restrictor length in SFC-PID for different column temperatures and pressures. Conditions: packed-capillary column, 150 x 0.32 mm I.D., packed with 5 μ m LiChrosorb RP-18; column temperature, 40°C; mobile phase, 0.48 mol% methanol and 0.24 mol% formic acid; injection volume, 60 nl; sample, butanoic acid, 45 ng in ethanol; helium flow rate, 2 ml min^{-1}; detector temperature, 200°C; lamp energy, 10.2 eV; lamp intensity, high.

three PAHs was analysed. The PAHs were dissolved in ethanol, but as can be seen in the chromatogram the peak of naphthalene is disturbed by a solvent peak (Fig. 10). Therefore, the MDL for this compound could not be determined. For the other two compounds, phenanthrene and pyrene, the MDLs were 12 and 20 pg, respectively. The MDL for phenanthrene is 100-fold lower than that found in LC-PID (cf. above) and is in the same order as the MDL found with GC-PID [1]. The calibration curve for phenanthrene showed good linearity (see Table III); that is, use of modified carbon dioxide does not deteriorate the detector performance.

For a further demonstration of the potential of SFC-PID fatty acids were used as test analytes. For the separation of these compounds a strongly modified mobile phase is needed making it impossible to use FID

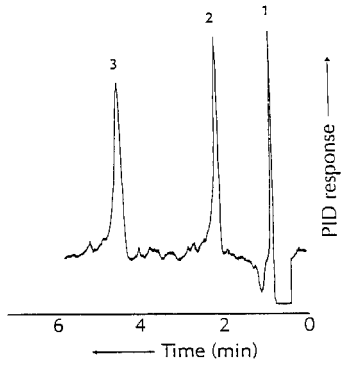

Figure 10. SFC-PID of some PAH's. Conditions: packed-capillary column, 150 x 0.32 mm I.D., packed with 5 μm LiChrosorb RP-18; column temperature, 54°C; restrictor length, 250 mm; pressure, 152 bar; mobile phase, 1.5 mol% methanol; injection volume, 60 nl; sample, (1) naphthalene, 200 pg, (2) phenanthrene, 220 pg and (3) pyrene, 290 pg in ethanol; helium flow rate, 1 ml min^{-1}; detector temperature, 200°C; lamp energy, 10.2 eV; lamp intensity, high.

detection. The acids also lack a chromophoric group necessary for UV detection. Formic acid was used as modifier in combination with methanol to obtain sufficient polarity of the mobile phase. The highly polar mobile phase used to separate the acids even allowed the use of pure water as injection solvent without a negative effect on the separation. In Fig. 11 the separation of butanoic, hexanoic, heptanoic and octanoic acid is shown. Obviously analysis of these rather polar compounds can be achieved without derivatisation. The calibration curves and the MDL values for these and some longer-chain fatty acids are included in Table III. Linearity is good; the detection limits are significantly higher than those found for phenanthrene and pyrene. This is due to the different molecular structures of these classes of compounds and consequently, different ionisation efficiencies [1].

TABLE III

MINIMUM DETECTION LIMITS (MDL) OF THE SFC-PID SYSTEM*.

Compounds	MDL (ng)	Calibration curve $y = a (\sigma_a) x + b (\sigma_b)$	R^2
Phenanthrene[I]	0.01	y = 1.47 (0.02) x - 7.0 (4.5)	0.999
Butanoic acid[II]	5.5	y = 0.16 (0.01) x + 5.0 (1.8)	0.995
Hexanoic acid[II]	3.5	y = 0.40 (0.01) x + 8.6 (2.6)	0.998
Heptanoic acid[II]	2.5	y = 0.88 (0.02) x + 6.1 (8.9)	0.995
Octanoic acid[II]	3.0	y = 1.31 (0.05) x + 7.7 (3.6)	0.995
Decanoic acid[III]	6.0	y = 1.77 (0.03) x - 5.0 (2.1)	0.998
Dodecanoic acid[III]	4.0	y = 2.33 (0.05) x - 0.4 (0.3)	0.999
Tridecanoic acid[III]	3.5	y = 2.17 (0.04) x - 0.3 (0.2)	0.999

* Conditions: LiChrosorb RP-18 column, 150 x 0.32 mm I.D.; injection volume, 60 nl; helium flow rate, 2 ml min^{-1}; lamp energy, 10.2 eV; lamp intensity, high; detector temperature, 200°C unless otherwise stated.
I: Conditions: column temperature, 54°C; mobile phase, 1.5 mol% methanol; pressure, 152 bar; restrictor length, 250 mm. II: Conditions: column temperature, 52°C; mobile phase, 0.48 mol% methanol, 0.24 mol% formic acid; pressure, 110 bar; restrictor length, 400 mm; detector temperature, 225°C. III: Conditions: column temperature, 54°C; mobile phase, 1.0 mol% methanol, 0.50 mol% formic acid; pressure, 152 bar; restrictor length, 250 mm.

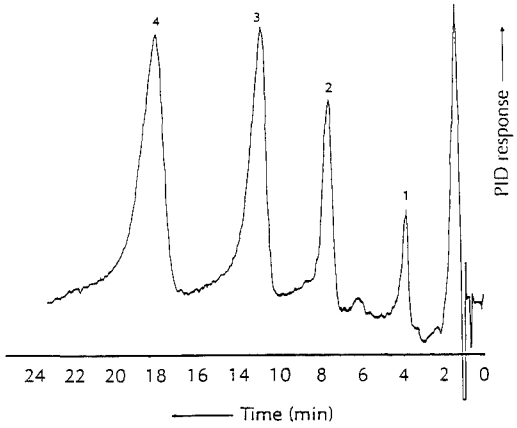

Figure 11. SFC-PID of some short-chain fatty acids. Conditions: packed-capillary column, 150 x 0.32 mm I.D., packed with 5 μm LiChrosorb RP-18; column temperature, 52°C; restrictor length, 400 mm; pressure, 140 bar; mobile phase, 0.48 mol% methanol, 0.24 mol% formic acid; injection volume, 60 nl; sample, (1) butanoic acid, (2) hexanoic acid, (3) heptanoic acid and (4) octanoic acid, all compounds 30 ng in ethanol; helium flow rate, 3 ml min^{-1}; detector temperature, 200°C; lamp energy, 10.2 eV; lamp intensity, high.

Finally, a mixture of three organosulphur/phosphorus pesticides, sulfotepp, disulfoton and ethion,was analysed (Fig. 12). They could be detected down to a level of 1 ng. Admittedly, this is much higher than reported for packed-capillary SFC with thermionic detection (ca. 50 pg; [16]). However, the result clearly illustrates the potential of the PID as a detector for widely varying classes of compounds. During this research two PID lamps were used as the 10.2 eV lamp already used in the LC-project lossed intensity during the SFC work. The new lamp is still in use after a working period of eight months. During the whole project (IC and SFC, two years) the PID broke one time down as the inlet in the ionisation

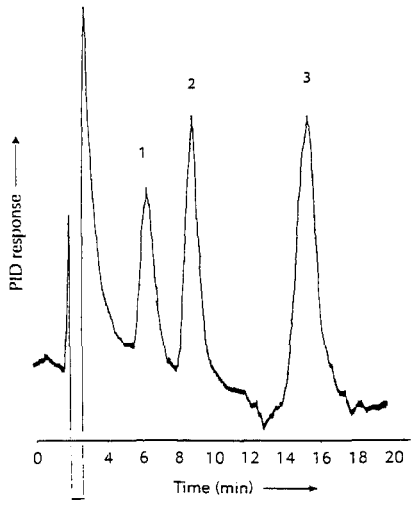

Figure 12. SFC-PID of some organo sulphur/phosphorus pesticides. Conditions: packed-capillary column, 210 x 0.32 mm I.D., packed with 5 μ m LiChrosorb RP-18; column temperature, 52°C; restrictor length, 280 mm; pressure, 133 bar; mobile phase, 0.97 mol% methanol; injection volume, 60 nl; sample, (1) sulfotepp, 23.7 ng, (2) disulfoton, 12 ng and (3) ethion, 24.7 ng , in ethanol; helium flow rate, 3 ml min^{-1}; detector temperature, 260°C; lamp energy, 10.2 eV; lamp intensity, high.

chamber was broken. Working with the PID in SFC is quite simple, the system can be used for months without changing any component, in LC the system is robust and care should be taken with the positioning of the interface capillary.

CONCLUSIONS

The present study shows that the optimum conditions for photoionisation detection in combination with packed-capillary LC are: a

10.2 eV lamp, a high lamp intensity, a helium flow rate of about 40 ml min^{-1} and a high temperature (between 200-250°C). The minimum detection limits for ketones, aldehydes and amides are in the low nanogram range. Although, for several analytes, detectability was 2 - 3 orders of magnitude better than reported in the literature, it is still true that the sensitivity of LC-PID is relatively low compared with GC-PID, the main problem being the quenching of the ionisation radiation due to the absorption of photons by the mobile phase. However, by combining LC-PID with, e.g., on-line trace enrichment the sensitivity problem can largely be solved. Besides, the tedious sample clean-up sometimes required for GC-PID, can then be avoided. Future work should be directed at exploring the practicality of such procedures.

Combining packed-capillary SFC with PID is a highly successful approach; the performance of the detector is almost the same as in GC. Under optimum conditions (10.2 eV lamp, high lamp intensity, helium flow rate of about 2 ml min^{-1}, temperature of 200°C) the MDLs for phenanthrene and pyrene are about 10 pg. Since strongly modified mobile phases can be used it is possible to perform separations of underivatised fatty acids and organosulphur/phosphorus pesticides down to the 2 - 5 ng level. Actually, this is the main - and extremely important - advantage of the PID over the more conventional FID.

One should of course realize that the main problem of (packed-capillary) SFC, i.e., the rather low injection volumes which are permitted, is not solved by utilizing another detector. A second problem with injection in SFC is the choice of the injection solvent. With injection volumes of typically ca. 60 nl and MDLs in the low-nanogram range, detection limits in the samples offered for analysis are in the 10 -100 ppm range. It is therefore interesting to briefly quote the developments made with regard to large-volume injections in SFC by using solid-phase extraction columns. With such an on-line set-up, sample injection volumes can be increased at least 1000-fold and no injection solvent is introduced into the detector [17]. In principle, this means that MDLs can

be improved down to in the range of 10 -1000 ppb. On-going research is directed at the further exploration of this approach.

Finally, it be interesting to mention that during the present study three PID lamps were used because the 10.2 eV lamp used for the LC work started to lose intensity during the SFC-orientated studies. The new lamp is still in use after a working period of 8 months. During the total work period, the PID broke down once because the inlet in the ionisation chamber broke. Working with the PID in SFC is especially simple; the system could be used for several months on end without exchanging any part of the total set-up.

ACKNOWLEDGEMENTS

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DETERMINATION OF THE ANTI-HIV DRUG 2'-β-FLUORO-2',3'-DIDEOXYADENOSINE IN BIOLOGICAL FLUIDS BY REVERSED-PHASE HPLC

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ABSTRACT

2'-β-Fluoro-2',3'-dideoxyadenosine (F-ddA) is a synthetic dideoxynucleoside analogue that has been designed to overcome the acid stability problems of the anti-AIDS drug didanosine. F-ddA is also a clinical candidate and will be tested in AIDS patients upon completion of its preclinical evaluation. We have developed a straightforward reversed-phase HPLC method to measure both F-ddA and its deaminated metabolite, 2'-β-fluoro-2',3'-dideoxyinosine, in plasma and urine. This method employs an adenosine deaminase inhibitor to prevent sample degradation, an internal standard for quantitation, and C_{18} solid-phase extraction to isolate and concentrate the fluorinated dideoxynucleosides. Gradient HPLC analysis on a reversed-phase phenyl column with UV detection at 260 nm gives a limit of quantitation of 50 ng/ml (0.2 μM) for both analytes. This assay has been applied to preclinical studies in rats and monkeys to determine drug stability, disposition, metabolism and plasma kinetics.

INTRODUCTION

2'-β-Fluoro-2',3'-dideoxyadenosine (F-ddA, Figure 1) is a synthetic analogue of dideoxyadenosine (ddA) in which the 2'-β-hydrogen of the dideoxyribose sugar has been replaced by a fluorine atom (1). This modification retains the anti-HIV activity of ddA, confers acid stability, and reduces the rate of degradation by adenosine deaminase (ADA) on the fluorinated derivative (1-4). Both F-ddA and its deaminated catabolite, 2'-β-fluoro-2',3'-dideoxyinosine (F-ddI, Figure 1) possess *in vitro* activity and potency against HIV that is similar to that of 2',3'-dideoxyinosine (ddI, didanosine, Videx), a drug approved by the FDA to treat AIDS (1,2,5). These purine dideoxynucleosides all exhibit almost complete protection against the cytopathic effects of HIV in infected ATH8 cells over the concentration range 5-100 μM (6).

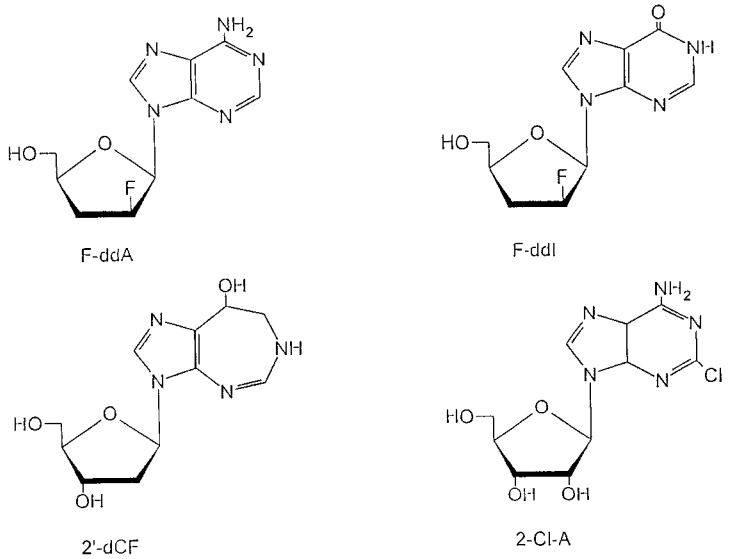


FIGURE 1. Structures of various nucleosides.

The hydrolytic instability of ddI toward acidic conditions ($t_{1/2}$ = 30 sec at pH 1) (2) complicates oral administration, which is preferred because chronic dosing is required (7,8). F-ddA offers possible advantages in this regard because of its better oral bioavailability (9), which results from its acid stability, and its potentially greater central nervous system (CNS) penetration by virtue of its higher lipophilicity (log P = -0.183 versus log P = -1.242 for ddI)(10). This latter property is important because the CNS can be a sanctuary for HIV as well as a site of significant physiologic damage as shown by the wide occurrence of AIDS-related dementia (11,12). For these reasons, the NCI has undertaken the preclinical studies necessary to bring F-ddA to clinical trial.

Chromatographic methods for the bioanalysis of anti-HIV agents, and antiviral agents in general, have recently been reviewed (13). Most HPLC methods for the measurement of purine dideoxynucleosides in biological samples employ solid-phase extraction to isolate and concentrate the compounds of interest (14-19). Reversed-phase HPLC is then combined with UV detection to achieve a limit of quantitation in the 25-200 ng/ml range. (14-21). Fluorescence detection following precolumn fluorogenic derivatization has also been used in conjunction with solid-phase extraction and reversed-phase HPLC for the analysis of ddA and ddI (22). Although this latter procedure is potentially more sensitive than UV-based analyses, it is also more complicated.

This report describes a reversed-phase HPLC method to measure both F-ddA and its primary catabolite, F-ddI, in plasma and urine at submicromolar concentrations. Solid-phase extraction is used to remove protein, isolate the fluorinated dideoxynucleosides from the biological matrix in high yield and concentrate the analytes. The fluorinated dideoxynucleosides are then separated by gradient elution on a phenyl reversed-phase column and measured by their UV absorption. We have employed this method to determine plasma and urine drug and catabolite levels during preclinical studies in rats and monkeys.

MATERIALS

Reagents and Chemicals

F-ddA (NSC-613792) and F-ddI (NSC-616290) were supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program (DTP), NCI (Bethesda, MD). The adenosine deaminase inhibitor, 2'-deoxycoformycin (2'-dCF, NSC-218321) (23) was obtained from the Drug Synthesis and Chemistry Branch, DTP, NCI (Bethesda, MD). 2-Chloroadenosine (2-Cl-A) was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade methanol, acetonitrile and water, as well as certified 1.00 N sodium hydroxide solution, were bought from Fisher Scientific (Fair Lawn, NJ). Spectrophotometric grade DMSO and sodium azide were purchased from Aldrich Chemical Co., (Milwaukee, WI), while monobasic potassium phosphate was from Mallinkrodt (St. Louis, MO). All chemicals and reagents were used without further purification, although the purity of F-ddA, F-ddI and 2-Cl-A was confirmed by HPLC analysis before use. Phosphate buffer was vacuum filtered through a 0.45 μm nylon membrane before mixing with acetonitrile. The mobile phase was then continuously degassed by sparging with helium.

Apparatus

Chromatographic analysis was performed using two separate modular HPLC systems. One configuration consisted of an LKB 2150 pump with a low pressure mixing valve and an LKB 2152 LC controller (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Samples were injected via a Gilson Model 231 autosampler (Gilson International, Middleton, WI) and UV absorbance was monitored with a Gilson Model 116 variable wavelength UV detector. The other HPLC system included a Spectra System P2000 binary pump, an AS3000 autosampler (Spectra-Physics Analytical, San Jose, CA) and a Waters model 994 programmable photodiode array detector (Waters Associates, Milford, MA). Peak areas were integrated, processed and archived using a WINner/386 chromatography data system running

on a Compaq Deskpro 386 computer (Compaq Computer Corporation, Houston, TX) interfaced to Spectra-Physics SP-4200 and SP-4400 integrators.

METHODS

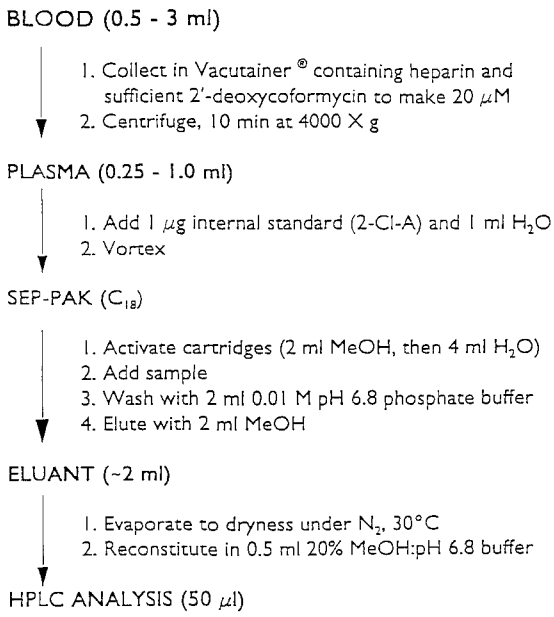
HPLC Analysis

Separations were performed on either a 3.9 x 150 mm Waters Novapak 4 μm phenyl column or a 4.6 x 250 mm Hypersil 5 μm Phenyl-2 column (Shandon Scientific, Cheshire, UK). The analytical column was preceded by a 4.6 x 30 mm Brownlee Spheri-5 Phenyl precolumn (Applied Biosystems Inc., San Jose, CA). A 20-min linear gradient from 1% to 7% acetonitrile in 0.01 M pH 6.8 phosphate buffer was used with the Waters column, while a 22-min linear gradient from 1% to 18% acetonitrile was employed for the Hypersil column. The flow rate was 1 ml/min and UV detection was at 260 nm for all analyses.

Sample Preparation

The standard sample workup procedure is outlined in Scheme I. Blood was collected in 5 or 15 ml Vacutainer® tubes (Becton Dickinson, Rutherford, NJ) containing heparin and a sufficient amount of 2'-dCF to make the final concentration 20 μM in adenosine deaminase inhibitor. Plasma was then separated from blood cells by centrifugation at 4000 X g for 10 min. Urine was diluted 500X before processing. An aliquot of sample (0.25 to 1.0 ml), depending on availability, was then mixed with 1 μg 2-Cl-A internal standard and 1.0 ml water. The sample was loaded onto a C_{18} Sep-Pak Classic cartridge (Waters Associates, Milford, MA), preactivated with 2 ml methanol and 4 ml water. The cartridge was washed with 2 ml 0.01 M, pH 6.8 phosphate buffer and components of interest were eluted with 2 ml methanol. After the methanol eluant was evaporated to dryness under nitrogen at 30°C using an N-Evap analytical evaporator (Organomation Assoc., South Berlin, MA), the residue was reconstituted in 0.5 ml 20% methanol in 0.01

STANDARD WORKUP PROCEDURE



SCHEME I

M, pH 6.8 phosphate buffer. Fifty μl aliquots were injected for HPLC analysis.

Processed Sample Stability

Standards with F-ddA, F-ddI and 2-Cl-A, each at 1 μg/ml concentration, were prepared in 0.01 M, pH 6.8 phosphate buffer and in buffer mixed v/v with 10% acetonitrile, 10% methanol or 20% methanol. The samples were stored at room temperature in closed vials. Aliquots of each were taken immediately and at several times over a twelve day period for HPLC analysis. In a separate experiment, standards with 2 μg/ml each F-ddI and 2-Cl-A were prepared in phosphate buffer with or without 0.1% sodium azide. These were treated in the manner described above.

Calibration Curves, Recovery and Precision

Stock solutions of F-ddA, F-ddI and 2-Cl-A in DMSO were stored at room temperature. Standards were prepared by adding appropriate amounts of stock solutions to blank plasma, preincubated for 5 min with 2'-dCF, or to blank, diluted urine. Standard curves were generated by least-squares linear regression fit of the log of the peak area ratio of the dideoxynucleoside to the 2-Cl-A internal standard versus the log of the analyte concentration.

Analyte recovery and precision were determined at three concentrations for monkey plasma and at one concentration for rat plasma. For each experiment sufficient 2'-dCF was added to thawed plasma to make it 20 μM and the sample equilibrated for 5 min at room temperature. The appropriate amount of F-ddA and F-ddI was added to this plasma and the sample mixed. For 5.0 ml spiked human plasma, four 1.0 ml aliquots were taken at each concentration. For 2.0 ml spiked rat plasma, three 0.50 ml aliquots were used. The spiked plasma aliquots and a blank plasma (1.0 ml for human, 0.5 ml for rat) with no internal standard were all treated by the standard sample workup procedure. Dideoxynucleoside standard solutions with the appropriate amount of internal standard were prepared in triplicate in 20% methanol in 0.01 M, pH 6.8 phosphate. These absolute standards and processed plasmas were then analyzed by HPLC. Recovery was calculated by dividing the mean of the absolute peak areas of the spiked plasma samples, corrected for blank, by the mean of the absolute peak area of the appropriate dideoxynucleoside standards. The relative standard deviation of the peak area ratios of the spiked plasma samples at each concentration level was used as a measure of precision.

Plasma Protein Binding

The bound and free fractions of F-ddA and F-ddI were determined at two concentrations in both rat and monkey plasma. The two compounds were tested separately to prevent possible

binding competition. For each experiment, fresh plasma with 20 μM 2'-dCF was spiked with a dideoxynucleoside at the appropriate level, allowed to equilibrate for at least 5 min at room temperature and then ultrafiltered in three 0.50 ml aliquots by centrifuging at 1900 X g in Amicon Centrifree micropartition units (W.R. Grace & Co., Beverly, MA). Blank plasma ultrafiltrate, direct standards in 0.9% NaCl solution and ultrafiltered standards were also prepared. The ultrafiltered plasma samples and the direct and ultrafiltered standard solutions were then analyzed by HPLC. The absolute peak areas of the F-ddA and F-ddI were used to calculate membrane holdup and plasma protein binding at each concentration level according to the following formulas.

$$\% \text{ Membrane holdup} = 100(1-P_{ufs}/P_{ds})$$

$$\% \text{ Bound} = 100[1-(P_{puf}-P_{bluf})/P_{ufs}]$$

P_{ufs} is the mean peak area of the ultrafiltered standards and P_{ds} is the mean peak area of the direct standards. P_{puf} is the mean peak area of spiked plasma ultrafiltrate and P_{bluf} is the mean peak area of the blank plasma ultrafiltrate, if any.

Plasma Stability

F-ddA stability was determined in plasma from three different monkeys and in pooled rat plasma. F-ddI stability was measured in plasma from one monkey and in pooled rat plasma. For experiments using monkey plasma, 5.0 ml plasma from freshly drawn blood was warmed to 37°C in a Dubnoff metabolic shaking incubator (Precision Scientific, Chicago, IL). For the study of F-ddA and F-ddI in pooled rat plasma, a 4.0 ml aliquot was used. The sample was spiked at 10 μg/ml with the appropriate dideoxynucleoside and kept in the shaker bath. At various times, 0.50 ml aliquots were taken and added to 1.0 ml chilled water containing 1.03 μg 2-Cl-A internal standard. For samples containing F-ddA, 2'-dCF was also added at this point to prevent further deamination. Samples were then immediately processed by the usual procedure for analysis by HPLC. For F-ddA experiments the peak area ratio of F-ddA to 2-Cl-

A versus time was fit to a monoexponential decay curve using the Inplot program (GraphPad Software, San Diego, CA). Appearance of F-ddI, as measured by peak area ratio to internal standard versus time, was fit to an exponential association curve. No weighting was used.

Stability of F-ddA in rat plasma was evaluated by a similar procedure. A 1.0 ml aliquot of fresh rat plasma was heated to 37°C in an Eppendorf Model 5320 thermostated block heater (Brinkman Instruments, Westbury, NY) and then spiked with 20 μg/ml F-ddA. A 50-μl aliquot was taken at predetermined times and added to 0.45 ml water along with internal standard and 2'-dCF. Each sample was heated to 95°C for 1 min, cooled and ultrafiltered in an Amicon Centrifree micropartition unit. Absolute peak areas of F-ddA and F-ddI versus time were then curvefit using Inplot software.

In Vivo Studies

The rat studies were conducted at Battelle Memorial Institute (Columbus, OH) as part of comparative dideoxynucleoside cardiotoxicity study (24) under Contract NO1-CM3-7834 to the Developmental Therapeutics Program, DCT, NCI. Female Sprague-Dawley rats (200-250 gm) were administered intravenous bolus doses of 2.5 to 250 mg/kg F-ddA in 0.9% NaCl solution. Blood samples (approximately 1 ml) were collected from each rat 2 min after dosing and immediately mixed with EDTA and 50 μl, 0.6 mM 2'-dCF. Plasma was separated from cells by centrifugation and then frozen until analyzed. Plasma from untreated animals was used for spiked standards to generate the appropriate calibration curves.

An adult male rhesus monkey weighing 6.7 kg was administered 20 mg/kg F-ddA as a 3-min intravenous push into the right saphenous vein as part of a protocol with the Pharmacology and Experimental Therapeutics Section (Pediatric Oncology Branch, DCT, NCI) to determine drug pharmacokinetics and metabolism in primates. Blood was drawn before dosing and at predetermined

times after treatment through a catheter placed into the left jugular vein as described in "Sampling". The resultant plasma was frozen and stored at -20°C until analysis. General procedures for both rat and monkey care, housing and treatment were in accord with published guidelines (25).

RESULTS AND DISCUSSION

The aim of this work was to develop a straightforward HPLC method suitable for use in preclinical studies to measure F-ddA and F-ddI at submicromolar concentrations in plasma and urine.

Chromatography

Reversed-phase HPLC has been widely applied for the analysis of endogenous nucleosides in biological samples (26,27). Because of their more lipophilic nature, dideoxynucleosides and their analogues are even more suited to analysis by this mode of HPLC (13). Although F-ddA and F-ddI were more strongly retained on a C_{18} column, a better separation from endogenous interferences in biological samples was achieved on a phenyl reversed-phase column. This better suitability of a phenyl column has also been noted for the analysis of ddA and ddI (8,21), which have chromatographic retention similar to their fluorinated analogues. Several phenyl packing materials were evaluated and two columns were found to give suitable results - the Waters Novapak phenyl and the Hypersil Phenyl-2. The latter is packed with an end-capped, bonded 5-μm spherical silica which maintained better peak shape and separation after multiple sample injections and prolonged use. With these columns, there were minimal interferences at the retention times of the compounds of interest in either plasma or urine from monkeys (Figure 2A) or rats (Figure 3A).

Since it was desirable to determine both F-ddA and its F-ddI metabolite for pharmacology studies, and since these two dideoxynucleosides differed substantially in their chromatographic retention (Table 1), elution with a linear acetonitrile gradient was

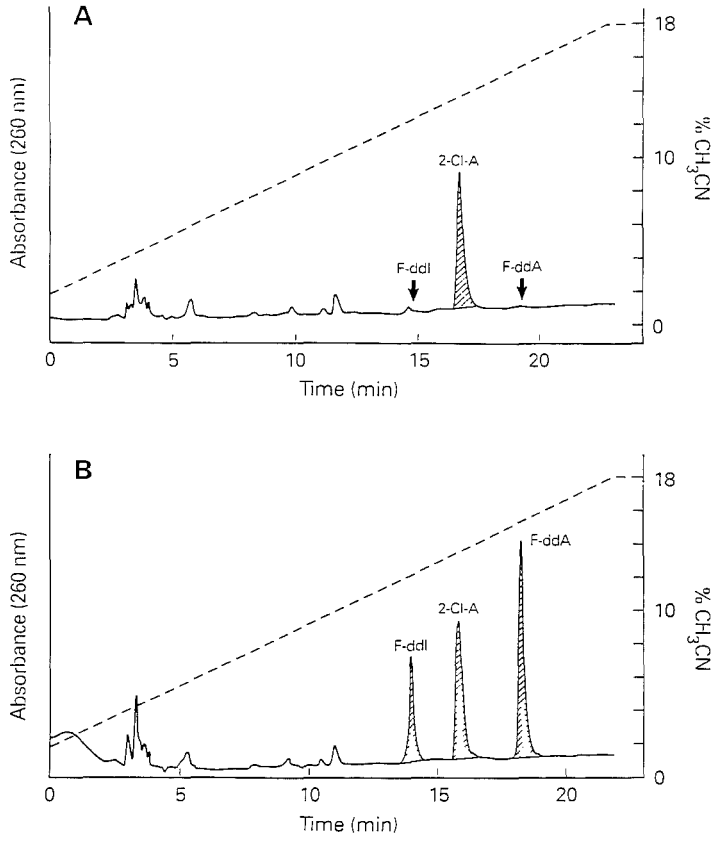


FIGURE 2. Gradient HPLC analysis of A) blank rat urine and B) rat urine spiked with F-ddA (2 μg/ml), F-ddI (2 μg/ml) and 2-Cl-A internal standard (1 μg/ml) on a 4.6 X 250 mm Hypersil Phenyl-2 column and Brownlee Spheri-5 phenyl cartridge precolumn. The dashed line shows the gradient of acetonitrile in the mobile phase. The bold arrows in chromatogram A) mark the expected elution position of the indicated analytes. See HPLC Conditions in Methods for full details.

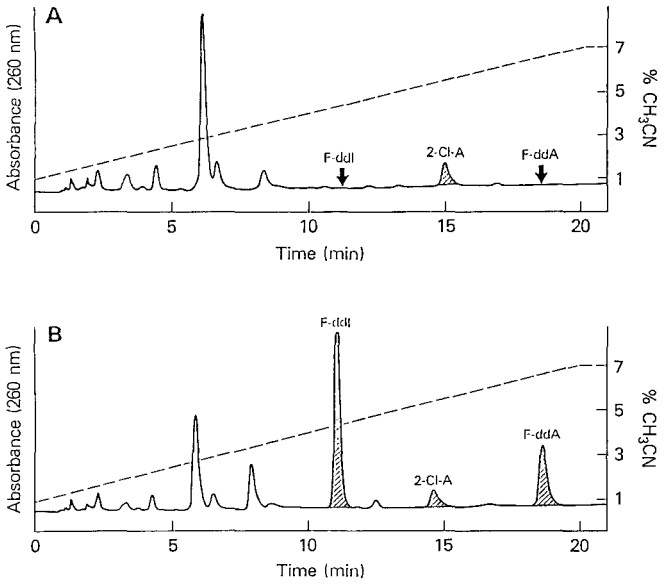


FIGURE 3. Gradient HPLC analysis on a 3.9 X 150 mm Waters Novapak phenyl column and Brownlee Spheri-5 phenyl cartridge precolumn of A) pretreatment monkey plasma and B) plasma from the same monkey 15 min after a 20 mg/kg intravenous dose of F-ddA. The measured concentration of F-ddA was 3.61 μg/ml and that of F-ddI was 17.8 μg/ml. The dashed line shows the acetonitrile gradient program.

employed to achieve an analysis cycle of 25 min. This included a 22-min gradient to final concentration, a 1-min reverse gradient to original conditions and a 2-min equilibration period (Figure 3). This latter 2-min interval, although not adequate for thermodynamic equilibrium, was sufficient to obtain reproducible retention if the column was initially conditioned by running a gradient cycle before the first injection. UV absorption at 260 nm was chosen for detection of the analytes because the greatest sensitivity was needed for F-ddA, and this wavelength is intermediate to the λ_{max} of both F-ddA and the 2-Cl-A internal standard (Table 1).

TABLE 1

Chromatographic Properties of the Analytes

Compound	RRT (Novapak)	RRT (Hypersil)	λ_{max} (nm)
F-ddA	1.25	1.10	259
F-ddI	0.78	0.88	247
2'-dCF	0.45	0.70	282
2-Cl-A	1.00	1.00	262

Internal Standard

2-Cl-A was selected as an internal standard for several reasons. Besides being commercially available, it was chemically similar to the compounds of interest in terms of UV absorption and chromatographic behavior (Table 1). Initially, stability problems were encountered with 2-Cl-A when it was left at room temperature in phosphate buffer overnight for automated HPLC analysis. Partial decomposition was noted; and, when 2-Cl-A standards in only phosphate buffer were kept at room temperature for two days, complete decomposition of 2-Cl-A and the appearance of a new, more polar peak was observed. 2-Cl-A stability was then evaluated in 10% acetonitrile in phosphate buffer as well as in 20% methanol in phosphate buffer. Standards were more stable in buffered acetonitrile, but possessed very poor chromatographic peak shape upon HPLC analysis. However, the standards in 20% methanol were stable for 12 days and the chromatography was not adversely affected. To determine if the above stability problem was due to microbial digestion, 1% sodium azide (a microbicide) was added to buffer spiked with 2-Cl-A. After 12 days at room temperature, little degradation of 2-Cl-A had occurred, confirming that the original problem was probably due to microbial contamination. Reconsti-

tuting plasma extract residues (vide infra) in 20% methanol in phosphate buffer ensures that processed samples are stable overnight at room temperature, and for at least a week, if frozen.

Sample Preparation

The sample preparation procedure in Scheme I is a modification of a method previously used for other purine dideoxynucleosides (7,28). The adenosine deaminase inhibitor, 2'-dCF, is added directly to the blood collection tubes before sampling to stop immediately any *in vitro* conversion of F-ddA to F-ddI. After plasma is separated from blood cells, 1 μg 2-Cl-A is added to a sample aliquot, giving an internal standard concentration in the middle of the expected analyte range. Then, since the dideoxynucleosides are more lipophilic than the endogenous nucleosides in plasma, a C_{18} solid-phase extraction is used to isolate and concentrate the compounds of interest. The solid-phase extraction step also serves to deproteinize the sample. The degree of sample concentration, of course, depends on the initial sample aliquot and the volume of 20% methanol in phosphate buffer used to reconstitute the residue. Typically, a volume corresponding to 10% of the original sample is used for an individual analysis.

F-ddA and F-ddI recovery using the isolation procedure of Scheme I was essentially complete for a 0.1 to 10 μg/ml range in monkey plasma (Table 2). Recovery of the 2-Cl-A internal standard, which was evaluated at the 1 μg/ml level usually used for the assay, was also comparably high. Precision, determined simultaneously with recovery, was better than 7% RSD for both F-ddA and F-ddI over all concentrations in monkey plasma. The recovery and precision in rat plasma were evaluated at 1 μg/ml for all three compounds, and the results were similar (Table 2).

Quantitation

Standard curves were evaluated for both plasma and urine from monkey and rat. These standard curves were based on the peak

TABLE 2

Analyte Recovery and Measurement Precision

Concentration	Mean Recovery (%) F-ddA	F-ddI
Monkey Plasma (n=4)		
100 ng/ml	102 ± 7	95.4 ± 14
1 μg/ml	97.2 ± 8	103 ± 12
10 μg/ml	95.4 ± 4	95.1 ± 4
Rat Plasma (n=3)		
1 μg/ml	110 ± 6	107 ± 3
Concentration	**Precision (RSD, %) F-ddA**	**F-ddI**
Monkey Plasma (n=4)		
100 ng/ml	4.0 (0.156)[a]	6.5 (0.0973)
1 μg/ml	6.2 (1.33)	6.4 (0.657)
10 μg/ml	2.8 (14.9)	2.2 (6.18)
Rat Plasma (n=3)		
1 μg/ml	10.4 (1.24)	8.8 (1.22)

[a] Mean peak area ratio

area ratios of F-ddA or F-ddI to internal standard and were linear over the range of spiked standards (0.050 to 10 μg/ml). Both the F-ddA and F-ddI standard curves had correlation coefficients better than 0.999 for all the matrices studied. For plasma standards that covered the entire concentration range, calculated concentrations were closer to actual values for low levels (< 250

ng/ml) if a log concentration *versus* log area ratio linear regression was utilized. The limit of quantitation (S/N $\geq$ 5) for this method was 50 ng/ml (~0.2 μM) for both F-ddA and F-ddI.

Protein Binding

Protein binding was determined individually for each compound by centrifugal ultrafiltration in both rat and monkey plasma. This is an important therapeutic parameter because only free drug can penetrate the blood-brain barrier and reach HIV sequestered in the brain (11). Plasma protein binding of F-ddA and F-ddI was essentially zero for the monkey (Table 3). A small percent of protein binding was observed for F-ddA and F-ddI in rat plasma (Table 3). Protein binding of this extent, however, can usually be ignored when the pharmacokinetics of a compound is being determined. No ultrafiltration membrane interaction or holdup was observed for either F-ddA or F-ddI. It should also be noted that error associated with the measured plasma protein binding, which has been estimated from the sum of the RSD's of the absolute peak areas in plasma and standard solution for each compound, appears large because the percent of bound drug is so small. The parent drugs, ddA and ddI, were also observed to have no significant plasma protein binding (20,28,29).

Stability in Plasma

The stability of both F-ddA and F-ddI was investigated in fresh plasma at 37°C to determine what precautions might be required during sampling and workup. 2',3'-Dideoxyadenosine, the parent compound for F-ddA, was rapidly degraded by plasma adenosine deaminase (ADA) and had a half-life of less than 10 min in fresh rat plasma (28,30). The deamination kinetics of F-ddA was evaluated in plasma from individual monkeys as well as in pooled rat plasma. F-ddA was deaminated to form F-ddI, but with a much longer half-life of at least 4 hr in monkey plasma (Figure 4). A similar result was observed for of F-ddA in pooled rat plasma,

TABLE 3

Plasma Protein Binding

Concentration	Plasma Protein Binding (%)	
	F-ddA	F-ddI
Monkey plasma		
1 μg/ml	9.4 ± 11.1	8.2 ± 11.1
250 ng/ml	4.0 ± 10.6	0 ± 14.1
Rat plasma		
1 μg/ml	15.7 ± 2.4	15.8 ± 3.3
250 ng/ml	13.7 ± 8.1	7.3 ± 8.7

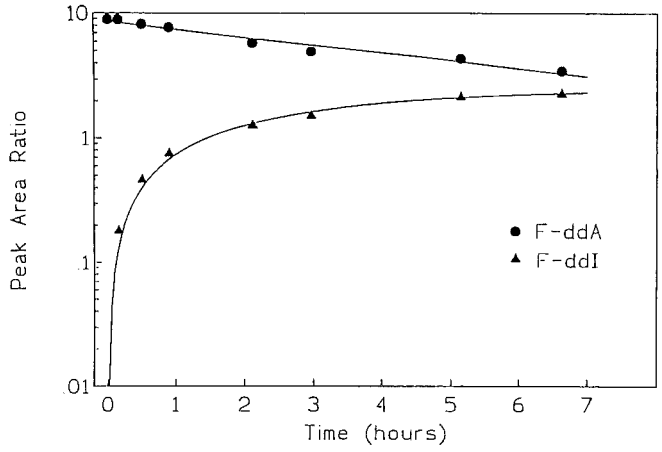


FIGURE 4. Stability of F-ddA (○) in fresh monkey plasma at 37°C and corresponding formation of F-ddI (△). Deamination of F-ddA was curvefit to a monoexponential decay, shown by the solid line, which led to a half-life of 4.8 hr. The appearance of F-ddI, represented by the dashed line, was fit to an exponential association curve. Both fits were performed using GraphPad Inplot software with no weighting.

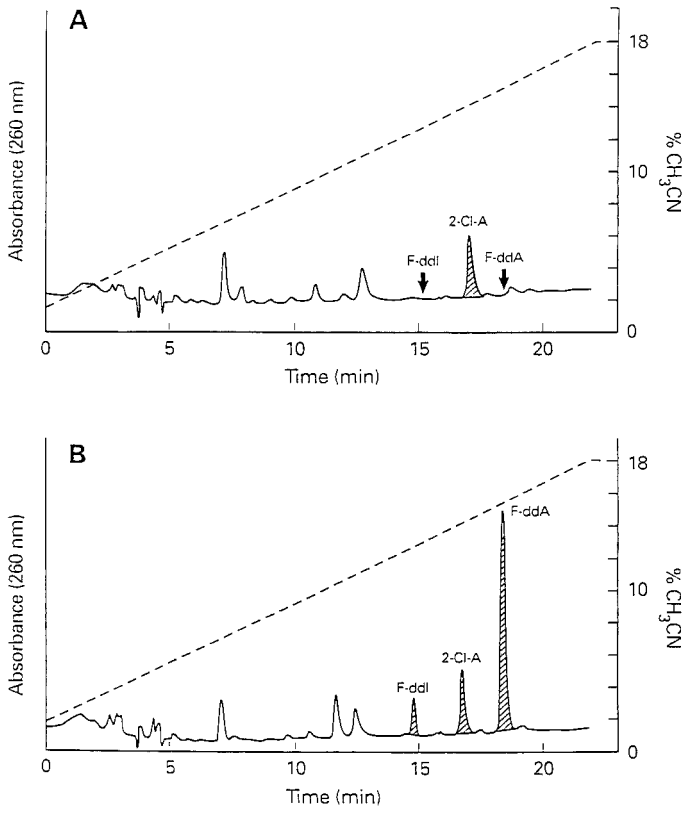


FIGURE 5. Gradient HPLC analysis on a 4.6 X 250 mm Hypersil Phenyl-2 column and Brownlee Spheri-5 phenyl cartridge precolumn system of A) pretreatment rat plasma and B) rat plasma obtained 2 min after a 12.5 mg/kg intravenous bolus dose of F-ddA. The concentration of F-ddA was 9.6 μg/ml and that of F-ddI was 3.35 μg/ml. The dashed line shows the mobile phase acetonitrile gradient.

where the half-life was 4.1 hr. Although F-ddA was a much poorer substrate for adenosine deaminase than ddA, use of 2'-dCF as an ADA inhibitor was still required during blood sampling to prevent further deamination and distortion of actual F-ddA and F-ddI blood levels. F-ddI was much more stable than F-ddA in both rat and monkey plasma, showing less than a 15% loss after 24 hr at 37°C. This was not surprising since the ddI parent showed similar stability in human plasma (28).

Application of the Method

This method has been used to document plasma concentrations of F-ddA and F-ddI following dosing at different levels and by various schedules during a comparative cardiotoxicity study of dideoxynucleosides in Sprague-Dawley rats (24). Figure 5B shows a representative chromatogram of the plasma of a rat that received F-ddA as an intravenous bolus dose. As expected, but in contrast to the rapid deamination observed with ddA, rats dosed with F-ddA had much higher levels of unchanged drug than of the F-ddI metabolite (24). This slower deamination rate of F-ddA is significant because the dideoxyinosine catabolite is more polar and presumably less able to cross the blood brain barrier. Therefore, higher levels of F-ddA relative to F-ddI may be beneficial for CNS drug penetration. The greater resistance of F-ddA to metabolic clearance from the plasma also means the drug should persist for a longer time after administration. This property may be advantageous in devising a dosing regimen. This assay has also been applied in a study of the bolus dose plasma kinetics of F-ddA in rhesus monkeys. Although deamination to F-ddI was the major route for clearance of this drug from plasma, concentrations of F-ddA corresponding to those required for *in vitro* activity persisted for an extended period (Figure 3).

Conclusion

A straightforward reversed-phase HPLC method that measures both F-ddA and F-ddI at submicromolar sensitivity in biological

samples has been developed and analytically evaluated. This method has a quantitative range of 0.2 - 10 μM (0.05 - 10 μg/ml) and a measurement precision of better than 10%, making it suitable for use in pharmacokinetic studies. Since solid-phase extraction forms the basis of the isolation procedure from the biological matrix, full assay automation using a robotic sample preparation unit is possible. This may be of value in adapting this method for the analysis of HIV-infected human samples during a future clinical trial of this anti-HIV drug.

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CONSEQUENCES OF ION-EXCHANGE AND SORPTION EFFECTS ON pH WAVES PROPAGATING IN A STRONG-BASE ANION-EXCHANGE COLUMN

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ABSTRACT

When pH buffers are used in frontal anion exchange chromatography, it is known that important transient pH variations may occur, even if pHs are identical in preequilibration and feed. This effect was earlier studied with the aid of equilibrium theory extended to the case where dissociation reactions are involved. In the present paper, the sodium acetate/acetic acid system has been revisited but accounting for sorption effects. Sorption experiments were performed to determine the ratio between stationary and mobile phases as a function of ionic concentration. Column experiments with respectively alkaline and acid acetate buffers have been performed and modelled (computer program Impact). Sorption effects have been taken into account; a good prediction of pH and acetate breakthrough curves has thus been achieved.

INTRODUCTION

Frontal ion-exchange chromatography is a well-developed technique, increasingly applied to extraction and purification of molecules of biological interest

(amino-acids, proteins etc.) [1]. These molecules are very sensitive to pH and , a constant pH value must be imposed in the course of separation: pH buffers are used to achieve this goal. But chromatographic processes generally comport a step where buffer ionic strength is modified. For example, ionic concentration is significantly increased to elute sorbed species. It is commonly believed that the use of a pH buffer imposes pH regardless of any properties of the ion-exchanger. Unfortunately, a modification of ionic concentration may transiently bring important pH alterations as a consequence of ion exchanges between buffer components and adsorbed species.

An experimental and theoretical study of this phenomenon has been proposed by Helfferich and Bennett [2-4]. The first part of this work was concerned with systems composed of a weak acid and its sodium salt (sodium acetate/acetic acid; sodium carbonate/carbonic acid). For each system, two solutions of different ionic concentration were successively injected into a column of strong-base anion exchanger [2] and important pH excursions in the column outflow were observed. The second part of the work dealt with a three-component exchange involving a weak acid and its sodium salt with an additionnal species not undergoing a reaction. The chosen system was sodium acetate/acetic acid/sodium chloride with the same type of ion-exchanger as in the first part [3]. The theoretical interpretation of experiments was based on the so-called "equilibrium theory" essentially developed by Klein, Helfferich and Tondeur [5-10]. The main assumptions of this type of model are: plug-flow inside the column (hydrodynamic dispersion is neglected); no shrinking, no swelling (the volume of each phase is assumed constant); thermodynamic equilibrium attained at any point of the column at any time (local equilibrium assumption); isothermal behavior; description of ion-exchange equilibria by means of constant binary separation factors. From these assumptions,

in the case of a coherent wave (instantaneous modification of the composition of the solution entering the column), the resolution of mass balances by the method of characteristics allows the prediction of the number and natures of concentration propagating waves. The first systems (sodium salt/weak acid) were shown to generate two waves, identified as a "sodium wave" and an "ion-exchange wave", and the three-component system three waves, a sodium wave and two ion-exchange ones.

The comparison of equilibrium theory predictions with experimental results [2] shows that this theory allows a semi-quantitative prediction of solute concentration variations observed at the column outflow. Theory fails to precisely describe experiments because of limitations due to hydrodynamic and thermodynamic assumptions and moreover so important reactions as sorption of neutral species or penetration of electrolytes into the resin are mentioned but not taken into account.

A more complete approach to physico-chemical interactions should improve agreement between model and experiments. It is worth underlining that equilibrium theory was first worked out for systems involving only adsorption or ion-exchange reactions and was extended later to cases in which homogeneous reactions like dissociation or heterogeneous reactions like precipitation/dissolution must be considered [11-16]. But it turns out to be very complex and even useless for systems involving too many species and reactions.

Another approach consists in using equilibrium theory on the one hand for a semi-quantitative treatment of problems: analysis of involved species and reactions, computation of wave number and nature [12,13]. On the other hand, a computer code in which physicochemical interactions are coupled with hydrodynamics may be helpful. The Impact computer code worked out by Jauzein et al. [17] has been used in this work.

In this paper, a study about the propagation of pH and acetate waves in a strong-base anion-exchanger column is presented. The column behavior is investigated when the column is equilibrated with a pH buffer and then fed with a solution of the same buffer at a different ionic concentration. The sodium acetate/acetic acid system developed by Helfferich and Bennett is revisited with experimental complements in particular about acetate breakthrough and sorption. Results are modelled by means of the Impact computer program and sorption effects are taken into account.

MODELLING OF FLOW AND OF PHYSICOCHEMICAL INTERACTIONS

Flow Description

To describe flow and mass-transfer inside a fixed bed of total volume V_t, it is important to define precisely which phases are taken into account [18] and what their respective volumes are. The bed is represented by two phases (Figure 1), that are:

- one mobile phase or fluid phase of volume V_m, composed of the volume accessible to solution and thus to all ions by convection and diffusion: that is the sum of external or interstitial volume V_e and of internal resin volume accessible to cations V_{ia}.
- one stationary zone of volume V_s including the motionless resin-bound water (volume V_i) from which cations are excluded (except hydronium ions produced by immobile water hydrolysis) and the resin itself, with its true solid phase volume V_r.

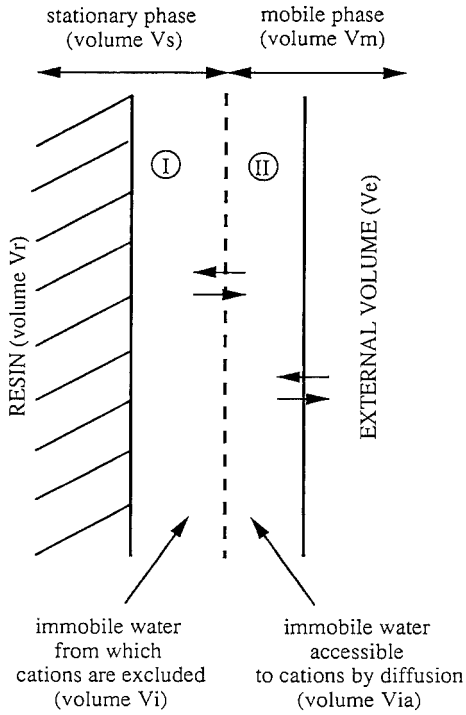

FIGURE 1 : Schematic representation of the different zones of the resin bed

It is important to point out that the borderline between the two zones is located somewhere inside the resin and not right between interstitial and apparent (V_{ap}) resin volume. The relations between these volumes are:

$$V_{ap} = V_r + V_i + V_{ia} \quad (1)$$

and $$V_t = V_{ap} + V_e = (V_r + V_i) + (V_{ia} + V_e) = V_m + V_s \quad (2)$$

Usually, the frontier between mobile and stationary zones is imposed by geometric constraints: external or intersticial fluid transported by convection on the one hand and internal fluid accessible to ions by diffusion on the other hand. Here a

distinction is made between fluid accessible to cations and fluid inaccessible to cations. The frontier is determined by ionic exclusion: it depends not only on the porous medium geometry but also on the total ionic concentration of the mobile solution.

This description is based on the assumption of local equilibrium. The fluid velocity is slow enough so that mass transfer limitations between phases may be neglected.

Fluid flow in the mobile zone is assumed to be convective-dispersive and one-dimensional. It is modelled by mixing cells in series: the bed is represented by a network composed of J basic identical elements with a constant geometry [18]. In each element, the mobile and stationary phases are respectively located in the volumes V_m/J and V_s/J. The two parameters V_m and J are deduced from sodium breakthrough (see experimental section).

Physicochemical Interactions

For the studied system, three types of reactions are involved [17]: (1) dissociation of neutral species in the mobile phase (homogeneous reaction), (2) anion exchange, (3) sorption of neutral species (heterogeneous reactions). Let R be the number of independent reactions, these reactions can be written in terms of stoichiometric relations as:

$$\sum_{j=1}^{N} \nu_{ij} A_j = 0 \qquad i = 1 \text{ to } R \qquad (3)$$

where N is the number of chemical species, ν_{ij} is the stoichiometric coefficient for species A_j in the reaction i (positive for products, negative for reactants). A_j is a

species in a given phase: a species present in two different phases is given by two different numbers or symbols. For instance, hydroxyl ion in the mobile zone is represented by OH^- (species A_1 for example) as hydroxyl ion in the stationary zone is represented by $\overline{OH^-}$ (species A_2 for example).

Equilibria are described by mass-action laws:

$$K_i = \prod_{j=1}^{N} a_j^{\nu_{ij}} \qquad (4a)$$

where a_j is the activity of A_j. As a first approximation activities are assimilated to concentrations ; ν_{ij} is the required exponent for homogeneous reactions, it is replaced by zero for species in excess or with an activity equal to unity (pure precipitate).

$$K_i = \prod_{j=1}^{N} (A_j)^{\nu_{ij}} \qquad (4b)$$

(A_j): concentration of A_j

All concentrations are expressed in moles per liter of mobile phase, the species being either in the mobile or in the stationary phase.

Set of Assumptions

The set of assumptions can be summed up to:

(1) Ion-exchange, dissociation and sorption equilibria are reached at any point anytime (local equilibrium).

(2) Cations are partially excluded from the interior of the resin.

(3) Neutral species penetrate into the resin.

(4) The bed volume is constant (swelling or shrinking does not exceed 5% in general).

(5) Mass transfer in the bed is dispersive-convective in the flow direction.

(6) The system is isothermal.

(7) Each phase is electrically neutral.

(8) Activity coefficients equal unity.

(9) The separation factors of binary ion-exchanges defined in terms of concentrations are constant.

BEHAVIOR OF THE SODIUM ACETATE/ACETIC ACID SYSTEM

Species and Equilibria

This system is relatively simple, giving rise to four independent reactions:

- water dissociation

$$H_2O = H^+ + OH^- \qquad K_w = (H^+)\,(OH^-) \quad [mol/l]^2 \qquad (5)$$

- acetic acid dissociation

$$AcOH = AcO^- + H^+ \qquad K_a = \frac{(AcO^-)\,(H^+)}{(AcOH)} \quad [mol/l] \qquad (6)$$

- anionic exchange between acetate and hydroxyl ions:

$$AcO^- + \overline{OH^-} = \overline{AcO^-} + OH^- \qquad K_{AcO,OH} = \frac{\overline{(AcO^-)}}{(AcO^-)}\,\frac{(OH^-)}{\overline{(OH^-)}} \qquad (7)$$

(adimensional)

- sorption of undissociated acetic acid:

$$AcOH = \overline{AcOH} \qquad K_D = \frac{\overline{(AcOH)}}{(AcOH)} \quad \text{(adimensional)} \qquad (8)$$

To be rigorous in what concerns sorption, two additional species and reactions must be considered to describe dissociation occuring in the stationary phase:

$$\overline{H_2O} = \overline{H^+} + \overline{OH^-} \qquad \overline{K_w} = \overline{(H^+)}\ \overline{(OH^-)} \tag{9}$$

$$\overline{AcOH} = \overline{AcO^-} + \overline{H^+} \qquad \overline{K_a} = \frac{\overline{(AcO^-)}\ \overline{(H^+)}}{\overline{(AcOH)}} \tag{10}$$

(mol/l of mobile phase)^2

(mol/l of mobile phase)

The following relations between the sorbed species can then be written:

$$\overline{(AcOH)} = \frac{\overline{(AcO^-)}\ \overline{(H^+)}}{\overline{K_a}} = \frac{\overline{(AcO^-)}}{\overline{(OH^-)}}\ \frac{\overline{K_w}}{\overline{K_a}} \tag{11}$$

From equations (6) and (7):

$$\overline{(AcOH)} = K_{AcO,OH}\ \frac{\overline{K_w}}{\overline{K_a}}\ \frac{(AcO^-)}{(OH^-)} = K_{AcO,OH}\ \frac{\overline{K_w}}{K_w}\ \frac{K_a}{\overline{K_a}}\ (AcOH) \tag{12}$$

Let us assume the continuity of dissociation constants from one phase to another one. K_w and K_a (resp. $\overline{K_w}$ and $\overline{K_a}$) are relative to homogeneous reactions and should be referred to the phase in which they occur. Thus to express equality between $\overline{K_w}$ and K_w (resp. $\overline{K_a}$ and K_a), it is necessary to introduce the volume ratio V_m/V_i, which gives $\left[\frac{V_m}{V_i}\right]^2 \overline{K_w} = K_w$ and $\left[\frac{V_m}{V_i}\right] \overline{K_a} = K_a$.

Reporting eq. (8) into (12), the Donnan constant K_D becomes:

$$K_D = K_{AcO,OH}\ \frac{\overline{K_w}}{\overline{K_a}}\ \frac{K_a}{K_w} \cong \frac{V_i}{V_m}\ K_{AcO,OH} \tag{13}$$

Finally, there are only four independent reactions (5) to (8). On the whole, there are:

- 5 species in the mobile phase ($N_l = 5$): AcOH, AcO^-, H^+, Na^+, OH^-,
- 3 species in the stationary phase ($N_S = 3$): $\overline{AcO^-}$, $\overline{OH^-}$, $\overline{AcOH}$

Water is eliminated since its activity equals unity. Sodium ion is present as an inert

species. The reaction scheme can be written under the form of a stoichiometric matrix with 4 rows and 8 columns:

$$\nu_{4,8} = \begin{array}{c} \begin{array}{cccccccc} H^+ & OH^- & AcOH & AcO^- & Na^+ & \overline{AcO^-} & \overline{OH^-} & \overline{AcOH} \end{array} \\ \begin{bmatrix} 1 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & 0 & -1 & 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & -1 & 0 & 1 & -1 & 0 \\ 0 & 0 & -1 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} \end{array} \begin{array}{l} (5) \\ (6) \\ (7) \\ (8) \end{array} \quad (14)$$

mobile species stationary species

The rank of this matrix equals the number of independent reactions R = 4. The matrix (14) can be separated in two submatrices, associated with mobile and stationary species, the ranks of which being respectively $R_l = 3$ and $R_s = 2$. Following the rules enounced by Schweich et al. [12,13], the number of Stoichiometric Inert Species or SIS is the number of species minus the number of independent reactions:

$$n_{SIS} = N - R = 4 \quad (15)$$

The number of Stoichiometric Inert Species in the mobile phase, known as stoichiometric tracers is $N_l - R_l = 2$, these stoichiometric tracers are:

- electroneutrality

$$S_1 = (AcO^-) + (OH^-) - (H^+) - (Na^+) = 0 \quad (16a)$$

- sodium, as a true inert species:

$$S_2 = (Na^+) \quad (16b)$$

In the same way, the number of SIS in the stationary phase is $N_s - R_s = 1$. There is only one stationary SIS, that is actually the electroneutrality in the stationary phase:

$$S_3 = (\overline{OH^-}) + (\overline{AcO^-}) - N_E = 0 \quad (16c)$$

where N_E is the exchange capacity expressed in moles per liter of mobile phase. The fourth SIS is a combination associating species in the mobile phase and in the stationary phase. The total acetate mass balance is a typical example:

$$S_4 = (AcOH) + (AcO^-) + (\overline{AcO^-}) + (\overline{AcOH}) = (Ac) \quad (16d)$$

Consequences of Physicochemistry on the form of Breakthrough Curves

Stoichiometric Matrix and Number of Fronts The number of fronts is obtained from the stoichiometric matrix [12,13], that is the number of combined SIS:

$$F = R_I + R_S - R \qquad (17)$$

This number is sometimes called "sorption variance" of the transport problem [11,14]. Moreover, the existence of stoichiometric tracers (SIS depending on the mobile phase exclusively) may give birth to unretarded waves travelling at fluid velocity, if the stoichiometric tracer concentration in the initial solution and in the feed solution are different.

In the case of the sodium acetate/acetic acid system, sodium ion is the only stoichiometric tracer affected by a concentration variation at the time when the feed solution is introduced into the preequilibrated column. It can be used as a flow tracer to determine of the different zone volumes.

The number of retarded fronts is the same as in [2] despite the occurence of the sorption reaction (one new reaction and one new species are added simultaneously), the SIS number remains unchanged. Given the linear relation between AcOH and $\overline{AcOH}$, S_4 can be written directly as a function of only three species:

$$S_4 = (AcOH)\,(1+ K_D) + (AcO^-) + (\overline{AcO^-}) \qquad (18)$$

As a conclusion, when a strong-base anion-exchange column equilibrated with a sodium acetate/acetic acid buffer is submitted to a step-wise injection of the same buffer at a different concentration, two waves are observed: an indifferent wave travelling at fluid velocity across which sodium concentration varies and a retarded one in which sodium concentration remains unchanged as reactive species concentrations vary simultaneously. These waves are separated by a constant

composition zone or intermediate plateau zone. Knowledge of the composition of this zone and of the nature and location of the retarded wave allows the construction of the breakthrough curves.

Composition Calculation on the Intermediate Zone and Position of the Retarded Wave Before presenting the computation of the intermediate plateau zone composition with Impact, let us recall the basic principles of an a priori calculation with the equilibrium theory. In this problem, the retarded front is self-sharpening ($K_{AcO,OH}$ greater than unity) and the ion-exchange wave travels at the velocity $\mathbf{U}$:

$$\mathbf{U} = \frac{\mathbf{U}_0}{1 + \frac{\Delta(\overline{Ac})}{\Delta(Ac)}} \qquad (19)$$

where: $\mathbf{U}_0$ is the fluid velocity (m/s), $(\overline{Ac})$ and (Ac) are the total acetate concentration in the mobile and stationary phases, respectively (mol/l of mobile phase) and Δ represents the difference between the concentrations in the feed solution and in the intermediate zone.

Since the first wave is indifferent, the stationary phase composition remains unchanged from the initial plateau zone to the intermediate one. Conversely, the total ionic concentration in the mobile phase varies because of sodium step. This new sodium concentration (feed concentration) is reported into electroneutrality (16a), which enables us to calculate the mobile phase composition in the intermediate zone.

Case 1: Alkaline Medium The column is equilibrated with a dilute acetate buffer at alkaline pH and fed with a concentrated alkaline acetate buffer. The whole acetate

is dissociated and there is no sorption effect. Matrix (14) reduces to N = 6 species and R = 2 reactions:

$$\nu_{2,6} = \begin{bmatrix} H^+ & OH^- & AcO^- & Na^+ & \overline{AcO^-} & \overline{OH^-} & \\ 1 & 1 & 0 & 0 & 0 & 0 & (5) \\ 0 & 1 & -1 & 0 & 1 & -1 & (8) \end{bmatrix} \quad (20)$$

now $N_I = 4$, $R_I = 2$, $N_S = 2$, $R_S = 1$ (same SIS and wave numbers).

The propagation velocity of the ion-exchange wave is:

$$\mathbf{U} = \frac{\mathbf{U}_0}{1 + \dfrac{\Delta(\overline{AcO^-})}{\Delta(AcO^-)}} \quad (21)$$

The position of the exchange wave is in terms of reduced eluted volume:

$$V' = L\,\Omega\,\frac{\mathbf{U}_0}{\mathbf{U}} \quad (22)$$

where L and Ω are the column length (m) and section (m^2) respectively.

Case 2: Acid Medium The column is equilibrated with a dilute acetate buffer at acid pH and fed with a concentrated one at acid pH. Acetic acid is now partially dissociated and all species and reactions must be considered (stoichiometric matrix (14)).

Although theory predicts the existence of an ion-exchange wave independently of sorption effects, the variation of acetate ion concentration in each phase between final and intermediate zones is almost zero. Thus in the absence of sorption, the ion-exchange wave would travel at a velocity very close to fluid velocity. Consequently, the intermediate zone would have a so short duration that it could not be experimentally detected.

If sorption effects are taken into account, $\Delta(\overline{Ac})$ becomes important because of the sorption of undissociated acid, and wave velocity is strongly affected:

$$U \equiv \frac{U_0}{1 + \frac{\Delta(\overline{AcOH})}{\Delta(AcOH)}} \cong \frac{U_0}{1 + K_D} \qquad (23)$$

This relation predicts the occurence of an intermediate zone with a significant length depending only on sorption effects.

Computation with IMPACT

Impact has been worked out [17] with the set of assumptions previously presented. It allows the computation of equilibrium in a batch system as well as the computation of breakthrough curves and profiles in a flow system.

To compute breakthrough curves one must supply:

- the elementary reactions (species, stoichiometric coefficients, equilibrium constants),
- the mechanisms composed of sets of elementary reactions. For column experiments, two mechanisms are required: one including reactions in the mobile phase and one including all the reactions. The former allows the computation of the equilibrium composition of preequilibrant and feed solutions and the latter the equilibrium composition of the two phases along the bed.
- the flow network (number of mixing cells in series J),
- reference compositions of preequilibrant and feed solutions and of the stationary phase. From these reference compositions (corresponding here to experimental values), Impact computes the equilibrium composition of both phases at initial and

final states, that gives actually initial and limit conditions. Impact is then ready to compute all the required breakthrough curves and profiles.

EXPERIMENTS

Experiments were made in two steps: to begin, penetration of electrolytes into the resin as a function of ionic concentration has been measured and then column experiments were performed as an illustration of the presented theory.

Penetration of Cations into a Strong-Base Anion Exchange Resin

The chosen resin is a gel type strongly basic anion-exchange resin Dowex SBR-P with a granulometry 0.6-0.8 mm. This resin is placed in a glass column (diameter: 1.4 cm; bed length: 18 cm). Penetration of cations is evaluated by measuring the volume accessible to a sodium chloride solution by a residence time distribution measurement.

For convenience, volume fractions V_x/V_T will be denoted by ε_x, with x being either m, e, i or ia (see eq. (2)).

A typical experiment consists in stabilizing the column at a given ionic concentration with a sodium chloride feed, the resin being saturated with chloride. At t=0, the feed concentration is slightly modified and the system response measured in the column outflow and amplified (with a conductimetric cell and a zero-annulation conductimeter). The mean residence time of sodium ion in the column is deduced from the mean position of the step response, after correction of

apparatus dead volumes: the total volume accessible to sodium ion ($V_m=V_e+V_{ia}$) is thus obtained for each feed concentration.

The same experiments are repeated, the column being saturated with acetate ion and fed with sodium acetate. For both series, ε_m is plotted against the mobile phase concentration (Figure 2).

When ionic concentration tends towards zero, cations are totally excluded: the external porosity ε_e is given by the intersection point of both curves with the ordinate axis (Fig. 2). In the same fashion, a measurement of the total penetration would give the total porosity:

$$\varepsilon_t = \frac{V_t - V_r}{V_t} = \varepsilon_e + \varepsilon_{ia} + \varepsilon_i \qquad (24)$$

The internal porosity β is deduced from ε_t, it is defined as usual by :

$$\beta = \frac{\varepsilon_t - \varepsilon_e}{1 - \varepsilon_e} \qquad (25)$$

In Figure 2, both experimental curves intercept the ordinate axis at the same point: $\varepsilon_e=0.39$ (close to 0.40, usual value for spherical beads).

For the highest sodium chloride concentration (1.9 mol/l), curve 1 begins to reach a plateau: electrolyte penetration is almost totally achieved and the total porosity is about 0.65 (this value is certainly a little underestimated, it could reasonably reach 0.70 and even more). It corresponds to an internal porosity $\beta=0.43$. Acetate, unlike sodium chloride indicates for the same concentration an accessible porosity $\varepsilon_m=0.55$; this suggests that penetration is not yet total. In the following, the value obtained with chloride will be used for K_D estimation.

Column Experiments

The apparatus was composed of a tank containing the feed solution, a peristaltic pump, a small volume cell containing the bulbe of a pH electrode and a fraction

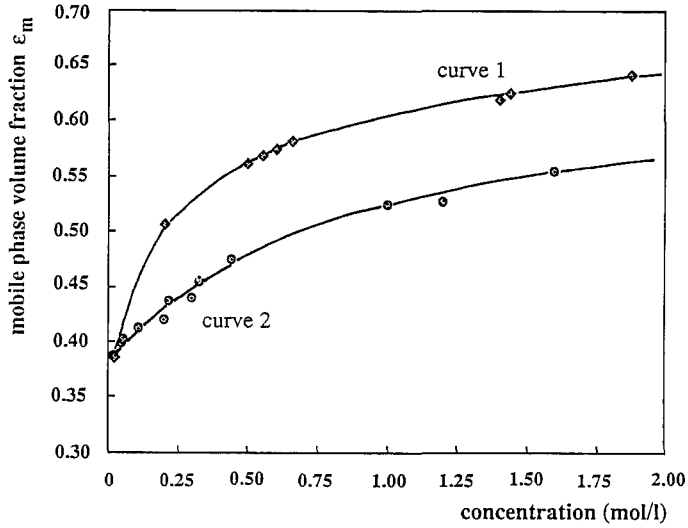

FIGURE 2 : Variations of the mobile phase volume fraction with concentration
◇ curve 1: sodium chloride
⊙ curve 2 : sodium acetate

collector. The characteristics of the system are: total exchange capacity 1.22 eq per liter of column; bed volume: 45.4 ml; flowrate: 2 ml/mn; column diameter: 1.4 cm. The dead liquid volume between injection and detection (independently on porous medium) was evaluated to 8 ml. pH was measured with a high-alcalinity electrode. Analysis were made by atomic absorption spectrometry for sodium ions (λ = 330.3 nm) and by ion chromatography for anions (Dionex 4000 I, CS2 column, eluent: sodium borate for total acetate).

The experiments performed at alkaline and acid pH are presented in Table 1.

The raw values of elution volume are corrected by substraction of the dead volume and reported to the mobile phase volume. Experimental curves of Figure 3 and 4 are thus obtained and compared with the results predicted by equilibrium model and by numerical simulations.

TABLE 1: Experimental compositions of the preequilibrant and feed solutions for Cases 1 and 2.

	Case 1: alkaline pH		Case 2: acid pH	
	Preequilibrant	Feed	Preequilibrant	Feed
total acetate (mol/l)	0.016	0.480	0.104	1.068
sodium (mol/l)	0.022	0.530	0.048	0.504
pH	11.75	12.70	4.66	4.71

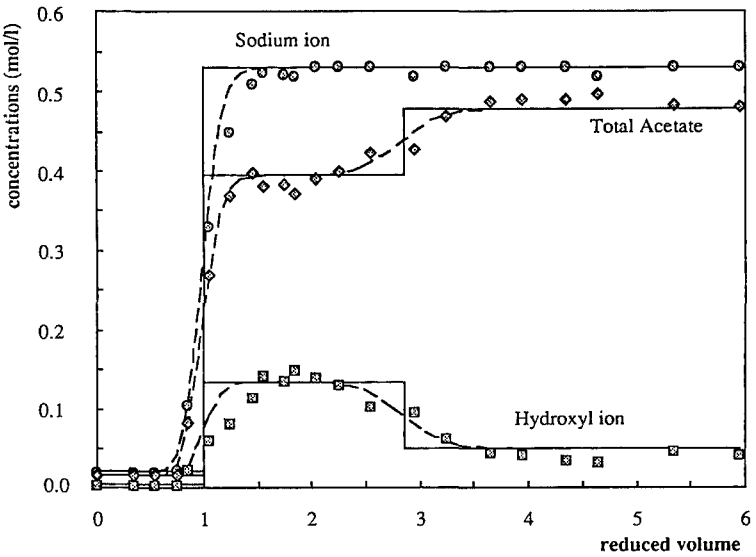

FIGURE 3a : Case 1 - Experimental and modelled breakthrough curves of total acetate, sodium ion and hydroxyl ion

Experimental curves : ◇ total acetate, ◎ sodium ion , ▨ hydroxyl ion
Modelled curves : — Equilibrium Theory predictions, – – Impact results

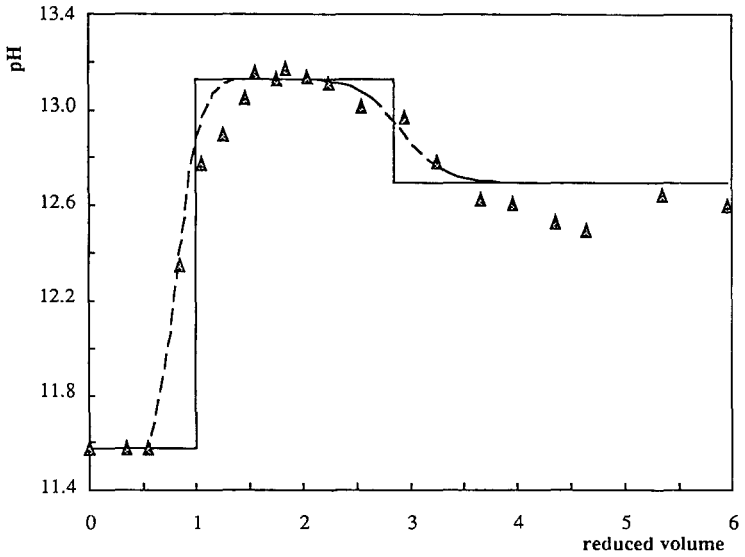

FIGURE 3b : Case 1 - Experimental and modelled variations of pH in the column effluent

Experimental curve : ▲ pH
Modelled curves :— Equilibrium Theory predictions,– – Impact results

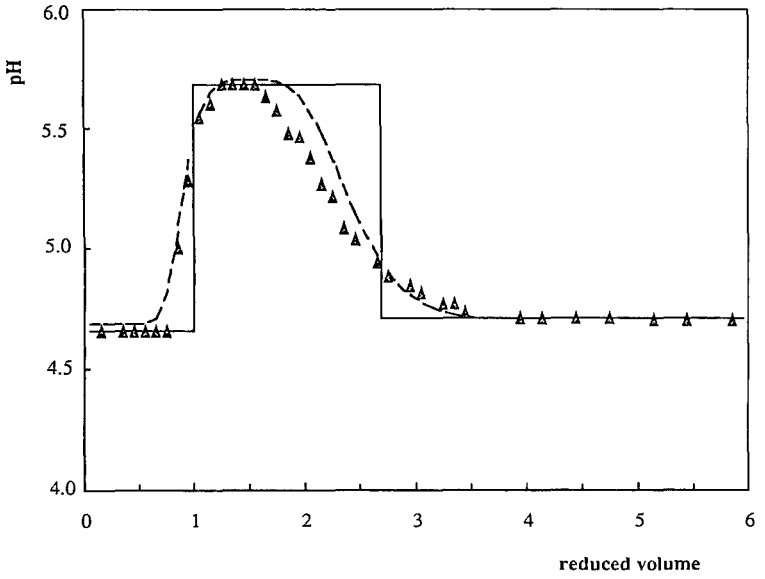

FIGURE 4a: Case 2- Experimental and modelled breakthrough curves of total acetate and sodium ion.

Experimental curves : ◇ total acetate, ◎ sodium ion
Modelled curves : — Equilibrium Theory predictions, – – Impact results

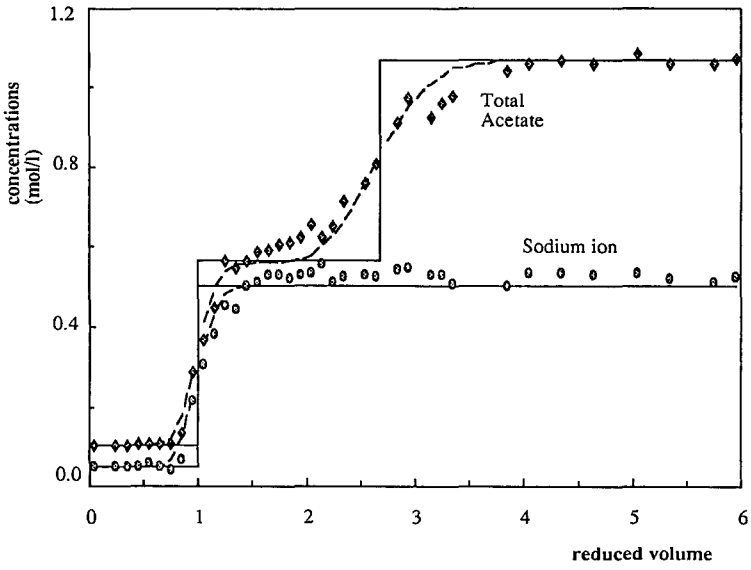

FIGURE 4b : Case 2 - Experimental and modelled variations of pH in the column effluent
Experimental curve : ▲ pH
Modelled curves :— Equilibrium Theory predictions,— — Impact results

Hydrodynamic Dispersion As a first approximation, hydrodynamic dispersion is estimated by mean of the inflection tangent method applied to the sodium concentration curve. J equals 53 in Case 1 and 71 in Case 2 (values used in simulations).

RESULTS AND DISCUSSION

The discussion will not focus on the occurence of a pH excursion in a strong-base anion exchanger [2] but rather on the comparison between experimental data and the results obtained from equilibrium theory and from simulations.

TABLE 2 Experimental and computed composition of the intermediate zone and of the position of the ion-exchange wave

	Case 1: alkaline pH		Case2: acid pH	
	experiment	simulation	experiment	simulation
Composition of the intermediate zone				
total acetate (mol/l)	0.395	0.395	0.580	0.560
pH	13.14	13.13	5.63	5.71
Position of the retarded wave				
reported to V_m	2.875	2.850	2.680	2.475

The experimental and computed composition of the intermediate zone and position of the ion-exchange wave are presented in Table 2. Dissociation and exchange constants were taken from litterature: $K_w = 1.0\ 10^{-14}$ (mol/l)^2, $K_a = 1.76\ 10^{-5}$ mol/l, $K_{AcO,OH} = 2.95$ [2]; K_D was estimated, a convenient K_D value is 1.475.

Let us compare this K_D value with the result of equation (13). For a concentration of 0.59 mol/l of total acetate (mean value between preequilibrant and feed concentration in case 2), one can read ε_m=0.48 on Curve 2 (Fig. 2). That gives ε_i=0.22 (with ε_t=0.70) and K_D=1.35. This calculated K_D is not far from the value used in simulations. The difference may be due to different reasons: experimental error (difficulties in evaluating ε_m), assumptions leading to a simple K_D expression (13) in particular continuity assumptions, or perhaps a specific adsorption of acetate ion.

The work made by Helfferich et al. [2] has shown that equilibrium theory offers a good prediction of the number of waves and of the intermediate zone pH. The exchange wave velocity is fairly good predicted in Case 1 but the experimental

wave is more dispersed than the expected shock. In Case 2, it was shown that ion-exchange was almost non-existent and equilibrium theory indicates that the ion-exchange wave travels at about fluid velocity. Nevertheless, a broad intermediate zone is experimentally observed that theory fails to predict: actually this wave is mainly due to sorption effects and these effects are neglected in the theory premises.

The point is that in Case 1 the retarded wave is really an ion-exchange wave, as in Case 2 it is a combined ion-exchange/sorption wave. Its location is imposed by the sorption phenomenon, as anion-exchange determines the intermediate zone composition.

The use of Impact, in which sorption and hydrodynamic dispersion are taken into account, allows a very good agreement between experiments and predictions. In this way, it is shown that the assumptions constituting the model bases have an excellent prediction power in what concerns intermediate zone composition, ion-exchange wave velocity and hydrodynamic dispersion.

In particular, the preliminary experiments realized about electrolyte penetration into resin give the volumes of each phase with a fair precision. From that point, a reasonable estimation of a sorption constant used in a simple linear sorption law can be made and the prediction of the ion-exchange wave position is really improved ; especially in Case 2.

To obtain a perfect agreement between experimental and computed values, a sensitivity study of the different factors would have to be made. For instance the intermediate zone composition depends uniquely on sodium feed concentration as the ion-exchange wave velocity is sensitive simultaneously to sodium feed concentration, total exchange capacity and ion-exchange constant.

CONCLUSION

1. The study of the penetration of electolyte into a strong anion-exchanger allowed the determination of the mobile phase volume, from the point of total exclusion to the one where total penetration of cations is almost reached. The variations of the mobile phase volume fraction were plotted against concentration for sodium chloride and for sodium acetate. The volume phase ratio allowed an estimation of K_D.

2. When a strong anion exchange column is preequilibrated with a sodium acetate/acetic acid solution and fed with a solution of the same buffer at a different concentration at the same or at a close pH, a transient pH-acetate coupled variation is observed along the fixed bed: pH presents an excursion up to one unit and acetate concentration in the effluent varies in two steps.

3. In the case of alkaline solutions, the retarded wave position and the intermediate zone composition depend only on ion-exchange. In the case of acid buffers, the wave location is a function of Donnan equilibrium as the intermediate zone composition is fixed by ion-exchange. An a priori estimation of the Donnan constant is made assuming that undissociated acetic acid concentration in the stationary phase is determined by the acetate ion concentration in the same phase. Consequently, the sorption constant depends directly on the ion-exchange constant.

A model based on these premises offers a very good prediction of breakthrough curves.

LIST OF SYMBOLS

Ac	total acetate
A_j	chemical species j

a_j	activity of species A_j
J	number of mixing cells
K_a	acetic acid dissociation constant (mol/l of mobile phase)
$\overline{K_a}$	dissociation constant of acetic acid in volume V_i (mol/l of mobile phase)
$K_{AcO,OH}$	separation factor of acetate and hydroxyle ions (-)
K_D	Donnan constant of AcOH sorption (-)
K_i	conditional thermodynamic constant of reaction i
K_w	water dissociation constant (mol/l of mobile phase)^2
$\overline{K_w}$	dissociation constant of immobile water (volume V_i) (mol/l of mobile phase)^2
L	column length (m)
N	number of chemical species
R	number of independent reactions
υ	ion exchange wave velocity (m/s)
V'	reduced volume (reported to V_m) where the exchange wave appears (-)
υ_0	fluid velocity (m/s)
V_{ap}	apparent resin volume (l)
V_e	external volume (l)
V_i	volume of immobile water (l)
V_{ia}	internal volume accessible to cations (l)
V_m	volume of mobile phase (l)
V_r	volume of the resin skeleton (l)
V_s	volume of stationary phase (l)
V_t	total column volumn (l)
(X)	concentration of species X (mol/l of mobile phase)
$\overline{(X)}$	concentration of X in the stationary phase (mol/l of mobile phase)
β	internal porosity (-)
ε_e	fraction of external volume (V_e/V_t) (-)
ε_i	fraction of internal volume (V_i/V_t) (-)
ε_{ia}	fraction of internal volume accessible to cations (V_{ia}/V_t) (-)
ε_m	fraction of mobile phase volume (V_m/V_t) (-)
ε_t	total porosity (-)
ν_{ij}	stoichiometric coefficient for species A_j in reaction i
Ω	column section (m^2)

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CHROMATE AS A MOBILE PHASE FOR THE DETERMINATION OF ANIONS USING CETYL-TRIMETHYLAMMONIUM-COATED COLUMNS AND INDIRECT PHOTOMETRIC DETECTION

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ABSTRACT

A suitable indirect photometry chromatographic system for the separation and detection of common inorganic and organic anions was made by dynamically coating an octadecyl-bonded silica column with cetyltrimethylammonium ions and using potassium chromate as the eluent. The effect of chromate ion concentration on the capacity factor indicates an ion-exchange mechanism. The detection limit of the anions F^-, Cl^-, NO_2^-, Br^-, and NO_3^-, corresponding to a signal-to-noise ratio of 3, were 0.29, 0.55, 0.72, 0.83 and 0.97 ppm, respectively. A linear response was observed between peak heights and injection volume of a solution with 1.19 ppm F^-, 2.22 ppm Cl^-, 2.88 ppm NO_2^-, 4.99 ppm Br^- and 3.88 ppm NO_3^-. This can further lower the detection limit of the anions by about 9 fold when a volume of 175 μL is injected. This system has been applied to the trace analysis of NO_3^- in tap water. A concentration of 1.97 $\pm$ 0.10 ppm NO_3^- was detected. This system was also found applicable for the separation of organic acids (formate, acetate, propionate and butyrate) and may also be used for the separation of a mixture of organic and inorganic anions.

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INTRODUCTION

Ion-exchange chromatography (IEC) has been widely used for the separation of ionic species. A major breakthrough in IEC was the introduction of conductivity detectors coupled with a dual-column into the system (1). This technique, called ion chromatography, is used for the determination of ions which do not absorb in the uv-vis region. Most recently, Small and Miller (2) reported the concept of indirect photometric chromatography (IPC) for the determination of these ions. This technique uses a single-column and a uv-vis detector. A light-absorbing reagent is added to the mobile phase. This reagent maintains a background signal and competes with the analyte ions for the active sites on the column. When the analyte ions elute, they are indirectly detected as negative peaks because the concentration of the light-absorbing reagent in the analyte bands decreases. The mechanism of IPC has been reported in the literature (2). The selection of light-absorbing reagent is important in determining the sensitivity and selectivity of the separation. Although several reagents such as benzoate, phthalate, salicylate, naphthalenesulfonate and other carboxylate salts have been successfully employed (2-8), the use of potassium chromate has not been described. This reagent possesses fine ion-exchange capability, good chromatographic selectivity, and large molar absorptivity (see discussion for details). In addition, chromate ion has tendency to absorb in wavelength regions above 254 nm which reduces interference from ultraviolet absorbing substances and therefore its use as an eluent is preferred over phthalate and other benzene derivatives, which are the most widely used in indirect photometric chromatography.

In indirect photometric chromatography, the capacity of the ion-exchange column is important in determining the sensitivity, speed and efficiency of separation (6). Several commercially available ion-exchange resins each with a different capacity have been reported (2-8). However, by coating a reverse-phase column with ion pairing reagents (which would not desorb in aqueous eluents)

under various solvent composition and/or reagent concentrations, resin with a variety of capacities can be prepared (9-15). In addition, this approach possesses a great flexibility with regard to choice of columns, eluents, and ion-pair reagents for optimum separation.

This paper describes the results of a study of the behavior of potassium chromate as an eluent for an indirect photometric method for the separation and detection of common inorganic anions using ODS-bonded silica column coated with cetyltrimethylammonium ions. It also confirms the applicability of this system for the separation of a mixture of organic anions.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Perkin Elmer Series 3 Liquid Chromatograph equipped with a variable wavelength LC-65T UV detector/oven, an LC1-100 integrator and a 20 μL (or 175 μL) Rheodyne sample injector (Model 7125, Berekely, CA). The column (5 cm x 4.6 mm i.d.) was packed with 3 micron Spherisorb ODS-bonded silica (Regis, Morton Grove, IL). A prefilter SSI. (Alltech Associates, Inc., Deerfield, IL) with a 0.2 micron filter was located between the injector and the column. The void volume of the system was calculated by using the peak of injected water. The uv-vis spectra were determined on a double beam Hitachi U-2000 spectrophotometer fitted with 1-cm cells. All pH measurements were performed with an ALTEX Model 3560 Digital pH meter and Corning combination glass electrode. The flow rate was fixed at 1.0 mL min^{-1} and a wavelength of 328 nm was selected throughout the work unless otherwise mentioned.

Chemicals and Reagents

Cetyltrimethylammonium bromide (CTAB) and potassium chromate were obtained from Aldrich Chemical Company, Inc. All other chemicals used were

of analytical grade. The chemicals were used without further purification. The stock solution of 0.15 M CTAB was prepared in deionized water and filtered through a 0.45-μm membrane (Rainin Instrument Co., Inc., Woburn, MA). Stock solution of 0.10 M CrO_4^{2-} was also prepared in deionized water. Eluents were prepared by dilution of the CrO_4^{2-} stock solution and then filtered through a 0.45-μm membrane filter before using. The pH was adjusted to 7.00 with aqueous hydrochloric acid. Stock solutions of the test solutes were prepared by carefully weighing the sodium salt of the compound in a volumetric flask and then diluting it with deionized water. The working concentrations for the mixture of inorganic anions were as follows: fluoride (9.5 ppm), chloride (17.7 ppm), bromide (40.0 ppm), nitrite (23.0 ppm), nitrate (31.0 ppm). The working concentrations for the mixture of organic anions were as follows: formate (22.5 ppm), acetate (29.5 ppm), propionate (73.0 ppm), butyrate (87.0 ppm).

Preparation of the Column

The ODS-bonded silica column was coated with CTA^+ ions by passing an aqueous solution of 0.10 M CTAB at a flow rate of 0.50 mL min^{-1} through the column for 2 h, followed by washing with deionized water for 30 min. After the above dynamic modification, the column is equilibrated with potassium chromate until a steady baseline with high absorbance background is reached.

Following multiple separations the column was regenerated by washing with deionized water for 10 min at 1 mL min^{-1}, followed by pure methanol until all surfactant was removed (about 1 h), and then with deionized water until all methanol was removed (about 1 h).

RESULTS AND DISCUSSION

The absorption spectrum from 200 to 450 nm of 0.5 mM potassium chromate solution showed absorption maxima at 370.5 and 272.5 nm with molar absorptivities of 4.5 x 10^3 and 3.3 x 10^3 L cm^{-1} mol^{-1}, respectively. A detection wavelength of 328 nm (ϵ = 1.0 x 10^3 L cm^{-1} mol^{-1}) was selected throughout the

work unless otherwise mentioned. This wavelength is not that of maximum sensitivity but was selected to keep the absorbance of chromate within the back-off range of the detector. However, the wavelength of maximum sensitivity can be used when working with very low concentrations of potassium chromate or when comparing the absorbance of the mobile phase in the presence and absence of the analyte (16).

The effect of chromate ion concentration on the retention time of F^-, Cl^-, NO_2^-, Br^-, and NO_3^- ions was examined in the range of 0.05 - 0.50 mM. The plots of log k′ against log $[CrO_4^{2-}]$ are shown in Figure 1. Increasing the chromate concentration resulted in a linear decrease in capacity factor for all five anions. Considering the competition of the solute (X^-) and chromate ions for the CTA^+ sites on the stationary phase, i.e. the ion-exchange mechanism, then:

$$(CTA^+)_2CrO_4^{2-}{}_s + 2X^-_m \leftrightharpoons 2CTA^+X^-_s + CrO_4^{2-}{}_m \qquad (1)$$

The designations s and m refer to stationary and mobile phases, respectively. The equilibrium expression in terms of concentrations of the species in the preceding equation is:

$$K = \frac{[CTA^+X^-]_s^2\ [CrO_4^{2-}]_m}{[(CTA^+)_2CrO_4^{2-}]_s\ [X^-]_m^2} \qquad (2)$$

Where K is the selectivity coefficient. An expression for capacity factor of the solute, k′, can be obtained by rearranging this equation to

$$\frac{[CTA^+X^-]_s}{[X^-]_m} = \left(\frac{K\ [(CTA^+)_2\ CrO_4^{2-}]_s}{[CrO_4^{2-}]_m}\right)^1/2 = k' \qquad (3)$$

The logarithm of this becomes

$$\log k' = constant - \frac{1}{2}\log\ [CrO_4^{2-}] \qquad (4)$$

where the constant = 1/2 {log (K)($[CTA^+{}_2CrO_4^{2-}]_s$)}.

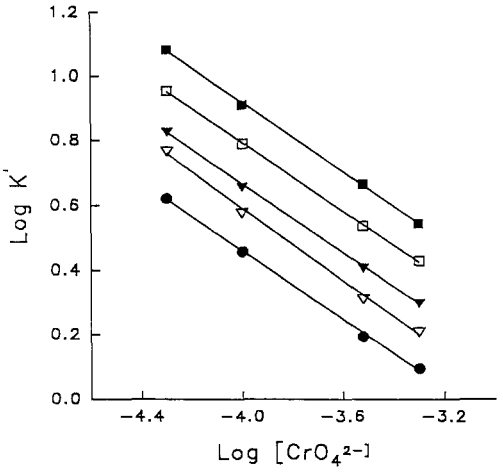

Figure 1: Effect of chromate ion concentration on the capacity factor. Conditions: eluent pH 7.0; fow rate 1.0 mL min^{-1}; detection, uv absorbance at 328 nm; injection volume, 20 μL. Line identities and solute concentrations: (●) = F^- (9.5 ppm), (▽) = Cl^- (17.7 ppm), (▼) = NO_2^- (23.0 ppm), (□) = Br^- (40.0 ppm) and (■) = NO_3^- (31.0 ppm).

Thus if the retention behavior of the anions is similar to that of ion-exchange chromatography, the slope of the plot of log k′ against log $[CrO_4^{2-}]$ should give a slope equal to 0.50. The results gave an average slope of the lines equal to 0.58 $\pm$ 0.05, which supports the ion-exchange mechanism. This is confirmed by the fact that the elution order of a mixture of these ions increases with their size (Figure 2).

The variation of capacity factor as a function of temperature was studied from 25°C to 40°C. The plot of In k′ against 1/T (van't Hoff plot) yields straight lines (Figure 3), therefore the proposed ion-exchange mechanism remains unchanged over the temperature range studied. In addition, a more significant increase was observed in the capacity factor of Br^-, NO_2^- and NO_3^- ions than that of F^- and Cl^-. This differences in slopes is related to the differences in the thermodynamic distribution behavior of these anions. In general, the overall changes in the capacity factor of the anions studied is low over the temperature

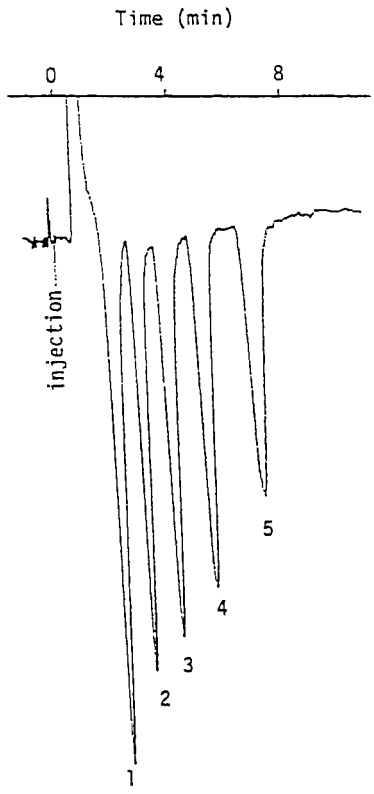

Figure 2: Separation of a mixture of inorganic anions on ODS-column coated with CTA^+. Conditions: eluent 0.5 mM potassium chromate. Other conditions and solute concentrations as for Figure 1. Peak identities: (1) = F^-, (2) = Cl^-, (3) = NO_2^-, (4) = Br^-, (5) = NO_3^-.

range studied. Therefore, experiments can be performed without careful temperature control.

In an attempt to improve the column efficiency as reported by other workers (17), the effect of 2-propanol added to the aqueous mobile phase was studied. This resulted in loss of some of the CTA^+ coated on the column as indicated by a strong positive absorbance due to the increase in concentration of

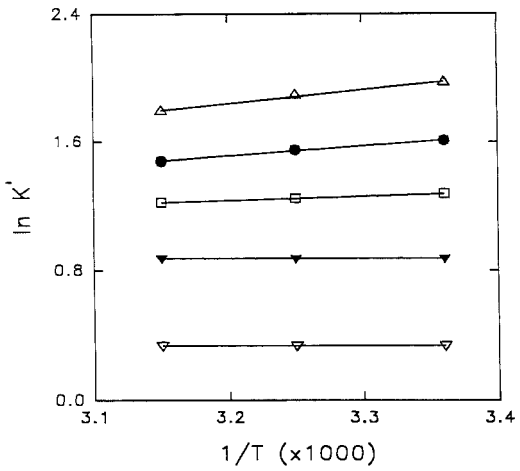

Figure 3: Effect of column temperature on the capacity factors of the investigated anions. Conditions and solute concentrations as for Figure 2. Line identities: (▽) = F^-, (▼) = Cl^-, (□) = NO_2^-, (●) = Br^- and (△) = NO_3^-.

the chromate ion in the mobile phase. When equilibrium was reestablished, i.e. a stable baseline was obtained, a mixture of NO_2^-, Br^- and NO_3^- ions was injected. A decrease in retention time was observed (Table 1). This was expected due to the decreased ion-exchange capacity of the column. To evaluate the role of adding small amounts of the modifier to the mobile phase on the efficiency of the column, the number of theoretical plates (N) from the peaks obtained in the presence of 3% of 2-propanol were compared to those in the absence of the modifier. The values of N were calculated from the commonly used equation:

$$N = 5.54 \left(t_R / W_{0.5} \right)^2$$

The results are shown in Table 1. Little improvement in column efficiency was obtained, therefore 2-propanol was not added to the mobile phase.

To determine the stability of the column, repeated injections of a mixture of F^-(9.5 ppm), Cl^-(17.7 ppm), NO_2^-(23.0 ppm), Br^-(40.0 ppm), and NO_3^-(31.0

TABLE 1

Variation of Capacity Factor and Column Efficiency in the Presence of 2-Propanol

	Zero% 2-Propanol		3% 2-Propanol	
Anion	K′	N	K′	N
NO_2^-	6.10	985	5.27	1024
Br^-	8.32	982	7.02	998
NO_3^-	11.41	1086	8.84	1141

0.5 mM potassium chromate at pH 7.00 and 1.0 mL min^{-1}; wavelength 328 nm.

ppm) were made on a freshly coated ODS-column with CTA^+ and a continuous flow of 0.5 mM chromate (flow rate 1 mL min^{-1}) over a period of 30 hours. No significant changes in retention times over the duration of the experiment were observed. When the column was used continuously over two weeks, a drift in the base line and a broadening in the peaks were observed. This problem was overcome by inverting the column.

The sensitivity of the method was investigated by preparing various calibration plots for 20 μL injections of the anions using 0.5 mM CrO_4^{2-} as the mobile phase. Plots of the peak height against concentration injected were linear over the range up to 14.4 ppm F^-, 17.7 ppm Cl^-, 23.0 ppm NO_2^-, 40.0 ppm Br^- and 31.0 ppm NO_3^- studied (Figure 4). Reproducible results can be obtained in low ppm ranges (Table 2). The detection limits of the anions F^-, Cl^-, NO_2^-, Br^- and NO_3^-, corresponding to a signal-to-noise ratio of 3, were 0.29, 0.55, 0.72, 0.83 and 0.97 ppm, respectively. These detection limits are comparable with

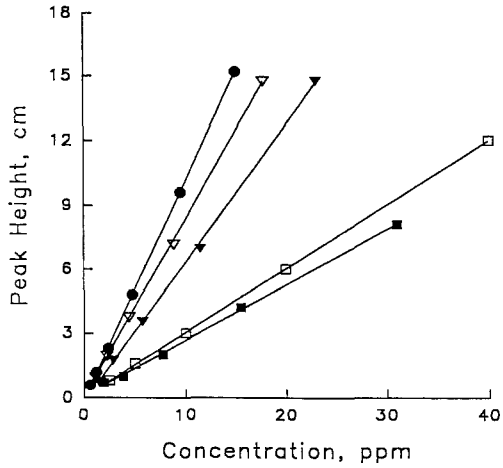

Figure 4: Calibration curves for anion determination with CTA^+-coated column. Conditions as for Figure 2. Line identities: (■) = NO_3^-, (□) = Br^-, (▼) = NO_2^-, (▽) = Cl^- and (●) = F^-.

TABLE 2

Repeatability of Anion Determinations

Anion	Conc.[a], ppm	RSD[a*] %	RSD[b*] %
F^-	9.5	3	
	1.2	5	0.6
Cl^-	17.7	0.4	
	2.2	6	1.1
NO_2^-	23.0	1.1	
	2.9	7	0.9
Br^-	40.0	3	
	5.0	5	1.1
NO_3^-	31.0	2	
	3.9	3	0.8

* Relative standard deviation of 6 samples
a 20 μL injected volume
b 175 μL injected volume

Chromatographic conditions as in Table 1

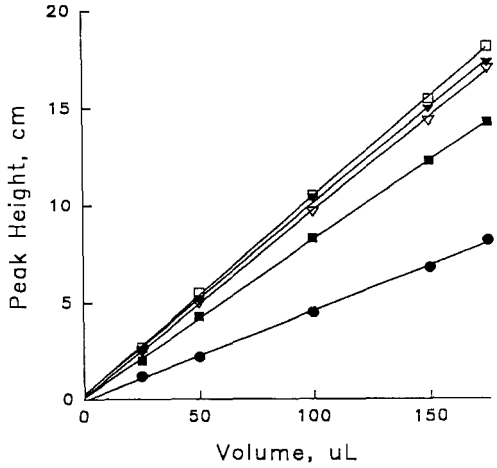

Figure 5: Relationship of detector response to injection volume. Conditions as for Figure 2. Line identities and solute concentrations: (●) = F^- (1.19 ppm), (■) = NO_3^- (3.88 ppm), (▽) = Cl^- (2.22 ppm), (▼) = NO_2^- (2.88 ppm) and (□) = Br^- (4.99 ppm).

those reported using conductivity (18-22) or indirect refractive index methods (23).

A linear response between peak heights and injection volume for the anions (1.19 ppm F^-, 2.22 ppm Cl^-, 2.88 ppm NO_2^-, 4.99 ppm Br^- and 3.88 ppm NO_3^-) was observed up to an injection volume of 175 μL studied (Figure 5). In addition, reproducibility significantly increased when 175 μL was injected in comparison to 20 μL for the same analyte concentration (Table 2). Therefore, the injection of 175 μL lowers the detection limit by about nine fold compared to a volume of 20 μL. Provided that volumes larger than 175 μL do not destabilize the baseline and the large solvent peak does not interfere with early eluting peaks, then this approach of improving the detection limit can be extended to even larger injection volumes.

This system was used to analyze NO_3^- in tap water. A mobile phase of 0.5 mM $CrO4^{2-}$ at pH 7.02 was used and a sample of 175 μL was injected. A

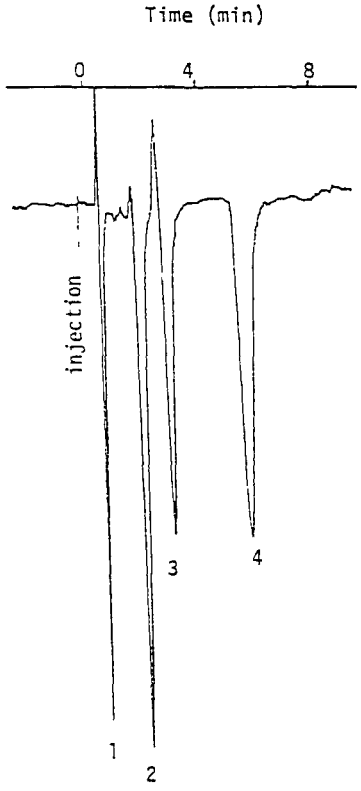

Figure 6: Separation of a mixture of organic anions on ODS-column coated with CTA^+. Conditions as for Figure 2. Peak identities and solute concentrations: (1) = formate (22.5 ppm), (2) = acetate (29.5 ppm), (3) = propionate (73.0 ppm), (4) = butyrate (87.0 ppm).

concentration of 1.97 ± 0.10 ppm NO_3^- was detected based on the calibration-curve method using peak height.

The ODS-bonded column dynamically coated with CTA^+ and loaded with CrO_4^{2-} ions was tested for its ability to separate a mixture of organic anions (formate, acetate, propionate and butyrate). Figure 6 illustrates that a complete separation of the four species was obtained in less than 6 minutes. In addition,

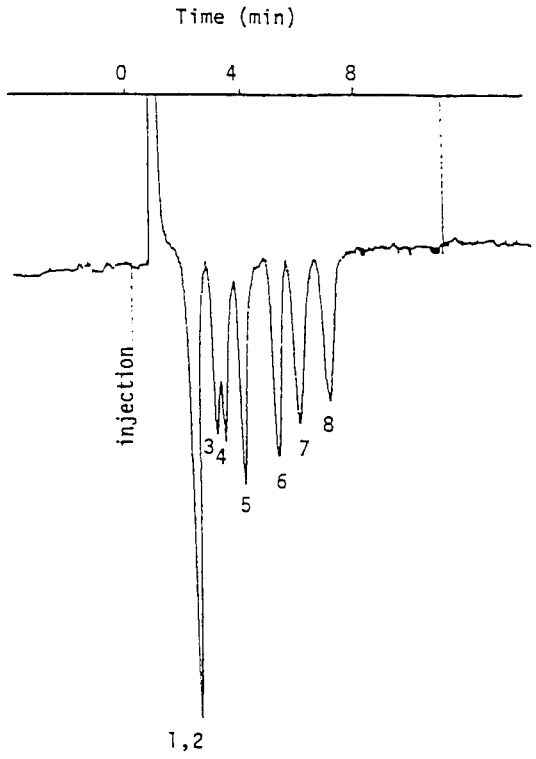

Figure 7: Separation of a mixture of inorganic and organic anions on ODS-column coated with CTA^+. Conditions as for Figure 2. Peak identities and concentrations: (1) = fluoride (9.5 ppm), (2) = acetate (29.5 ppm), (3) = chloride (17.7 ppm), (4) = propionate (73.0 ppm), (5) = nitrite (23.0 ppm), (6) = bromide (40.0 ppm), (7) = butyrate (87.0 ppm), (8) = nitrate (31.0 ppm).

the elution order as expected based on the size of these ions. When a mixture of the inorganic anions, fluoride, chloride, bromide, nitrite, and nitrate, and the organic anions acetate, propionate, and butyrate was injected, a complete overlap of fluoride and acetate, a partial overlap of chloride and propionate, and a complete separation of the other anions was obtained (Figure 7). Further studies to improve this separation and to include other organic anions are underway.

CONCLUSIONS

Coating an octadecyl-bonded silica column with cetyltrimethylammonium ions and using an aqueous solution of potassium chromate as the mobile phase provides a method that is comparable to that of other HPLC methods reported for the separation and determination of inorganic anions in low ppm concentration levels. In addition, this system is applicable for the separation of organic anions and may be used for the separation of mixtures of organic and inorganic anions This approach to detect trace anions may be attractive because ODS-columns are readily available and used by most high-performance liquid chromatographers.

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SEPARATION OF RAT LIVER CYTOPLASMIC tRNAs BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Experiments aimed at developing a chromatographic map of cytoplasmic tRNA population from rat liver are described. HPLC tRNA fractionation by a linear gradient of acetonitrile/ammonium acetate, results in a chromatogram with more than 60 significant peaks, having area peak percentages ranging from 0.001 to 5.0. This paper presents evidence that each peak corresponds to a specific tRNA species. Specificity of tRNA-peak assignment was established by charging total tRNA with a mixture of 19 unlabeled aminoacids plus a radioactive aminoacid, and isolating the radioactive aminoacylated iso-tRNAs by gel electrophoresis, followed by HPLC acetonitrile gradient. The radioactive aminoacyl-tRNA species were eluted as well-behaved peaks. Here we describe the analysis of $tRNA^{Trp}$, which gave 2 well separated radioactive peaks, most likely corresponding to $tRNA^{Trp}$ isoacceptor species postulated by the wobble hypothesis. Taken together, the present data validate this procedure for monitoring quantitatively and qualitatively tRNA cell population.

INTRODUCTION

New quantitative and qualitative tRNA patterns have often been observed in different tissues, in cancer cells, under different growth conditions, and during cell differentiation or transformation (1-5).

Because of these observations, it has been suggested that changes in tRNA population might be mechanistically involved in the regulation of gene expression (6-8). On the other hand, at present, only laborious and time-consuming procedures are available for fractionating the complex molecular mixture represented by a normal tRNA cell population. This represents a noteworthy obstacle to the understanding of the role of tRNA in the translational regulation of gene expression.

Taking advantage of the highly resolving power of HPLC, recently we succeeded in establishing a simple and highly reproducible procedure for fractionating the numerous cellular tRNA species (9,10). Rat liver tRNA population could be resolved into as many as 60 peaks using a linear gradient of acetonitrile/ammonium acetate. In order to validate this procedure as a reliable means of studying the correlation between tRNA cell composition and translational efficiency, and, in general, tRNA biology (11-13), qualitative characterization of the chromatographic peaks was carried out in the present study.

MATERIALS AND METHODS

Materials. L-[3,4-^{3}H]proline, L-[3,4(n)-^{3}H]valine (26 Ci/mmol), L-[3-^{3}H]threonine (18 Ci/mmol), L-[5-^{3}H]tryptophan (29 Ci/mmol), L-[4,5-^{3}H]lysine (75 Ci/mmol), L-[3,5-^{3}H]tyrosine (54 Ci/mmol), L-[4-^{3}H]phenylalanine (28 Ci/mmol), L-[methyl-^{3}H]methionine (15 Ci/mmol) were from Amersham, UK. Enzymes were from Boehringer, Mannheim, except where differently stated.

tRNA Isolation. Hepatic cytoplasmic tRNA was extracted and purified from Wistar male albino rats as already described in detail (14, 15).

In vitro tRNA Aminoacylation. Purified rat liver tRNA was deacylated by incubation in 0.1 M Tris-HCl, pH 9.5, at 37°C for 1 hr (16). Then the tRNA solution was brought to pH 7.5 and reacylated. The conditions for aminoacylation were: 0.3 M Tris-HCl, pH 7.6, 0.1 M KCl, 0.02 M $MgSO_4$, 0.01 M dithiothreitol, 0.01 M ATP, 0.5 mM each of 19 unlabeled aminoacids, 200 μCi labeled aminoacid, and 7.5 mg/ml aminoacyl-tRNA synthetases as crude enzyme extract (17). The final volume was 3.0 ml. After incubation for 45 min at 37°C, the reaction was terminated by adding an equal volume of phenol, and phases were separated by centrifugation for 10 min at 10,000 rpm. [^{3}H]aminoacyl-tRNA was ethanol-precipitated from the aqueous phase, collected on a GF/C filter (2.5 cm, Whatman), freed from residual unreacted radioactive aminoacid by thorough washing with ethanol and eluted from the filter.

Gel Electrophoretic Separation of tRNA. After *in vitro* aminoacylation, [^{3}H]aminoacyl-tRNA was purified by 2 dimensional polyacrylamide gel electrophoresis. Electrophoretic conditions were identical to those described by Kuchino (18). At the end of the run, the gel was assayed for [^{3}H]-radioactivity. The [^{3}H]aminoacyl-tRNA was eluted and concentrated by repeated extraction with sec-butyl alcohol, precipitated with 3 vols ethanol, centrifuged, liophylized, dissolved in 0.05 M ammonium acetate, pH 6.6, and analyzed by using the HPLC gradient of acetonitrile/ammonium acetate described in the next section.

HPLC Fractionation of tRNA. Separation of tRNA species was carried out on a Perkin Elmer liquid chromatograph equipped with an LC-100 column oven, an LC-55B spectrophotometric detector with a 254nm filter and a Shimadzu C-R64 Chromatopac. The column was a RP-18 LiChrosorb (250mm x 4mm I.D.; particle size 5μm) pre-fitted with a 7μm guard column, packed with the same material as the analytical column. Column and precolumn were from Merck (Darmstadt, Germany). Elution was performed by a linear gradient using buffer A (0.05 M ammonium acetate, pH 6.6) and solution B (50% acetonitrile) which increased B by 1% every 10 min. The chromatographic run was carried out at 37°C at a flow rate of 0.5 ml/min (5,9,10).

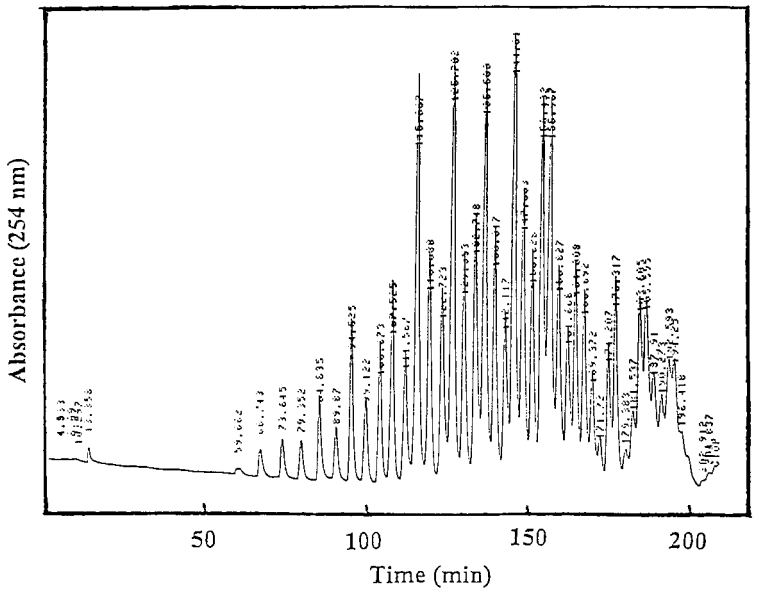

FIGURE 1 - Chromatogram of rat liver cytoplasmic tRNA obtained by acetonitrile/ammonium acetate gradient. Ninty $A_{260\ nm}$ tRNA dissolved in ammonium acetate 0.05 M, pH 6.6, were applied to a C_{18} column.

RESULTS

Fig. 1 illustrates a typical tRNA fractionation that was obtained by a linear gradient of acetonitrile/ammonium acetate. In this experiment tRNA was resolved into more than 60 peaks of which 37 had an area peak percentage higher than 0.5%. In the validation of this methodology, the qualitative identification of the numerous peaks of Fig. 1 was undertaken as the first step, in order to obtain a precise chromatographic map of the tRNA species. The possibility of using *in vitro* or *in vivo* aminoacylation to characterize the HPLC gradient procedure has already been examined. A series of experiments where radioactive proline, methionine, valine, threonine, tryptophan, lysine, tyrosine, phenylalanine were singly used as labeled aminoacid in the tRNA aminoacylation reaction *in vitro* (or

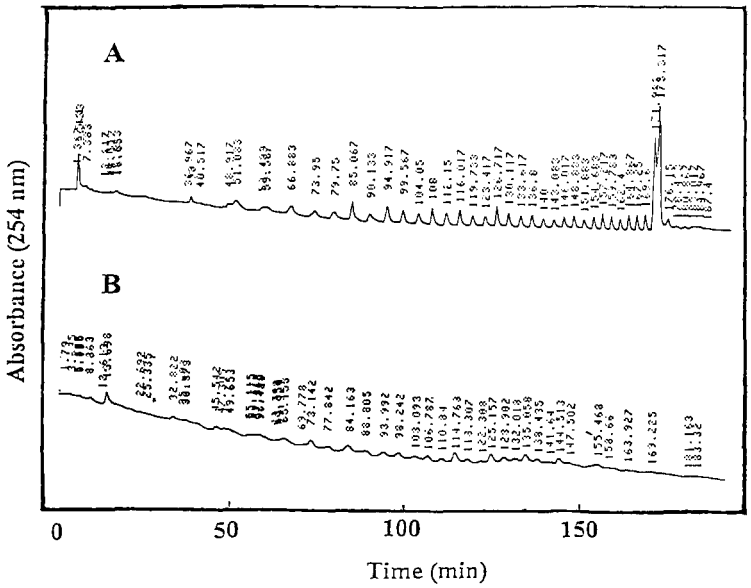

FIGURE 2 - A) Chromatogram by acetonitrile/ammonium acetate gradient of L-[5-^{3}H]tryptophan-tRNA aminoacylated in vitro and isolated by 2-dimensional gel electrophoresis. L-[5-^{3}H]tryptophan tRNA (2.6 $A_{260\ nm}$) was applied to a C_{18} column.
B) Background chromatogram of the acetonitrile/ammonium acetate gradient. At zero time 100 µl ammonium acetate 0.05 M, pH 6.6, were applied to a C_{18} column.

in vivo) was carried out, but results were unsatisfactory (10; Kanduc and Bracalello, unpublished). In fact, the major part of radioactivity eluted fast at the beginning of the chromatographic run and did not coincide with any UV-absorbing peak. That was interpreted as indicative of a possible detachment of the radioactive aminoacid from charged tRNAs during chromatographic separation by the acetonitrile/ammonium acetate gradient (10). However, these results dictated that peaks be assigned by a different qualitative procedure. Consequently, the following experimental design was undertaken. Total rat liver tRNA was deacylated and aminoacylated with a mixture of 19 unlabeled aminoacid plus a

TABLE 1

Comparative Analysis of L-[5-^{3}H]Tryptophan-tRNA Retention Times with Respect to Chromatogram of Rat Liver tRNA Population.

	1st peak	2nd peak
Retention times of *in vitro* amino acylated L-[5-^{3}H]tryptophan-tRNA	171.98-171,20[a] (2)	173.32-174.21 (2)
Retention times of corresponding peaks from chromatograms of rat liver tRNA population[b]	171.66±0.38 (5)	174.05±0.59 (5)

[a]Data are reported as mean ± S.D. with the number of determinations given in parentheses. Where only two experiments were carried out, the figures indicate the range.
[b]Validation of the determination of the two $tRNA^{Trp}$ peaks was carried out by comparing the retention times values obtained when pure L-[5-^{3}H]tryptophan-$tRNAs^{Trp}$ were chromatographed (see Fig. 2), to those relative to chromatograms of rat liver tRNA population (see Fig. 1).

radioactive one. Then the [^{3}H]aminoacyl-tRNAs were separated by two-dimensional gel electrophoresis, eluted from the gel and chromatographed by the HPLC acetonitrile/ ammonium acetate gradient. If each peak corresponded to single tRNA species, by applying specific [^{3}H]aminoacyl-tRNAs on the column, a limited number of peaks should be found. More exactly, one should recover a number of peaks corresponding to the number of the isoaccepting tRNA species relative to the examined radioactive aminoacid. Fig. 2 demonstrates that is the case: when L-[5-^{3}H]-tryptophan-tRNA was isolated by gel electrophoresis and chromatographed, only two UV-absorbing peaks with retention time 172 and 173 were detected. The precision of the separation was determined by comparing the retention times values of the two peaks obtained by chromatography of L-[5-^{3}H]tryptophan-tRNA to the equivalent peaks (i.e. peaks having the same retention time) from chromatograms of entire hepatic tRNA population: the relative data are reported in Table 1. Taken together, the data from Fig. 2 and Table 1 demonstrate unequivocally that the single peaks derive from single tRNA species and clearly show the high accuracy of the method. Similar specific resolutions

were also obtained for iso-tRNAs relative to other aminoacids (manuscript in preparation). We are currently in the process of deriving a complete high performance map of rat liver cytoplasmic tRNAs.

DISCUSSION

Transfer RNAs form a large family of 80-90 molecules with physico-chemically related properties. The multiplicity of tRNA species for a single aminoacid is explained by the degeneracy of the genetic code and the wobble in the third anticodon site. In the present work tryptophan-tRNA was resolved into two peaks: possibly that might indicate the two isoaccepting species caused by the wobble in the third anticodon site. The significance of the great number of isoaccepting species might consist in the speeding up protein synthesis by the use of alternative tRNAs. This would mean a regulatory role of tRNA in gene expression and modulation at translational level. On the other hand, central to any hypothesis or experimental design on tRNA role is the possibility of quantitatively and qualitatively monitoring the complex mixture of tRNAs during the various phases of cell cycle as well as under different growth and development conditions. So far, this possibility has remained beyond the reach of experimental probing, mainly because of the difficulty of efficiently separating the various tRNA species.

This study represents the completion of the first phase of a program directed toward the development of HPLC tRNA mapping as a method of studying the role of tRNA changes in gene regulation. The experiments here reported validate HPLC acetonitrile/ammonium acetate gradient as methodology having the requirements of reproducibility and specificity, and able to generate a complete tRNA chromatographic map. Data of Table 1 are an example of the high specificity by which this procedure resolves tRNAs into chromatographic peaks corresponding to specific tRNA species. Another important feature of this technique, inherent to the determination of a possible regulatory role to tRNA, is its ability to monitor the minor tRNA species that appear to play an important role

in modulating the rate of protein synthesis (19). Thus tRNA fractionation by this HPLC procedure may add a dimension to comparative analyses of the relative distribution patterns of tRNA species in order to assess eventual tRNA involvement in modulation and regulation of gene expression at translational level.

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DETERMINATION OF TRACE AMOUNTS OF SULFIDE IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION AFTER DERIVATIZATION WITH 2-AMINO-5-N,N-DIETHYLAMINOTOLUENE AND IRON(III)

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ABSTRACT

A fluorometric high-performance liquid-chromatographic method is described for the determination of sulfide in human serum. The sulfide ion had been derived into a fluorescent compound with 2-amino-5-N,N-diethylaminotoluene and Fe (III) under an acidic condition. The compound was extracted into 2-octanol as an ion-pair with sodium 1-octane sulfonate. The extract was separated by a reversed phase method (column, Inertsil ODS-2, 250mm ×4.6mm i.d.;mobile phase,90%(V/V) acetonitrile aqueous solution containing sodium 1-octanesulfonate as a counter ion reagent; flow rate 0.5cm^3·min^{-1} and detected fluorometrically (Ex. 640nm; Em. 675nm). The sulfide ion could be determined over the range from 4.4×10^{-10}M to 4.4×10^{-7}M. The coefficient of variation at 4.4×10^{-8}M of sulfide was 3.2%(n=7). The range of concentration of sulfide ion in human serum were from 3.04×10^{-8}M to 2.24×10^{-8}M by this method.

INTRODUCTION

A simpler and more specific colorimetric method based on the formation of Methylene Blue by the reaction of sulfide with N,N-dimethyl-p-phenylenediamine in the presence of an oxidizing agent. This method is widely used for the detection of sulfide in many samples since it was reported by Fisher in 1883(1- 5). Recently, Kloster and King (6) have obtained twice sensitivity of the Methylene Blue method by converting the methyl groups into ethyl groups and found that n-propylene blue method(7) was slightly higher sensitivity than that of Ethylene Blue method. But these methods were not successful to detect ppb levels of sulfide ion in bloods because of insufficiency of the sensitivity.

Reversed-phase HPLC determination of sulfide in an aqueous matrix was reported(8) using 2-indo-1-methylpyridinium chloride as a precolumn ultra-violet derivatization reagent.

The calibration graph(340nm) was rectilinear for 0.04~ 50ug of sulphide ion with 20ul of sample. The sensitivity of this method was poor for the detection of sulfide ion in bloods.

In recent years, the fluorescent properties of thionine were applied to the spectrofluorometric determination of sulfide(9). The thionine is the substuted compounds of the four methyl groups in methylene blue with hydrogen atoms Thus, the method for the detection of fluorescence of thionine produced in this reaction (9) and the method by the HPLC with fluorometric detection(10) were suggested. However, the former method was influenced considerably by excess iron ion. In the latter method, many disadvantages have been

pointed out, such as tailing problems and shorten the lifetime of columns (9) because of thionine has high adsorptivity onto the stationary phase in columns.

We had already reported a high sensitive methd for the determination of sulfide ion by HPLC with fluorometric tection. The method solve any problems of shorten of the li fetime of columns by using a 2-amino-5-N,N-diethylamino-toluene and the solvent extraction(11).

Thus, the applicability of the proposed method to the detection of sulfide ions in human sera using trace amounts of blood samples without any complicated pretreatments was studied.

EXPERIMENTAL

Reagent

All of the chemicals used were of analytical-reagent grade unless stated otherwise. Doubly distilled water was used in all experimentals. A standard sulfide solution was prepared by dissolving 6 g of crystals of sodium sulfide ($Na_2S \cdot 9H_2O$) in 10% zinc acetate with distilled water purged with N_2. The solution was stored in dark polyethylene bottle and was standardized by titration before use (11).

2-amino-5-N,N-diethylaminotoluene hydrochloride was purchased from Tokyo Kasei Kogyo (Tokyo) and the reaction solution was prepared by dissolving 2 g of it in 100 cm^3 of 0.70N sulfuric acid. This solution was effective for 2 weaks in cool and dark.

An approximately 1 $\times 10^{-4}$M iron (III) chloride solution was prepared by dissolving 0.135g of iron chloride ($FeCl_3 \cdot 6H_2O$) in 500cm^3 of 0.1N sulfuric acid. Counter-ion solution (0.1M) was pepared by dissolving 10.8g of sodium 1-octansulfonate in 500 cm^3 of water. The mobile phase was prepared by dissolving 1.08g of sodium 1-octansulfonate in 500cm^{3}of 90% (V/V) of acetonitrile aquaous solution. The acetonitrile was of HPLC-grade and purchased from Tokyo Kasei Kogyo (Tokyo). All mobile phase were de-gassed under vacuum in an ultrasonic bath just before use.

Apparatus

The HPLC with a fluorescent detector (Model F1050) was supplied by Hitachi Corporation with a pump (Model 576, GL Science Inc.) with connecting a loop injector obtained from Ryeodyne. A "Chromato-integrator"(Model 200) was supplied by Hitachi Corporation as a recorder. A separation column was supplied by GL Science Inc. A test tube mixer for extractions was supplied by Taiyo Science Industry Inc., and Centrifugal separator (SCT5BA) from Hitachi Corporation.

Sample pre-treatments

Human whole blood were obtained from the ante-cubital vein of healthy volunteers (subjects) in our laboratory. The human whole blood were immediately poured into centrifuge tubes, and were allowed to stand for two hours at room temperature. After the sample was solidified, the clots formed in the tube was removed with a thin glass stick. After that, those were centrifuged for 10 min at 2000G and classified as sera samples.

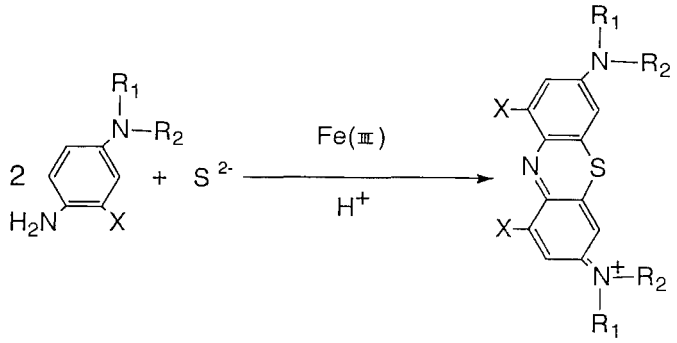

FIGURE 1 Fluorogenic reaction of sulfide with 2-amino-5-N,N-diethylamino toluene and Fe^{3+} to form thionine derivative

Recommended procedures

Pipete 4 cm^3 of sample solution containing sulfide ion, add 0.4cm^3 of a reaction solution and 0.2cm^3 of iron chloride solution in the measuring flask and dilute the solution to 5 cm^3 with pure water. Add 0.5cm^3 of counter-ion solution and 1 cm^3 of 2-octanol, and then extract the compound by mixing for 10 minutes. Since then, the compound was centrifuged at 1820G. HPLC analysis were performed at a 0.5cm^3 min^{-1} of flow rate of mobile phase, maximum excitation wavelength set at 640nm and fluorescent detection wavelengthset at 675nm(12).

Formation reaction and calibration curve

The thionine derivative formation reaction(Fig.1) proceeded under an acidic and excess amounts of oxidizing agents such as, iron(Ⅲ).

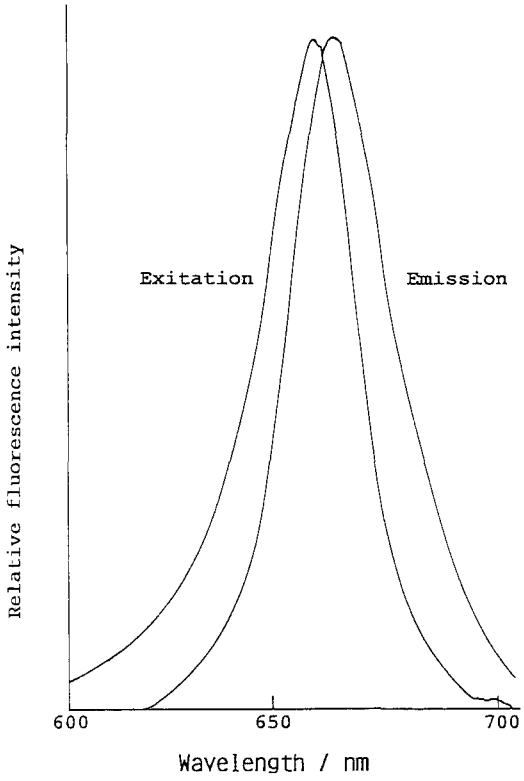

FIGURE 2 Fluorescence spectra of thionine in water
A:Excitation spectrum, B:Emission spectrum.

The detection wavelength was chosen from the fluorescence spectral data of a reaction solution we had already reported (12). The excitation and emission wavelengths maximum of the fluorescence using 2-amino-5-N,N- diethylaminotoluene appeared at 661nm, and 675nm, respectively (Fig.2).

The proposed method was studied using the excitation wavelength at 640nm and the emission wavelength at 675nm considering to obtain the maximum S/N ratio.

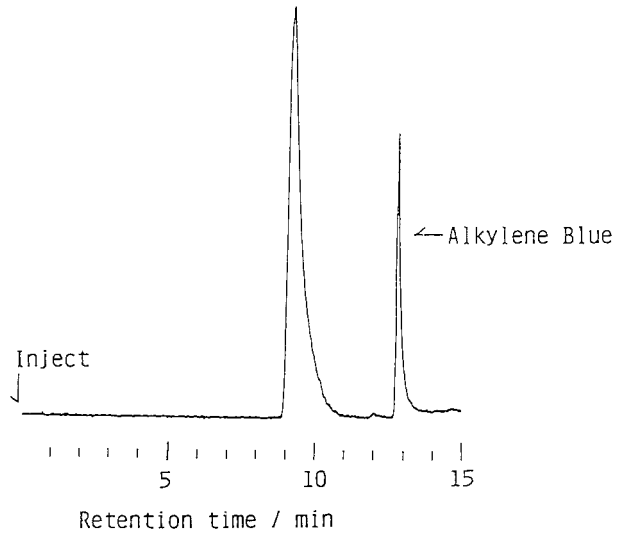

FIGURE 3 Typical chromatogram
Derivatized standard solution containing 0.056ng/10ul sulfide. Chromatographic condition; column:Inertsil ODS-2(250mm ×4.6mmi.d.),mobile phase; 90%(V/V) acetonitrile aqueous solution containing sodium 1-octanesulfonate as a counter-ion reagent, flow rate:0.5$cm^3 \cdot min^{-1}$, injection volume;10ul.

The calibration curve using optimum conditions (12)was linear over the range from 4.4×10^{-10}M to 4.4×10^{-7}M sulfide (γ=0.999, Blank:3.6×10^{-9} M). The precision for seven replicate determination at the 4.4×10^{-8}M level was 3.2% of relative standard deviation.

Typical chromatogram

The chromatograms (Fig.3) obtained from 2-octanol phase showed a large peak at 9 minutes of retention time, and an another peak of thionine derivative compound (analyte) at 13 minutes of retention time.

The second peak was propotional to the concentration of sulfide ion injected, but the first peak was not proportional to one. So, the first peak was the reaction reagents oxidized and the second peak was the thionine.

RESULTS AND DISCUSSION

Effect of diverse ions

There are more than 20 kinds of amino acid compounds in a human body. We investigated the influences of some amino acids containing sulfur, especially methionine, cysteine, cystine.

The results are shown in Table 1.

The 10^4 times amino acids coexisted in the sample were determined by this method based on peak area which was proportional to the concentration of sulfide ion. It can be concluded that those amino acids did not react with the reaction reagents under this condition from the experimental

TABLE 1 Influences of sulfur-containing amino acids on HPLC measurement of sulfide ion

Amino acids	Molecular formula	Error (%)
l-Metionine	$CH_3S(CH_2)_2CH(NH_2)COOH$	-2.17
l-Cysteine	$HSCH_2CH(NH_2)COOH$	-0.45
l-Cystine	$HOOCCH(NH_2)CH_2SSCH_2CH(NH_2)COOH$	+2.68

Amino acids: 1×10^{-4}M
Sulfide ion: 1×10^{-8}M

results that the measured values by coexistence agreed with the measured value by absence within±3%.

We examined the effect of some inorganic ions that might be present in a human blood. Van Hoff's artificial seawater (physiological saline) was used because the saline was used often as dilution solution for blood. As the relative error was less than 10% in the presence of 10^7-10^3 times ions, we have considered that there is no influences of inorganic ions on this method (Table 2).

Application to human sera

This proposed method is so useful to detect sulfide ion sensitively without any influenes of coexisting diverse ions. In order to make sure that the proposed method is practicable to determine the sulfide ion in a human blood. The human blood (sera) samples that obtained from healthy subjects of both sexes between the age of 21 and 24 years were analyzed by

TABLE 2 Effect of other ions on the determination of sulfide

Ion	Added as	Ion/S^{2-}	Error (%)
Cl^-	NaCl	10^9	-9
CO_3^{2-}	Na_2CO_3	10^3	+5
SO_4^{2-}	Na_2SO_4	10^8	-3
$H_2PO_4^-$	NaH_2PO_4	10^3	+6
NO_3^-	$NaNO_3$	10^3	-5
SiO_3^{2-}	Na_2SiO_3	10^3	-10
K^+	KCl	10^7	+7
Mg^{2+}	$MgCl_2$	10^8	+5
Ca^{2+}	$CaCl_2$	10^8	-6

S^{2-}:1.25ng·cm^{-3}

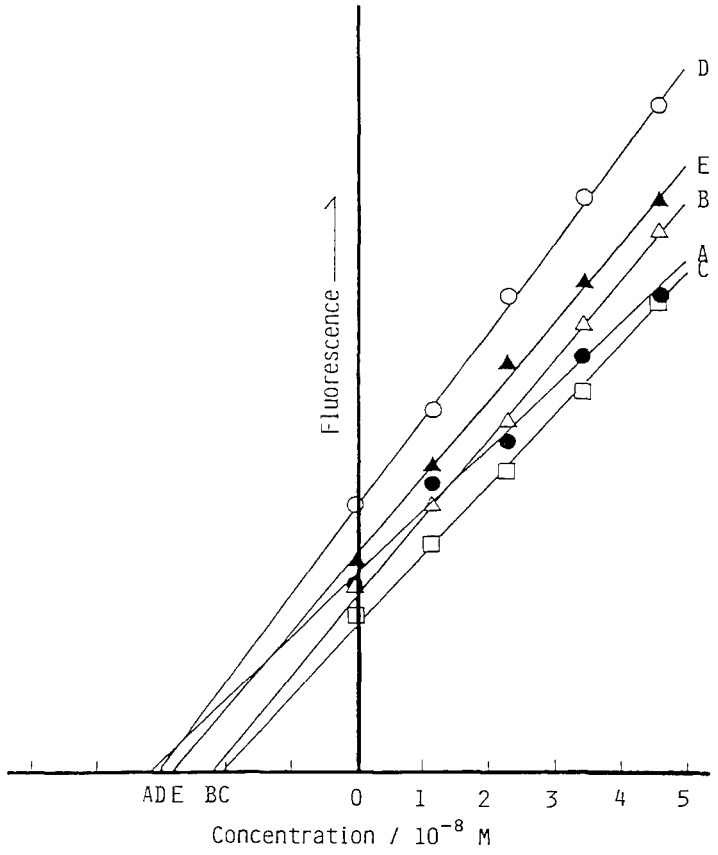

FIGURE 4 Determination of sulfide in human serum by standard addition method

this method with a standard addition method. To the 5 sulfide standard solutions (0 $\sim$4.4 $\times 10^{-8}$ S^{2-}M) 100ul of seram samples were added. And the 5 mixed solutions were analyzed without any complicated pretreatment(See Fig.4 and Table 3).

From these results it became clear that 10^{-8}M levels of sulfide ion in serum can be determined by this method.

TABLE 3 Determination of sulfide ion in human serum

Sample	Sex	Age(Y)	Sulfide concentration (M)
A	Male	24	3.04×10^{-8}
B	Male	23	2.15×10^{-8}
C	Male	22	2.04×10^{-8}
D	Female	22	2.93×10^{-8}
E	Female	21	2.76×10^{-8}

However, it's so difficult for us to obtain any diseased blood samples from AIDS, B-type hepatitis, and C-type hepatitis which are serious problems today. That's one of reasons we couldn't study the relation between concentrations of sulfide ion in human sera and physical conditions for human body.

Although the determination of sulfide in whole blood has been reported previously(13-16), no data exist for the sulfide concentration in human sera as published data. Furthermore, the concentration of sulfide in whole blood reported previously were obtained from human sera of hydrogen sulfide poisoned human.

Although the existence of endogenous sulfide as a normal microcomponent of mammals is evident, significant details of physiology are not clear because of lack of quantitative informations; in short, the normal level of sulfide ion in blood could not be estimated because of the sensitive limitations of measurment.

In conclusion, the HPLC method using 2-amino-5-N,N-diethylamino toluene method has described here is so

sensitive as to be able to determine the sulfide ion in biological materials like human sera without any complicated pretreatments.

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DETERMINATION OF NEUTRAL LIPIDS IN REGULAR AND LOWFAT EGGS BY HIGH PERFORMANCE TLC WITH DENSITOMETRY

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ABSTRACT

A simple quantitative HPTLC method was developed for determination of cholesterol esters, triacylglycerols, free fatty acids, and cholesterol in hens' egg yolk. Lipids were extracted with chloroform-methanol (2:1), the extract was passed through a glass wool column, and the eluate was chromatographed on laned preadsorbent silica gel HPTLC plates with bracketing standards using the Mangold developing solvent for the latter three lipid classes or a modified Mangold solvent for cholesterol esters. Lipids were detected with phosphomolybdic acid spray reagent, and zones were quantified by densitometric scanning. The method was used for the analysis of four regular and four lowfat eggs, and it was found that there was no significant difference between the two types of eggs except for a lower concentration of free fatty acids in the lowfat eggs. There was no significant difference between the mean cholesterol content of either type of eggs in this study and 18 regular eggs analyzed in 1987. Fractions containing monoacylglycerols, diacylglycerols, and nonpolar hydrocarbons were identified in yolk from both types of eggs, but amounts were not quantified.

INTRODUCTION

In an earlier paper (1), a densitometric HPTLC method was developed for determination of cholesterol in egg yolk and was used to survey the cholesterol content of 18 individual eggs. In this study, the HPTLC method was modified by using phosphomolybdic acid (PMA) detection reagent instead of cupric acetate-phosphoric acid reagent for quantification and was extended to the identification and quantification of triacylglycerols, free fatty acids, and cholesterol esters, in addition to cholesterol, in egg yolk. The applicability of the method was demonstrated by performing comparative quantitative analyses of these neutral lipid fractions in regular eggs with no nutritional information listed on the carton compared to eggs purchased in a carton labeled as containing "less than 4% saturated fat" and having a list of nutritional data. It was found that the two types of eggs differed significantly only in the concentration of the free fatty acid fractions.

EXPERIMENTAL

Analyses were performed on Whatman laned preadsorbent high performance silica gel plates using sample preparation (1) and quantitative TLC (2) procedures similar in general to those described earlier. Mixed and individual neutral lipid standards were obtained from Matreya (Pleasant Gap, PA), and standard solutions were prepared in chloroform-methanol (2:1) solvent at a concentration of 0.200 ug/ul of each lipid.

Lipids were extracted from 100 mg egg yolk samples with 2 ml of chloroform-methanol (2:1) in a small vial, and

extracts were passed through a glass wool column held in a disposable pipet. Column effluents were evaporated to dryness under nitrogen and reconstituted in 3.00 ml of chloroform-methanol (2:1) for determination of cholesterol esters and free fatty acids. The reconstituted column effluent was further diluted 1:1 with chloroform-methanol (2:1) for determination of cholesterol (0.25 ml + 0.25 ml of solvent), and a 16-fold dilution of the original reconstituted column effluent was required for determination of triacylglycerols (0.20 ml + 3.00 ml of solvent).

The mobile phases used for development of 10 x 20 cm Whatman (Clifton, NJ) LHP-KDF high performance, preadsorbent silica gel plates in a Camag twin-trough chamber were the Mangold solvent, petroleum ether (37.5-52°C)-diethyl ether-acetic acid (80:20:2) (solvent 1), for determination of cholesterol, triacylglycerols, and free fatty acids, and n-hexane-petroleum ether-ethyl ether-glacial acetic acid (50:20:5:1) (solvent 2) for cholesterol esters. Lipids were detected by spraying the plate with 5% ethanolic PMA and heating at 110-120°C for 5-10 min. A series of sample and standard aliquots between 1.00 and 16.0 ul were applied to each plate using 10 and 25 ul Drummond (Broomall, Pa) digital microdispensers, and quantification was performed by reflectance scanning at 700 nm of the sample and standard zones of each lipid with the closest matching areas using a Shimadzu Model CS-930 densitometer. A response factor was calculated from the area and weight of the scanned standard zone and was used to calculate the weight of lipid in the

sample zone (2). Lipid concentrations are reported in units of mg/g of yolk.

RESULTS AND DISCUSSION

The PMA reagent detected the lipids as flat dark blue bands on a light yellow background. The Mangold solvent (solvent 1) gave excellent resolution of the lipid fractions in standards and samples with the following respective R_f values (Figure 1): cholesterol (sterols), 0.37; oleic acid (free fatty acids), 0.43; triolein (triacylglycerols), 0.74; cholesteryl oleate (cholesterol esters), 0.90. The cholesterol ester zones were located too close to the solvent front to allow their measurement by scanning, so solvent 2, which provided an R_f value of 0.67 (Figure 2), was used for their analysis.

The determination of lipids in the yolks of four regular and four lowfat eggs yielded the values shown in Table 1. It can be seen that the regular eggs had higher concentrations of each of the lipids except cholesterol, for which the regular eggs had a lower value. Student's t-test was used to compare the means between the two types of eggs ($P<0.05$ was considered to indicate a significant difference), and it was found that only the free fatty acid concentrations were significantly different ($P=0.024$). The spread of values found for the concentration of lipids in individual eggs of both types was quite consistent, with coefficient of variation (relative standard deviation) values ranging from 13 to 25% except for free fatty acids in the lowfat eggs (52%).

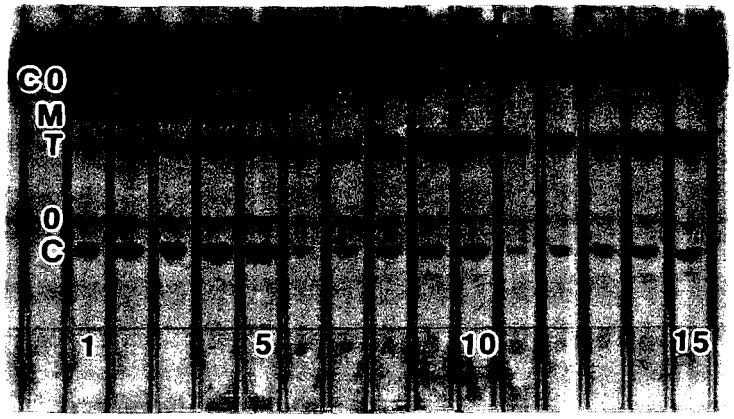

FIGURE 1. Photograph of chromatograms of lipid standards and egg yolk extracts on an HPTLC silica gel plate developed with the Mangold solvent (solvent 1) and detected with PMA reagent. Lanes 1-5 contain 2, 4, 8, 12, and 16 ul aliquots of a solution of Matreya Nonpolar Lipid Mix B having concentrations of 0.2 ug/ul each of cholesteryl oleate (CO), methyl oleate (M), triolein (T), oleic acid (O), and cholesterol (C). Lanes 6-10 and 11-15 contain 2, 4, 8, 12, and 16 ul of two different regular egg yolk extracts diluted for analysis of triacylglycerols.

TABLE 1

LIPID CONTENT (MG/G YOLK) OF REGULAR AND LOWFAT EGGS AS DETERMINED BY THE QUANTITATIVE HPTLC METHOD

Lipid Class	Mean	Range	SD
(Regular Eggs)			
Cholesterol	16.7	14.7-19.6	2.2
Free fatty acids	4.00	2.79-5.10	1.0
Triacylglycerols	216	180-271	43
Cholesterol esters	2.32	1.78-2.89	0.53
(Lowfat Eggs)			
Cholesterol	19.4	14.8-22.3	3.2
Free fatty acids	1.77	0.717-2.94	0.93
Triacylglycerols	190	150-247	42
Cholesterol esters	1.72	1.54-2.06	0.23

SD = standard deviation for 4 replicates in each case

The carton containing the lowfat eggs analyzed in the present study lists a cholesterol content of 390 mg/100 g. This is presumably the average concentration on a whole-egg basis, so it is impossible to directly compare our value of 19.4 mg/g of yolk. The carton also claims that the eggs contain less than 4% saturated fat in the yolk. This value was probably determined by gas chromatographic analysis of the transesterified lipids in whole eggs. We did not examine fatty acid methyl esters and cannot comment on the percentage of saturated fatty acids in our samples. Therefore, our TLC results cannot be compared to the stated value for saturated fat in the lowfat eggs we analyzed.

Analysis of 18 eggs for cholesterol performed in 1987 [1] gave a mean of 15.2 mg/g, a range of 9.72-26.2, and a standard deviation of 4.7. This mean cholesterol concentration is lower than both the current regular or lowfat eggs, but the values are not statistically different based on Student's t-test. It is interesting that any modified feeding protocols instituted by hen breeders over the intervening 7 years to lower cholesterol levels apparently did not have this effect in the samples we analyzed.

The earlier HPTLC method for identification of lipids and quantification of cholesterol in egg yolk (1) was modified by use of the Mangold solvent system (solvent 1) for determination of cholesterol, free fatty acids, and triacylglycerols and a new mobile phase (solvent 2) for cholesterol esters, in place of the Skipski double-development system and chloroform-ethyl acetate (94:6). In addition,

cupric acetate-phosphoric acid was replaced by PMA as the detection reagent. Solvent 2 is the best mobile phase we have found to date for qualitative and quantitative determination of cholesterol esters in complex lipid mixtures. As can be seen in Figure 2, it also provides excellent resolution of triacylglycerols (R_f 0.32) and could be used for their densitometric quantification after dilution of the yolk extracts so that the scan areas of the samples were closely matched to the areas of the standards.

It was proven earlier (1) by use of preparative silica gel TLC to separate the sterol fraction followed by argentation- and RP-TLC and capillary gas chromatography to resolve and identify the individual sterols that cholesterol was the only sterol present in egg yolk extract. Therefore, cholesterol could be reliably quantified using a pure cholesterol standard. Similarly, pure standards of oleic acid, triolein, and cholesteryl oleate were used as markers and quantitative standards for analysis of free fatty acids, triacylglycerols, and cholesterol esters,respectively, in this study.

Calibration curves (peak area vs standard weight) prepared by spotting 2.0, 4.0, 8.0, 12.0, and 16.0 ul (400 ng to 3.20 ug) of standards for the four lipids quantified had typical linear correlation coefficients (R values) greater than 0.99. This strong linear correlation allowed quantification to be carried out reliably by comparing the areas of single sample and standard zones on the same plate if the areas of the two zones matched closely and were within the linear calibration range.

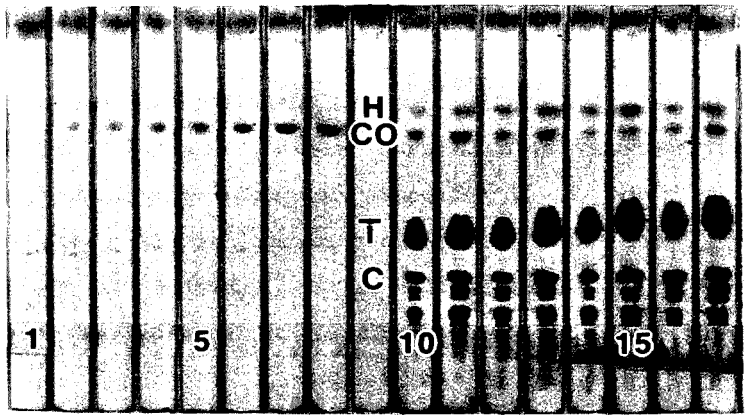

FIGURE 2. Photograph of chromatograms used for determination of cholesterol esters on a silica gel plate developed with solvent 2 and detected with PMA. Lanes 1-8 contain 1, 2, 3, 4, 5, 6, 7, and 8 ul aliquots of a 0.2 ug/ul standard solution of cholesteryl oleate (CO). Lanes 10 and 11, 12 and 13, 14 and 15, and 16 and 17 contain 9 and 15 ul, respectively, of yolk extract from four different regular eggs, in which zones of hydrocarbons (H), cholesterol esters (CO), triacylglycerols (T), cholesterol (C), and the more polar lipids and phospholipids below cholesterol can be seen.

Besides the four lipid fractions quantified, additional faint zones were found in yolk chromatograms developed with solvent 1 at R_f values of 0.32, 0.40, and 0.92. These zones probably represented monoacylglycerols, diacylglycerols, and nonpolar hydrocarbons, respectively. Phospholipid zones were also detected in the preadsorbent and streaking just beyond the preadsorbent-silica gel interface. With solvent 2, nonpolar hydrocarbons were detected above the cholesterol esters at R_f 0.76 (Figure 2). In our earlier analyses of egg eggs using the Skipski solvent system, free fatty acids were

not identified in yolk samples as they were in the present study. Methyl oleate, the marker for methyl esters, was resolved between the cholesterol oleate and triolein standards (Figure 1) but was not detected in egg yolk chromatograms in either this or the earlier study (1).

In summary, the HPTLC method described allows quantification of cholesterol, free fatty acids, triacylglycerols, and cholesterol esters in egg yolk with accuracy and precision that are adequate for routine analytical use. It is more selective than previously reported methods for lipid analysis based on spectrophotometry, and the ability to spot multiple samples on a single plate provides a rapid analysis with high sample throughput. Solvent consumption is very low because of the nature of the microextraction procedure and use of the twin trough chamber.

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SIMULTANEOUS DETERMINATION OF NICOTINE AND COTININE IN UNTREATED HUMAN URINE BY MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, precise, accurate and sensitive method for the simultaneous determination of nicotine and cotinine by direct injection of human urine was developed and validated. This method utilizes nontoxic, nonflammable and relatively inexpensive sodium dodecylsulphate (SDS) as a mobile phase and eliminates the need for pretreating the urine. Optimum conditions for the separation of cotinine and nicotine, the column efficiency, and the elution of non-alkaloid components in urine were investigated. Nicotine and cotinine in untreated human urine were separated and quantitatively eluted using 0.20 M SDS and 3% 2-propanol at a pH of 4.60 and column temperature at 40 °C. Retention times for nicotine and cotinine were: 10.85 and 9.48 minutes respectively, and all components in the urine sample were eluted within 15 minutes. This method may be suitable for use in hospitals, research centers, or in a physician's office.

INTRODUCTION

Nicotine accounts for nearly 95% of the total alkaloid content in commercial tobaccos (1). When nicotine is metabolized, 70% of it is converted to cotinine

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(2). Nicotine has a very short half-life and its excretion may be used to identify recent exposure (3). However, cotinine may indicate cigarette smoke exposure during the previous two to three days since it has a half-life of between 16 and 19 hours in various biological fluids (4,5).

A recent report on the detection of cotinine in semen, blood and urine, described the highest cotinine levels in urine and observed cotinine in 100% of the urine samples obtained from smokers (4). The authors also noted that cotinine concentrations increased with an increase in the number of cigarettes smoked per day or in the amount of passive smoke exposure. The presence of cotinine in urine has proved to be an effective marker of tobacco smoke exposure due to its sensitivity and specificity (2,5,6).

A simple and rapid analytical method for the simultaneous determination of nicotine and cotinine in urine would aid in identifying people who smoke or are exposed to environmental tobacco smoke, quantifying the correct dosage of nicotine for those who may need it for the therapeutic treatment of ailments such as Alzheimer's disease (7), or investigating the pharmacokinetics of non-tobacco-derived nicotine systems used in smoking cessation programs (8).

Various chromatographic methods have been developed and are used for the analysis of nicotine and its metabolites in bodily fluids. Nicotine and cotinine concentrations have been determined using high-performance liquid chromatography (2,3,9-15) and gas chromatography (7,8,16-18). A major drawback of these methods is the time-consuming derivatization, or solvent or solid-phase extraction process. An additional problem is the use and disposal of the toxic solvents and chemicals used for the derivatization or extraction processes.

Direct injection of urine eliminates time-consuming extraction procedures and avoids a protein precipitation step. Micellar chromatography is suited for direct injection because the proteins are solubilized by the micellar aggregates in the mobile phase and, therefore, are eluted with the void volume (19-21).

The purpose of this study was to develop a procedure for the simultaneous quantification of nicotine and cotinine by direct injection of human urine into a

micellar high-performance liquid chromatographic system. The applicability of this method to the assessment of human exposure to nicotine via tobacco use was demonstrated.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Perkin Elmer Series 3 Liquid Chromatograph equipped with a variable wavelength LC-65T UV detector/oven, an LC1-100 integrator and a 20 μL Rheodyne sample injector (Model 7125, Berkeley, CA). The column (25 cm long and 4.6 mm i.d.) was packed with 5 micron Econosphere CN-bonded silica (Alltech, Deerfield, IL). A precolumn (25 cm long and 4.6 mm i.d.) packed with 50 micron silica gel (Alltech, Deerfield, IL) was located between the pump and sample injector to saturate the mobile phase with silica to minimize dissolution of the analytical column packing. The void volume of the system was calculated by using the peak of injected water. All pH measurements were performed with an ALTEX Model 3560 Digital pH meter and Corning combination glass electrode. The flow rate was fixed at 1.0 mL min^{-1}, the wavelength was set to 260 nm and the column temperature was maintained at 40.0 °C unless otherwise mentioned. The chart speed was maintained at 5 mm min^{-1} and at 40 mm min^{-1} when the column efficiency was studied. A double beam Hitachi U-2000 uv-vis spectrophotometer fitted with 1 cm cells was used for absorbance measurements.

Chemicals and Reagents

Sodium dodecylsulphate and 2-propanol were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Nicotine sulfate [(L-)-methyl-2-(3-pyridyl) pyrrolidine-sulfate] and cotinine [1-methyl-5-(3-pyridyl)-2-pyrrolidinone] were received from Sigma Chemical Company (St. Louis, MO). These chemicals were used as received. The stock solution of 0.20 M SDS was prepared in deionized water and filtered through a 0.45-μm membrane (Rainin Instrument

Co., Inc., Woburn, MA). The mobile phase was prepared in 0.05 M Na_2HPO_4 and adjusted to the desired pH with H_3PO_4. Stock solutions of 1140 ppm nicotine and 1100 ppm cotinine were prepared in methanol. Standard solutions of 36.29 ppm Nicotine and 79.00 ppm cotinine were prepared by appropriate dilution of the stock solutions with 0.10 M SDS. The stock solutions and the standard solutions were refrigerated when not used. The stability of nicotine and cotinine in the standard solutions was monitored each day by measuring their absorbance at 260 nm.

Preparation of Standards in Human Urine

Urine specimens were obtained from healthy nonsmoker and smoker volunteers. Each specimen was immediately acidified to a pH of 2.00 with phosphoric acid and filtered through a 0.20 μm Acrodisc syringe filter (Rainin Instrument Co., Inc., Woburn, MA). When the analysis was not to be performed immediately, the samples were frozen at -4.0 °C and thawed before using.

Blank urine samples containing 0.20, 0.53, 1.04, 1.98 and 2.85 ppm nicotine and 0.21, 0.35, 0.77, 1.51 and 2.87 ppm cotinine were prepared by spiking 2.00 mL of urine with the appropriate amounts of the standard solutions of nicotine and cotinine. These solutions were prepared fresh each day.

RESULTS AND DISCUSSION

Studies to determine optimal conditions for separation and elution of nicotine and cotinine were performed using the alkaloids dissolved in surfactant only. Once optimal conditions were determined, applicability of this method was validated using untreated urine from a smoker.

Separation of Nicotine and Cotinine

The effect of pH on the retention of nicotine and cotinine was examined in the range of 3.00 - 7.00. The capacity factor decreased slightly when the pH of the mobile phase increased (Figure 1). On the basis of pK_a values (pK_{a1} = 3.15, pK_{a2} = 7.85) nicotine and cotinine are predominantly cationic throughout

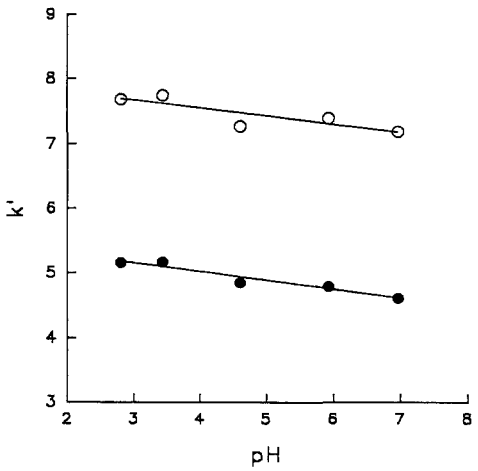

Figure 1: Dependence of capacity factor on pH of the mobile phase with 0.10 M SDS in 0.05 M phosphate buffer and 3% 2-propanol at 40.0 °C. (○) = nicotine and (●) = cotinine.

most of the pH range studied. A significant contribution from electrostatic interaction would cause an increase in retention time as the mobile phase pH increased. Therefore, the contribution from the hydrophobic interaction of the neutral species of nicotine and cotinine with the micelle and the stationary phase may be the predominate force. Based on these results, the analysis of nicotine and cotinine can be performed without careful adjustment of the pH of the mobile phase.

Figure 2 shows the dependence of the capacity factor on the surfactant concentration in the range of 0.01 - 0.20 M at pH 4.60. Increasing the SDS concentration to 0.20 M resulted in an approximately five-fold decrease in capacity factor for both nicotine and cotinine. This is most likely due to an increase in the number of micelles in the mobile phase as the concentration of surfactant increases (22). When the results were replotted as 1/k′ against [SDS], a linear relationship was obtained (Figure 3). This behavior also is expected

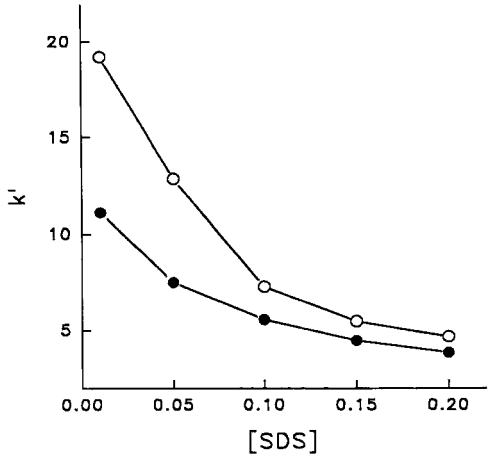

Figure 2: Dependence of capacity factor on [SDS] of the mobile phase containing 3% 2-propanol at pH 4.60 and 40.0 °C. (○) = nicotine and (●) = cotinine.

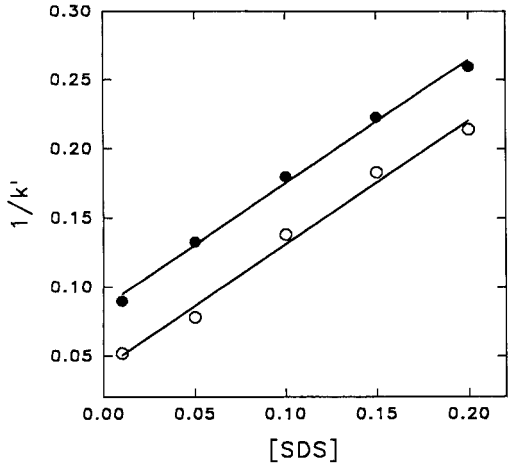

Figure 3: Variation of 1/k′ with [SDS] of the mobile phase under conditions as for Figure 2. (○) = nicotine and (●) = cotinine.

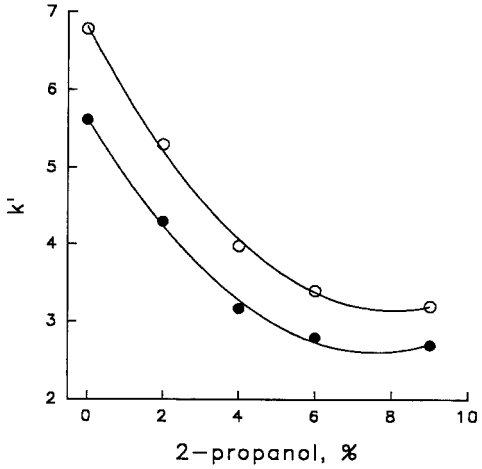

Figure 4: Dependence of capacity factor on percent of 2-propanol: 0.15 M SDS and pH 4.60. (○) = nicotine and (●) = cotinine.

when hydrophobic interaction is the predominant factor contributing to the retention time (22).

When the percentage of 2-propanol in the mobile phase was increased from 0.0 to 9%, the retention time decreased sharply between 0 and 4% and was less affected by greater concentrations (Figure 4). Increasing the percentage of 2-propanol decreases the concentration of micelles in the mobile phase and, as a consequence, the retention time should increase. The decrease in retention time may be related to a decrease in the polarity of eluent and a subsequent increase in solubility of nicotine and cotinine.

In an attempt to improve the column efficiency as reported by other workers (23), the effect of 2-propanol added to the mobile phase was studied. The number of theoretical plates (N) and the asymmetry ratios (B/A) from the observed peaks were compared. The values of N were calculated from the following equation which corrects for the asymmetry of skewed peaks (24):

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

where: t_R is retention time,

$W_{0.1}$ is the peak width measured at 10% peak height,

B/A is the asymmetry ratio.

The results are shown in Table 1. The presence of 3% of 2-propanol doubled the plate count of nicotine and improved its peak symmetry as well as that of cotinine.

The variation of capacity factor with temperature was studied from 22.0 to 54.0 °C (under [SDS] = 0.20 M, pH = 4.60 and 3% 2-propanol). The plot of ln k′ against 1/T (van't Hoff plot) was linear with a more significant increase observed in the capacity factor of cotinine than nicotine. This difference in slopes is related to the difference in the thermodynamic distribution behavior of these species. In general, the overall changes in the capacity factor of nicotine and cotinine is low over the temperature range studied. Therefore, experiments can be performed without careful temperature control.

The effect of column temperature on both the peak symmetry and column efficiency was also studied. Again an improvement was seen in both peak symmetry and efficiency as the temperature was increased from 20.0 to 54.0 °C (Table 2).

Elution Studies on Urine Matrix

Previously reported investigations (19-21) on the use of micellar chromatography have not examined the optimum conditions for eluting urine. To determine conditions which minimize elution time of non-alkaloid components in urine, the following studies were performed.

When the surfactant concentration was increased from 0.01 to 0.20 M, the retention time of the last eluted species in the blank urine sample decreased to 6.64 min (Figure 5). In the presence of 0.20 M SDS, temperature (from 22.0 to

TABLE 1

Variation of Efficiency and Asymmetry
with 2-propanol Concentration

	Nicotine		Cotinine	
2-propanol, %	N	B/A	N	B/A
0	825	2.71	2024	1.88
3	1969	1.40	2538	1.52
6	2032	1.42	2601	1.49
9	1976	1.52	2539	1.55

0.15 M SDS at pH 4.60 and 1.0 mL min^{-1}; temperature 40.0 °C; wavelength 260 nm.

TABLE 2

Variation of Efficiency and Asymmetry
with Temperature

	Nicotine		Cotinine	
Temperature, °C	N	B/A	N	B/A
20.0	1126	2.15	1325	2.11
43.0	1984	1.36	2323	1.46
54.0	1737	1.42	2201	1.53

0.15 M SDS and 3% 2-propanol at pH 4.60 and 1.0 mL min^{-1}; wavelength 260 nm.

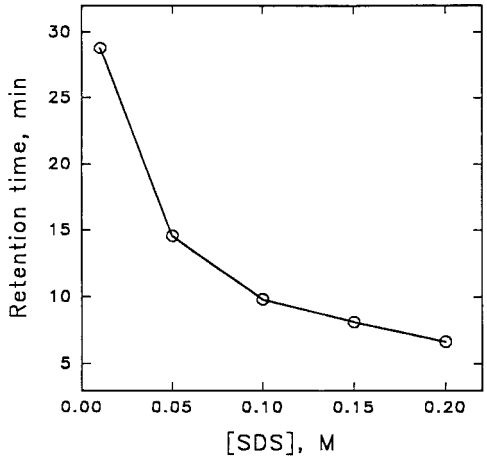

Figure 5: Variation of the retention time of the last species of the urine matrix on [SDS] of the mobile phase under conditions as for Figure 2.

54.0 °C) studied had no effect on the elution of components in urine (average retention time of 6.89 ± 0.80 min). The retention of the last eluted peak was slightly dependent on the acidity of the mobile phase. It increased from 9.68 to 10.78 min when the pH of the mobile phase (containing 0.10 M SDS, 3% 2-propanol and column temperature of 40.0 °C) was increased from 2.80 to 7.00.

Direct Sample Injection

Based on these studies a mobile phase consisting of a solution containing 0.20 M SDS and 3% 2-propanol at a pH of 4.60 (column temperature 40 °C) was chosen as optimum for quantitation of nicotine and cotinine in untreated urine. Under these conditions retention times for nicotine and cotinine were: (10.85 ± 0.33 minutes) and (9.48 ± 0.28 minutes) respectively, and all components in the urine sample eluted within 15 minutes (Figure 6). It is worth mentioning that elution of blank urine and that of a smoker were run for about three hours without noticing additional peaks. In addition, no significant changes in retention

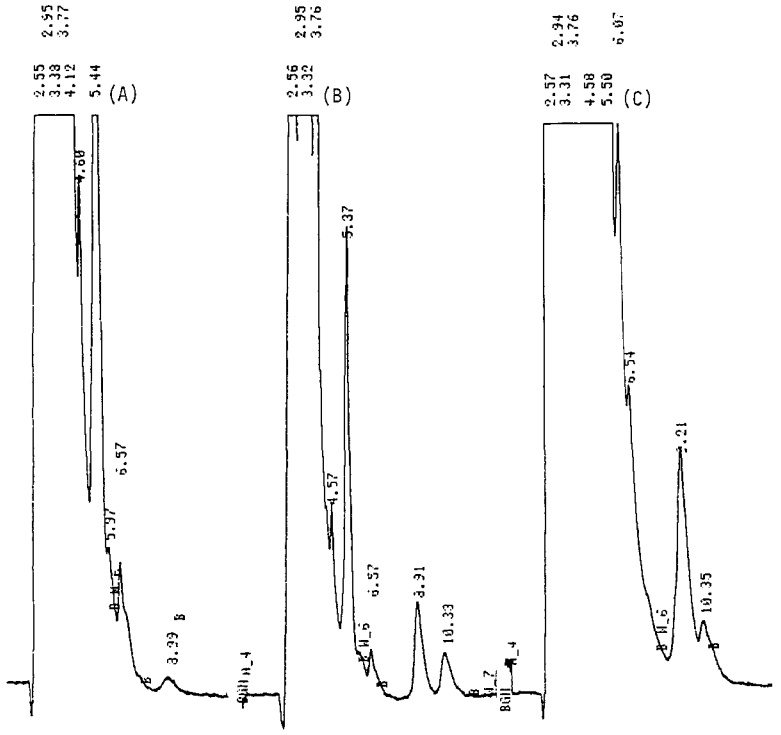

Figure 6: Typical chromatograms of (A) a non-smoker's urine (blank urine) (B) blank urine spiked with 1.23 ppm nicotine and 1.42 ppm cotinine; (C) a smoker urine. Conditions: 0.20 M [SDS], pH 4.60, 3% 2-propanol and 40.0 °C.

time, pressure, or carry over peaks from one injection to another were observed during this study.

The recovery of nicotine and cotinine from spiked urine was studied by adding different quantities of these species to blank urine samples. The results are shown in Table 3. The average percent recovery for nicotine was 100 $\pm$ 3 over a 0.53 - 1.98 ppm concentration range and the recovery for cotinine was 101 $\pm$ 2 over a 0.77 - 2.87 ppm concentration range. Based on these results both alkaloids can be quantitatively recovered.

TABLE 3

Recovery of Nicotine and Cotinine from Urine Samples

Nicotine			Cotinine		
Conc. (ppm)	Recovery %	Average Recovery	Conc. (ppm)	Recovery %	Average Recovery
0.53	109 106 100	105±4	0.77	106 100 104	103±3
1.04	100 90 97	96±4	1.51	103 97 100	100±3
1.98	97 97 101	98±2	2.87	99 100 97	99±1

0.20 M SDS and 3% 2-propanol at pH 4.60 and 1.0 mL min^{-1}; temperature 40 °C; wavelength 260 nm.

The sensitivity of the method was investigated by preparing calibration plots of 20 μL each of nicotine and cotinine in urine. Plots of the peak height against concentration injected were linear from 0.20 to 2.85 ppm nicotine and 0.21 to 2.87 ppm cotinine with correlation coefficients greater than 0.9996. The typical linear relationship for the calibration curve can be expressed by the following regression equations:

$$\textit{Peak Height} = 3.265\ [\textit{nicotine}] - 0.006 \quad .....(2)$$

$$\textit{Peak Height} = 5.268\ [\textit{cotinine}] + 0.001 \quad .....(3)$$

TABLE 4

Precision and Accuracy of Concentration Measurements of Nicotine and Cotinine in Urine Samples

Nicotine				Cotinine			
Conc. (ppm)	Average±SD	CV	E_r %	Conc. (ppm)	Average±SD	CV	E_r %
0.20	0.19±0.02	10.5	-5.0	0.21	0.20±0.02	10.0	-5.0
0.53	0.54±0.01	1.9	1.9	0.35	0.34±0.02	5.9	-2.9
1.04	1.03±0.03	2.8	-1.0	0.77	0.81±0.03	3.7	5.2
1.98	1.90±0.06	3.1	-4.0	1.51	1.48±0.03	2.0	-2.0
2.85	2.87±0.06	2.1	0.71	2.87	2.89±0.03	1.0	0.70

0.20 M SDS and 3% 2-propanol at pH 4.60 and 1.0 mL min^{-1}; temperature 40 °C; wavelength 260 nm. Four replicates at each concentration.

where the peak height is in cm and the concentration is in ppm. The minimum detectable levels of nicotine and cotinine were found to be 0.18 ppm and 0.11 ppm based on a signal-to-noise ratio of 3 with an injection volume of 20 μL. These detection limits are comparable to those values reported for nonsmokers (3).

The precision and accuracy of this technique was determined by making replicate measurements of five different standards within a concentration range of 0.20 to 2.85 ppm nicotine and of 0.21 to 2.87 ppm cotinine. Very low relative standard deviations with low relative errors were obtained (Table 4).

Analytical Applications

The amount of nicotine and cotinine in a pooled 24-h urine sample from a healthy smoker volunteer using the optimal conditions described was

determined. A 25.00 mL sample was acidified to pH 2.00 and filtered before injection. Using the regression equation from the calibration curve and recovery values, the amount of nicotine and cotinine in the urine was determined to be: 0.677 μg nicotine/mL and 5.483 μg cotinine/mL. These results are comparable to those reported for a smoker (2-4).

CONCLUSIONS

The micellar liquid chromatographic method described is a simple, precise, accurate and sensitive method for the simultaneous determination of nicotine and cotinine in human urine. It utilizes a mobile phase which is nontoxic, nonflammable and relatively inexpensive, and eliminates the need for pretreating the urine. A complete analysis can be obtained within 15 minutes. Therefore this method is suitable for use in clinical and pharmacokinetic studies, or in a physician's office.

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A STABILITY-INDICATING HPLC METHOD FOR THE DETERMINATION OF BENZALKONIUM CHLORIDE IN PHENYLEPHRINE HCl 10% OPHTHALMIC SOLUTION

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ABSTRACT

A high-performance liquid chromatographic (HPLC) procedure employing ultraviolet (UV) detection for the analysis of benzalkonium chloride (BAK) in Phenylephrine HCl 10% ophthalmic solution is reported. The method requires no sample pretreatment and is sensitive, accurate, and reproducible. The peak area versus BAK concentration is linear over the range of 50-150% of its label claim of 0.05 mg/mL. The mean absolute recovery of BAK using the described method is 103.2 ± 0.6%, (mean ± SD, n = 10). A stress study with heat, acid, base and UV radiation indicates that the method is stability-indicating with no interference from drug, excipients or their degradation products.

INTRODUCTION

Phenylephrine HCl 10% is an alpha sympathetic receptor agonist producing mydriasis of short duration and vasoconstriction. Its indications of use include

prevention of posterior synechiae associated with uveitis and as a mydriatic prior to intraocular surgery [1,2]. Phenylephrine HCl 10% is preserved with benzalkonium chloride, which is a mixture of alkylbenzyldimethylammonium chlorides of the general formula $[C_6H_5CH_2N(CH_3)_2R]Cl$. "R" represents a mixture of alkyls with the $n\text{-}C_{12}H_{25}$, $n\text{-}C_{14}H_{29}$, and $n\text{-}C_{16}H_{31}$ homologues comprising the major portion.

Several HPLC methods exist for the determination of BAK in ophthalmic solutions [3-9], however, these formulations do not contain phenylephrine. When using these methods, a separation problem exits because phenylephrine is 2000 times more concentrated than BAK. Recently, our laboratory investigated several developmental parameters in the separation of BAK from sulfacetamide in Vasocidin® ophthalmic solution [10]. This work has expanded to include quantitating BAK in the presence of high phenylephrine concentrations. This manuscript describes a sensitive, accurate, and reproducible, ion-pair reversed-phase HPLC method for the determination of BAK in an ophthalmic solution containing phenylephrine. Moreover, this method was determined to be stability-indicating.

According to the USP XXII quidelines, analytical methods for the quantitation of major components of bulk drug substance or preservatives in finished pharmaceutical products fall under Assay Category I [11]. Data elements required for Assay Category I include precision, accuracy, selectivity, range, linearity, and ruggedness. The method for BAK in Phenylephrine HCl 10% ophthalmic solution satisfies all of these requirements.

EXPERIMENTAL

Chemicals and Reagents

Phenylephrine HCl 10% ophthalmic solution was formulated at IOLAB Corporation (Claremont, CA, USA).

Phenylephrine HCl and benzalkonium chloride were USP grade. HPLC grade acetonitrile, 1 **N** sodium hydroxide, hydrochloric acid, and ACS reagent grade potassium phosphate monobasic, monohydrate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Hexane sulfonate, sodium salt, HPLC grade, was purchased from Eastman Kodak (Rochester, NY, USA). The water was deionized and distilled. All reagents were used without further purification.

Apparatus

The chromatographic system consisted of a Waters model 600E system controller and pump, a WISP 712D autosampler, and a Waters 486 variable-wavelength UV detector set at 215 nm (Waters Associates, Milford, MA, USA). A stainless-steel μBondapak™ phenyl column (30 cm x 3.9 mm, 10 μm, Waters Associates) was maintained at ambient temperature.

Mobile Phase

The mobile phase consisted of acetonitrile - buffer (65:35 V/V), where the buffer was comprised of 50 **m**M potassium phosphate monobasic, monohydrate and 57 **m**M hexane sulfonate, sodium salt, adjusted to pH 6.3 with 1**N** NaOH. The mobile phase was filtered through a 0.45 μm filter and degassed for 30 minutes. The flow rate was 1.8 mL/minute with a typical operating pressure of **ca.** 80 bar. Under these conditions, the retention times of the C_{12} and C_{14} homologues of BAK were 7.5 and 12.1 minutes, respectively.

Preparation of BAK Solutions

A BAK Stock Solution was prepared by accurately weighing BAK (W_S) into a tared volumetric flask (V_1) and diluting to volume with water.

A BAK Standard Solution was prepared by pipeting the BAK Stock Solution (V_2) into a volumetric flask (V_3) and diluting to volume with water.

System Suitability

The system suitability results were calculated according to Chromatography <621> of the USP XXII from typical chromatograms [11]. The instrument precision as determined by six successive injections of a BAK Standard Solution should provide a relative standard deviation (RSD) not greater than 1.0%. The column efficiency, when calculated using the C_{14} peak should be greater than 2000 theoretical plates. The tailing factor for any homologue should not exceed 2.0 at 5% peak height. Finally, the resolution between peaks must be greater than 1.5.

The Test Solution (Phenylephrine HCl 10% ophthalmic solution with no sample work-up) is used to verify that the method meets all of the suitability limits.

Stress Study

The specificity of the method was studied through the analysis of stressed Test Solutions containing the label claim of BAK (0.05 mg/mL) and stressed Placebo Solutions (Test Solution without BAK). The stressed samples were subjected to heat, acidic, basic and UV light environments.

Five mL aliquots of the Test and Placebo Solutions were sealed in transparent glass containers and exposed to a UV radiation source (200-400 nm, 40 mWatt/cm^2) for 16 days. Other 5.0 mL aliquots were adjusted to either pH 2 with concentrated HCl or pH 12 with 50% NaOH and sealed in glass containers with equal head space and stored at 88°C for up to 16 days.

Data Acquisition

The peak areas of the C_{12} and C_{14} homologues of BAK were measured using a PE Nelson 900 series interface and down-loaded to PE Nelson Turbochrom 3 workstation (Perkin-Elmer Corporation, Cupertino, CA, USA). The chromatographic data was automatically processed for peak area followed by an unweighted linear regression analysis.

Calculations

The BAK content of the Test Solution was calculated according to the individual BAK homologue peaks. The total BAK content was obtained by combining the BAK homologue concentrations of the Test Solution. The response factor, RF_i, for each BAK homologue peak is:

$$RF_i = \frac{W_S \times F \times P_i \times V_2}{V_1 \times V_3 \times PA_{Si} \times 100}$$

where W_S is the quantity (mg) of the BAK standard used, F is the purity factor (mg/mg) of the BAK standard, P_i is the composition (%) of any homologue in the BAK standard, V_1 is the volume of the flask used to prepare the BAK Stock Solution, V_2 is the pipetted volume of the BAK Stock Solution, V_3 is the volume of the volumetric flask used to prepare the BAK Standard Solution, and PA_{Si} is the peak area of the corresponding homologue of the BAK Standard Solution.

The concentration of any BAK homologue, C_{Ti}, is:

$$C_{Ti}\ (mg/mL) = RF_i \times PA_{Ti}$$

where RF_i is the response factor of any homologue peak and PA_{Ti} is the peak area of the corresponding homologue of the Test Solution. The total BAK content, C, is:

$$C\ (mg/mL) = \Sigma C_{Ti}$$

where ΣC_{Ti} is the sum of the BAK homologue concentrations calculated in the above section.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms obtained from a 50 μL injection of a Standard, Test Solution and Placebo are illustrated in Figure 1 (a-c), respectively. The retention times of the C_{12} and C_{14} homologues of BAK were 7.5 and 12.1 minutes, respectively. The overall chromatographic run time was 20 minutes.

System Suitability

The column efficiency for the C_{14} homologue of BAK was 2500 theoretical plates. The tailing factors of the C_{12} and C_{14} homologues were 1.4 and 1.3, respectively. The resolution was 5.9 between the C_{12} and C_{14} peaks. The instrument precision, determined by 6 replicate injections of the BAK Standard Solution, exhibited a RSD of 0.4%.

Precision and Accuracy

The precision (RSD) and accuracy (relative error, RE) were determined by analyzing Placebo Solutions spiked with BAK, in replicates of six, ranging from ca. 50-150% (23.5-80 μg/mL) of its label claim in Phenylephrine HCl 10% (Table 1).

Recovery

The recovery of BAK was determined by comparing the concentration found in Phenylephrine HCl 10% to that of the BAK Standard Solution. The mean found recovery of BAK over the range of 50-150% its label claim was 103.2 ± 0.6% (mean ± SD, n=10, Table 2).

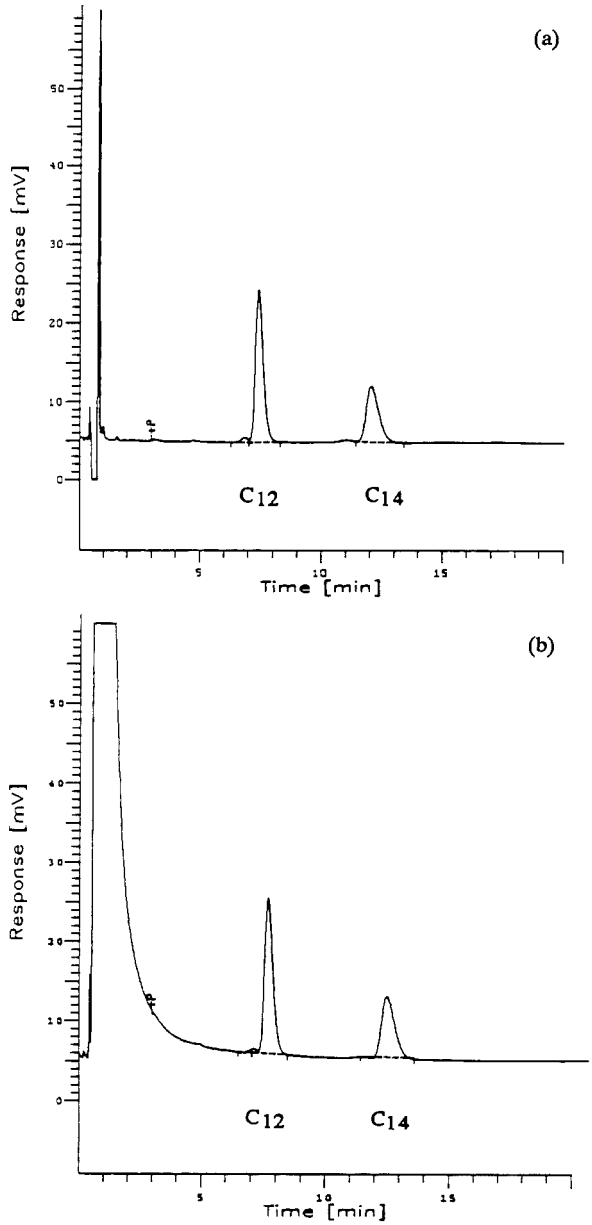

FIGURE 1. Typical chromatograms of (a) a Standard Solution of BAK, (b) Phenylephrine HCl 10% ophthalmic solution containing BAK and (c) Phenylephrine HCl 10% ophthalmic solution not containing BAK.

(continued)

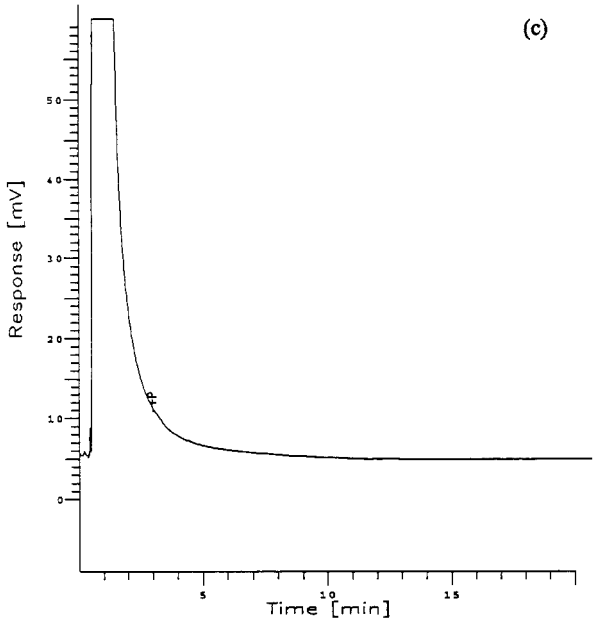

FIGURE 1 (Continued).

TABLE 1

Accuracy and Precision of BAK in Phenylephrine HCl 10%

Nominal Conc. (μg/mL)	n	Mean Found Conc. (μg/mL)	%RSD	%RE
23.50	6	24.29	0.61	3.4
37.50	6	39.01	0.47	4.0
47.00	6	48.55	0.45	3.3
56.40	6	58.03	0.46	2.9
80.00	6	82.73	0.20	3.4

TABLE 2

% Recovery of BAK in Phenylephrine HCl 10%

% Label Claim of BAK in Phenylephrine HCl 10%	% Recovery
50	102.6
50	103.3
75	104.1
75	104.2
100	103.1
100	103.1
125	102.8
125	102.2
150	103.4
150	103.2

Linearity

A linear response in peak area for BAK over the range of 50-150% of its label claim in Phenylephrine HCl 10% was observed. The correlation coefficients were 1.000 (n=6).

Stress Study

Phenylephrine HCl 10% ophthalmic solution was stressed with heat, acid, base, and UV radiation for up to 16 days or until approximately 10% degradation of phenylephrine was achieved. The acid stressed samples were adjusted to pH 2 with concentrated HCl and heated at 88°C for 16 days. No degradation was observed for the acid stressed samples under the described conditions. The base stressed samples were adjusted to pH 12 with 50% NaOH and heated at 88°C for 65 hours. Phenylephrine degradation of 9.2% was observed for the base stressed samples under the described conditions. Ultraviolet light stressed samples were placed in the path of a UV lamp at 40 mWatt/cm^2 for 16 days. No degradation was observed for the UV stressed samples under the described

conditions. Furthermore, no interfering peaks at the retention times for the BAK homologues were observed in any of the stressed sample.

Conclusion

The described assay for the analysis of BAK in an ophthalmic solution containing phenylephrine HCl is sensitive, accurate, and reproducible. Furthermore, the method is stability-indicating with no interference from phenylephrine HCl or excipients or their degradation products under the described stress conditions.

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HPLC DETERMINATION OF MORPHINE-HYDROMORPHONE-BUPIVACAINE AND MORPHINE-HYDROMORPHONE-TETRACAINE MIXTURES IN 0.9% SODIUM CHLORIDE INJECTION

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ABSTRACT

High performance liquid chromatography procedures have been developed for the assay of morphine-hydromorphone-bupivacaine and morphine-hydromorphone-tetracaine mixtures in 0.9% sodium chloride injection. The separation and quantitation of morphine-hydromorphone-bupivacaine was performed on a phenyl column at ambient temperature using a mobile phase of 50:50 v/v 0.02M phosphate buffer pH 6.0-acetonitrile at a flow rate of 1.0 mL/min with the detection of all three analytes at 235 nm. The separation was achieved within 20 min with sensitivity in ng/mL range for each analyte. Morphine, hydromorphone and bupivacaine were linear in 5.0-51, 5.1-51.7 and 5.0-50 μg/mL ranges, respectively. Accuracy and precision were in the range 0.51-1.89 and 0.02-0.50%, respectively, for all three analytes and the limit of detection was close to 250 ng/mL for each component based on a signal to noise ratio of 3 and 20 μL injection. The separation and quantitation of morphine-hydromorphone-tetracaine was achieved on a silica column at ambient temperature using a mobile phase of 75:25 v/v 0.01 M phosphate buffer, pH 4.0-methanol at a flow rate of 1.0 mL/min with detection of the three analytes at 235 nm. The separation was achieved within 20 min with sensitivity in the ng/mL range for each analyte. Morphine, hydromorphone and tetracaine were

linear in the 4.95-49.6, 5.07-50.69 and 5.0-50.17 μg/mL ranges, respectively. Accuracy and precision were in the range 0.07-2.93% and 0.01-0.40%, respectively, for each analyte. The limit of detection was near 330 ng/mL for each compound and was based on a signal to noise ratio of 3 and a 20 μL injection.

INTRODUCTION

Morphine-hydromorphone-bupivacaine (A) and morphine-hydromorphone-tetracaine (B) mixtures are administered by epidural block for the treatment of pain. Interest in our laboratories in the stability and compatibility of the drug mixtures over time in 0.9% sodium chloride injection required the development of HPLC methods. A search of the literature indicated that HPLC methods were not available to assay all three compounds either in mixture A or mixture B concurrently in a single injection.

Hydromorphone, morphine and opiates, in general, have been analysed by HPLC with electrochemical (ECD) or UV detection (1-4) and by radioimmunoassay (RIA)(5,6). The HPLC methods are not as sensitive as RIA, but are more specific and often used in the analysis of the compounds. The HPLC-ECD method involved separation of hydromorphone and morphine on an octylsilane column equipped with an octadecylsilane guard column (1). The mobile phase consisted of 15:85 v/v absolute methanol-50 mM dibasic sodium phosphate pH 3.5 containing 3 mM octanesulphonic acid. The electrode potential was set at +600 mV vs Ag/AgCl. The HPLC-UV method for hydromorphone was achieved on an octadecylsilane column using a mobile phase of 40:1:0.5:58.5 v/v/v/v acetonitrile-anhydrous acetic acid-sodium dodecyl sulphate-water (3). The flow rate was 1.5 mL/min and the detector wavelength was 280 nm. Morphine has also been analysed using UV detection

with an HPLC system consisting of an octadecylsilane column operating at a flow rate of 0.8 mL/min and 26.5:73.5 v/v acetonitrile-0.8mM sodium dodecyl sulphate in 10 mM monobasic phosphate buffer mobile phase (4). The wavelength for detection of the morphine was 210 nm. The RIA method utilized a commercially available RIA kit and detected hydromorphone in the 10-40 ng/mL range with a 0.1 mL serum sample (5,6).

Assay methods for bupivacaine include HPLC (7,8) GC (9,10) and amperometry (11). HPLC and GC are the most commonly reported methods for bupivacaine. The HPLC separation was achieved on an octylsilane column using a 92:8 v/v 10mM monobasic phosphate buffer pH 2.4-tetrahydrofuran mobile phase with detection at 210 nm. The flow rate was set at 1.6 mL/min. The GC procedure used nitrogen sensitive detection and was utilized in pharmacokinetic studies of bupivacaine.

Tetracaine has been analysed by derivative UV spectrophotometry (12) and HPLC (13,14). The spectrophotometric method was used to determine a cocaine and tetracaine mixture. A reversed phase HPLC method used an octadecylsilane column with a 70:30 v/v methanol-phosphate buffer pH 7.2 mobile phase and UV detection (13). An ion-pair chromatographic procedure used a mobile phase composed of acetonitrile, phosphate buffer, sodium chloride and tetrabutylamminium hydrogen sulphate (14). An octadecylsilane column was used for the separation and the tetracaine was detected at 294 nm.

In this paper, isocratic HPLC assays are presented for the simultaneous analysis of morphine, hydromorphone and bupivacaine (Mixture A) and

morphine, hydromorphone and tetracaine (Mixture B) in 0.9% sodium chloride injection. Mixture A was separated on a phenyl column using a buffered aqueous-acetonitrile eluent while Mixture B was separated on an underivatised silica column using a buffered aqueous-methanol eluent. Both separations were achieved within 20 min with sensitivities in the ng/mL range.

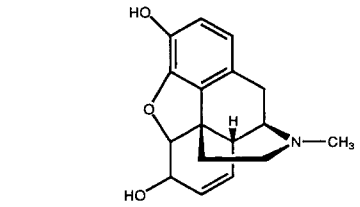
EXPERIMENTAL

Reagents and Chemicals

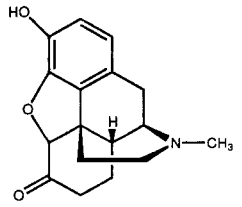
The structural formulae of the compounds studied are shown in Figure 1. Tetracaine and bupivacaine were purchased as their hydrochloride salts from Sigma Chemical Co. (St. Louis, MO 63178, Lot No. 102H0648 and 38F0507, respectively). Hydromorphone hydrochloride (Lot No. 31000144) was purchased from Knoll Pharmaceutical Company (Whippany, NJ 02101). Morphine sulfate reference standard was purchased from the United States Pharmacopeia (Rockville, MD 20852). Acetonitrile and methanol (J.T. Baker, Phillipsburg, NJ 08865) were HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell GA 30076). Monobasic potassium phosphate, potassium hydroxide and concentrated phosphoric acid were Baker analysed reagents.

Instrumentation

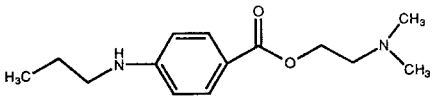
The chromatographic separation were performed on an HPLC system consisting of a Water Model 501 pump (Milford, MA 01757), an Alcott Model 728 autosampler (Norcross, GA 30093) equipped with a 20 μL loop, a Beckman Model 163 variable wavelength UV-VIS detector (Fullerton, CA 92634) and a Hewlett Packard Model 3395 integrator (Palo Alto, CA). Separation of Mixture



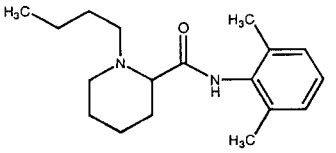
MORPHINE



HYDROMORPHONE



TETRACAINE



BUPIVACAINE

Figure 1 - Chemical structures of compounds studied.

A was achieved on a 30 cm phenyl column (3.9 mm i.d., 10 μm particle size, Waters μ Bondapak, Milford, MA 01757). The mobile phase consisted of 50:50 v/v 0.02M aqueous monobasic potassium phosphate, pH 6.0 (adjusted with 1N potassium hydroxide) -acetonitrile. The separation of Mixture B was accomplished on a 22 cm underivatized silica column (4.6 mm i.d., 5μm particle size, Brownlee Silica, Applied Biosystems, Inc., San Jose, CA 95134). The mobile phase consisted of 0.01M aqueous monobasic potassium phosphate pH 4.0 (adjusted with 10% phosphoric acid) - absolute methanol. The mobile phases were filtered through a 0.45 μm nylor-66 filter (MSI, Westborough, MA 01581) and degassed by sonication prior to use. The flow rate was 1.0 mL/min and the detector was set at 235 nm for both mixtrues.

Preparation of Standard Solutions

Combined standard solutions containing morphine, hydromorphone and tetracaine and morphine, hydromorphone and bupicacaine were prepared by accurately weighing 5 mg each of morphine sulfate, hydromorphone hydrochloride and tetracaine hydrochloride in a 100 mL volumetric flask and 5 mg each of morphine sulfate, hydromorphone hydrochloride and bupivacaine hydrochloride in a separate 100 mL volumetric flask. Fifty mL of 0.9% sodium chloride injection was added to each mixture and the flasks were shaken vigorously for 2 minutes folowed by addition of 0.9% sodium chloride to volume. These standard solutions, along with 1:10, 4:10 and 6:10 dilutions, gave solutions containing 5, 20, 30, and 50 μg/mL of each of the drugs as their respective salts. Four point calibration curves were constructed for each analyte using the analytical conditions established for each mixture. Additional dilutions (2:10 and 8:10) of each of the two combined standard solutions were prepared in 0.9% sodium chloride injection to serve as spiked samples for each analyte to determine accuracy and precision. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration in μg/mL.

RESULTS AND DISCUSSION

The goal of this study was to develop isocratic HPLC assays for the analytes of Mixture A and Mixture B in 0.9% sodium chloride injection within a 20 min time interval. Stability studies of these mixtures would require that assay procedures quantitate each analyte with reasonable accuracy and precision.

There were no reports in the scientific literature describing the separation of either combination of drugs in a single mixture. Initial studies to separate both analyte mixtures involved the use of an octadecylsilane column with various mobile phases containing acetonitrile-aqueous phosphate buffer. It was observed that there was great difficulty in separating the three components of either Mixture A or B within the desirable time interval. In addition, morphine and hydromorphone eluted close to the solvent front. When an ion pairing agent was added to the mobile phase, morphine and hydromorphone were retained longer and thus separated from the solvent front, but tetracaine or bupivacaine eluted late (> 30 min). Octylsilane columns were also investigated and essentially gave the same data as the octadecylsilane columns. With both octadecylsilane and octylsilane columns, tetracaine and bupivacaine eluted as broad peaks with extensive tailing. A deactivated octylsilane column provided sharp peaks for all the analytes but separation of either mixture was not accomplished within 20 min. Our attention turned towards a phenyl column. One commercial phenyl column gave good separation of the components, but at higher analyte concentrations gave rise to split peaks. However, another brand of phenyl column provided a very good separation of the components in Mixtures A and B. The best separations were obtained with a 50:50 v/v 0.02M aqueous phosphate buffer pH 6.0-acetonitrile. The run times for Mixture A and Mixture B were 20 and 25 min, respectively. An underivatized silica column was investigated to see if even faster run times could be obtained. Good separation of both Mixtures A and B were obtained using an aqueous phosphate buffer pH 4.0 with either acetonitrile or absolute methanol as organic modifier.

It was observed that there was better resolution and a faster turn-around time (< 20 min) when methanol was used as the organic modifier. Good separations were otobtained for both mixtures using a 75:25 v/v aqueous monobasic phosphate buffer pH 4.0-absolute methanol mobile phase. The run time was 25 min for Mixture A and 20 min for Mixture B. Since we were looking for run times of 20 min or less, we decided to use the phenyl column with a mobile phase of 50:50 v/v 0.02M phosphate buffer pH 6.0-acetonitrile to assay Mixture A and a silica column with a mobile phase of 75:25 v/v 0.01M phosphate buffer-methanol for the analysis of Mixture B. Typical chromatograms showing the separation of the analytes in Mixtures A and B under their respective analytical conditions are shown in Figures 2 and 3.

From earlier studies in this lab, it was shown that morphine and hydromorphone gave maximum UV absorbance at 235 nm in a acetonitrile phosphate buffer solvent system. Studies indicated that 210, 230 and 280 nm could also be used to detect bupivacaine and 235 and 294 nm for tetracaine. In acetonitrile - phosphate buffer mobile phases, the UV absorption maxima for tetracaine were 235 nm and 315 nm. Since there was good absorption of all analytes at 235 nm, the wavelength for detection of the analytes in both imxtures was set at 235 nm.

The HPLC method for Mixture A showed concentration versus absorbance linearity for morphine, hydromorphone and bupivacaine in the 5.0-51, 5.1-51.7 and 5.0-50 μg/mL ranges, respectively, at 235 nm. Table 1 gives the analytical figures of merit for each of the analytes in Mixture A. The HPLC method for Mixture B showed concentration versus absorbance linearity for

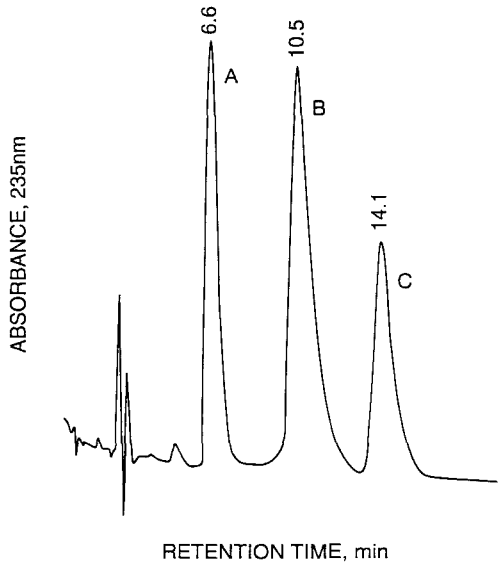


Figure 2 - Typical HPLC chromatogram of morphine (A), hydromorphone (B) and bupivacaine (C) on a phenyl column with 50:50 v/v 0.02 M phosphate buffer pH 6.0-acetonitrile. See Experimental Section for assay conditions.

morphine, hydromorphone and tetracaine in the 4.95-49.6, 5.07-50.69 and 5.0-50.17 μg/mL ranges, respectively, at 235 nm. Table 1 also gives the analytical figures of merit for each of the analytes in Mixture B. A photodiode array detector (Model 990, Waters associates, Milford, MA 01757) was used to verify that none of the degradation products of the analytes in either Mixture A or B (analysed under their respective analytical conditions) interfered with the quantitation of each drug at 235 nm. These experiments were performed on solutions of each drug in 0.9% sodium chloride injection after they had been degraded for 6 hr at 80°C in both 1.0 N hydrochloric acid and 1.0 N sodium hydroxide.

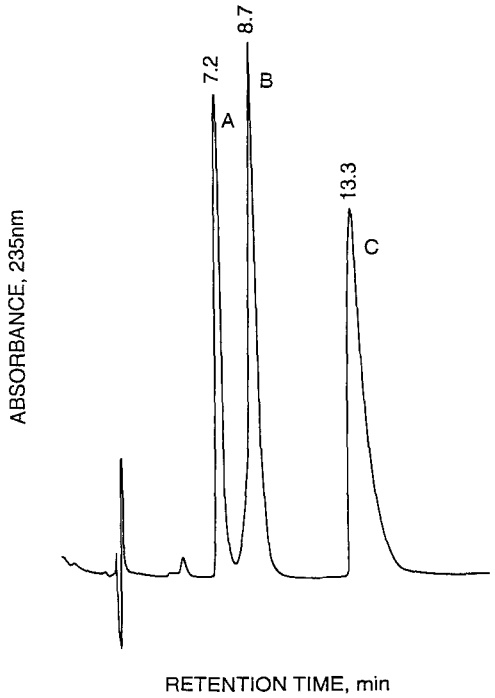


Figure 3 - Typical HPLC chromatogram of morphine (A), hydromorphone (B) and tetracaine (C) on a silica column with 75:25 v/v 0.01 M phosphate buffer pH 4.0-methanol. See experimental Section for assay conditons.

Percent error and precision of the methods were evaluated using spiked samples containing each analyte. The results for Mixture A and Mixture B are shown in Table 2. The results indicate that the procedures give acceptable accuracy and precision for the analytes in both mixtures.

Intra-day variabilities for morphine, hydromorphone and bupivacaine (Mixture A) expressed as % RSD were 0.6, 0.95 and 0.96% (n=6), respectively. Inter-day variabilities of the assay for these drugs were in the 0.6-

Table 1

Analytical Figures of Merit for Morphine-Hydromorphone-Bupivacaine and Morphine-Hydromorphone-Tetracaine

Mixture	r^{2a}	System Suitability[b]	LOD ng/mL[c]	k'	Theoretical Plates[d]	Tailing Factor[e]	Rs
A							
Morphine	0.9975	0.60	258	1.34	1480	1.5	
							3.01
Hydromorphone	0.9993	0.95	253	2.31	1069	1.7	
							5.69
Bupivacaine	0.9998	0.96	254	4.97	2050	1.3	
B							
Morphine	0.9979	1.85	330	2.02	3006	1.2	
							2.63
Hydromorphone	0.9991	1.03	338	2.74	2053	1.1	
							4.87
Tetracaine	0.9996	1.49	334	5.15	1386	1.7	

[a] Range examined from 5.1-51 μg/mL morphine (n=9), 5.17-51.7 μg/mL hydromorphone (n=9) and 5.0-50.2 μg/mL bupivacaine for Mixture A at 235 nm and 4.9-49 μg/mL morphine (n=9), 5.0-50.6 μg/mL hydromorphone (n=9) and 5.0-50.1 μg/mL tetracaine (n=9) for Mixture B at 235 nm.

[b] Mean RSD % of 6 replicate injections at 51.0 μg/mL morphone, 51.7 μg/mL hydromorphone and 50.3 μg/mL bupivacaine for Mixture A at 235 nm and 49.5 μg/mL morphine, 50.7 μg/mL hydromorphone and 50.2 μg/mL tetracaine for Mixture B at 235 nm.

[c] Limit of Detection, S/N=3.

[d] Calculated as $N = 16 (tr/w)^2$.

[e] Calculated at 10% Peak Height.

TABLE 2

Accuracy and Precision Using Spiked Drug Samples

Mixture	Concn Added, μg/mL	Concn Found, μg/mL[a]	Percent Error	RSD (%)
A				
Morphine	10.21	10.30 ± 0.004	0.88	0.04
	40.84	41.05 ± 0.135	0.51	0.33
Hydromor-	10.35	10.46 ± 0.052	1.06	0.50
phone	41.40	41.81 ± 0.083	0.99	0.20
Bupivacaine	10.06	10.25 ± 0.016	1.89	0.16
	40.22	40.65 ± 0.006	1.07	0.015
B				
Morphine	9.89	9.82 ± 0.001	0.71	0.01
	39.56	40.72 ± 0.165	2.93	0.41
Hydromor-	10.14	10.22 ± 0.009	0.79	0.09
phone	40.55	40.87 ± 0.070	0.79	0.17
Tetracaine	10.03	9.96 ± 0.012	0.70	0.12
	40.14	40.17 ± 0.006	0.07	0.015

[a] Mean ± standard deviation based on n = 3.

3, 0.95-1.6, 0.96-1.5 (n = 18 over 3 days) ranges, respectively. Intra-day variabilities for morphine, hydromorphone and tetracaine (Mixture B) expressed as % RSD were 1.58, 1.38 and 1.72% (n = 6), respectively. Inter-day variabilities of the assay for the analytes were in the 1.58-2.8, 1.0-2.7 and 1.49-2.55% (n = 12 over 3 days) ranges, respectively.

In summary, a phenyl column with an aqueous 0.02M pH 6.0 buffer-acetonitrile mobile phase was shown to be suitable for the separation and quantitation of a morphine-hydromorphone-bupivacaine mixture (A) in 0.9% sodium chloride injection. It has also been shown that a silica column with an

aqueous 0.01M phosphate buffer pH 4.0-methanol mobile phase is amenable for the separation and quantitation of a morphine-hydromorphone-tetracaine mixture (B). This study suggests that the above listed HPLC methods can be used to investigate the chemical stability of the three analytes in either Mixture A or B.

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DIRECT DETERMINATION OF APOLIPOPROTEINS IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

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ABSTRACT

A simple and sensitive high performance liquid chromatographic (HPLC) method is described for the direct quantitation of apolipoproteins (apo) A-I, A-II and B in plasma. The apolipoproteins are well resolved, which allows for quantitation of apo A-I, A-II and B and calculation of the ratio of A-I/A-II. Only 10 μl is required for a single determination of these three apolipoproteins. Compared to other methods requiring separation of HDL by ultracentrifugation and delipidation of apoproteins with organic solvents, this method avoids any loss of apolipoproteins. The HDL in supernatant of plasma precipitated with phosphotungstic acid/magnesium chloride reagent contained essentially all of the plasma apo A-I and apo A-II. However, this supernatant also contained about 37% of plasma apo B, suggesting incomplete precipitation of apoproteins in very low and low density lipoproteins. Thus, this HPLC method can rapidly determine three apolipoproteins in a small volume of plasma without any loss and also can detect incomplete separation of apolipoproteins of very low and low density lipoproteins from HDL fraction.

* CFAR Contribution Number 2231.

INTRODUCTION

High density lipoproteins (HDL) play a central role in cholesterol transport and metabolism via their interaction with lecithin-cholesterol acyl transferase (1). Low concentrations of HDL in the plasma are associated with increased risk of atherosclerotic coronary artery disease (2). The protein fraction of HDL is composed of a number of chemically and immunochemically distinct polypeptides referred to as apolipoproteins (apo), of which apo A-I and apo A-II are the predominant species (80-90% of the total protein) (3). Recently, studies with transgenic mice have elucidated the importance of apoproteins A-I and A-II in the development of atherosclerosis. Warden et al. (2) showed that fatty streak atherosclerotic lesions were formed in transgenic mice which exhibited elevated HDL levels and overexpressed apo A-II. Transgenic mice overexpressing human apo A-I also showed elevated plasma HDL but the development of aortic fatty streaks was reduced, even when the mice were maintained on atherogenic diets high in fat and cholesterol (4-6). This suggests that the ratio of apo A-I to apo A-II in HDL is an important criterion indicative of pathogenesis of atherosclerosis.

A number of different methods have been developed to measure apo A-I and A-II levels in HDL. These include immunoassays (1,7,8), capillary electrophoresis (9) and high performance liquid chromatography (HPLC). The latter includes both reversed-phase (10,11) and gel exclusion chromatography (12-14). All of the above methods, with the exception of an immunoturbidimetric assay (7), require purification of the HDL fraction from plasma by ultracentrifugation. Further, most of these methods (1, 7, 8, 10, 11, 13) require delipidation of the apolipoproteins with some organic solvent(s). Both of these steps introduce the possibility of sample loss.

Kinoshita et al. (13) solubilized the HDL fraction with sodium dodecyl sulfate (SDS) and then separated apolipoproteins by using a gel exclusion HPLC column. We have standardized conditions to apply this technique directly to plasma samples and to the HDL fractions

in the supernatant after precipitating the very low density lipoproteins (VLDL) and low density lipoproteins (LDL) fractions with a standard precipitating reagent. Our method eliminates the need to separate HDL by ultracentrifugation and delipidation with organic solvents. Thus, there is no loss of apolipoproteins due to preparatory steps. There is an added advantage that very small samples (10 μl plasma or supernatant) are required for analysis of the major apolipoproteins (A-I, A-II and B) and the determination of the ratio of apo A-I to apo A-II.

MATERIALS AND METHODS

Reagents

Apolipoprotein A-I, A-II, B calibration serum (from human serum) was obtained from Boehringer Mannheim Biochemica (Lot No. 162794-66, exp. 31.Jul.94, Laval, Quebec, Canada). This serum contained (mg/dl) 163 apo A-I, 49 apo A-II, and 96 apo B. Phosphotungstic acid/magnesium chloride reagent ($Pta/MgCl_2$) (procedure no. 352-4, lot no. 52H-6132) for precipitating LDL and VLDL fractions from plasma and total cholesterol reagent (procedure no. 352, lot no. 033H6129) were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

The calibration serum was reconstituted with 1 ml distilled and deionized water. Aliquots (10 μl) were removed and diluted with 190 μl of 0.1 M phosphate buffer, pH 7.0, containing 0.2% SDS. The stock solution was frozen at -20°C. The diluted calibration serum was stored at 4°C and analyzed within two days.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a model P200 Spectra Series binary pump (Spectra-Physics Analytical Canada Ltd., Toronto, Ontario, Canada) equipped with a Rheodyne model 712NS syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), and a Datajet single-

channel integrator (Spectra-Physics). The apolipoproteins were detected with a variable wavelength ultraviolet detector (Model UV2000, Spectra-Physics) set at 280 nm. Data was also captured to a Trillium 386SX IBM compatible computer using "Winner-on-Windows" software package (Spectra-Physics Canada Ltd.).

The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% SDS; the flow rate was 0.33 ml/min. The solvent was filtered through a 0.45 um filter (Millipore Canada, Mississauga, Ontario, Canada) under vacuum, then allowed to sit overnight to remove the foaming from the SDS.

Analysis was performed on a TSK-Gel G3000SW gel filtration HPLC column (7.5 mm i.d. X 60 cm, particle size 10 um, TosoHaas, Montgomeryville, PA, U.S.A.), equipped with a TSK-Gel SW guard column (7.5 mm i.d. X 7.5 cm, particle size 10 um, TosoHaas).

Assay procedures

Blood samples were taken with heparinized syringes from the vena cava of adult male guinea pigs. The blood samples were centrifuged at 3000 rpm for 15 minutes at 4°C to obtain plasma.

Samples of plasma (30 μl) were placed in 1.5 ml polypropylene centrifuge tubes (Eppendorf, Hamburg, Germany), the required volume of phosphate buffer containing SDS was added to each tube, and the tubes were capped and heated at 60°C for five minutes to delipidate the apolipoproteins. Aliquots (10 μl) of each sample were injected onto the HPLC column.

Quantitation of apolipoproteins in the HDL fraction of plasma samples was performed as follows. Plasma samples (125 μl) were incubated with 25 μl of Sigma Pta/$MgCl_2$ reagent for five minutes at room temperature to precipitate LDLs and VLDLs, and the tubes centrifuged for ten minutes at 3000 rpm. The supernatants (30 μl) were transferred to 1.5 ml Eppendorf tubes and the required volume of phosphate buffer containing 0.2% SDS was added. Samples were heated as for plasma and 10 μl aliquots were analyzed by HPLC. The amounts of apoproteins in biological samples were quantitated by comparison to peak areas obtained by injecting known amounts of standard apolipoproteins.

Analytical variables

The relationship between area count response and injection size was determined using the diluted calibration standard. Aliquots of 2, 5, 10 and 15 μl, corresponding to 0.62, 1.54, 3.08 and 4.62 μg of total apoproteins A-I, A-II and B, respectively, were injected onto the HPLC column, and the correlation coefficient was determined for each apoprotein.

Reproducibility of injection was determined by triplicate analyses of 10 μl aliquots of plasma samples by HPLC.

RESULTS AND DISCUSSION

Chromatography

Elution with sodium phosphate buffer containing 0.1% SDS resulted in well-resolved peaks for the three main apoproteins. The HPLC chromatogram for the apolipoprotein calibration standard is shown in Figure 1 (a). The retention times for apoproteins B, A-I and A-II were 33.0, 39.9 and 54.2 minutes, respectively. The identities of the three major apolipoproteins were confirmed by injecting purified standards from human sera (Sigma Chemical Co., cat. no. A9284 (A-I), A8909 (A-II), A 9910 (B)) onto the HPLC column. A shoulder eluting just after apo B may represent some of the minor apolipoproteins (apo E, A-IV, B48).

Kinoshita et al. (14) found that the relation between the peak area of each apolipoprotein fraction separated by HPLC and the volume of sample applied to the column was linear for protein contents of 9 to 72 μg. This was confirmed in the present study with both the calibration standard and the plasma samples for smaller protein concentrations of 0.3 to 2.3 μg (r^2=0.999).

Reproducibility of injection was determined by injecting 10 μl of the same sample three times, and calculating the percent standard deviation in the area counts for the resulting peaks. The variation in peak areas was found to be 0.7%, 1.0% and 1.1%, for apolipoproteins A-I, A-II and B, respectively.

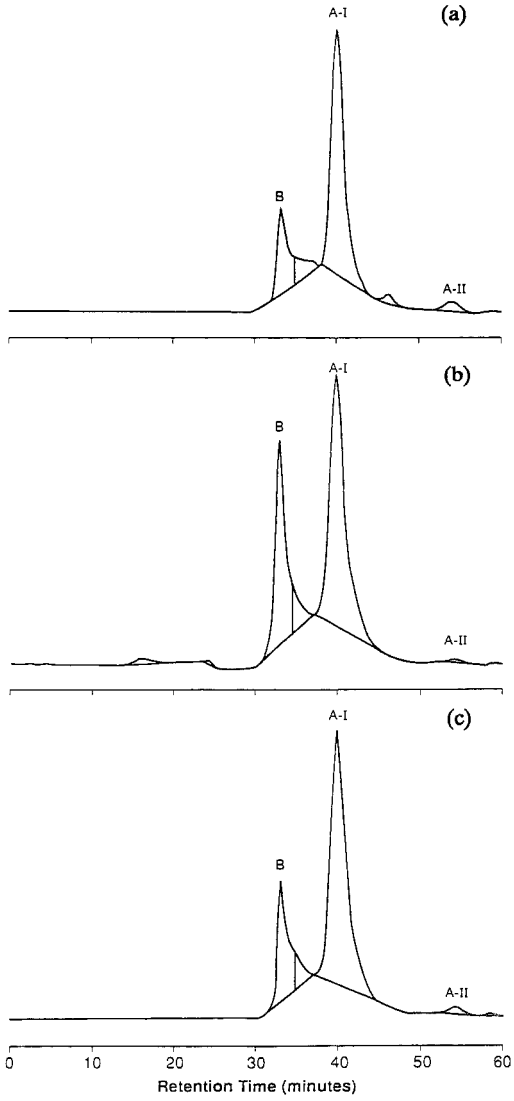

FIGURE 1.

HPLC separation of apolipoprotein from (a) calibration standard, (b) guinea pig plasma and (c) its HDL fraction. Chromatographic separation conditions are given in the text.

TABLE 1

Effect of Sodium Dodecyl Sulphate Concentration on Solubilization of Apolipoproteins

Guinea pig #	Total cholesterol (mg/dl)	SDS[1] in buffer (%)	Dilution[2] (x)	Apoproteins A-I (mg/dl)	Apoproteins A-II (mg/dl)	Apoproteins B (mg/dl)	Ratio of A-I/A-II
1	52.2	0.1	5	48.8	5.6	77.0	8.7
		0.2	10	51.1	6.7	71.6	7.6
		0.2	15	68.1	9.9	91.9	6.9
		0.5	5	39.0	5.2	56.9	7.5
2	67.8	0.2	15	52.3	6.0	61.7	8.7
		0.2	20	68.0	9.4	86.4	7.2
3	289.0	0.2	15	74.5	105.3	23.9	4.4
		0.2	20	85.2	126.3	29.2	4.3
		0.5	15	76.6	101.5	23.7	4.3

[1] Sodium dodecyl sulphate.
[2] Times (x) plasma was diluted with phosphate buffer containing SDS.

Analysis of biological samples

Guinea pig plasma samples and HDL samples (prepared by precipitation of LDLs and VLDLs) directly assayed by our method produced clear separation of apolipoproteins A-I, A-II and B as shown in Figure 1, (b) and (c), respectively. The amounts of apolipoproteins A-I, A-II, and B in plasma of guinea pigs containing 52.2 to 289 mg/dl cholesterol are shown in Table I. It is apparent that the use of 0.2% SDS solutions maximized the amounts of each of the above three apolipoproteins. A 20-fold dilution of plasma with 0.2% SDS compared to 15-fold dilution also markedly increased the amounts of apoproteins determined. The use of 0.5% SDS solution had a tendency to decrease the levels of various apolipoproteins, particularly for samples low in total cholesterol.

The levels of apolipoproteins A-I and A-II determined in guinea pig plasma were very similar to the values obtained by analyzing the HDL fraction prepared by Pta/$MgCl_2$ precipitation of

TABLE 2

Amounts of Apolipoproteins Present in Guinea Pig Plasma and HDL Fraction

	Apolipoproteins (mg/dl)			
Guinea pig #	A-I	A-II	B	A-I/A-II
Plasma				
4	47.6	4.0	40.3	11.9
5	43.7	6.3	47.9	6.9
6	44.3	6.6	47.0	6.7
HDL				
4	47.2	4.6	15.4	10.3
5	44.6	6.0	16.7	7.4
6	41.3	5.6	18.0	7.4

VLDL and LDL fractions (Table 2). However, there was about 37% of the plasma apo B present in the HDL fraction, although HDL particles are not known to contain any apo B (15). Thus, the HDL fraction prepared by precipitation of VLDL and LDL fractions is not pure in respect of the apolipoproteins.

Summarily, we have developed an HPLC method for clear separation and quantitation of apolipoproteins A-I, A-II and B directly in plasma without any fractionation by ultracentrifugation or delipidation by organic solvents. This procedure requires a very small sample of plasma and does not incur any losses in the preparatory steps. It can be used for the determination of the apo A-I to apo A-II ratio and for detecting any apo B contamination of HDL fractions.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PRAZIQUANTEL IN PLASMA AND TISSUES OF CULTURED FISH FOR RESIDUE AND PHARMACOKINETIC STUDIES

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ABSTRACT

A simple method for the determination of praziquantel in fish plasma and tissues by HPLC is presented. The samples were extracted with acetone, the organic layer then being separated with diethylether-hexane and evaporated to dryness. The lower limit of quantification was 5, 15, and 20 µg/kg, for muscle, liver, and plasma, respectively.

INTRODUCTION

The pseudophyllide cestode Eubothrium crassumw occurs in the intestine of salmonids in both fresh and sea water (1). The presence of this cestode in farmed, Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) has become an increasing problem in Norwegian fish farming (2, 3) and presumably also in

many other countries. Drug treatment against this parasite therefore plays an important role in fish farming. Praziquantel (PQ) is a drug known for its broad spectrum activity against trematodes and cestodes (4, 7). In Norway, appropriate withdrawal periods after end of treatment have been established for this drug, and the fishery control authorities carry out both pre-slaughter and post-slaughter control of drug residues.
Various assay procedures for studying praziquantel levels in serum and other body fluids have been reported: radiometry (8), fluorimetry (9), gas chromatography (10), and biological assay (11). A HPLC method for the determination of praziquantel in serum (12) and tissues (13) has also been published. The latest method (13) is based on UV detection, and is time-consuming and requires rather large amounts of reagents.
This paper describes simple and rapid extraction and clean-up procedures for the determination of PQ in fish plasma and tissues. The method is suitable for pharmacokinetic studies and residue analyses of this (antiparasitic) compound in Atlantic salmon and rainbow trout. This method is reliable and sensitive. There is no interference from the tissue matrix in the chromatographic analysis, which requires only small quantities of chemical reagents.

MATERIALS AND METHODS

Materials and Reagents

Samples of muscle, liver, and plasma of salmon and rainbow trout were used.
All chemicals and solvents were of analytical or HPLC grade. Praziquantel (Droncit vet. "Bayer"), 2-cyclohexylcarbonyl-4-oxo-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a] isoquinoline, was donated by Bayer Kjemi A.S. (Oslo, Norway). Stock solutions (1mg/ml) of PQ were prepared by dissolving the compound in a small amount of acetone and diluting to volume with water. Working standards were prepared by dilution with distilled water. The solutions stored in refrigerator are stable for five days.
Spin-X centrifuge filter units from Costar (Cambridge, MA, USA) were also employed.

Chromatographic Conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 410 Bio solvent delivery

system, an ISS 100 sampling system equipped with a Lauda RMT6 cooler (12°C) from Messgeräte Werk Lauda, (Lauda Königshafen, Germany), and a LC 235C Diode Array detector (Perkin-Elmer, Norwalk, CT, USA). The detector was operated at 205 nm. The integration was carried out using the software programme Omega-2 (Perkin-Elmer), which was operated on an Olivetti M300 personal computer connected to a BJ-330 printer (Canon).
The analytical column (stainless steel, 15 cm x 4.6 mm ID) and guard column (stainless steel, 2 cm x 4.6 mm ID), were packed with 5 µm particles of Supelcosil LC-ABZ (Supelco, Bellefonte, PA, USA).
The mobile phase was a mixture of water-acetonitrile (60:40 for muscle and 61:39 for liver and plasma). The flow rate was 1.0 ml/min for 3 min followed by 0.8 ml/min for 6 min. Between each 25 µl injection, the column was washed for 5 min with 100% acetonitrile with a flow rate of 1.5 ml/min, followed by the mobile phase with a flow rate of 1.8 ml/min for 7 min and 1.0 ml/min for 3 min. The samples were injected at intervals of 26 min.

Sample pretreatment

Plasma. The pretreatment of plasma samples was as follows: to 250 µl plasma were added 100 µl water (or standard) and 1 ml acetone. The mixture was homogenized for approx. 10 sec., and 50 µl 1 M NaOH and 2 ml diethylether-hexane (3:2) were added. The sample was mixed for approx. 10 sec. and then centrifuged for 3 min. (3000 rpm.). The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness at 60°C under a stream of nitrogen. The dry residue was dissolved in 200 µl methanol - solution A (70:30). Solution A was 0.02 M 1-heptane sulfonic acid sodium salt (Supelco, USA)-0.01 M di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany), made by dissolving 4.45 g/l heptane sulphonate and 1.779 g/l di-sodium hydrogen phosphate 2-hydrate in c. 750 ml of water when preparing 1 litre of solution. The pH was then adjusted to 6 with 2 M phosphoric acid and the solution made up to volume with water. After 0.5 ml of hexane had been added, the sample was again whirlimixed. After centrifugation for 3 min, the hexane layer was discharged. To 100 µl of the methanol-based phase was added 100 µl of 0.01 M phosphoric acid (corresponding to 400 µl dilution). The sample was then mixed. The water based phase was filtered through a Costar Spin-X centrifuge filter unit with a 0.2 µm nylon membrane by centrifugation for 4 min. at 10000 rpm. (5600g). Aliquots of the filtrate (25 µl) were injected into the HPLC system.

Muscle. The stepwise procedure for the pretreatment of muscle samples is shown in Fig.1.
The tissue sample, 3 g of muscle, was weighed into a 50 ml centrifuge tube with a screw cap (NUNC, Roskilde, Denmark). Volumes of 300 µl water (or standard) and 4.7 ml acetone were added. The mixture was homogenized for approx. 6 sec. in an Ultra-Turrax TP 18/2 (Janke & Kunkel KG, Ika Werk, Staufen, F.R.G.), and then centrifuged for 3 min. (5000 rpm). Four ml of the supernatant (corresponding 1.5 g muscle) were transferred into a glass-stoppered centrifuge tube and 5 ml diethylether-hexane (3:2) added. The sample was shaken vigorously for 10 sec., and centrifuged for 3 min. at 3000 rpm. The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness at 60°C under a stream of nitrogen. The dry residue was dissolved in 400 µl methanol-solution A (70:30), and kept in a freezer (-20°C) for 5 min. After centrifugation for 3 min. (3000 rpm.), 300 µl water, was added to 300 µl of the methanol-based phase which was then mixed (corresponding to 800 µl dilution). The water-based phase was filtered through a Spin-X centrifuge filter. Aliquots of the filtrate (25 µl) were injected into the HPLC system.

Liver. The samples of liver tissue (3g) were homogenized and extracted as described for muscle tissue. The upper layer (acetone, diethylether, hexane) was transferred to another glass-stoppered tube, and 50 µl 1 M NaOH were added. The sample was shaken vigorously for 5 sec., and centrifuged for 2 min. The upper layer was transferred to another glass-stoppered tube. The organic layer was evaporated to dryness. The dry residue was dissolved in 600 µl methanol-solution A (70:30). After 1 ml of hexane had been added, the sample was again whirlimixed. After centrifugation for 3 min, the hexane layer was discharged, and 160 µl of 0.02 M phosphoric acid was added to 240 µl of the methanol-based phase (corresponding to 1 ml dilution). The sample was again mixed for 3 sec. and filtered through a Spin-X centrifuge filter.
Aliquots of the filtrate (25 µl) were injected into the HPLC.

Calibration curves and recovery studies

The calibration curves for PQ were obtained by spiking plasma, liver and muscle tissue samples with standard solutions to yield 20, 50, 100, 200, 300, 500, 1000, and 2000 ng/ml, 20, 50, 100, 150, and 200 ng/g, and 5, 10, 20, 50, 100, 150 and 200 ng/g of PQ for plasma,

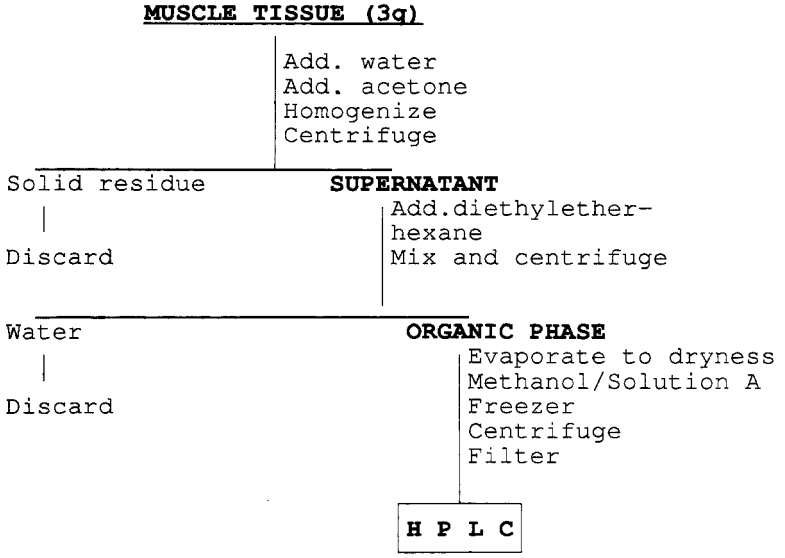

FIGURE 1

Extraction and clean-up procedure for praziquantel from fish muscle.

liver, and muscle, respectively. Duplicate samples were used. The recovery rates were determined by comparing results of analysis of the spiked plasma, liver and muscle samples with those of standard solution. The linearity of the standard curves for PQ in plasma, liver and muscle were tested using peak-height measurements.

RESULTS AND DISCUSSION

Chromatograms of clean plasma, liver, muscle and spiked samples are shown in Figure 2. The standard curves were linear in the investigated areas; 20 - 2000ng/ml, 20 - 200 and 5 - 200 ng/g PQ in plasma, liver and muscle respectively. The linearity of the standard curves was 0.9997 for plasma and 0.9996 for liver and muscle, respectively, when using the external standard method of calculation. The precision and recovery for PQ from plasma, liver and muscle were also calculated and are shown in Table 1.

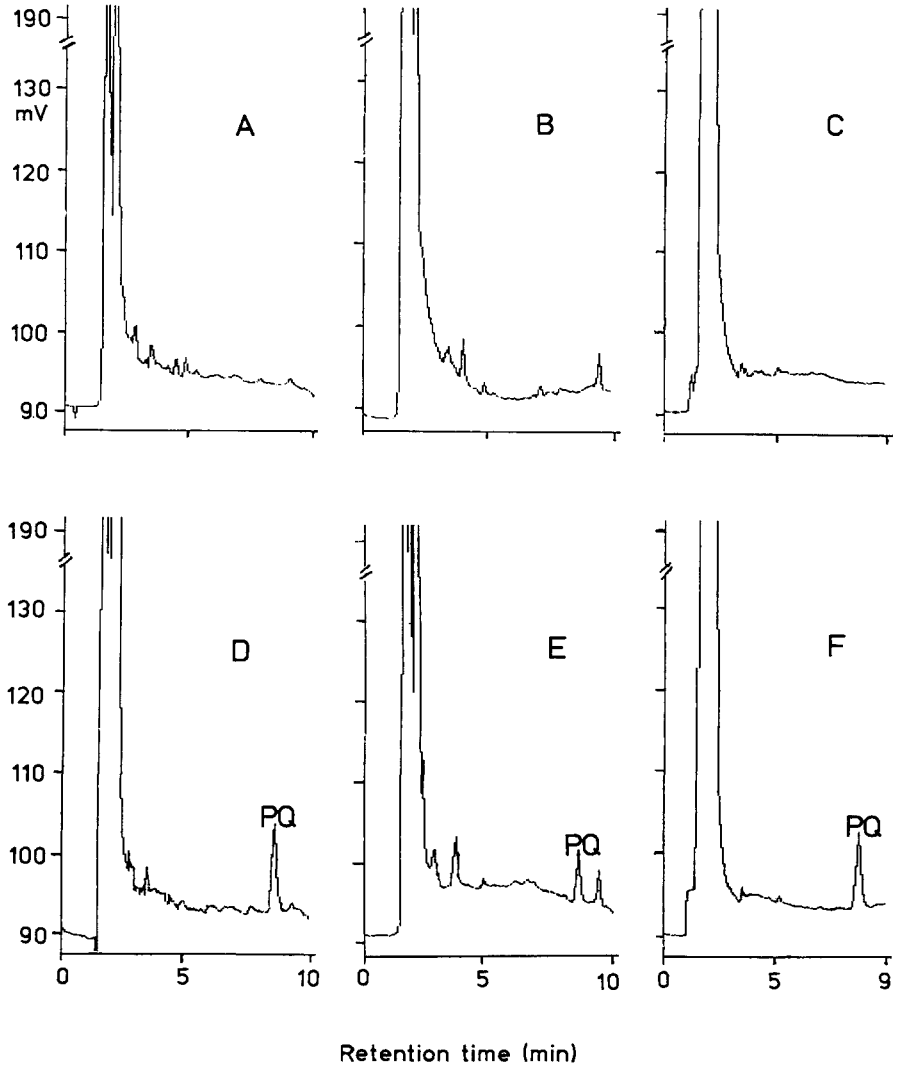

FIGURE 2

Chromatograms of extracts from fish plasma, liver and muscle.
A: drug-free plasma, **B**: drug-free liver, **C**: drug-free muscle, **D**: plasma spiked with PQ (500 ng/ml), **E**: liver spiked with PQ (200 ng/g), **F**: muscle spiked with PQ (200 ng/g).

TABLE 1.

Recovery and repeatability for PQ from spiked samples of plasma, liver and muscle.

Tissue	No. of samples	Amount in spiked samples (µg/ml-g)	Recovery % PQ Mean	S.D.
Plasma	8	0.05	92	1.3
(250 µl)	8	2.00	91	1.6
Liver	8	0.02	82	0.9
(3g)	8	0.10	81	0.8
Muscle	8	0.02	99	0.8
(3g)	8	0.10	100	0.5

S.D.= standard deviation

The extraction procedures were validated, and showed good recovery of PQ. The recovery of PQ varied from 91 to 92, 81 to 82, and 99 to 100% for plasma, liver and muscle, respectively. The precision of these recovery studies varied from 1.3 to 1.6, 0.8 to 0.9, and 0.5 to 0.8% for PQ in plasma, liver and muscle, respectively, the results also show that the precision and accuracy of the quantification of PQ are good.

The muscle clean-up procedure was applied to liver and plasma. Unfortunately, minor residues of endogenous compounds in the liver and plasma extracts interfered with PQ in the chromatogram. These impurities were efficiently removed from plasma by washing with NaOH. However, although this procedure also removed most impurities from liver, some remained, and the liver samples were therefore diluted to 1 ml, with somewhat reduced sensitivity as a consequence. Minor modifications of the mobile phase were therefore necesary. The limit of quantification was 5 and 15 ng/g PQ for muscle and liver, respectively, and 20 ng/ml PQ for plasma. No interference was seen during analysis, when calibrating the curves, and when performing recovery studies.

PQ, is a pyrazino isoquinoline derivative with low solubility in water. Its solubility increases in acetone, but residues of the endogenous compound also

increase. In the event of insufficient samples of liver or muscle (3 g) it is advantageous supplement the difference with water.
The method was tested under practical conditions by analysing about 100 samples from different rainbow trout, with no interfering peaks being observed.
This study has shown that residues of the antiparasitic compound PQ in plasma, liver and muscle may be determined using minimal sample manipulation. The cost of chemicals and the manual work-up procedures are also reduced compared to previously published methods. An experienced technician can carry out sample clean-up of about 30 plasma and muscle samples, and about 18-24 liver samples, per day. The assay shows good precision when using the external standard method. The method is robust, simple, and sufficiently sensitive, with good recovery. The quantification is linear over a wide concentration range. The amount of solvents required is minimized, and pretreatment of tissues by liquid-liquid extractions combined with centrifugation filters, is preferable to solid-phase extraction columns when performing the pretreatment manually. The chromatographic system was specific with regard to PQ.

ACKNOWLEDGEMENTS

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ANALYSIS OF TRIACYLGLYCEROLS OF BORAGE OIL BY RPLC IDENTIFICATION BY COINJECTION

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ABSTRACT

Borage oil is an interesting oil because it is rich in gamma linolenic acid (γLn 18:3 Z6, Z9, Z12). However, since triacylglycerol standards with γLn chains do not exist , there is a problem identifying these particular triacylglycerols. Because the hyphenated techniques (GC-MS, LC-MS) do not give sufficient resolution for unequivocal identification, an easier methodology is described here. It consists of using another oil of known composition as a standard mixture. Use of this method is shown for the assignment of triacylglycerols in borage oil.

INTRODUCTION

Oils found in biological fluids or in foods are complex mixtures which have always been studied by the analytical chemist (1). The challenge of separation for such mixtures is different depending on the class of compounds of interest. Thus, the separation techniques employed are very different if information is desired about one

class with regard to another (i.e. tocopherols, sterols, carotenoids, triacylglycerols) or about one or more specific compound.

One of the most important classes are the triacylglycerols, the main constituents of oils and fats. Many papers have already been published on this class, proposing different chromatographies, such as capillary gas chromatography (2-4), planar chromatography (5-7), argentation chromatography (8), or reversed phase liquid chromatography (9-11), with treatment or not of the sample.

Whatever the technique used, after obtaining the separation from direct injection of a given oil or fat, the last step consists of identifying the triacylglycerols. Due to the complexity of all the chromatograms, an alternative analytical method consists of preseparation and isolation of different fractions of triacylglycerols as a function of their total unsaturation number. In subsequent stage, each fraction can be analysed and characterized (in terms of triacylglycerol identification either by NPLC, RPLC or capillary gas chromatography). Many methods have been employed to answer this question, such as injection of triacylglycerol standards, use of predictive diagrams (12-13), hyphenated techniques (gas or liquid chromatography coupled with mass spectrometer detector) (3,14). The characterization of oils and fats after a treatment of the sample such as a transesterification of triacylglycerols into methyl esters has also been used (1,15,16). However, these methods do not allow for the easy identification of triacylglycerols with similar structures (i.e., same total number of carbon atoms and same total number of double bonds in each chain, but differing in the distribution along the chain) because their retention times are close together.

The method for the identification that has been developed in our laboratory consists in enriching the unknown oil with an oil of known composition. The comparison of the chromatograms of this mixture and of the pure oils allows a more accurate identification.

MATERIALS AND METHODS

Samples

The oils have been bought in supermarkets or kindly donated by the dermatological or cosmetological research center of Pierre Fabre (Gigoulet, France) or the Christian Dior (Orléans, France) and used without further purification. The injection solvent was acetone.

Solvents

Acetonitrile and methylene chloride were HPLC grade (Merck, Darmstadt, Germany; Carlo-Erba, Milan, Italy) and filtered through a 0,5 µm Millipore filter (Whatman, Hillsboro, OR, USA).

Apparatus

The chromatographic system consisted of a Model 1050 pump (Hewlett Packard, Palo-Alto, CA, USA), a model 7125 injection valve with a 20 µl loop (Rheodyne, Cotali, CA, USA) and a Model Sedex 45 light-scattering detector (Sedere, Alfortville, France).The column temperature was controlled using a Croco-cil oven (Cluzeau, Sainte-Foy-la-Grande, France) thermostated with water by means of a Model UCF10 cryostat with a precision of 0.1°C (Julabo, Seelbach, Germany, supplied by Touzart et Matignon, Vitry-sur-Seine, France). The mobile phase flow-rate was 1 ml / min. A Brownlee Spheri 5 ODS (5µm) 250 x 4,6 mm column was used (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA).

RESULTS AND DISCUSSION

Reversed phase liquid chromatography (RPLC) was the separation technique employed. The first step taken was to find simple conditions which would allow the separation of critical pairs of triacylglycerols (i.e. with the same total number of carbon atoms and the same total number of double bonds, differing in the distribution of these unsaturations sites within the three chains). To solve this problem, a systematic study of standards of homogenous triacylglycerol mixture on different stationary phases and with mobile phases of different nature and composition (11,17). The results of this study allowed us to propose optimum conditions for the separation of triacylglycerols with only one column and a binary mobile phase which was suitable for forty oils and fats (18-19).

The composition of many of the analyzed oils were already well known. However, other oils were also studied which have never been characterized. Few standards of triacylglycerols are commercially available, so their use gives little information for the identification of other unidentified triacylglycerols. In order to get further information, the use of hyphenated techniques such as gas chromatography or

liquid chromatography coupled with mass spectrometry is an alternative. It necessitates working with both electron impact as well as chemical ionization mode instrumentation. Initially, a GC-MS system was used in our laboratory: however, after identification of the triacylglycerols of an oil by this technique, each peak had to be matched with the corresponding peak in RPLC. But there are two limitations to this method of identification. First, the retention order is not the same in GC as in RPLC. Second, highly unsaturated compounds give very large peaks. This leads to poor chromatographic separation and a loss of information in MS. A priori, these two restrictions could be avoided by using LC-MS systems. However, some problems remain, particularly when the chromatogram has two closely spaced peaks with very different peak heights. In this case, the identification of minor peaks could be either very difficult or impossible. Thus, this latter technique can only give suitable identification of the well separated peaks for any analysed oil.

This is the case of the borage oil. It is an unusual oil because of its high content in gamma-linolenic acid (γLn 18:3 Z6, Z9, Z12) (1,20,21). Since commercial standard triacylglycerols with γLn chains do not exist , there is a problem identifying triacylglycerols which contain at least one of this residue. One possible method for analysing the triacylglycerol residues consists of using statistical calculations. However, this oil cannot be indexed simply by correlation of area percentage of peaks and this statistical manipulation: although its fatty acid composition is known, it is not possible to deduce its corresponding triacylglycerol composition by such a calculation. Indeed, pure statistical treatments lead to the following results : the more abundant a fatty acid, the more abundant the corresponding triacylglycerol, which is not always experimentally observed (22). The resulting calculations still could lead to erroneous conclusions as to the fatty acid composition. If the fatty acid composition of borage oil is considered, the triacylglycerols present could be various combinations of γ linolenic acid and linolenic acid (such as LnLnγLn and γLnγLnLn for example or OγLnγLn and OLnLn) independent of their relative amounts. This problem occurs because even under the best possible experimental separation conditions (11), these particular triacylglycerols are not totally resolved. Thus a second possible alternative to the identification of the triacylglycerol residues might be through the use of a MS detector. But, in this case, LC-MS could not distinguish between these two different triacylglycerols of each critical pair of peaks due to the similarity of the mass spectra of each.

Consequently, another strategy has been used to identify the major components of borage oil (Figure 1 whose comments about identification will be given later). This was done by spiking the sample oil with another oil of known composition. This method was previously used successfully in a similar situation to identify isomeric triacylglycerols

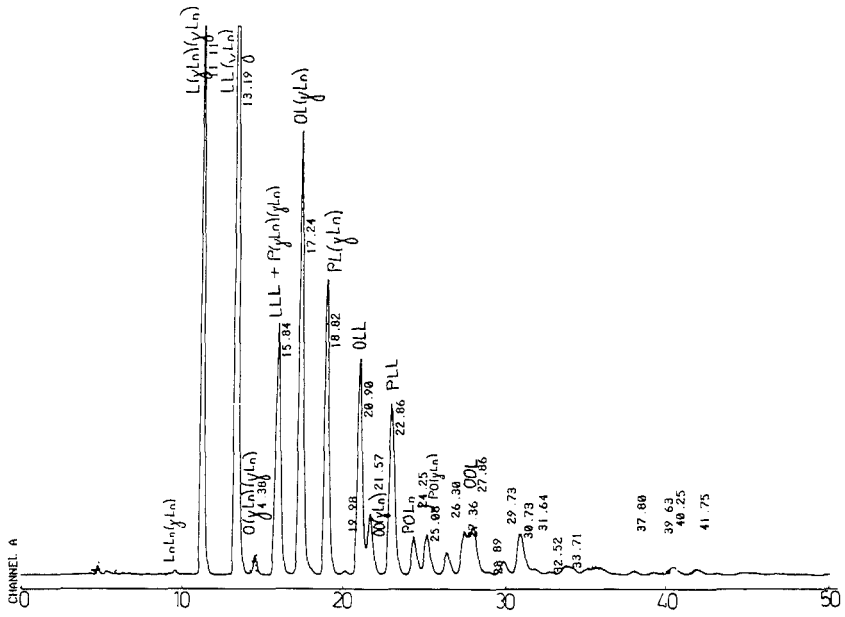

Figure 1: Chromatogram of the borage oil.
Column: Brownlee Spheri 5 ODS
Mobile phase: MeCN / CH_2Cl_2 68 / 32
Temperature: 21°C
P : palmitic acid (16:0); O : oleic acid (18:1, Z9); L : linoleic acid (18:2, Z9, Z12), Ln : linolenic acid (18:3, Z9, Z12, Z15); γLn : gamma linolenic acid (18:3, Z6, Z9, Z12).

with residues possessing cis and trans ethylenic double bonds (23). In our case, major problem occured during the choice of any oils or fats to use. Black current stone oil is one which also contains a significant content of γLn fatty acid (1,24,25). Thus, black current stone oil can be considered as a standard triacylglycerols with γLn residues similar to those found in borage oil (Figure 2). As the black current stone oil also has a large amount of Ln fatty acid, which is not the case with the borage oil, linseed oil which is rich in Ln but is free of γLn was used to distinguish triacylglycerols which possess at least a Ln residue (1).

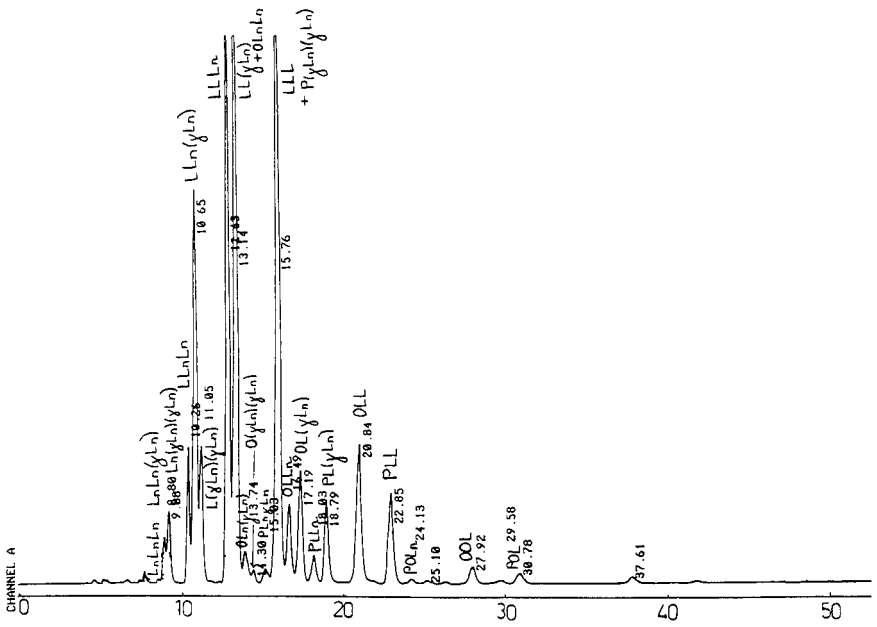

Figure 2: Chromatogram of the black current stone oil. For conditions, see Figure 1.

When the three chromatograms obtained from the previous described conditions (11,18) were compared, all the retention times were similar so that assignment of the peaks of the borage oil remained difficult. In addition, over several analyses, small shifts in the retention time of a given compound could be observed . To avoid this problem, mixtures of the oils (black current stone oil and linseed oil, black current stone oil and borage oil, linseed oil and borage oil) were made. The comparison of the respective heights of each peak of the mixtures with those of the pure non enriched oils allowed assignment of each peak to the correct triacylglycerol.

Identification of the borage triacylglycerols containing Ln and L fatty acids.

The first mixture studied was that of the two known oils taken as references, i.e. linseed oil and black current stone oil (Figure 4). The comparison of the fingerprint of each pure oil allowed confirmation of the peaks corresponding to triacylglycerols having

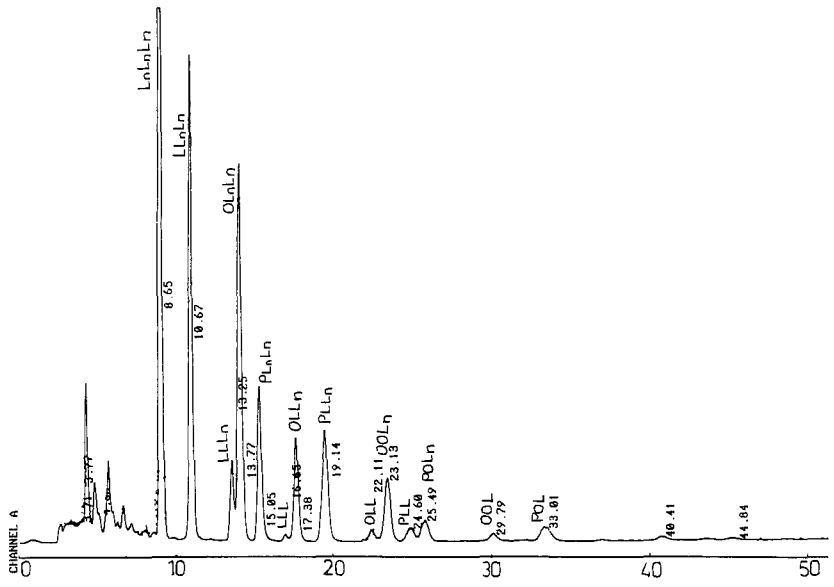

Figure 3: Chromatogram of the linseed oil. For conditions, see Figure 1.

the Ln fatty acid without the γLn fatty acid. Thus, in the chromatogram of the mixture (Figure 4), all the peaks whose intensity has increased in comparison with those in the chromatogram of the pure black current stone oil (Figure 2) are triacylglycerols which are found in the linseed oil. They are marked by an arrow on the chromatogram.

The chromatogram corresponding to a mixture (borage and linseed oils) is shown in Figure 5. The comparison with the non-enriched oils (Figure 1 and 3) allowed the conclusion that:

- The peaks to 8.7, 10.68, 13.25 and 15.05 min are due respectively due to the triacylglycerols LnLnLn, LLnLn, LLLn and PLnLn. They are not present in the pure borage oil.

- The intensities of the peaks at 16.61, 22.09 and 24.23 min have increased in comparison with those in the chromatogram of linseed oil (Figure 3). So, these peaks correspond respectively to LLL, OLL and PLL present in the two oils.

- Lastly, the peaks at 11.55, 18.12 and 19.85 min are in the pure borage oil but not in the linseed oil. They are identified later in this article.

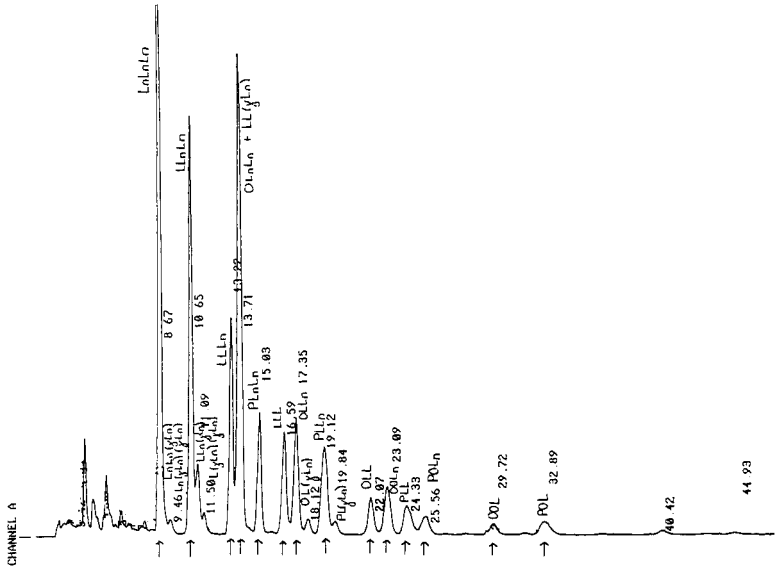

Figure 4: Chromatogram of the linseed oil enriched by black current stone oil.
For conditions, see Figure 1.

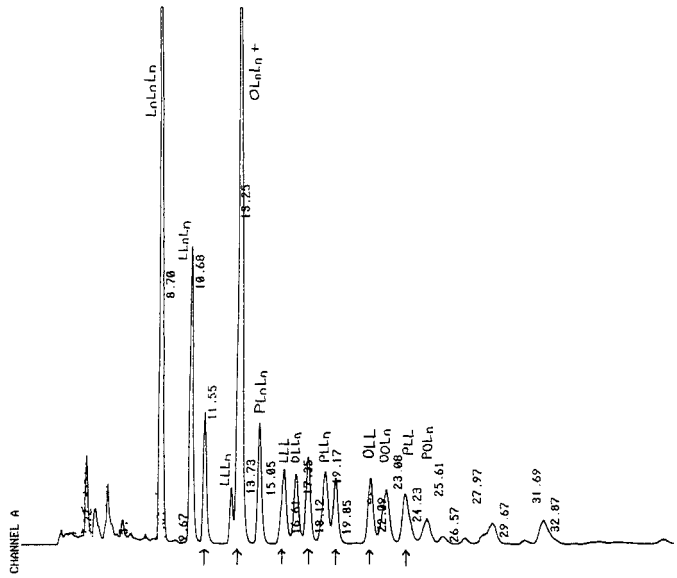

Figure 5: Chromatogram of the linseed oil enriched by borage oil .
For conditions, see Figure 1.

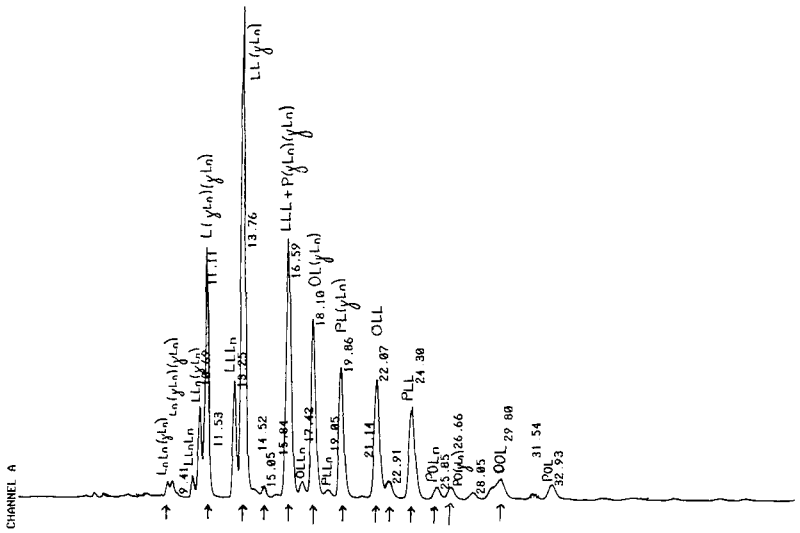

Figure 6: Chromatogram of the black current stone oil enriched by borage oil For conditions, see Figure 1.

- The intensity of the peak at 13.73 min has also increased in comparison with those in the chromatogram in Figure 3. So, one might be lead to believe that the triacylglycerol OLnLn is one of the compounds of borage oil. However, this peak is one of the most important in the chromatogram and it is known that there is little Ln fatty acid in borage oil. This did not seem correct. A reexamination of the chromatogram of mixture of black current stone oil and linseed oil (Figure 4) shows that OLnLn has the same retention time as LLγLn. Thus, it is probably this last triacylglycerol which could be in the borage oil, as it will be proven with the next mixture.

Identification of triacylglycerols containing γLn fatty acid.

The chromatogram of the mixture of borage and black current stone oil is given in Figure 6. The comparison with the chromatogram of black current stone oil (Figure 2) indicates that the peaks at 11.11, 13.76, 18.10 and 19.86 min are respectively due to LγLnγLn, LLγLn, OLγLn and PLγLn are also found in the borage oil.

In order to confirm the identifications, we have drawn the diagram of retention log k' versus the number of double bonds (DB) in the triacylglycerols (12,13). Goiffon et al have shown that the substitution of one residue by another has the same effect whatever the triacylglycerol, so for the four following triacylglycerols XXX- XXY-

XYY-YYY, log k' vs DB must be on a straight line. If we consider the evolution of log k' values in triacylglycerols (or homologues), the log k' vs the number of carbon atoms (n_C) are not always perfect straight lines (26). However, in a small range of n_C values this non linearity does not lead to the non validity of the Goiffon diagram. Thus, for the three following triacylglycerols classes [PLL-PLγLn-PγLnγLn], [LLL, LLγLn-LγLnγLn], [OLL-OLγLn-OγLnγLn] composed by solutes whose identification was made by the above described spiking method, the log k' vs DB plots are straight lines, confirming the assignments.

CONCLUSION

The identification of triacylglycerols in an oil by its enrichment with other oils of known composition is an efficient and accurate method. As for any identification with a standard, the assignment of a peak of an unknown based on the same retention time compared to those of the chosen standard does not lead to unambiguous assignments. To confirm an identification it is imperative to proceed to some verification such as a change of experimental conditions or the examination of the chromatograms gained from the use of multiple standards.

The choice of reference oils must be governed by the following considerations : as the qualitative composition in fatty acids is directly connected to the composition of the triacylglycerol, the knowledge of fatty acid composition of any non identified oil is needed to permit the correct selection of the reference oil which possesses the wanted triacylglycerols necessary to help in the identification of the unknown oil. Moreover, this also allows the unambiguous elimination of some possible triacylglycerols by absence of their corresponding peak.

By using this spiking technique, the correct assignment of triacylglycerols to the main components of borage oil has been possible. The results are in agreement with the assignment proposed by Aitzetmüller et al (25) with a short wavelength UV detection, on columns giving different selectivities. Thus, the information obtained after analysis of mixtures allowed the assignment of the peaks seen in the borage oil chromatogram. The mixture with the black current stone oil allowed the identification of most of the triacylglycerols containing γLn fatty acid. This mixture alone does not allow the unambiguous identification of all the compounds. To complete the assignment, it is also necessary to examine the mixture containing linseed oil which showed the presence of triacylglycerols such as LLL, OLL or POL. This methodology was extended to confirm the identification of triacylglycerols constituting some forty oils we have analysed.

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HPLC ANALYSIS OF TOXIC ADDITIVES AND RESIDUAL MONOMER FROM DENTAL PLATE

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ABSTRACT

Methyl methacrylate polymer, polysulphone, polycarbonate and Cleafil^R are widely used as the dental plate. During fabrication, there is a potential for toxic compound residue of methyl methacrylate, N,N-dimethyl p-toluidine, benzoylperoxide, benzoic acid, bisphenol A, 4,4'-dichlorodiphenylsulfone, triethyleneglycol dimethacrylate and bis phenol A diglycidylmethacrylate. This raises a safety concern as these compounds exhibit carcinogenicity and mutagenicity. The elution of these compounds to water, organic solvents and serum is determined to evaluate a risk to the patients. Analysis is by HPLC using a newly developed and sufficiently endocapped ODS column combined with UV detection. The eluted amount is generally proportional to the hydrophobicity of the organic solvent. Serum extracts hydrophilic compounds as well as hydrophobic compounds. Risk to the patients exposed by these compounds is not so significant.

INTRODUCTION

The leaching of chemicals from various denture base resins into the solvents (water, methanol, acetone, tetrahydrofuran (THF) and serum) was investigated. Two types of polymethylmethacrylate (PMMA) resins, Acron^R (heat-curing) and Yunifast^R (chemically activated), and polysulfone (PS), polycarbonate (PC) resins and Cleafil^R were used in the study. Eluted methyl methacrylate (MMA), N,N-dimethyl p-toluidine (DMPT), benzoylperoxide (BPO), benzoic acid (BA), bisphenol A (bis A), 4,4'-dichlorodiphenylsulfone (DCDPS), triethyleneglycol dimethacrylate (TEGDMA, 3G) and bisphenol A diglycidylmethacrylate (bis GMA) in the solvent were determined. In serum as well as saliva extraction, BPO transformed immediately into BA due to

enzymes, therefore BPO exists as BA in serum. Thus BA was determined as BPO in serum extraction.

Residual monomer and several kinds of additives caused hazardous effects to the patient such as mutagenicity, carcinogenicity or cytotoxicity. Therefore, the author studied for the time course elution and total elution amount of these chemicals. A high performance liquid chromatography (HPLC) determination was carried out using a newly fabricated and completely endocapped C-18 column combined with UV detection. Additionally, the author studied for the procedure to prevent the elution of these compounds as well as the risk to the patients.

MATERIALS AND METHODS

Polymethylmethacrylate dental material (Yunifast^R and Acron^R), PS, PC and Cleafil^R were prepared at 1 mm thickness plate according to the instruction of the manufacturers. After fabrication, Yunifast^R and Acron^R were kept in air for one week at 37^{o}C and thereafter kept into polyethylene bag at room temperature. These were used for the experiment. Other reagents used were HPLC use grade.

EXPERIMENTAL PROCEDURE

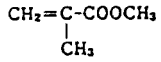
Each one gram of Yunifast^R and Acron^R was immersed in 20 ml aliquot of water, methanol, acetone, THF and serum in a glass-stoppered flask and allowed to stand for 24 hr at room temperature. The solvent was decanted and MMA, DMPT, BPO, BA, bis A, DCDPS, 3G and bis GMA in the solvent were determined by HPLC. Thereafter, to the identical flask were added 20 ml of the fresh solvent and extraction and HPLC analysis were repeated in the identical manner to attain the time course elution as well as the total elution amount.

In case of analysis, the solvents was not removed by vacuum evaporation by heating in order to prevent vaporization of MMA. Furthermore, the mixture solution of acetonitrile and water at the ratio of 1:1 was added to the extraction solvent at an identical volume in order to deposit polymer and oligomers. The supernatant was sampling, filtrating with the filter of pore size of 0.45 μm and 20 μl were applied to HPLC.

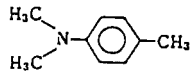
In case of PS, PC and Cleafil^R, bis A, DCDPS, 3G and bis GMA were determined in the identical manner mentioned above. Solvents were not removed by evaporation in order to prevent polymerization.

Chemical structure of MMA, DMPT, BPO, BA, bis A, DCDPS, 3G and bis GMA is presented in Figure 1.

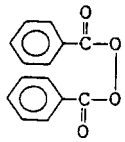
MMA :



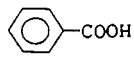
DMPT :



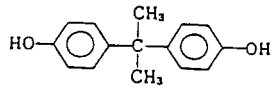
BPO :



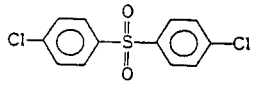
BA :



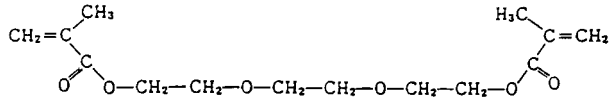
Bisphenol A^{R} :



DCDPS :



TEGDMA :



BisGMA :

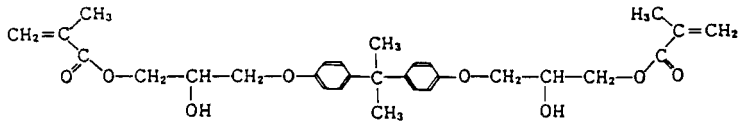


FIGURE 1 Chemical structure of compounds

ANALYTICAL PROCEDURE

HPLC analysis of MMA, DMPT and BPO

A newly developed and successfully fabricated C-18 column of Capcell Pak^{R} SG-120 (4.6X250 mm) from Shiseido Co. Ltd. was used. This column was successful for

preventing a residual silanol effect by silicone coating. In general fabrication procedure of Capcell Pak^{R}, silanol was coated with silicone and thereafter C-18 was linked onto silicone, therefore residual silanol effect was totally diminished. Weakness of this column is that the capacity was less than that of the conventional silanol linked C-18 column. The eluent was the aqueous mixture of water and acetonitrile at the ratio of 52/48, flow rate of 1 ml/min and detection at 235 nm at room temperature. Retention time of MMA, DMPT, BPO was 6.3, 17.3 and 29.6 min, respectively.

HPLC analysis of bis A and DCDPS

Almost identical to the analysis of MMA, DMPT and BPO with the exception of eluent of aqueous mixture of water and acetonitrile at the ratio of 1/1 and the retention time of bis A and DCDPS was 6.2 and 21.9 min, respectively.

HPLC analysis of 3G and bis GMA

Almost identical to the analysis of MMA, DMPT and BPO with the exception of eluent of aqueous mixture of water and acetonitrile at the ratio of 45/55 and the retention time of 3G and bis GMA was 7.0 and 15.7 min, respectively.

HPLC analysis of BA

Almost identical to the analysis of MMA, DMPT and BPO with the exception of the use of the column of Capcell Pak^{R} C-18 AG-120 (4.6X250 mm) in place of SG-120. The eluent was the aqueous mixture of water and acetonitrile at the ratio of 4/1 at pH 3 with phosphoric acid. Retention time of BA was 5.2 min.

MMA, DMPT and BA from BPO in serum were recovered using a solid phase extraction (SPE) with C-18 column (resin weight 100 mg, void volume 120 μl) cited in reference 1.

RESULTS AND DISCUSSION

Selection of the most appropriate column for HPLC analysis

The author studied for several C-18 columns available in the market to attain the sufficient baseline separation and shorter elution time. As indicated in Figure 2, the

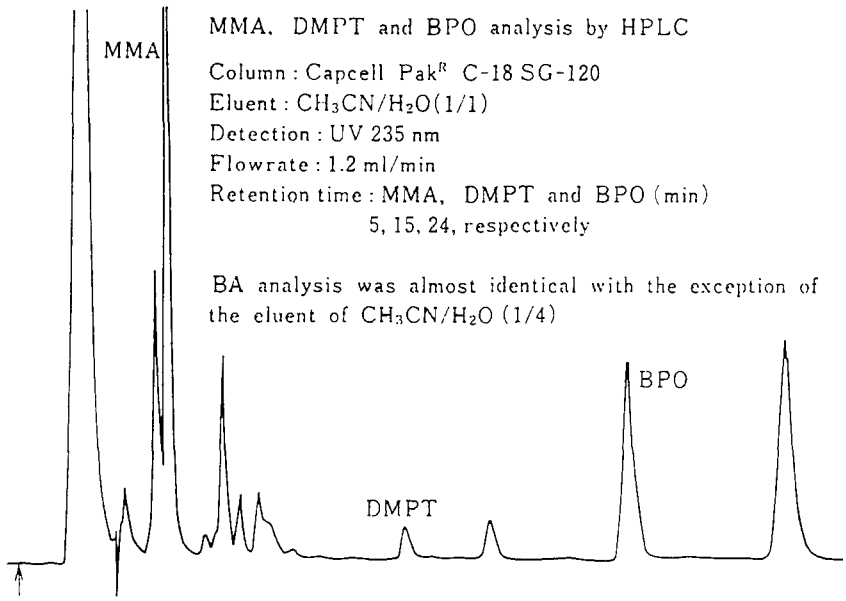


FIGURE 2 HPLC chromatogram of methanol extract of Yunifast^R

extract matrix is so complicated, therefore it was quite difficult to find out the appropriate column. Most of the C-18 column, even if they were endocapped, DMPT did not successfully elute or indicated a tailing phenomena.

Recently Capcell Pak^R of SG and AG-120 for basic and acid compounds, respectively, were available and the author tried to use them for the experiment. They indicated no tailing of DMPT and successful separation of desired compounds from admixtures. Therefore, this procedure was adopted to an official procedure of Japan dental material examination. Since then, several innovated columns other than SG and AG-120 columns such as UG-120 from Shiseido, L-column^R from Kagakuhin kensa kyohkai, Pegasil^R from Senshu kagaku were circulated in the market. UG-120 is the column combined the superiority of SG and AG-120, thus neutral, acidic and basic compounds can be separated using a single column. L-column is specially endocapped, thus almost or sometimes superior to Capcell Pak^R columns. However, due to specific endocapping procedure, it is difficult to fabricate the large volume size column for collection. Pegasil^R is also newly fabricated and the superior characteristics of this column is used the completely purified silanol totally free from residual heavy metals, therefore no effect due to residual heavy

metal was observed and basic compound as well as a chelating agent or clathrate compound containing metal or compound in the molecular were successfully eluted.

According to the advancement and innovation of the column fabrication technique, the author needs to revise the official method using one of these columns in place of SG or AG-120 for dental material examination currently performed.

Due to the use of Capcell Pak^{R} column in the current study, no salts addition into the elution for attaining the common ion elution effect was unnecessary and basic compound was successfully determined without any tailing phenomena. If insufficiently endocapped C-18 column was used, significant tailing or no elution of basic compounds in the worst case was observed.

Analysis of compounds from Yunifast^{R} and Acron^{R}

Typical chromatogram of methanol extract of Yunifast^{R} is presented in Figure 2. Total elution amount is presented in Table 1. Elution of every compound from Yunifast^{R} was observed excepting DMPT and BPO from water extract. Elution of every compound from Acron^{R} was also observed excepting MMA and BPO from water extract and BPO from serum extract. DMPT was not used for fabrication in Acron^{R} (Table 1).

Concerning the elution from Yunifast^{R}, BPO elution significantly increased with increasing hydrophobicity of organic solvents. DMPT elution from Yunifast^{R} also somewhat increased with increasing hydrophobicity of organic solvents, however no significant difference among organic solvents (Table 1). MMA was eluted into methanol in the greatest amount, indicating that more pliant material of Yunifast^{R} has no significant difference of swelling among organic solvents. It may be the reason the most hydrophilic MMA could be extracted in the greatest amount with the most hydrophilic solvent of methanol. Time course elution of MMA, DMPT and BPO is presented in Figures 3, 4 and 5.

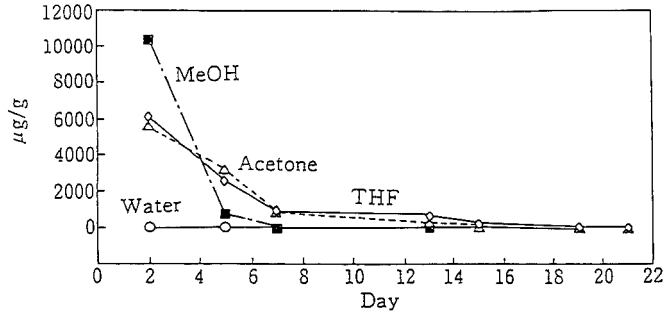
On the contrary, elution amount was parallel to swelling capacity of the solvents if the material is comparatively rigid and Acron^{R} was more rigid than Yunifast^{R}. Acetone indicated the greatest elution amount in MMA and BPO from Acron^{R} due to the greatest swelling capacity. A swelling capacity has an important factor to evaluate the elution amount if the material is rigid. An average swelling capacity (%) and Rockwell hardness (rigidity) of Yunifast^{R} and Acron^{R} were 798, 468 and 60, 84 (n=3), respectively, indicating Yunifast^{R} was more pliant.

In serum extraction, every compound was detected excepting BPO from Acron^{R} (Table 1). BPO was transformed into BA immediately when contacting with serum or saliva, therefore BPO was determined as BA. This is the first finding by the author.

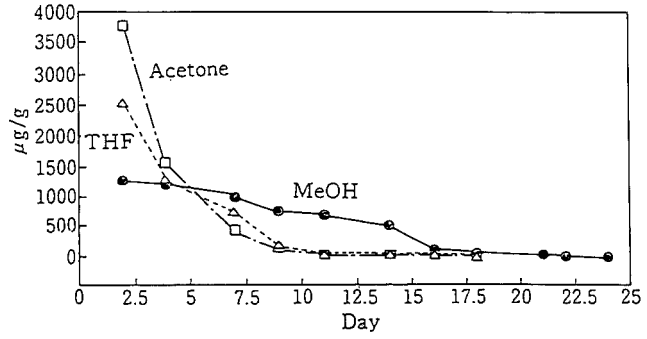
TABLE 1 MMA, DMPT and BPO from polyMMA Yunifast^{R} and Acron^{R}

Sample	Compound	Water	MeOH	Acetone	THF	Serum
Yunifast	MMA	84	11200	10400	10900	32
	DMPT	N.D.	347	373	396	67
	BPO	N.D.	38	796	1460	2
Acron	MMA	N.D.	5520	5920	4860	10
	DMPT		N.D. in all solvent			
	BPO	N.D.	4740	5470	4850	N.D.

Amounts (μg/g) shown are the average of three individual samples. All data refers to more than one week immersion excepting for three days serum immersion.

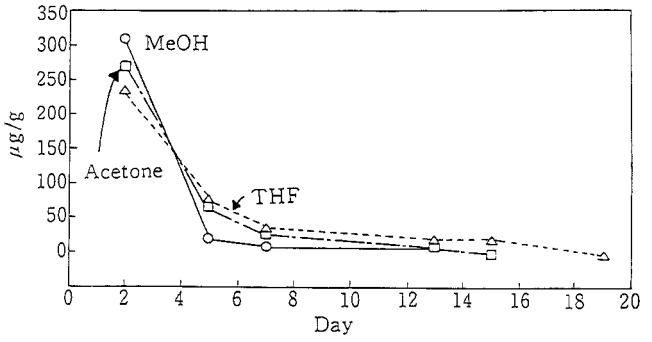


Elution of MMA from Yunifast



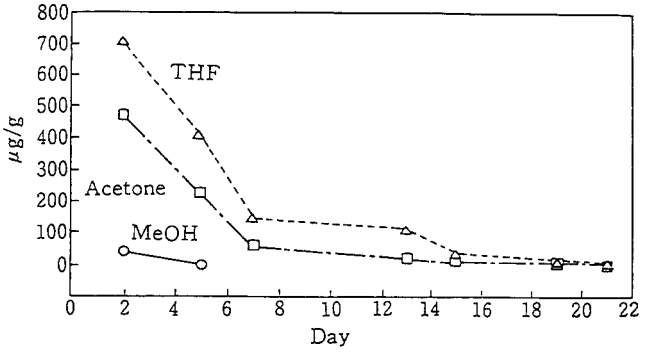
Elution of MMA from Acron

FIGURE 3 Elution time course of MMA from Yunifast^{R} and Acron^{R}

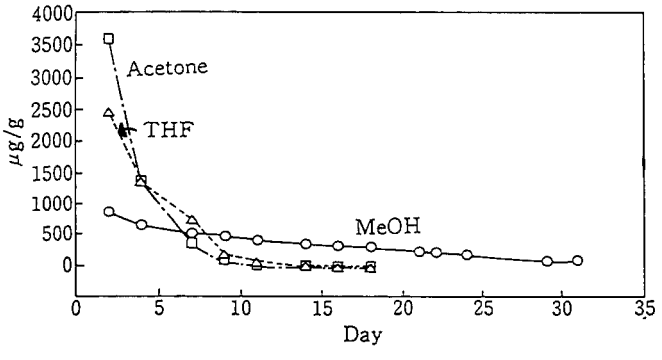


Elution of DMPT from Yunifast

FIGURE 4 Elution time course of DMPT from Yunifast^{R}



Elution of BPO from Yunifast



Elution of BPO from Acron

According to the time course elution of MMA and DMPT to serum, it is interesting that more hydrophobic compound of DMPT eluted greater than less hydrophobic compound of MMA with serum (refer to Table 1, Yunifast^{R} column). This is because serum contains wide range of polarity components from water (hydrophilic) to lipid (hydrophobic), so this phenomena was not surprising, but should be remembered.

Readers should keep in mind that serum was different from water or saline solution in the physical character as well as elution behavior or capacity when evaluating and considering the elution amount with blood or serum.

Serum extracted a greater amount from more pliant material of Yunifast^{R} than more rigid material of Acron^{R} (Table 1). Therefore the rigidity of the material, swelling capacity of solvents and hydrophobicity of the compounds of interest were the important factors to determine the elution amount. In case of BPO elution with serum, BPO in the surface of Yunifast^{R} was changed to BA when contacting with serum and BA was eluted. Due to the rigidity of Acron^{R}, serum supposed not to be penetrated into the interior of the material to extract BPO, therefore no elution was attained.

For international harmonization of the standards existing in individual country, there exists an International Standardization for Organization (ISO)/ Technical Committee (TC) 194, Biological evaluation of medical devices. In this document of part 9, Draft for International Standard (DIS) 10993-9, entitled "Degradation of materials related to biological testing", the extraction solvent *in vitro* from polymers recommends a phosphate buffer solution at pH 7.4 at 50 ^{o}C or 80 ^{o}C (2). Phosphate buffer at pH 7.4 is not an appropriate and representative solvent for evaluating the elution amount from polymer when contacting body fluids with polymers. So, elution with blood or serum was quite different from that with water, saline or phosphate buffer.

As presented herein, only MMA in Yunifast^{R} was extracted with water. This was quite different from serum elution because serum could extract MMA, DMPT and BPO as BA. The eluted amount with serum was greater than that with water with the exception of MMA with water from Yunifast^{R}. The author has no definite idea why MMA amount eluted with water was greater than that with serum in case of Yunifast^{R}. In case of Acron^{R}, serum extraction was greater than water extraction.

When comparing total amount used for polymer fabrication with eluted amount, MMA indicated the greatest elution amount, but eluted ratio compared with the added amount for fabrication was the least and most of MMA was eluted in the initial period in time course elution (Fig. 3). This is because MMA used for fabrication was almost completely polymerized and the residual MMA monomer was around 1 to 2 % used for fabrication.

On the contrary, ratio of DMPT and BPO elution was greater compared with the addition amount used for fabrication. This is because MMA with a lower boiling point

could be easily vaporized in the short period and did not remain in the polymer. DMPT and BPO with a higher boiling point could be difficult to be vaporized in the short period, thus remained in the long period in the polymer and could be extractable with solvents.

Water with less swelling capacity could not penetrate into the interior of the material and could not extract interior MMA. Only MMA remaining in the polymer surface was extractable.

From this result, the author considers the storage condition for materials after fabrication, especially for preventing vaporization of components. Otherwise easily vaporizable compounds could not be determined accurately and sometimes gave a comparatively less data. In one day after fabrication of Acron^{R}, MMA can also be extractable with water. The data in Table 1 is from one week storage at 37 ^{o}C after fabrication. Therefore, the elution data soon after fabrication will be greater than that in Table 1, indicating the storage condition after fabrication is an important factor to determine accurately.

On the other hands, MMA and other higher boiling point compounds can be diminishable by gently heating for a long period before shipping. As an alternative method to diminish the residual MMA before application to patients, it is recommended to immerse dental plate into a hot water to decrease the residual MMA and other hazardous compounds.

Analysis of compounds from PS and PC

Total elution amount is presented in Table 2. Total elution amount as well as elution time course from dental plate with several organic solvents were studied. Elution time course of bis A and DCDPS from PS and PC is presented in Figures 6 and 7.

In PS, both compounds could not be extracted with water and a few ppm of bis A was extracted with methanol and no elution of DCDPS was observed. In acetone and THF elution, elution of both compounds were observed and THF elution was greater than acetone elution, which was parallel to hydrophobicity of solvents.

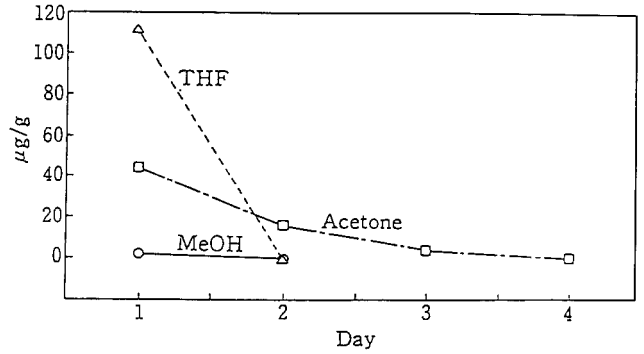
DCDPS was not used for fabrication of PC. In PC, no elution with water was observed. A few ppm elution with methanol was observed. Others were identical to PS. Total elution amount of PS was greater than that from PC, indicating PS was more pliant than PC.

The rigidity of PS and PC was greater than that of Yunifast^{R} and Acron^{R}, therefore the total elution amount of the former was less than that of the latter. Elution amount was parallel to the order of hydrophobicity of organic solvents and the swelling capacity of solvents was insufficient to penetrate into the interior of the

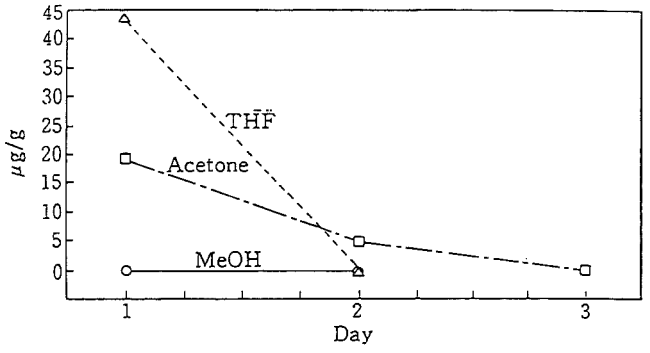
TABLE 2 Bis A and DCDPS from PS and PC

Sample	Compound	Water	MeOH	Acetone	THF
PS	bis A	N.D.	1.9	64.4	112.0
	DCDPS	N.D.	N.D.	24.1	43.5
PC	bis A	N.D.	5.6	22.5	37.0
	DCDPS		N.D. in all solvent		

Amounts (μg/g) shown are the average of three individual samples.



Elution of Bisphenol A from Polysulfone



Elution of 4, 4'-dichlorodiphenylsulfone from Polysulfone

FIGURE 6 Elution time course of bis A and DCDPS from PS

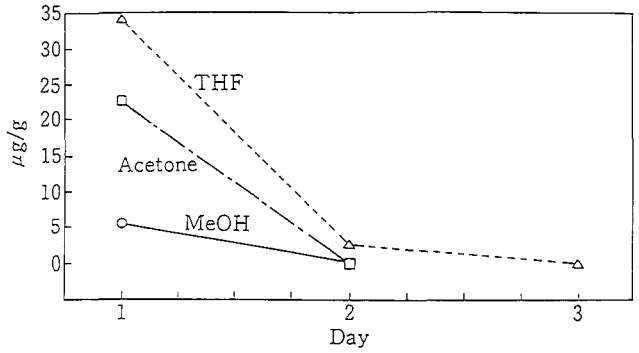


FIGURE 7 Elution time course of bis A from PC

polymer due to rigidity of PC and PS, therefore only residual compounds close to the surface was thought to be extracted in case of PS and PC.

On the contrary, Yunifast^R and Acron^R were more pliant than PS and PC, so the elution amount was greater. The more pliant material of Yunifast^R indicated a greater elution than that from more rigid material of Acron^R. If the material is comparatively pliant, the elution was mostly depend on hydrophobicity of solvents as well as swelling capacity of solvents. The latter was significant in the case of elution from Acron^R.

Analysis of compounds of 3G and Bis GMA from Cleafil^R

Total elution amount is presented in Table 3. Total elution amount as well as elution time course from dental plate with several organic solvents were studied.

In Cleafil^R, both compounds could not be extracted with water and extracted with methanol, acetone and THF. The extracted amount is parallel to the order of hydrophobicity of organic solvents. The elution time course was almost identical to bis A from PC presented in Figure 7.

Hazardous effect of MMA to human being

As MMA elution indicated the greatest amount, therefore it was studied the mutagenicity of MMA (Ames test with and without S9mix in Table 4). According to these results mutagenicity of MMA was not so significant.

TABLE 3 3G and Bis GMA from Cleafil^R

Sample	Compound	MeOH	Acetone	THF
Cleafil	3G	34.1	54.1	55.2
	Bis GMA	17.6	25.9	26.3

Amounts (μg/g) shown are the average of three individual samples.

TABLE 4 Mutagenicity of MMA

Method	note	result	reference
Ames test	5 strains, + or -S9mix S9mix (rat, hamster)	_	3
SCE assay	human lymphocyte	_	4
Ames test	5 strains, + or -S9mix	_	5

Additionally the followings were confirmed. Acute toxicity to human being, chronic oral toxicity to human being, carcinogenicity risk to human being, embryotoxicity and fetotoxicity to human being exposed with MMA were not problematic (6-9).

As a conclusion, acute toxicity, chronic toxicity, carcinogenicity, neurotoxicity, embryotoxicity and fetotoxicity were found not to be problematic. Potential to oral mucosa irritation was small (10-14). MMA, BPO and DMPT were reported to be allergen (15,16).

CONCLUSION

Yunifast^R and Acron^R of polymethylmethacrylate (polyMMA) dental plate were extracted with water, methanol, acetone, THF and serum. The extracted MMA, DMPT, BA, BPO, bis A, DCDPS, 3G and bis GMA were determined using HPLC. Elution of BPO from more pliant material of Yunifast^R increased with increasing hydrophobicity of solvent, indicating THF elution was the greatest. No significant difference of eluted amount of MMA and DMPT was observed excepting water and serum extraction. In the case of more rigid material of Acron^R, eluted amount with acetone indicating the greatest swelling capacity was the greatest, however there

was no significant difference among methanol, acetone and THF from Acron^R excepting water and serum elution. In water elution, only MMA from Yunifast^R was observed. In serum elution, elution of every compound was observed excepting BPO from Acron^R. As BPO was immediately transformed into BA, so BPO was determined as BA. Tiny amount of elution of bis A and DCDPS from PS and PC, and 3G and bis GMA from Cleafil^R of dental plate was observed and the elution order of these compounds was almost parallel to hydrophobicity of solvents.

Hazardous effect to the patients exposed by MMA, the greatest elution compound, was found not to be problematic.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(3), 627-632 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

FEBRUARY 13 - 15: PrepTech '95, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. Brian Howard, ISC Technical Conferences, Inc., 30 Controls Drive, Shelton, CT 06484-0559, USA.

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 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

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 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 14 - 16: 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.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

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

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

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 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

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

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

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

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

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

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

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

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

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

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

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

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

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

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.

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MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. 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.

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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.

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

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

2. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in HPLC of Biological Macromolecules, K. M.

Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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

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

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

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

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