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CONTINUOUS POST-COLUMN ION-PAIR EXTRACTION DETECTION OF SOME BASIC ORGANIC COMPOUNDS IN NORMAL-PHASE CHROMATOGRAPHY

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SUMMARY

Continuous post-column ion-pair extraction in a solvent-segmented system has been applied to the detection of three basic organic compounds (hydroxyatrazine, atropine and ergotamine) separated by means of normal-phase chromatography. Silica gel was used as stationary phase and chloroform containing methanol and butyric acid as mobile phase. The column effluent was mixed with an aqueous solution containing the fluorescent 9,10-dimethoxyanthracene-2-sulphonate (as its sodium salt) in a glass coil for at least 3 sec, when ion-pair formation and extraction into the organic phase occurred. The organic phase was monitored fluorometrically. The effects of mobile-phase composition, counter-ion concentration, flow-rates and extraction-coil material have been studied. Under optimized conditions, band broadening (measured as peak width at half height) due to the extraction detection system was $T_t = 9$ sec. As an application, the determination of hydroxyatrazine in urine samples is reported.

INTRODUCTION

Recently, publications have appeared[1,2] on the application of a continuous ion-pair extraction principle to the detection of solutes eluting from a reversed-phase column after their separation in a high-performance liquid chromatographic (HPLC) system. The apparatus consisted of standard Technicon equipment and employed the air-bubble principle to minimize band broadening[3]. Following this, some work was carried out on the application of solvent-segmented flow (*cf.* ref. 4) for the same purpose. Band broadening was found to be comparable with that in the air-segmented system[5].

All of the work mentioned above was carried out with HPLC systems in which the mobile phases eluting from the column were predominantly aqueous, the solute molecules of interest being continuously extracted as ion pairs into organic solvents

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such as chloroform and tetrachloroethane. These ion pairs were detected fluorometrically. This approach was shown to possess good sensitivity and excellent selectivity for the detection of basic compounds in complicated substrates[1].

The present report describes results of a further investigation of the post-column ion-pair extraction principle coupled with fluorescence detection. It has now been applied to normal-phase chromatography of some basic organic compounds on silica gel, using chloroform containing various amounts of an alcohol and a weakly ion-pairing aliphatic acid, as mobile phase[5].

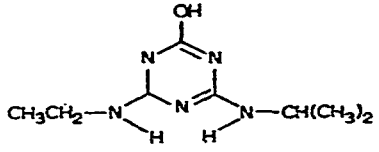
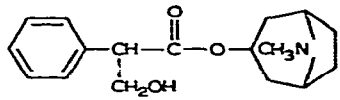
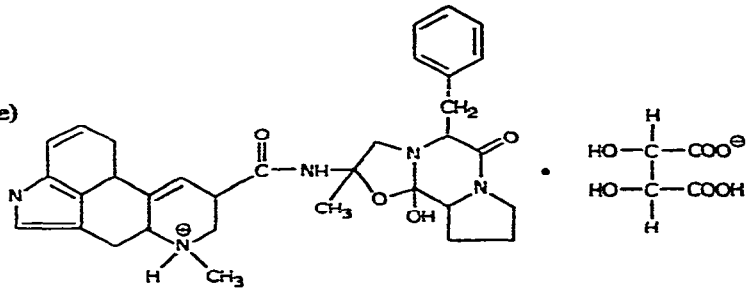
EXPERIMENTAL

Reagents

Table I lists the compounds studied together with their chemical structures. They were dissolved in methanol at concentrations of *ca.* 20–100 μg/ml. The fluorescent counter ion, 9,10-dimethoxyanthracene-2-sulphonate, as its sodium salt (DAS, Sandoz, Basle, Switzerland) was dissolved in doubly distilled water to a concentration of 10^{-4} *M*. All organic solvents and further chemicals were of analytical grade (J. T. Baker, Deventer, The Netherlands).

TABLE I

STRUCTURES OF THE COMPOUNDS STUDIED

Compound (Source)	*Structure*
Hydroxyatrazine (HA) (Ciba-Geigy, Basle, Switzerland)	
Atropine (Brocacef, Maarssen, The Netherlands)	
Ergotamine (hydrogen tartrate) (Sandoz, Basle, Switzerland)	

High-performance liquid chromatography

A Spectra-Physics (Santa Clara, Calif., U.S.A.) Model 8000 liquid chromatograph was employed. The analytical column was a 6 cm × 3.0 mm I.D. stainless-

steel column, pre-packed with 5-μm LiChrosorb SI 60 (Merck, Darmstadt, G.F.R.). The mobile phase consisted of 10% methanol dissolved in chloroform containing 0.1 *M* butyric acid; its flow-rate was maintained at 1.0 ml/min. A single-wavelength (254 nm) UV detector (Spectra-Physics) was placed between the outlet of the analytical column and the post-column extraction detector system.

Post-column detector system

Fig. 1 shows schematically the detection system set up for monitoring the effluent from the HPLC system. A Technicon (Tarrytown, N.Y., U.S.A.) pump was used to deliver the DAS-containing solution as well as to control the flow passing through the fluorometer (Fluoro-Monitor, Aminco, Silver Springs, Md., U.S.A.) equipped with a Corning-16 primary filter and a Wratten 2-E emission filter. Excitation and emission maxima of the extracted ion pairs were at 383 and 452 nm, respectively.

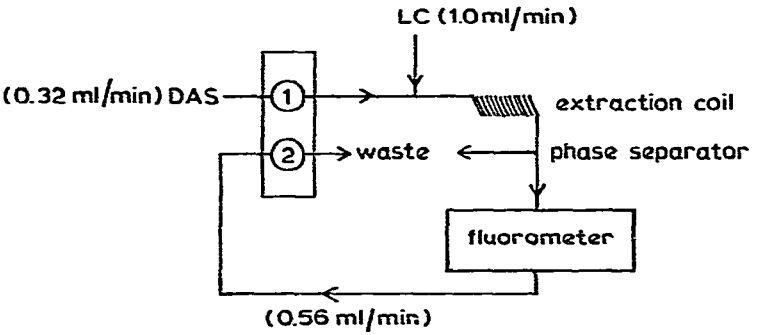


Fig. 1. Post-column ion-pair extraction system. 1, DAS (10^{-4} *M* in water, 0.32 ml/min); 2, flow-through fluorometer, 0.56 ml/min; LC flow-rate 1.0 ml/min. Extraction coil, 1.0 mm (I.D.) glass or Teflon.

Extraction of urine

Urine (50 ml) was spiked with 100 μg of hydroxyatrazine (HA). After the addition of 0.5 ml of an aqueous 10^{-2} *M* DAS solution, the mixture was stirred and the pH adjusted to 3.5–4.5 with 0.1 *M* H_3PO_4. Next, a single extraction was performed with 50 ml of dichloromethane–pentanol (4:1). The organic extract was dried over *ca.* 2 g of anhydrous Na_2SO_4 and then reduced in volume to 10.0 ml. For analysis, 10-μl aliquots, equivalent to 100 ng of HA, were injected into the HPLC system.

Determination of distribution coefficient

The distribution coefficient, *D*, for HA–DAS ion pairs was calculated by injecting 300 ng HA in the extraction system and determining the amount of HA extracted into the organic phase at a 1:1 ratio of organic to aqueous phase. This was done by collecting the organic phase passing through the fluorometer, concentrating this solution, re-injecting it and comparing the result with that for a standard HA solution. A correction was made for the loss of organic phase to waste.

RESULTS AND DISCUSSION

Extraction system

In the apparatus shown in Fig. 1, the aqueous DAS solution was mixed directly with the organic mobile phase eluting from the HPLC column, using a low-dead-volume mixing-tee similar to that constructed by Karlberg and Thelander[4]. As the two phases are immiscible, a regular pattern of organic and aqueous segments flowed from the mixing-tee, through the extraction coils. The volume of each segment was found to be *ca.* 1–2 μl. The two phases were then separated by a standard Technicon phase-separator, modified as shown in Fig. 2 to minimize dead volume. All of the aqueous phase plus a portion (*ca.* 50%) of the organic phase went to waste; the remaining organic phase was directed through the fluorometer for measurement. The considerable loss of organic extract was necessary for optimum detector performance (low noise levels). This loss did not affect detector sensitivity because the detector measures concentrations.

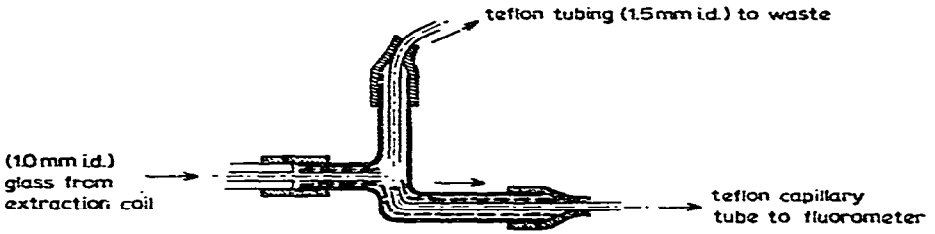


Fig. 2. Modified phase-separator. 2.0-mm O.D. Teflon tubing (dashed lines) was inserted through the lower portion of the glass phase-separator. A hole was cut into it to permit the aqueous phase to escape. A small length of 1-mm I.D. Teflon tubing (O.D. 1.5 mm) was inserted into the first tubing on the entrance side of the phase-separator. Teflon capillary tubing to the fluorometer was inserted into the other end of the 2.0-mm O.D. Teflon tubing, as shown.

Ion-pair extraction

By varying the length of the reaction coils from 6 to 160 cm, it was found that for the system studied equilibrium conditions were reached in less than 3 sec. This corresponds to a length of only 6 cm, which subsequently was used routinely.

The distribution coefficient of HA, as its DAS–HA ion pair, experimentally determined in the detector system was $D = 0.44$. Because of this relatively low value, the ratio of organic to aqueous phase could be expected to have a significant influence on the extraction of the ion pairs. When the flow-rate of the DAS (aqueous phase) was varied at a constant HPLC (organic phase) flow-rate, this was found to be true. Fig. 3 compares the relative sensitivities of the system under such conditions, for 1-m long Teflon and glass coils. It can be seen that at low DAS flow-rates, the percentage extraction into the organic phase, and thus the peak area recorded by the fluorometer, increased significantly, irrespective of the coil material used. Thus, for maximum sensitivity a low flow-rate of DAS should be used (0.32 ml/min in the present study). This observation is compatible with the results of band broadening studies (*cf.* below).

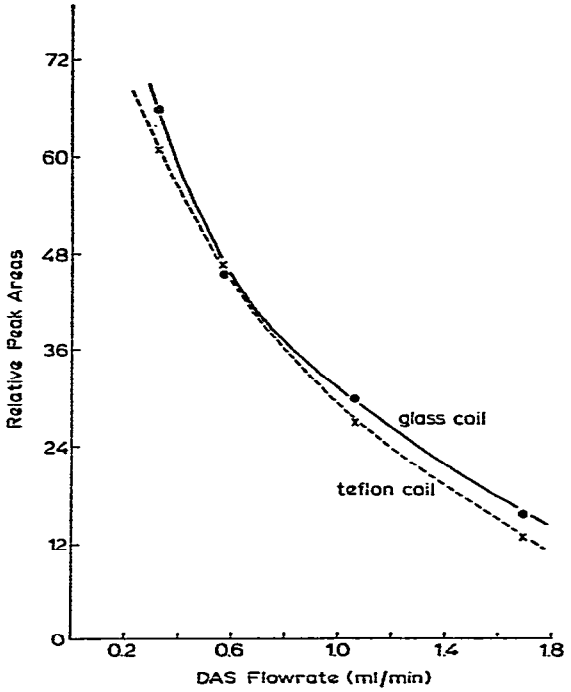


Fig. 3. Influence of DAS flow-rate on detector response for HA in glass (——) and Teflon (– – – – – –) coils. HPLC flow-rate 1.0 ml/min; mobile phase as described in the text.

Band broadening

Although no significant differences between glass and Teflon coils were observed as regards the percentage of extraction of the DAS–HA ion pairs, the opposite was true in the case of band broadening, as Fig. 4 illustrates for varying DAS, and constant HPLC flow-rate, with HA as the model compound. With glass coils, band broadening is seen to be almost independent of the flow-rate of the aqueous phase, whereas it strongly increases with decreasing flow-rate in the case of Teflon coils. The major factor responsible for this is undoubtedly the wetting phenomenon. As the ion pair to be detected is carried in the organic phase band broadening can be expected to be more severe in Teflon tubes with strong wetting of the organic segments on the wall; this is reflected in Fig. 4 at a low DAS flow-rate. The decrease in band broadening with a higher flow-rate of the aqueous DAS solution can be partly attributed to the increased rate of segmentation, resulting in a more efficient suppression of band broadening[7]. It might also be attributed in some degree to the higher total flow-rate, which results in changed flow characteristics, but here a better understanding of the Teflon system would be needed for a definite answer to be given.

It should be pointed out that the above results were obtained using coils with a length of 1 m, which is far in excess of the 6 cm required to establish ion-pair equilibrium. With such a short length of tubing, no significant difference between glass and Teflon coils would have been detected. For minimum band broadening, a DAS flow-rate of 0.32 ml/min with glass as the coil material was chosen. A low flow-

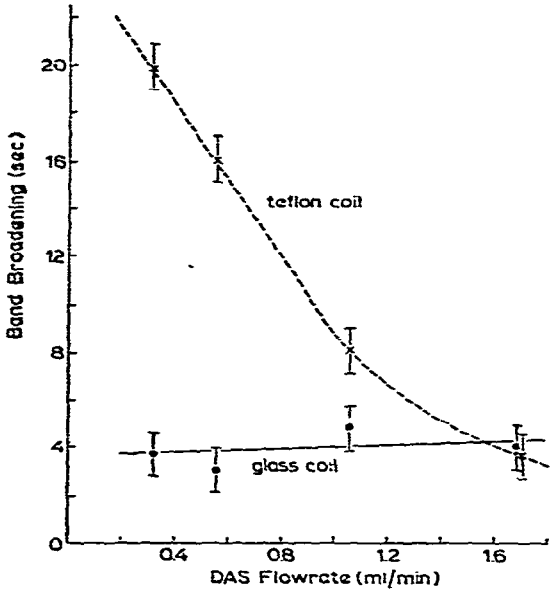


Fig. 4. Influence of DAS flow-rate on band broadening for HA in Teflon (– – –) and glass (——) coils. Band broadening measured as the increase in peak width at half height. HPLC conditions as in Fig. 3.

rate was preferred for maximum sensitivity. For the rest, varying the length of the coil from 6 to 160 cm did not effect a significant increase in band broadening. This indicates that, under non-wetting conditions, it should be possible to carry out rather time-consuming reactions in the post-column mode without undue band broadening. Under optimized conditions, band broadening (measured as peak width at half height) due to the extraction detection system was found to be $T_t = 9$ sec.

Detector sensitivity

Apart from the DAS flow-rate, several other parameters were investigated in order to obtain a better idea of the flexibility of the present detection system. The following observations were made.

The percentage of methanol in the mobile phase had a profound effect on the background signal. Fig. 5 shows the increase in baseline and noise when the methanol content varied from 0 to 30%. The increase in noise was not accompanied by a corresponding increase in extractability of the ion pairs of any of the compounds studied. Thus, the signal-to-noise ratio went down considerably at higher methanol concentrations. In other words, the most useful range was found to be 0–15% of methanol. For a reversed-phase system, it has been observed[5] that the addition of higher alcohols such as *n*-butanol or *n*-octanol considerably increased the extractability of hydroxy-containing compounds as their DAS ion pairs without significantly increasing baseline noise, so we carried out a similar study for the normal-phase system. When *n*-octanol was added to a mobile phase containing 10% of methanol, a considerable increase in noise and background fluorescence occurred. Over the range 0–20% *n*-octanol, noise increased 50-fold; besides, at 20% *n*-octanol, background fluorescence

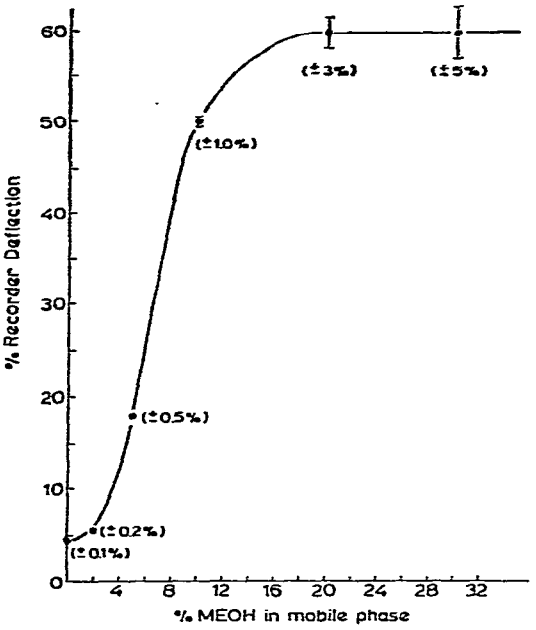


Fig. 5. Influence of methanol content of the mobile phase on noise and background fluorescence. DAS flow-rate, 0.32 ml/min. Mobile phase flow-rate, 1.0 ml/min. Numbers in the parenthese sindicate peak-to-peak noise. Measurements made at (or converted to) 10× attenuation.

was so high as to prevent the use of normal detector sensitivity, *i.e.*, 30–10× on the Aminco fluorometer. As the addition of the higher alcohols did not cause a significant increase of the peak heights of the DAS ion pairs of the several amines studied, our general conclusion must be that the alcohol content of the mobile phase should be kept as low as possible for best detector sensitivity. Evidently, this will somewhat limit one's choice of mobile phases for HPLC systems and, more importantly, will make it difficult to obtain even step gradient elution in a relatively polar (greater than 15% alcohol) solvent mixture.

Increasing the concentration of DAS was also found to increase baseline noise. For example, with HA, in the concentration range 10^{-5}–10^{-3} *M* DAS, a 10-fold increase in signal as well as in noise level was observed. That is, no real advantage accrued from using high concentrations of reagent. For routine operation, 10^{-4} *M* DAS solutions were used. For the rest, background noise was shown not to be affected by varying the flow-rate at any of the DAS concentrations used, while varying the pH of the aqueous phase from 2.5 to 6.5 had no effect on either the signal of the compounds studied or noise. The pH was 3.5 for routine use.

The detection limits for HA, atropine and ergotamine, as the DAS ion-pairs, are 30, 40 and 100 ng, respectively. They are thus of the same order of magnitude as the values previously reported[5] for some related compounds in a reversed-phase system. An improvement of at least one order of magnitude can be expected by using a monochromator instrument of the type PE-204.

Urine analysis

We analysed a urine sample spiked with 2.0 ppm of HA. Fig. 6 compares UV detection and post-column ion-pair extraction with fluorescence detection. As can be seen, the latter is much superior in selectivity and in ultimate detection limit, calculated to be less than 0.1 ppm by fluorescence, and greater than 1,0 ppm by UV, at a 3:1 signal/peak-to-peak noise ratio. Recovery was found to be *ca.* 85% with the extraction technique described.

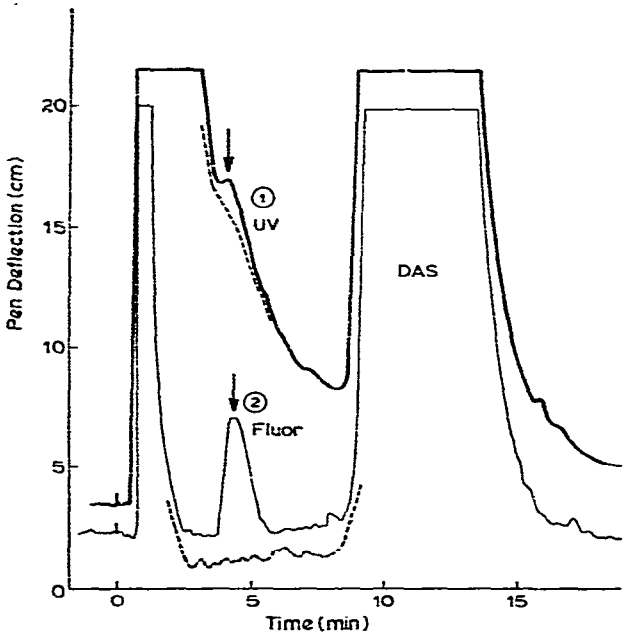


Fig. 6. Comparison of UV and fluorescence ion-pair extraction for the chromatographic detection of hydroxy-atrazine spiked in urine at 2.0 ppm. 1, UV results: 0.01 a.u.f.s.; dotted line indicates blank. 2, Fluorescence ion-pair results: 10× attenuation; dashed line indicates blank. DAS = response due to counter-ion. Mobile phase was 8% methanol in chloroform, 0.1 *M* in butyric acid. Arrow shows hydroxyatrazine peak. All other conditions as described in the experimental section.

The large peak following the DAS–HA peak in both chromatograms is due to free DAS extracted from the sample, which partitions to a certain extent into the organic phase. To eliminate this, it may be useful to use a non-fluorescent or non-UV-absorbing counter ion, such as long-chain aliphatic sulphonate for the sample extraction step. Some preliminary results along this line have been reassuring.

CONCLUSIONS

The extraction-detector principle is just as feasible and can be as useful for normal-phase chromatography as it is for the reversed-phase mode[5]. Although in the present study only one type of mobile phase (methanol in chloroform) and one reagent (DAS) have been used, recent experiments have shown that the method can be used for other organic solvent mixtures and with other ion-pairing reagents.

In addition to being complementary to the reversed-phase mode, the present system also has advantages to offer, such as a simpler design because post-column addition of organic solvent is not required. Residence times for maximum extraction of the ion pairs are distinctly shorter in the normal-phase mode, which again reduces band broadening. The choice of coil material (Teflon or glass) is critical with the normal-phase system, contrary to reversed-phase conditions. On the other hand, with proper choice of the reactor design, total band broadening can be kept somewhat smaller (normal phase, $T_t = 13$ sec, reversed phase, $T_t = 16$ sec*). The addition of increasing amounts of alcohol to the mobile phase was found to decrease considerably the signal-to-noise ratio in the normal-phase system, whereas no such detrimental effects were observed in the reversed-phase mode. The phase-separator is still the critical factor with regard to band broadening. Since a large organic-to-aqueous phase ratio is favourable in the present set-up, it would be more convenient to introduce electronic desegmentation techniques, which could drastically reduce the band-broadening effect.

For the rest, one must keep in mind that too strict a comparison of both systems should not be made, as differences in, for example, the distribution coefficients of the ion pairs, the composition of the mobile phase and/or the ratio of organic to aqueous phase may well be at least partly responsible for the various discrepancies noted.

Lastly, it should be realized that the prime value of the extraction detector coupled to normal-phase chromatography is its high potential for the handling of organic extracts such as those obtained in residue and trace analysis of complex matrices.

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* These values were determined using otherwise identical conditions; *i.e.*, using the same mixing-tees, coils, phase-separator, *etc.*