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ION-PAIR PARTITION CHROMATOGRAPHY IN THE ANALYSIS OF DRUGS AND BIOGENIC SUBSTANCES IN PLASMA AND URINE

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SUMMARY

Liquid–liquid chromatography based on the ion-pair partition technique gives separation systems of high efficiency when silica micro-particles are used as the support for the stationary phase. With 10-μm particles, plate heights of the order of 40–70 μm have been achieved with a linear velocity of 0.25 cm/sec. The retention in ion-pair partition systems is determined by the nature and concentration of the counter ion, and the properties of the mobile phase also have a major influence. It is often possible to predict the selectivity, and this can be controlled by varying the composition of the mobile phase.

This paper describes the application of ion-pair partition chromatography to the bioanalysis of drugs, drug metabolites and biogenic substances. Typical counter ions in the stationary phase were methanesulphonate and perchlorate for ammonium compounds and tetrabutylammonium for the separation of organic anions. Determinations by liquid chromatography were demonstrated for quinidine and dihydroquinidine, metanephrine and normetanephrine and for imipramine and its demethyl metabolite in plasma. A quaternary ammonium compound, QX-572, was determined in urine and chromatograms are shown for the isolation of indoleacetic and hydroxyindoleacetic acid in urine.

The methods have been used in routine analysis. Ultraviolet detection has permitted the determination of highly absorbing compounds down to the 10-ng level in plasma and urine.

INTRODUCTION

The quantitative determination of drugs, drug metabolites and endogenous substances in biological media often requires methods with a high degree of selectivity and sensitivity. The drug and its metabolites can be structurally closely related and in most instances are present in low concentrations. Endogenous substances will occur together with various compounds with similar chemical properties.

Most drugs and related biogenic substances are ionizable and some of them are present in aqueous solution only in ionized form, *e.g.* quaternary ammonium

compounds and sulphate conjugates. Ionized compounds can be extracted as ion pairs with suitable counter ions, as illustrated by eqn. 1:

$$Q^+_{aq} + X^-_{aq} = QX_{org} \quad (1$$

where, for example, Q^+ can be a protonized amine and X^- a sulphonate, a phenolate or an inorganic anion. This technique has found wide application in drug analysis owing to the superior selectivity and the possibility of an increased sensitivity for quantitative determinations. Schill[1] has surveyed the use of ion-pair extraction in drug analysis.

Ion-pair partition has also been applied successfully in liquid–liquid chromatography[2–7] and high efficiency has been achieved, particularly when silica microparticles are used as the support for the stationary phase. Recently, Knox and coworkers[8,9] demonstrated the use of ion-pair partition in liquid–liquid, adsorption and bonded-phase chromatography. Ion-pair partition chromatography in the reversed-phase mode has recently been reported by Wahlund[10] for the separation of carboxylates and sulphonates.

In this paper, high-performance ion-pair partition chromatography has been used in bioanalytical applications. The widely used drugs quinidine and imipramine have been analysed routinely at therapeutic levels in plasma. A potential antiarrhythmic drug, a quaternary ammonium compound, has been determined in plasma and urine samples in clinical tests. The biogenic amines metanephrine and normetanephrine have been analyzed in plasma samples and acid metabolites of indoleamines have been isolated from plasma and urine by liquid–liquid chromatography as tetrabutylammonium ion pairs.

Separation times of 5–10 min are common and strongly absorbing compounds have been determined down to the 10-ng level by means of ultraviolet (UV) detection.

EXPERIMENTAL

Chromatographic apparatus

The liquid chromatograph was a Milton Roy (Philadelphia, Pa., U.S.A.) mini-pump with a pulse dampener (Laboratory Data Control, Riviera Beach, Fla., U.S.A.; 711-47) and a Cecil (Cambridge, Great Britain) 212 UV spectrophotometer with an 8-μl flow cell or a Laboratory Data Control Model 1205 UV monitor. The injector was either a septum injector or a valve with a 100-μl loop. The separation column of precision-bore stainless steel (length 150 or 200 mm, 1/4 in. O.D., 4.5 mm I.D.) had end fittings of modified Swagelok connections. A pre-column (300 × 9 mm I.D.), used to improve the equilibration of the mobile phase, was packed with Porasil E (Waters Assoc., Milford, Mass., U.S.A.; 37–74 μm) loaded with stationary phase (1 ml per 2 g) by shaking for 1 h. Room temperature was used throughout.

Column packing and coating

The separation column was packed with 10-μm silica gel particles by the balanced density slurry technique[11]. A 50-ml stainless-steel cylinder was used as a reservoir for the slurry and tetrabromoethane–carbon tetrachloride–dioxan (4:3:3) was the slurry medium. The packed columns were evaluated by adsorption chro-

matography of a test solution; 3000–4000 theoretical plates ($k' = 2$–3) were obtained for the 150-mm columns.

The coating of the particles with stationary phase was performed by an *in situ* technique[6]. Initially, about 100 ml of acetone were pumped through the column at high velocity, then 50 ml of a mixture of stationary phase and acetone (3:1), supplied from the packing cylinder, were pumped through at a flow-rate of 2 ml/min, *n*-hexane saturated with stationary phase being the eluent. The cylinder was then disconnected and *n*-hexane passed through the column at an initial flow-rate of 1 ml/min (30 ml), gradually increasing to 4 ml/min until about 150 ml had been collected.

Mobile phase was equilibrated with stationary phase by mixing with a magnetic stirrer for more than 1 h. After phase separation, the organic phase was filtered through glass-wool and glass-fibre. About 50 ml of the mobile organic phase were passed through the pre-column before the separation column was connected and a further 50 ml were eluted. Usually the chromatographic system was recycled with mobile phase overnight, a small volume of stationary phase being present in the reservoir.

In most instances the columns were used for several hundred biological samples without re-coating. Stationary phase was washed off the separation column by rapidly eluting it with 150 ml of methanol before the coating procedure was repeated.

The amount of stationary phase loaded on the particles by this *in situ* coating technique averaged 35% of the total weight of the loaded particles.

Chemicals and packing material

Methylene chloride, 1-butanol and isobutanol (pro analysi; E. Merck, Darmstadt, G.F.R.) and *n*-hexane (certified A.C.S.; Fisher Scientific, Pittsburgh, Pa., U.S.A.) were used. Diethyl ether used in the extraction was of A.C.S. quality (Mallinckrodt, St. Louis, Mo., U.S.A.).

All reagent and buffer solutions were prepared with analytical-grade chemicals, except for methanesulphonic acid (puriss; Fluka, Buchs, Switzerland).

Indole-3-acetic acid (IAA), 5-hydroxyindole-3-acetic acid (HIAA), metanephrine and normetanephrine were purchased from Sigma (St. Louis, Mo., U.S.A.).

The drug compounds fulfilled the quality requirements of the Pharmacopeia Nordica.

The silica particles used in the separation columns were LiChrosorb SI 100 (average diameter 10 μm) (Merck) or Partisil 10 (average diameter 10 μm) (H. Reeve Angel, Clifton, N.J., U.S.A.).

Extraction technique

In the most common procedure, the biological sample was mixed with buffer and reagent solutions and extracted with organic solvent in a centrifuge tube. An aliquot of the organic phase was transferred into a silanized tapered centrifuge tube for evaporation under nitrogen. The residue was then dissolved in a small volume of mobile phase and an aliquot injected into the column. When disturbances by adsorption losses or degradation under the evaporation occurred, back-extraction into an aqueous phase and re-extraction into a small volume was used.

Quantitative evaluation

In the routine analysis of drug levels in plasma samples, peak heights were usually measured and the concentrations were obtained from standard graphs con-

structed by analyzing plasma samples spiked with known amounts of the drug. A standard graph was usually prepared daily.

RESULTS AND DISCUSSION

Selection of liquid phase system

It has been demonstrated in ion-pair partition chromatography that chromatographic separation conditions can be based on batch extraction data, from which retention data can be predicted[3,4,12,13]. Kraak and Huber[5] and Karger *et al.*[7] have recently shown good agreement between predicted and found retention data in liquid–liquid chromatography on micro-particles.

Our recent experience indicates, however, that in chromatographic systems with silica micro-particles as the support and ion-pair partition, although good agreement between batch and chromatographic data can be obtained in some instances, the discrepancy is usually large[14]. This means that a mixed partition mechanism or differences in the properties of the aqueous phase in the column and in the batch experiments occur. There is also evidence for a much higher counter ion concentration in the aqueous stationary phase in the column than in the aqueous phase used for coating and for equilibration of the mobile phase[15]. This aspect will be discussed below.

Batch extraction data will, however, still be a basis for the construction of ion-pair chromatographic systems if the divergencies are taken into account. Adjustments of the retention behaviour are often easily made by minor changes to the composition of the mobile phase. Many of the applications of ion-pair partition chromatography discussed below will illustrate that a column loaded with a certain counter ion, in this instance perchlorate, can be used for the separation of ammonium compounds with different properties by varying the composition of the mobile phase. We use mostly 1-butanol, methylene chloride and *n*-hexane as solvent components, as they cover a wide range of solutes. *n*-Hexane is inert and non-solvating, the presence of 5% or more of 1-butanol has been shown to improve peak symmetry[3] and methylene chloride gives good extraction capacity and selectivity. As the ion-pair solvating ability is due to 1-butanol and methylene chloride, an increased proportion of these two solvents compared to *n*-hexane will decrease the retention times, while an increased amount of *n*-hexane will have the opposite effect. 1-Butanol has a superior solvating ability for compounds that contain hydrophilic substituents[16] but simultaneously gives a lower selectivity between two molecules that differ by one or more polar groups. With the inherent high efficiency of micro-particle columns, in most instances the selectivity is not the restricting parameter, at least in the analysis of plasma samples. If so, there is the possibility of changing the relationship between 1-butanol and methylene chloride in the mixture while keeping the retention constant for one of the sample components.

The same solvent components were employed for the separation of carboxylic acids and acid metabolites of biogenic amines, tetrabutylammonium being used as the counter ion in the aqueous stationary phase. Even in this instance one column can be used for the separation of compounds with a wide range of partition properties by varying the proportions between the solvent components. Changes in selectivity relationships have earlier been observed for such chromatographic systems[6].

In all of the applications of ion-pair partition chromatography described here, UV detection was used to follow the sample components in the eluent. Adequate sensitivity was achieved in most instances owing to the high absorbance of the compounds studied. Ion-pair partition chromatography, however, offers, the possibility of high sensitivity by choosing a suitable counter ion, independent of the detection properties of the sample components[3,13,17]. So far, the technique has not been combined with the efficiency of silica micro-particles, but there will be several applications of such systems.

In the following section, some applications of ion-pair partition chromatography to the bioanalysis of drugs and endogenous substances are described. Sample preparation and other aspects of the analytical method will be discussed briefly.

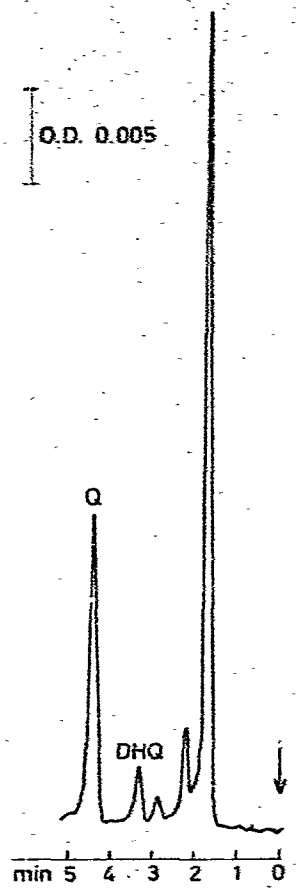
Bioanalytical applications

Determination of quinidine and dihydroquinidine in plasma. Quinidine is a divalent amine that has been used as drug for several years. Its determination in plasma is commonly performed by extraction and spectrofluorimetry, but a more selective method was obtained if an extraction of the amine from an alkaline plasma sample with diethyl ether was followed by a chromatographic procedure. An aliquot of the ether extract was evaporated and, after dissolution of the residue in the mobile phase, a portion of the solution obtained was injected into the chromatographic column. A chromatogram from a plasma sample is shown in Fig. 1; the separation time was about 5 min. The method permits the determination of 10 ng of quinidine in 1 ml of plasma with a standard deviation of less than 10%.

The diprotonated form of quinidine has a $pK'_{H_2A^{2+}}$ value of 4.3. This means that under the conditions used in the separation, quinidine is mainly present as a divalent cation in the aqueous stationary phase (0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$). Quinidine can be extracted into and migrate in the organic mobile phase as an ion pair in both monovalent ($HACl_{org}O_4$) and divalent form ($H_2A(ClO_4)_{2org}$), but the retention will have a constant value if the pH and the perchlorate concentration are not significantly affected by the sample applied[3]. It should be possible, however, to evaluate which form dominates under the actual conditions by batch extraction studies[18].

Quinidine is always contaminated with about 10% of dihydroquinidine, in which the vinyl substituent is replaced with ethyl. By ion-pair partition chromatography, they are separated with a separation factor of about 1.7 and it is possible to determine the two compounds separately by liquid chromatography.

Determination of a quaternary ammonium compound (QX-572) in plasma and urine. QX-572 is a potential antiarrhythmic drug which has been analyzed in plasma and urine; its chemical structure is shown in Fig. 2. Ion-pair partition offers the possibility of extracting aprotic ions such as quaternary ammonium compounds. In this instance, perchlorate was used as the counter ion in the extraction with methylene chloride and QX-572 was extracted quantitatively from the biological sample. After evaporation of an aliquot of the organic phase and dissolution of the residue in a small volume of mobile phase, a portion of the solution obtained was injected into the chromatographic column. Perchlorate was the counter ion in the aqueous stationary phase and a mixture of 1-butanol, methylene chloride and *n*-hexane was the mobile phase. A chromatogram from a urine sample is shown in Fig. 3. The sepa-



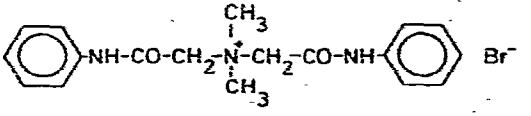


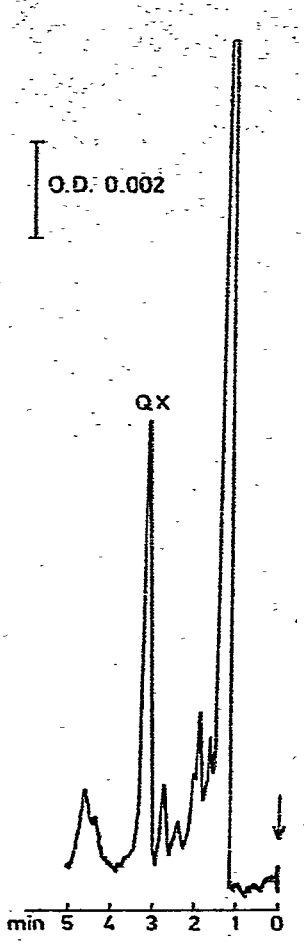
Fig. 1. Separation of dihydroquinidine (DHQ) and quinidine (Q) from plasma extract. Support: LiChrosorb SI 100 (10 μm). Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: 1-butanol–methylene chloride–*n*-hexane (1:7:2). Sample: extract from authentic sample containing 200 ng of quinidine in 1 ml of plasma (50 ng injected).

Fig. 2. Structural formula of QX-572.

ration time was about 4 min and, owing to the high UV absorption, the quaternary ammonium compound can be determined down to 10 ng in 1 ml of sample with a standard deviation of less than 10%.

This method illustrates the use of ion-pair partition in the determination of aprotic organic ions. In this instance, the procedure is facilitated by the fact that the same counter ion can be used in the extraction and in the chromatographic separation. The mobile organic phase has a much lower ion-pair solvating ability for QX-572 than has the methylene chloride used in the initial extraction.

In ion-pair partition methods, the counter ion used for quantitative extraction to an organic phase is often much more hydrophobic than counter ions that are suitable for the subsequent chromatographic separation. If no precautions are taken in such instances, serious disturbances can appear in the chromatographic separation,



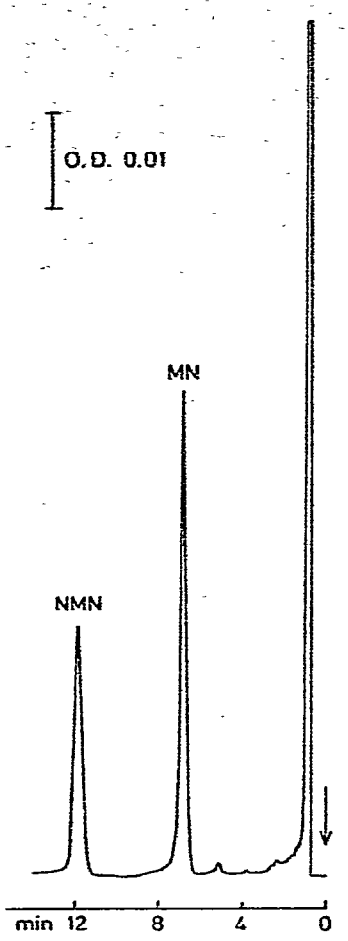


Fig. 3. Isolation of QX-572 from urine extract. Support: Partisil 10 (10 μm). Stationary phase: 0.2 *M* $HClO_4$ + 0.8 *M* $NaClO_4$. Mobile phase: 1-butanol–methylene chloride–*n*-hexane (1:1:8). Sample: extract from authentic sample containing 200 ng of QX-572 in 1 ml of urine.

Fig. 4. Separation of metanephrine (MN) and normetanephrine (NMN) from plasma extract. Support: LiChrosorb SI 100 (10 μm). Stationary phase: 0.25 *M* $HClO_4$. Mobile phase: isobutanol–methylene chloride (2:3). Sample: extract from plasma spiked with metanephrine and normetanephrine.

as there will be a discontinuous partition process. The ionic compound will migrate as ion pairs both with the counter ion in the stationary phase and with the counter ion used in the extraction. The concentration of the latter will gradually decrease along the column[19]. A displacement technique has been used with success to overcome such a problem in a bioanalytical method for acetylcholine[2].

Determination of metanephrine and normetanephrine in plasma. The analysis of adrenaline and noradrenaline in plasma requires a method of extremely high sensitivity, as the basic levels are in the region of 0.1 ng/ml. By introducing a tritiated methylene group into the 3-hydroxy substituent, the two biogenic amines are con-

verted into metanephrine and normetanephrine, respectively, which then have the inherent properties of high detector response by liquid scintillation counting[20].

The amines were extracted with an ion-pairing and adduct-forming agent, bis(2-ethylhexyl)phosphoric acid plus chloroform, back-extracted into an aqueous phase and finally re-extracted into a small volume of the organophosphoric acid in chloroform. This organic phase was injected into a chromatographic column with perchloric acid as the stationary phase and a mixture of isobutanol and methylene chloride as the mobile phase. The fractions of metanephrine and normetanephrine were collected with the aid of UV detection of added unlabelled amines and measured[21]. A chromatogram of an extract from a plasma sample is shown in Fig. 4. The two amines were well separated, which was desirable as a blank eluent fraction was collected between the two peaks in order to check that no contamination between the radioactive fractions had occurred.

The final extract that was injected into the column consisted of a mixture of the organophosphoric acid and chloroform. A high concentration of the organophosphoric acid gives a high degree of extraction and was necessary as the amines are difficult to extract. The higher solvating ability will, however, cause peak deformation in the chromatogram above a certain limit, but the 0.1 *M* concentration used in this method is well within the range where no disturbances are observed.

Determination of imipramine and demethylimipramine in plasma. Imipramine is an antidepressant drug which has an active metabolite, demethylimipramine (desipramine), which is also used as a drug. Most available analytical methods used so far have been of limited value owing to rather low sensitivity. Liquid chromatography with UV detection is very suitable for such compounds with tricyclic ring systems, owing to their high UV absorbance. Chromatographic systems have been described recently[8,9] for the separation of several tricyclic amines and a related compound, thioridazine, has been analyzed in plasma by liquid–liquid chromatography[22].

The present method comprises extraction of the amines as bases from alkaline plasma with diethyl ether, back-extraction into dilute sulphuric acid and finally re-extraction (after alkalization) into 100 µl of 1,2-dichloroethane, the resulting solution being injected into the chromatographic column. The extraction procedure was designed to purify and concentrate the extract without using evaporation to dryness, when losses due to adsorption on the glass walls would occur. 1,2-Dichloroethane forms a distinct organic phase in the bottom of the tube that is easy to collect in the syringe and causes no disturbances in the chromatogram. A chloro-analogue is used as internal standard to compensate for volume variations[14]. A chromatogram from an authentic plasma sample is shown in Fig. 5. The method permits the determination of 10 ng of each amine in a sample.

Aqueous methanesulphonic acid was used as the stationary phase, as it gives less hydrophobic ion pairs than perchlorate and is more suitable for these high-molecular-weight amines. Batch extraction data for the chloro-analogues have been determined and capacity factors calculated and compared with experimental values[14]. The latter are 5–15 times higher than the calculated values, which indicates that the aqueous phases on the column and in static partition experiments have different properties. Studies on such effects are in progress.

Determination of indole-3-acetic acid and 5-hydroxyindole-3-acetic acid in plasma and urine. An area that attracts much interest and where ion-pair partition

chromatography can play a useful role in the future is the analysis of acid metabolites of biogenic amines. Recently, the separation of a test solution of four acids as tetrabutylammonium ion pairs has been demonstrated[6].

Further studies on chromatographic systems with tetrabutylammonium as the counter ion have shown that the behaviour of the quaternary ammonium compounds in the aqueous stationary phase is different from that in batch partition experiments[15]. After coating the silica micro-particles with 0.1 *M* tetrabutylammonium in buffer solution, the stationary phase is not in equilibrium with a mobile phase saturated with the same solution outside the column. Tetrabutylammonium, which is bleeding in the mobile phase as ion pairs with buffer anions, is continuously withdrawn until a much higher concentration of the quaternary ammonium compound in the stationary phase is reached. Equilibrium concentrations in the column of about 0.3 *M* have been measured after washing off the stationary phase. The discrepancy between the nominal and the available concentration might be explained either by a selective adsorption of quaternary ammonium ions on to the support or by a change in the nature of the aqueous phase in the column. Accordingly, it is necessary to equilibrate the chromatographic column for a sufficiently long period that stable conditions are obtained. This is effected by passing mobile phase through, or by recycling if pre-

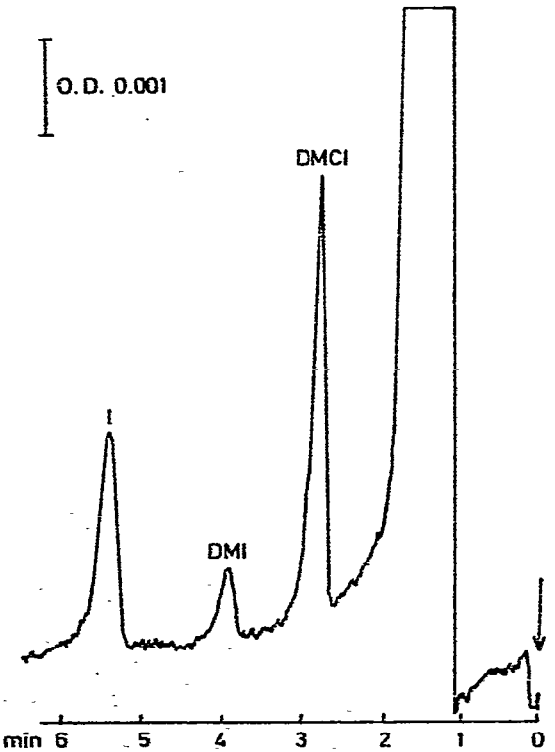


Fig. 5. Separation of imipramine (I) and its metabolite demethylimipramine (DMI) from plasma extract. Support: Partisil 10 (10 μm). Stationary phase: 0.1 *M* methanesulphonic acid. Mobile phase: 1-butanol–methylene chloride–*n*-hexane (6:25:19). Sample: extract from plasma containing imipramine (13 ng/ml of plasma), demethylimipramine (3.5 ng/ml) and the internal standard demethylchloroimipramine (DMCI, 25 ng/ml) (4 ml of plasma taken).

cautions are taken to ensure that the mobile phase is saturated continuously with aqueous phase present in the reservoir. Sufficient equilibration may be obtained overnight but may require a longer time, depending on the nature of the liquid phase system. The buffer substances used in the aqueous phase and the composition of the mobile phase will affect the extent of bleeding of tetrabutylammonium. The current studies also showed variations in column efficiency between stationary phases that contained different buffer substances[15].

The application of tetrabutylammonium columns to the analysis of acid metabolites of tryptamine and serotonin has recently started, with encouraging results. The technique seems to be competitive with ion-exchange on micro-particle resins and the separation times are much shorter for the ion-pair partition columns[23]. In the present studies, Tris buffer has been used, giving columns with good efficiency. The mobile phase consisted of 1-butanol, methylene chloride and *n*-hexane, and it

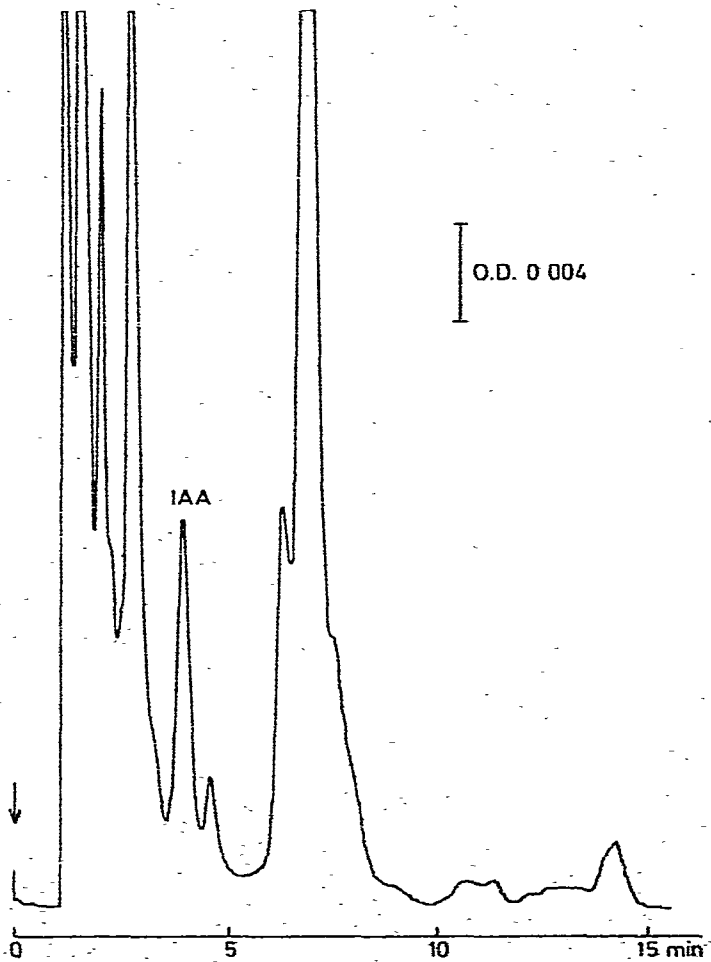


Fig. 6. Isolation of indole-3-acetic acid (IAA) from urine extract. Support: LiChrosorb SI 100 (10 μm). Stationary phase: 0.1 *M* TBA in Tris buffer (pH 8.3, $\mu = 0.5$). Mobile phase: 1-butanol–methylene chloride–*n*-hexane (3:5:12). Sample: extract from 1 ml of urine.

was possible to adjust the retention and affect the selectivity by varying the composition of the solvent mixture.

Indole-3-acetic acid was extracted from acidified plasma with diethyl ether and, after evaporation of an aliquot, the extraction residue was been dissolved in mobile phase and injected into the column. The substance was well separated from other components present.

Urine samples are much more contaminated by low-molecular-weight and polar compounds and, in the analysis of indole-3-acetic acid and 5-hydroxyindole-3-acetic acid, a back-extraction and re-extraction procedure was used. After extraction from acidified urine with diethyl ether, back-extraction into carbonate buffer solution (pH 9) was effected, followed by addition of acid and re-extraction into diethyl ether. An aliquot of this solution was collected and evaporated to dryness, and the residue was dissolved in mobile phase and injected into the column. The two acids were isolated by two different chromatographic runs owing to the large differences in capacity factors. It must be emphasized that the identity of the supposed peaks for

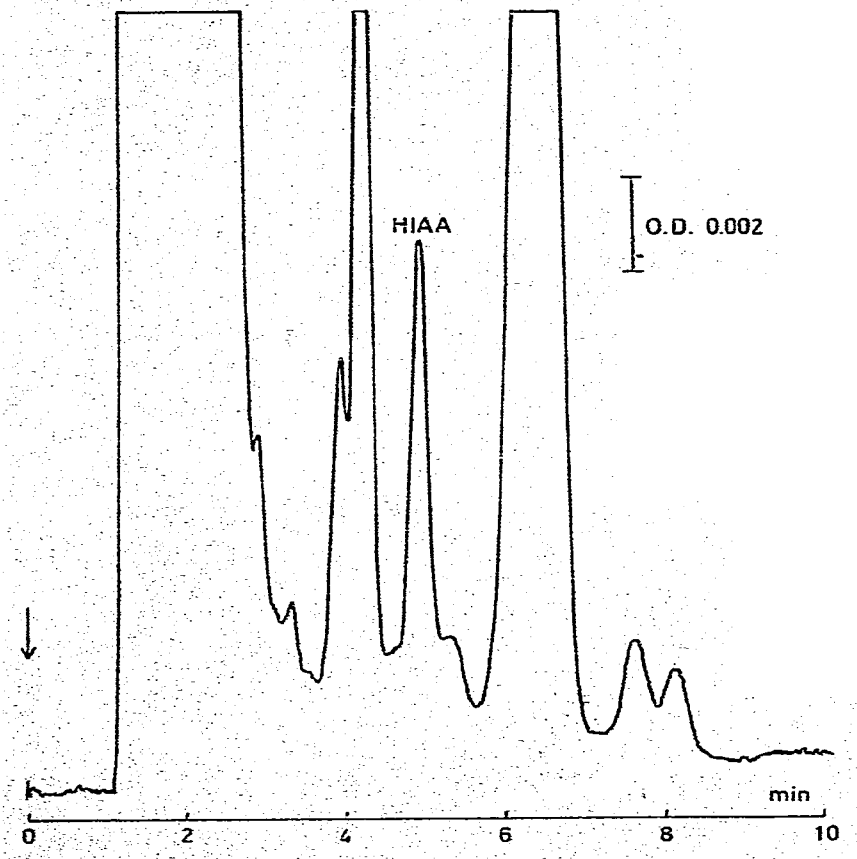


Fig. 7. Isolation of 5-hydroxyindole-3-acetic acid (HIAA) from urine extract. Support: LiChrosorb SI 100 (10 μm). Stationary phase: 0.1 *M* TBA in Tris buffer (pH 8.3, $\mu = 0.5$). Mobile phase: 1-butanol–methylene chloride–*n*-hexane (3:8:9). Sample: extract from 1 ml of urine.

the indoleacetic acids has not been confirmed by any independent technique. Fig. 6 shows a chromatogram for the isolation of indole-3-acetic acid and Fig. 7 for that of 5-hydroxyindole-3-acetic acid. In the latter separation, a more solvating organic phase was used. Contaminants in high concentration were eluted close to the 5-hydroxy compound. It should be possible to purify the extract further before injection.

Recently, ion-pair partition chromatography in the reversed-phase mode has been described for the separation of carboxylic and sulphonic acids[10]. This is a very promising technique in the bioanalytical field and will be an alternative to the straight-phase technique for acid metabolites of biogenic amines.

CONCLUSION

Ion-pair partition chromatography on silica micro-particles gives separation systems of high efficiency and selectivity. The technique has been used routinely in various bioanalytical applications. Drugs, drug metabolites and biogenic compounds have been determined in blood plasma and urine and the chromatographic systems have shown long-term stability and reliability. The field of application of ion-pair partition chromatography within the biomedical area will extend and the possibility of increasing the sensitivity for ionizable organic compounds by the use of suitable counter ions will be utilized in combination with high-efficiency chromatographic columns.

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