'ournal of Chromatography, 115 (1975) 423–436

:HROM. 8566

IIGH-SPEED ION-PAIR PARTITION CHROMATOGRAPHY IN PHARMA-:EUTICAL ANALYSIS

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Received June 26th, 1975)

SUMMARY

Ion-pair chromatography offers attractive possibilities in pharmaceutical analysis. The specificity of the separation systems can be varied over a wide range by appropriate selection of the stationary phase. The choice of a suitable counter-ion can also drastically improve the detection limit, permitting the determination of drug substances in low dosage and possibly of by-products or breakdown products. Ion-pair chromatography of tropane and ergot alkaloids has been investigated using picrate as counter-ion. Alumina, Kieselguhr and various grades of silica gel have been tested as supports. Partition properties studied in a batch procedure have been compared with the actual chromatographic conditions. Columns (10 cm) filled with silica gel (particle size, 5 μm; pore size, 1000 Å) show the best performance in the separation of hyoscyamine, scopolamine and ergotamine as picrate ion-pairs. Close control of pH and temperature is essential for reproducible separations. Improvements in detection limits between 100 and 300 times have been observed with these systems. Ion-pair extractions of these alkaloids from dosage forms can be used for sample preparation prior to injection on to the column. This provides an added degree of selectivity and sensitivity.

I' TRODUCTION AND THEORY

The best detectors currently available in high-pressure liquid chromatography (PLC) are spectrophotometric and also spectrofluorimetric and it seems logical t- refore to solve the current detection problems at least partially by ultraviolet (UV) a i fluorescence derivatization methods. Recent activities show indeed the increased i erest in such an approach[1,2]. Another important argument for derivatization is the s cificity of detection. This is an important factor for analysis in a complex matrix s h as polluted water sources, biological materials or complicated pharmaceutical f mulations where many interfering substances are encountered. By using specific o semispecific labelling reagents, derivatization can be employed as a clean-up step.

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We can distinguish roughly between two different types of derivatization HPLC: *in vitro*, before chromatographic separation is carried out ("pre-column derivatization"); and reaction after separation in a continuous flow-through mode where the reagent is mixed in the eluent stream ("post-column derivatization"). Applications of the first type have been discussed by Frei and Lawrence[1,2] and an example of the second type by Muusze and Huber[3].

Another technique is ion-pair separation which can be classified as a case of pre-column derivatization. It is well known from a number of publications[4] that, for example, basic organic molecules can be extracted as ion-pairs with acidic dyes such as bromothymol blue, methyl orange, picric acid, etc. The distribution equilibrium constant of ion-pair QX, D_{QX}, is determined by the extraction constant, E_{QX}, and the concentration of the counter-ion, $[X^-]$.

$$D_{QX} = E_{QX}\,[X^-] \tag{1}$$

E_{QX} on the other hand is defined by the ratio of the concentration of the ion-pair complex extracted into the organic phase to the concentrations, $[Q^+]$ and $[X^-]$, of the ions remaining in the aqueous phase; it is easily determined from batch experiments.

$$E_{QX} = [QX]_{org.}/[Q^+]_{aq.}\,[X^-]_{aq.} \tag{2}$$

It is clear that the extraction properties can be modified considerably by variation of the concentration of the counter-ion $[X^-]$ or of the pH which in turn determines the concentration of the protonated species, $[Q^+]$. Naturally the polarity of the organic phase also plays an important role. The theoretical bases for ion-pair chromatography has been discussed in detail by Eksborg and Schill[5] and is briefly mentioned here to aid interpretation of our data.

The stationary phase consists of the buffered aqueous phase containing the counter-ion X^-. The compounds to be separated are injected as ion-pairs on the column ("pre-column derivatization"). Based on the previously discussed variation possibilities, we can now select appropriate separation conditions. Assuming that the major mechanism is liquid–liquid distribution and that other phenomena such as adsorption can be neglected, the capacity factor, k', can be defined as in eqn. 3 where V_s is the volume of the stationary phase and V_m is the volume of the mobile phase.

$$k' = V_s/V_m\,E_{QX}\,[X^-] \tag{3}$$

The chromatographic behaviour could therefore be predicted under ideal conditions.

Schill and his co-workers[4] have shown several possibilities for ion-pair liquid chromatography. One example was the separation of quarternary ammonium ions as picrates on an acetylated cellulose support. In this study we are trying to apply similar principles to rigid particles which allow high-pressure operation. Belladonna alkaloids were chosen for this study because of their poor chromophoric properties and low-dose usage in pharmaceutical forms; hence an improvement in the detection limit achieved by attaching an excellent chromophore such as a picrate ion would greatly enhance the sensitivity. This is in contrast to some of the work of Karger and his co-workers[6,7] on thyroid hormones and sulpha drugs where counter-ions such as

perchlorate and tetrabutylammonium were used. There the specificity of separation of the systems was of primary interest. The specificity of the picrate system is also of course advantagous and is desirable in connection with the ergot alkaloids studied and present in the same pharmaceutical products. The ion-pair extraction step, which would permit isolation of the various active principles from the interfering substances, adds also to the specificity aspect.

EXPERIMENTAL

Reagents

All reagents and the solvent were of analytical grade (E. Merck, Darmstadt, G.F.R.) and were used without further purification. The stationary phase consisted of the appropriate amount of picric acid dissolved in a citrate buffer (Titrisol; E. Merck). The pH of these solutions was checked and if necessary adjusted with 5 *M* NaOH.

Instrumentation

A Model 1010 A (Hewlett-Packard) liquid chromatograph was used. The low-pressure columns (Silica gel 60, size A; E. Merck) were loaded with the stationary phase by means of a peristaltic pump (Watson-Marlow, Model MHRE 22). Loading of the high-pressure columns (10 × 0.3 cm I.D.) was carried out with another pump (Lewa, Model FL 1). A single-wavelength UV detector (DuPont, Model 842) was used at 254 nm. Samples were injected via a septum with 10-μl Hamilton syringes.

Procedure

The silica gel columns were packed with an equal-density slurry-packing procedure described earlier[8]. For other adsorbents a new slurry-packing technique[9] was applied. After packing, chloroform (twice the dead volume) was pumped through the column. The columns were then heated at 180° for 2 h and simultaneously flushed with a gentle stream of nitrogen. Silanized silica gel columns were treated at 120°. The columns (usually 10 cm long) were treated with *ca.* 10 ml of the stationary phase at a flow-rate of 0.5 ml/min followed by a flushing step with 20–40 ml of hexane. For equilibration, an eluent flow-rate of 0.2 ml/min was used.

The samples were injected on to the column as ion-pairs. The scopolamine and hyoscyamine ion-pairs were formed in 5 ml of buffer solution (pH 5 or 6) to which picric acid solution (40 mg of picric acid in 5 ml of buffer) had been added. The ion-pairs were extracted with 5 ml of chloroform. Ergotamine, which is poorly soluble in the buffer, was dissolved in chloroform and shaken with the picric acid solution for 2 h. The organic phase was then injected.

All the experiments were carried out in an air-conditioned laboratory. The columns were insulated from air currents and were sometimes thermostatted.

RESULTS AND DISCUSSION

Tests on low-pressure silica gel columns

Preliminary investigations showed that hyoscyamine and scopolamine can be extracted quantitatively as ion-pairs from a weakly acidic solution (pH 5) of picric

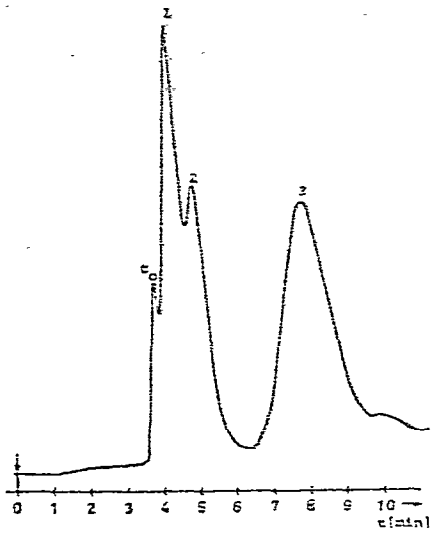

Fig. 1. Separation of hyoscyamine (peak 1, 2.35 μg), ergotamine (peak 2, 5 μg) and scopolamine (peak 3, 25 μg) on a low-pressure column of Merck silica gel. Mobile phase: chloroform saturated with 0.03 *M* picric acid in citric acid buffer at pH 5; flow-rate, 1.4 ml/min. Detector setting, 0.32 area units.

acid. The concentration of picric acid was 1.2×10^{-3} *M*. The solubility of picric acid in chloroform was minimal at this pH and it was assumed that chloroform would also be suitable as a mobile phase in ion-pair HPLC. Exploratory tests in this regard were carried out on pre-manufactured columns (E. Merck) packed with silica gel (pore size, 60 Å). These columns were operated at only a pressure of a few bars. 100 ml of a picric acid solution (pH 5, citric acid buffer) were loaded on to the dry column using a peristaltic pump and the column was equilibrated at a flow-rate of 2 ml/min. The picrate ion-pairs were directly injected on to the column. A separation is shown in Fig. 1. The sensitivity of detection (at a wavelength of 254 nm) was significantly enhanced. Separation of the non-retained component, t_0 (dodecylbenzene), hyoscyamine and ergotamine was not complete.

TABLE I

k' VALUES OBTAINED ON LOW-PRESSURE COLUMNS OF MERCK SILICA GEL AS A FUNCTION OF pH, PICRIC ACID CONCENTRATION AND ELUENT

Stationary phase		*Eluent*	*k'*		
pH	*Picric acid concentration (M)*		*Hyoscyamine*	*Ergotamine*	*Scopolamine*
5	0.01	chloroform	0.25	—	1.71
	0.03		0.14	0.3	1.08
6	0.03	chloroform	0.57	2.74	2.03
	0.03	chloroform–methylene dichloride (199:1)	0.28	0.91	1.34

Variation of the k' values in order to optimize the separation of these components was attempted by adjusting the concentration of picric acid, $[X^-]$, and the pH. The results are shown in Table I and can simply be explained in terms of eqn. 3. Increasing $[X^-]$ resulted as expected in a decrease in k'. An increase in pH results in a lower concentration of protonated alkaloid, $[Q^+]$, and hence in a lower rate of ion-pair formation, [QX]. This produces a decrease in E_{QX} which in turn yields a higher k' value. By increasing the polarity of the mobile phase, one can increase E_{QX} and hence reduce k'. This was demonstrated by other workers[5] for the separation of quarternary ammonium ions as picrates where small amounts of pentanol were added to the chloroform or methylene chloride. The modification possibilities via polarity variations are, however, limited due to an increase in the solubility of picric acid in the mobile phase which results in background detection problems.

With suitable optimizations of this system it was eventually possible to separate all the components shown in Fig. 1 (see Fig. 2). The smaller peak, 2a, in Fig. 2 was identified as the picrate ion-pair of ergotaminine [height equivalent to a theoretical plate (HETP) of hyoscyamine, 0.32 mm]. In general, the use of these Merck columns offers a low-cost procedure for ion-pair chromatography. However, with the specific picrate system used in this study, the column material was not sufficiently inert to ensure long-term usage.

Tests on high-pressure columns

Silica gel (pore size, 60 Å). For efficiency and reasons explained in the previous section, it was decided to continue investigations with high-pressure systems. Columns of silica gel SI 60 (particle size, 10 μm; E. Merck) were tried first since they were at that time the most widely used materials in our laboratories. A separation under otherwise identical conditions was basically possible but the columns were unusable after a few separations since the compounds were completely retained. In this experiment,

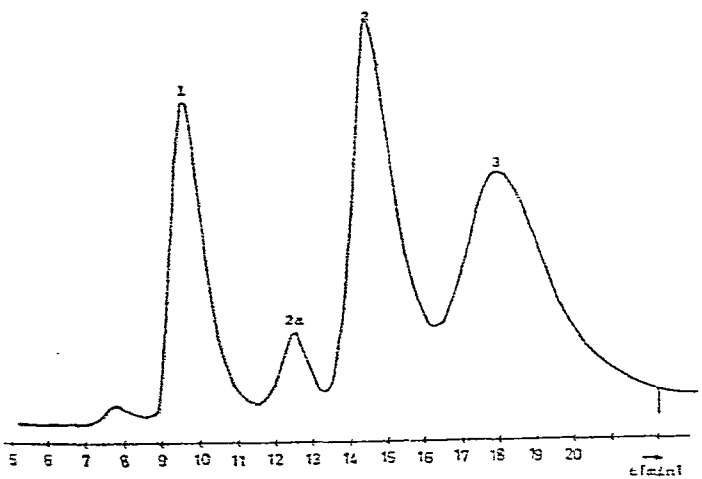

Fig. 2. Separation of hyoscyamine (peak 1, 2.35 μg), ergotamine (peak 2, 5 μg), scopolamine (peak 3, 25 μg) and ergotaminine (peak 2a) on a low-pressure column of Merck silica gel. Mobile phase: chloroform–methylene chloride (199:1) saturated with 0.03 *M* picric acid in buffer at pH 6; flow-rate, 1.25 ml/min. Detector setting, 0.32 area units.

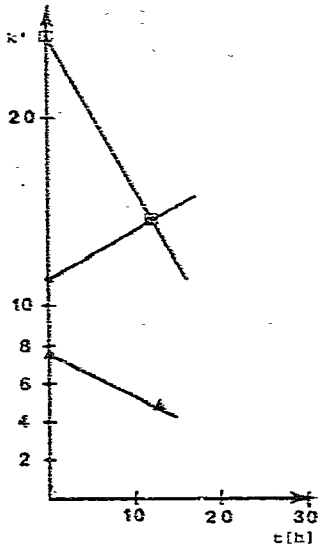

Fig. 3. Graph of k' against time of column usage with silanized silica gel as the support. ▲ = Hyoscyamine, □ = ergotamine and ● = scopolamine. Mobile phase: chloroform saturated with 0.03 *M* picric acid in buffer at pH 6; flow-rate, 0.8 ml/min. Column (10 × 0.28 cm I.D.) packed with silanized Merckosorb SI 60 (particle size, 10 μm).

columns (25 cm long) with a linear velocity of 1 cm/sec were used. As we show later, this can be attributed to mechanical instability of the system which was quite appreciable at a flow-rate of 0.4 cm/sec for 10-cm columns packed with silica gel (pore size, 100 Å). With the low-pressure Merck columns this problem was less obvious since a velocity of <0.1 cm/sec was used. Reloading of the same column was difficult and the use of a pre-column for better mobile-phase saturation did not solve the problem.

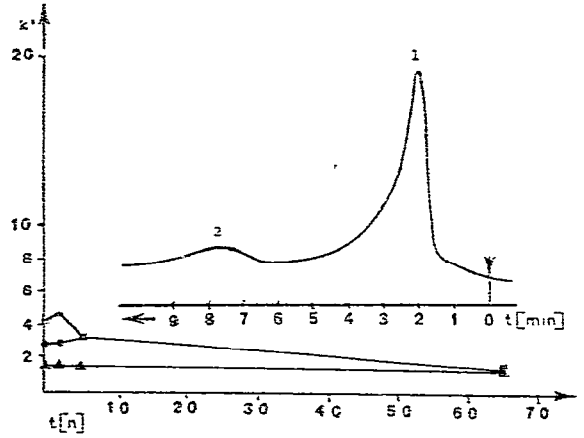

Fig. 4. Graph of k' against time of column usage with Merckosorb Alox T (particle size, 10 μm) as the support. ▲ = Hyoscyamine (peak 1), □ = ergotamine (peak 2) and ● = scopolamine (peak 2). Mobile phase as in Fig. 1; flow-rate, 0.5 ml/min. Column length as in Fig. 3.

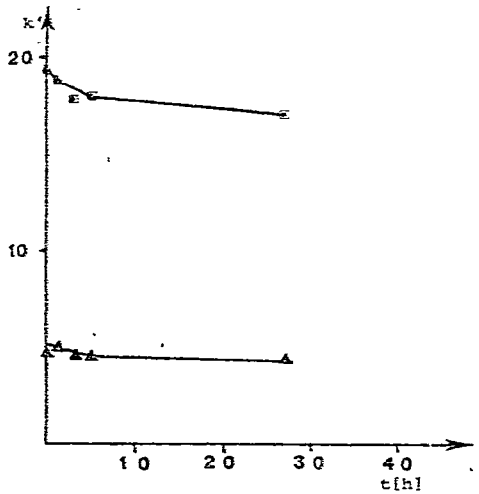

Fig. 5. Graph of k' against time of column usage with Kieselguhr as the support. ● = Scopolamine, □ = ergotamine and ▲ = hyoscyamine. Mobile phase as in Fig. 1; flow-rate, 0.8 ml/min. Column length as in Fig. 3.

Silanized silica gel. This support was studied as an alternative material with low adsorption strength. The stability of the system seemed to be rather poor as can be seen from Fig. 3. The first separation followed the same order observed on the other high-pressure systems, but then the α values (k'_{n+1}/k'_n, where n = peak number) changed drastically due to a loss of stationary phase. Other separation phenomena such as a reversed-phase effect may become dominant.

Aluminium oxide. Merckosorb Alox T (particle size, 10 μm) was also investigated. The separation of hyoscyamine and scopolamine is shown in Fig. 4. Ergotamine and scopolamine were separated at first, but after 5 h no further separation occurred. The strong tailing seen on the chromatogram can be attributed to interference from strong adsorption. The stability of the system seemed to be good, but after 65 h the selectivity was completely lost. All these disadvantages render alumina quite unsuitable as a support for this system.

Kieselguhr. Better results were obtained with Kieselguhr (Spherosil XOB; particle size 5–10 μm) as a support. After an initial period in which the k' values

TABLE II

k' VALUES OBTAINED ON HIGH-PRESURE COLUMNS OF KIESELGUHR AS A FUNCTION OF pH AND PICRIC ACID CONCENTRATION

The eluent was chloroform.

Stationary phase		*k'*		
pH	*Picric acid concentration (M)*	*Hyoscyamine*	*Scopolamine*	*Ergotamine*
5	0.03	5	19.2	19.2
	0.05	2.2	7.4	9.9
3	0.05	7.6	19.4	23.8

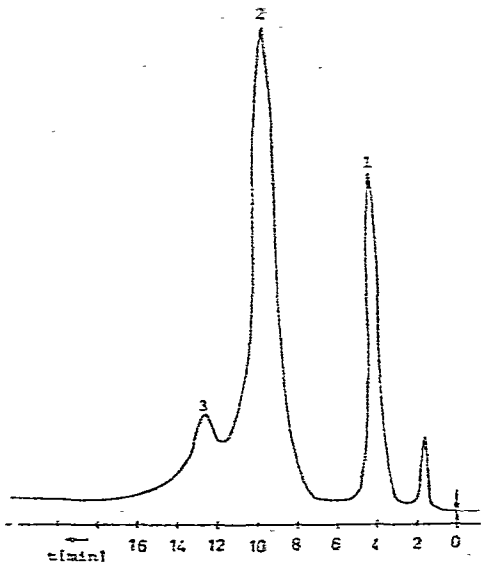

Fig. 6. Separation of hyoscyamine (peak 1, 1.14 μg), scopolamine (peak 2, 1.8 μg) and ergotamine (peak 3, 0.9 μg) on a Kieselguhr support. Mobile phase: chloroform saturated with 0.05 M picric acid in buffer at pH 5; flow-rate, 0.5 ml/min. Column length as in Fig. 3. Detector setting, 0.16 area units.

decreased slightly (Fig. 5), the retention behaviour was fairly stable over a longer period of time. The dry column was loaded with 0.03 M picric acid buffered to pH 5. Assuming that some stationary phase was lost in the first hours of operation of the fairly heavily loaded column and that this does not result in increased interference due to adsorption effects a decrease in k' is expected according to eqn. 3. In general the values of k' observed on Kieselguhr were larger than on silica gel or alumina supports. A reduction in k' could be effected by increasing the picric acid concentration or decreasing the pH (Table II). These data may again be explained according to eqn. 3.

Fig. 6 and Table II show that a separation of scopolamine and ergotamine can only partially be achieved by using a pH of 5. With a pH of 6 (Table II) a complete separation is possible. The order of elution did not change on varying the pH. The peaks were symmetrical. Addition of methylene chloride resulted in a decrease in the k' values which was difficult to reproduce, and at concentrations above 5% there were problems with the blanks. Reloading of the column by injection of picric acid solution was not possible. In conclusion, Kieselguhr seems to be a suitable support for ion-pair chromatography with the picrate system. A disadvantage, however, is that the quality of this material is never uniform and large batch variations make it difficult to reproduce column performance.

Since the pore size, although irregular, is considerably larger than for silica gel SI 60, it was decided to continue tests with grades of silica gel of larger pore size such as SI 100 or SI 1000. These also have the advantage of being obtainable in uniform qualities.

Silica gel (pore size, 100 Å). The same optimum conditions were applied to

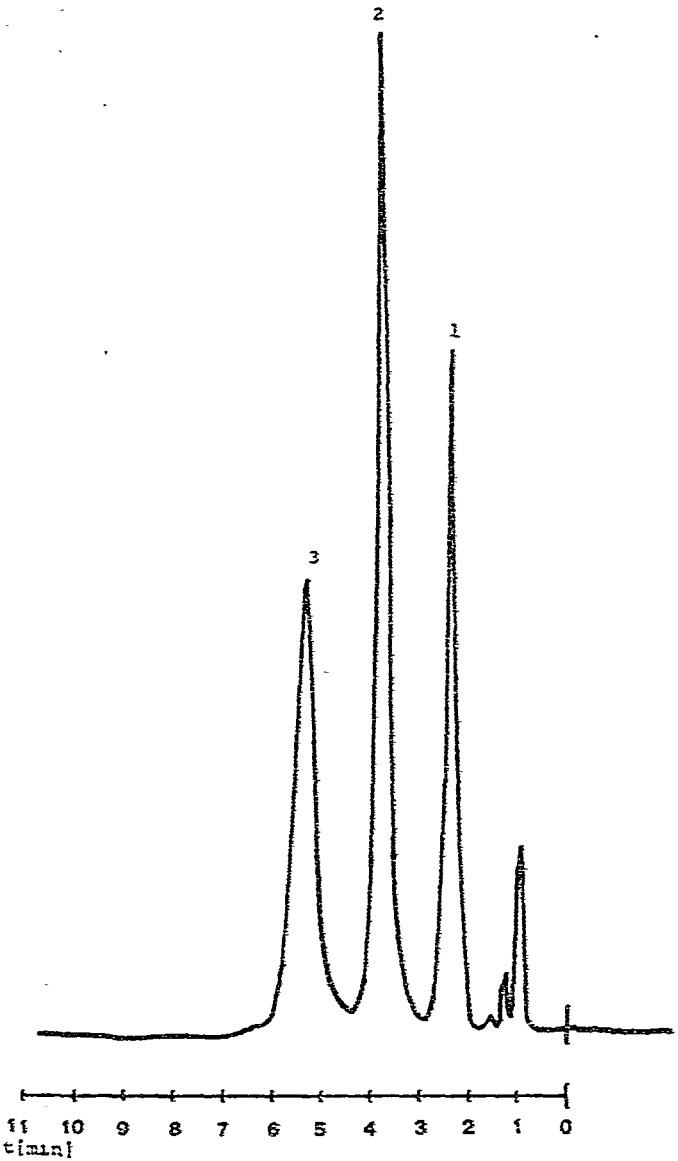

Fig. 7. Separation of hyoscyamine (peak 1, 0.9 μg), ergotamine (peak 2, 0.9 μg) and scopolamine (peak 3, 2.8 μg) on Merckosorb SI 100 (particle size, 5 μm). Mobile phase: chloroform saturated with 0.06 *M* picric acid in buffer at pH 6; flow-rate, 0.6 ml/min. Column length as in Fig. 3. Detector setting, 1 area unit.

Merck silica gel SI 100 except that the columns were loaded with 0.06 *M* picric acid solution. An average particle size of 5 μm was used to improve the plate height of the separation system. A typical separation of hyoscyamine, ergotamine and scopolamine in the same order of separation is shown in Fig. 7. The k' values were initially lower than for Kieselguhr (Fig. 8) but higher than for the Merck "low-pressure" columns. The component used for the determination of t_0 was dodecylbenzene.

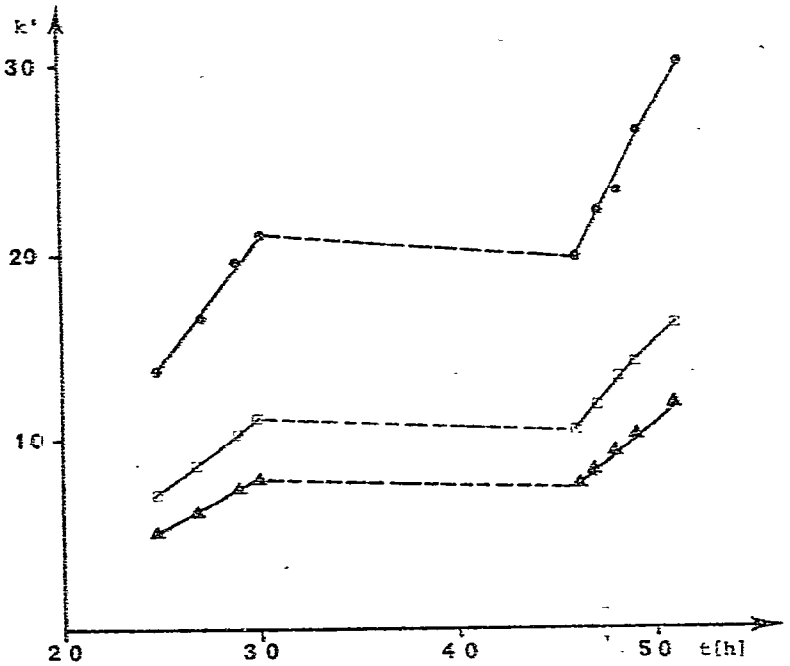

Fig. 8. Graph of k' against time of column usage with Merckosorb SI 100 (particle size, 5 μm) as the support. Mobile phase as in Fig. 7; flow-rate, 0.6 ml/min (linear column velocity, $u = 0.23$ cm/sec) (———) and 0.08 ml/min ($u = 0.04$ cm/sec) (– – –). Column length and alkaloids as in Fig. 3.

The stability of the system over an extended period of time was tested and the results are presented in Fig. 8. The flow-rate used for the actual separation was 0.6 ml/min (solid line) and for conditioning of the column overnight or over the weekend the flow-rate was reduced to 0.1 ml/min. Again the k' values increased rapidly with extended use at the higher flow-rate, rendering the columns unusable after a period of 6 h. When conditioning at the lower flow-rate, the column was stable. From these data it is clear that deterioration of the separation system occurs due to mechanical stress caused by the high flow-rates. On gradually eliminating the stationary phase via the bleeding process, adsorption became dominant and the ion-pairs were retained or even dissociated. Small injections of stationary phase resulted in a brief reversal of this trend, but this was followed by an even more rapid degradation of the column. Thermostatting of the column and the use of a pre-column, which was identical to the separation column, did not improve the situation either.

The α values, which indicate the actual thermodynamic difference in the solute–liquid phase interaction, remained constant (Fig. 9). Similar observations were made for the other supports (except silanized silica gel) where a regular change of k' with time occurred. The reproducibility of the α values was checked for three different columns and the values were found to be practically identical.

Silica gel (pore size, 1000 Å). In the hope of further improving the long term stability of this ion-pair system so that it could be used in routine analysis, silica gel SI 1000 (average pore size, 1000 Å; particle size, 5 μm) was studied under identical

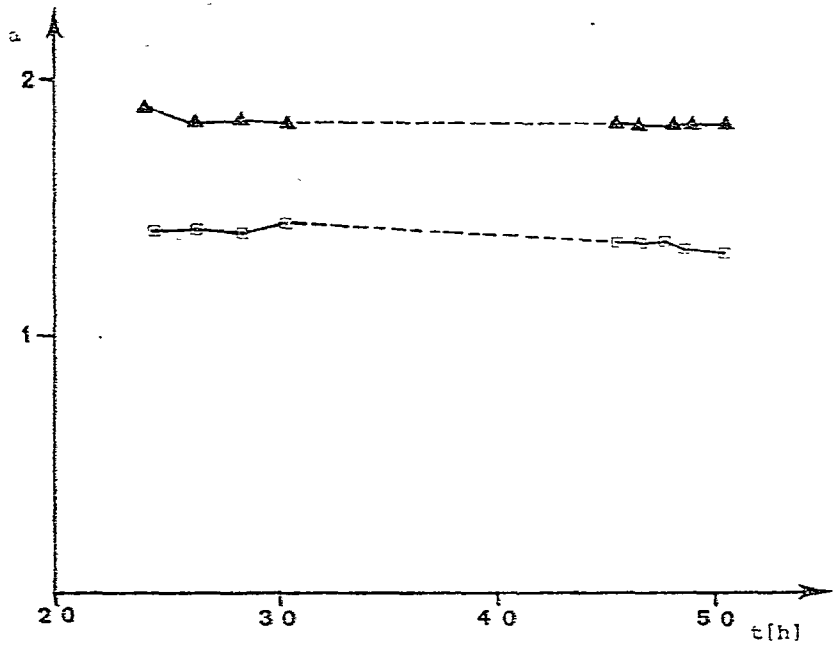

Fig. 9. Graph of α against time of column usage for scopolamine–ergotamine (▲) and ergotamine–hyoscyamine (□). *Conditions as in Fig. 8.*

conditions to SI 100. The k' values were considerably smaller ($\leqslant 1$) as can be seen from Fig. 10. This is to be expected due to the significantly smaller surface area of SI 1000 (SI 100, 300; SI 1000, 15 m^2/g) which results in a smaller value of V_s (see also eqn. 3). This fact permits separations at lower flow-rates of 0.2 ml/min within a couple

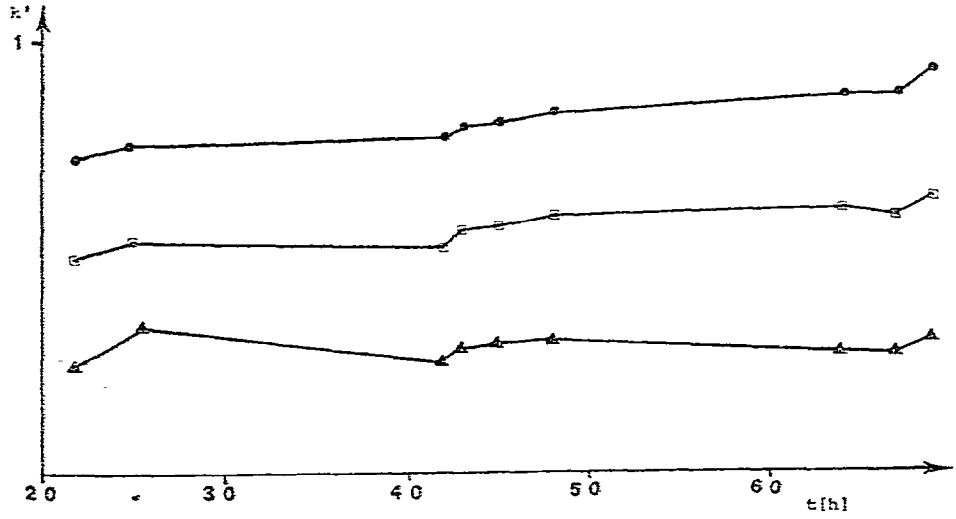

Fig. 10. Graph k' against time of column usage with Merckosorb SI 1000 (particle size, 10 μm) as support. Mobile phase as in Fig. 7; flow-rate, 0.2 ml/min (u = 0.08 cm/sec). Column length and alkaloids as in Fig. 3.

of minutes without loss of time and reduces the mechanical stress. An additional argument to the improved stability is a better retention of the stationary phase in the larger pores. Columns of both SI 100 and SI 1000 can be recuperated by washing with ethanol, drying in a stream of nitrogen at *ca.* 180° and reloading with 100 ml of the picric acid solution.

Applications to pharmaceutical products

The aim of this research was to develop a sensitive and selective method for the determination of pharmaceutical products. Fig. 11 shows the chromatogram of an ampoule solution. 10 ml of the ampoule solution were treated with 10 ml of a 0.05 *M* picric acid solution buffered to pH 6 and the ion-pairs were extracted with chloroform. 2 μl of this extract were injected on to the column. The selectivity of the system

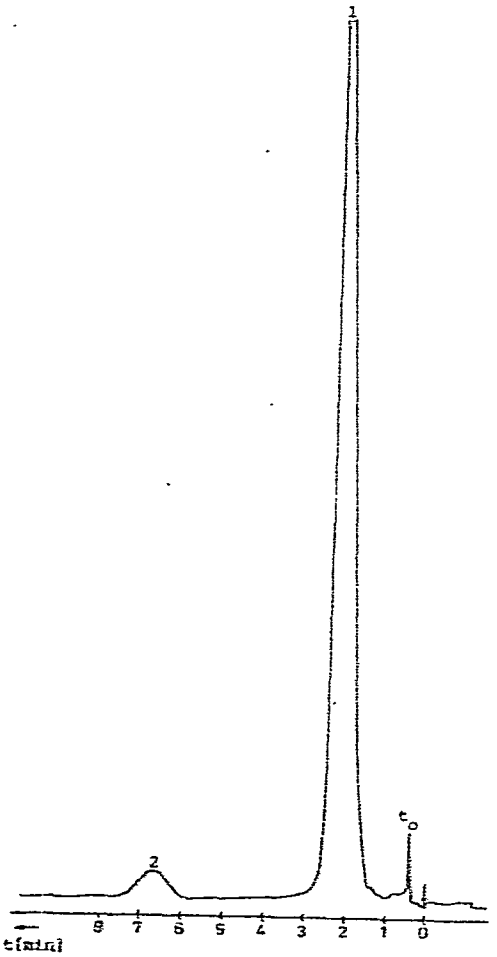

Fig. 11. Determination of hyoscyamine (peak 1, 1.25 μg) and scopolamine (peak 2, 0.1 μg) from ampoule solutions. Mobile phase: chloroform saturated with 0.05 *M* picric acid in buffer at pH 6; flow-rate, 0.8 ml/min. Detector setting, 0.08 area units.

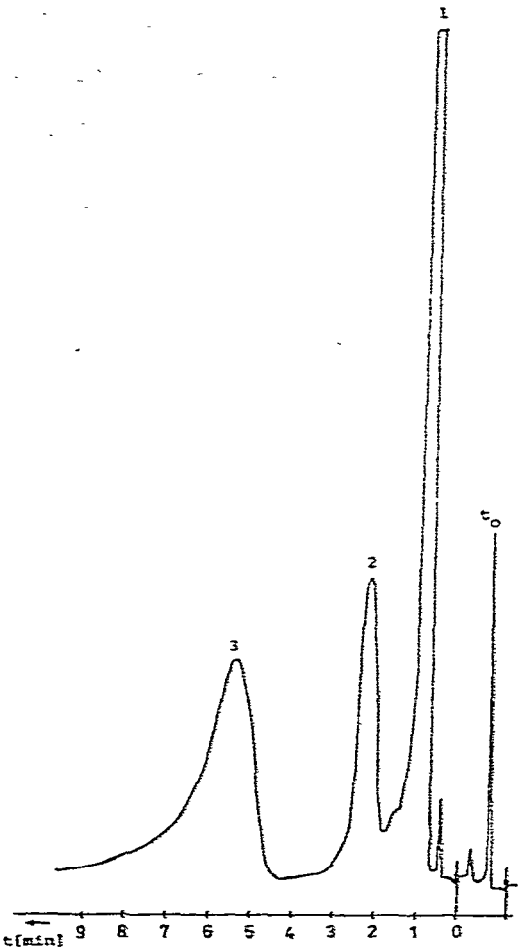

Fig. 12. Separation of a drug mixture corresponding to an actual tablet formulation. Peaks: 1 = phenobarbital (20.4 μg), 2 = hyoscyamine (0.2 μg) and 3 = ergotamine (0.66 μg). Conditions as in Fig. 11.

permits a baseline separation within a few minutes on a column of 10 cm length and the sensitivity allows a quantitative determination of scopolamine present in an excess of hyoscyamine. Using external standardization, the analytical results obtained for this sample were 90.7% of hyoscyamine and 6.8% of scopolamine, a total of 97.5% of the drug substance present.

In a second example (Fig. 12) a drug mixture corresponding to an actual tablet formulation was analyzed. Phenobarbital does not form an ion-pair but can be extracted with chloroform under analogous conditions. Here the specificity of the system permits the analysis of a drug mixture in one run within a few minutes. The small shoulder on the phenobarbital peak probably stems from ergotaminine and could be resolved further with optimization of the separation process. The interference of the phenobarbital could also be suppressed by detecting the ion-pairs at the wavelengths of 348 (hyoscyamine) and 402 nm (scopolamine) instead of using a single-wavelength detector at 254 nm.

CONCLUSIONS

From the experience gained with the ion-pair systems we conclude that this approach offers several advantages: it is complementary to true pre-column derivatization, in cases where reactive sites for substitution are lacking but where Lewis-acid or -base activity is apparent; the possibility of artefact formation is small; the selective extraction procedure via ion-pairs serves as a pre-clean-up step and enhances selectivity; the detection can be improved by choosing counter-ions which possess suitable chromophores or fluorophores; the system can be made to fit the actual separation problem; and finally the technique is non-destructive and could be used for preparative purposes, for example, in order to carry out structure elucidations.

Specifically, with regard to the picrate system, silica gel (pore size, 1000 Å) is best suited and sufficiently stable for routine applications in quality-control analysis. With the lower k' values, separations of greater sensitivity (narrower peaks) can be made in a shorter time using low flow-rates. Working with small particle sizes enhances the separation efficiency without causing too large a decrease in pressure. The possibility of using short columns of 10–15 cm length also permits work with lower flow-rates and minimizes the problems of decreases in pressure so that automated septum injection can be employed. The feasibility for re-conditioning such columns makes this method also attractive in cost.

The choice of the acid reagent used in the formation of the ion-pairs offers many possibilities. Preliminary tests with the same type of alkaloids using, for example, trinitrobenzenesulphonic acid have shown much promise. A comparison of chromatographically measured k' values with data obtained from batch experiments (static conditions) according to eqns. 2 and 3 is currently being made with selected systems.

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