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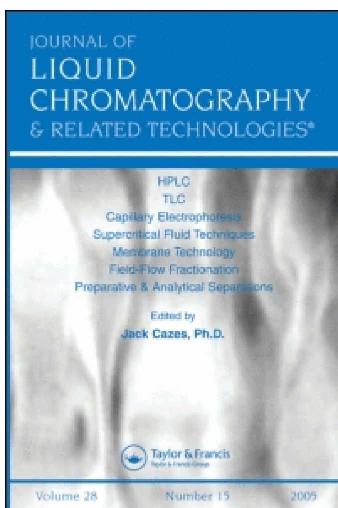

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Isocratic High-Performance Liquid Chromatography for the Simultaneous Separation of Eleven Tricyclic Imipramine-Derived Compounds of Therapeutic Interest

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ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SIMULTANEOUS SEPARATION OF ELEVEN TRICYCLIC IMIPRAMINE-DERIVED COMPOUNDS OF THERAPEUTIC INTEREST

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ABSTRACT

A rapid, specific, and sensitive high performance liquid chromatographic method for the simultaneous analysis of eleven tricyclic imipramine-derived drugs has been developed by using a Lichrosorb RP Select B column, an isocratic system at ambient temperature, and UV detection at 230 nm. The influence of acetonitrile and methanol content, the molarity of sodium acetate, and the effect of adding counter-ion in the mobile phase have been investigated. Excellent separation of a mixture of the eleven compounds was accomplished within 30 minutes. The method was applied to pharmaceutical dosage forms containing a single active ingredient; any of the other compounds can be used as internal standard since these drugs are structural analogues and are generally not found simultaneously. Satisfactory results were obtained both for the recovery and the coefficient of variation.

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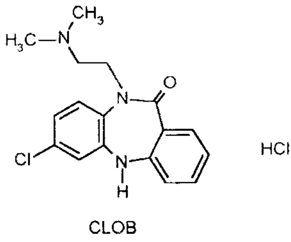

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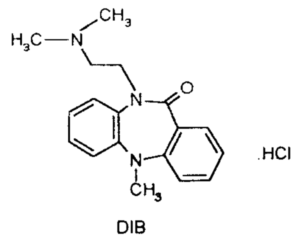

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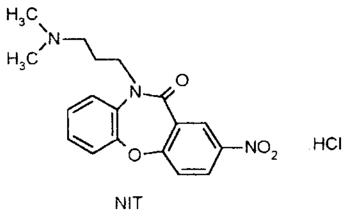

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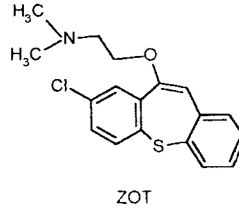
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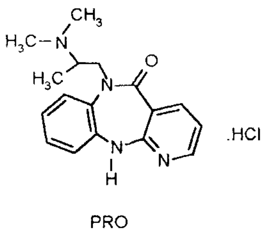

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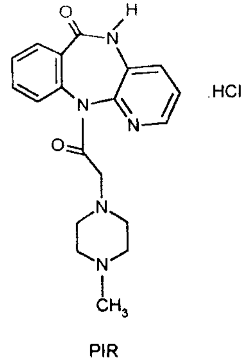

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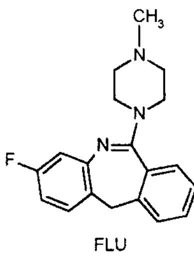
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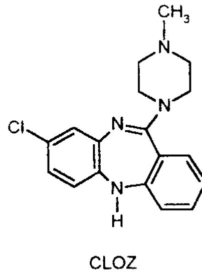
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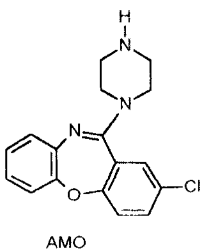
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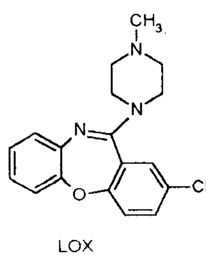
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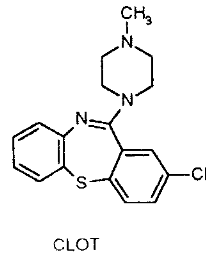
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INTRODUCTION

The discovery of the antidepressant activity of imipramine has inspired the synthesis of a large number of tricyclic compounds with a central seven-membered ring fused to two benzene rings on either side or to one benzene ring and one pyridine ring. The majority of these molecules exhibit antidepressant or neuroleptic properties, some of them are antimuscarinic or antihistaminic agents. Eleven members of this series which have been the subject of published clinical studies or which have been introduced into clinical practice are given in Figure 1, viz., clobenzepam (CLOB), dibenzepine (DIB), nitroxazepine (NIT), zotepine (ZOT), propizepine (PRO), pirenzepine (PIR), fluperlapine (FLU), clozapine (CLOZ), amoxapine (AMO), loxapine (LOX) and clotiapine (CLOT). Their closely related structure poses a problem of identity for the analyst as they have similar characteristics. Analytical methods which distinguish individual drugs within a certain chemical class of compounds would be of interest for quality control and clinical monitoring laboratories. For routine analytical applications, high performance liquid chromatography (HPLC) seems to be an attractive analytical approach, so we decided to use that method for solving the above problem.

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A number of papers dealing with HPLC of some of these eleven tricyclic molecules have been published. Most of these procedures have concentrated on the determination of individual compounds eventually with their metabolites as known constituents in a biological fluid, viz. DIB,[1,2] AMO with or without LOX,[3-5] CLOZ,[6-12] CLOT,[13] and PIR.[14-16] Few studies have examined the determination of a single compound in pharmaceutical dosage forms or a small group of some of these tricyclic compounds in synthetical mixtures.[17-20] The methods reported cannot simultaneously analyse more than two of these drugs. We previously reported[21] an HPLC method for the separation of ZOT, AMO, LOX, FLU, CLOT, NIT,and fluradoline hydrochloride, a tricyclic drug substance with analgesic and antidepressant activity. The method however could not be applied to the separation of the eleven above-mentioned molecules. We therefore investigated the chromatographic behaviour of the eleven compounds and established a method for their simultaneous separation with UV detection under isocratic conditions by using a reversed-phase Lichrosorb RP Select B column. Different parameters were tested in order to obtain good resolution of all compounds and select the right eluent composition for chromatographing any of these tricyclic compounds. The phase system was tested for its suitability for the analysis of PIR, PRO, DIB, AMO, CLOZ, LOX, and CLOT in pharmaceutical formulations.

Figure 1 (left). Structures of compounds studied.

EXPERIMENTAL

Equipment

Chromatography was performed on a modular isocratic system consisting of a LDC Constametric 3000 pump (Milton Roy company, Riviera Beach, FL, USA), a Rheodyne Model 7125 syringe loading sample injector (Rheodyne Inc., Cotati, CA, USA) fitted with a 20μL sample loop, a LDC SM 4000 variable-wavelength UV detector operating at 230nm and a Perkin-Elmer R50-10mv chart recorder at a chart speed of 300 mm./h (Norwalk, CT, USA). The time constant of the detector was set to 0.5 s and the sensitivity range setting depended upon the amount of sample.

Separations were carried out by using stainless-steel columns: a Lichrosorb RPSelect B Hibar (250 mm x 4.0 mm I.D.), particle size 5 μm (Merck, Darmstadt,Germany), a Lichrosorb RP-8 (120 mm x 4.6 mm I.D.), particle size 5 μm, purchased from CIL (Ste Foy-la-Grande, France), and a μBondapak C18 Waters (300 mm x 3.9 mm I.D.), particle size 10 μm (Waters Assoc., Milford, MA, USA). An Uptight guard column (20 x 2 mm I.D.) (Upchurch Scientific, Oak Harbor, WA, USA) packed with Perisorb RP-8 or RP-18, particle size 37-40 μm, was used to protect the analytical column.

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Chemicals and Reagents

All chemicals and reagents were used without further purification. The pure drugs were obtained as gifts from the following sources: PIR (Boehringer Ingelheim, Reims, France), PRO (UPSA, Agen, France), DIB (Sandoz, Basel, Switzerland), NIT (Ciba Geigy, Basel, Switzerland), and CLOB (Wander, Basel, Switzerland) as hydrochloride salts; ZOT (Fujisawa Pharmaceutical Co., Osaka, Japan), AMO and LOX (Lederle, Oullins, France), CLOT, CLOZ, and FLU (Sandoz, Rueil-Malmaison, France; Basel, Switzerland) as free bases. All the solutions prepared from these samples were kept in light-protected bottles at 4°C.

HPLC grade acetonitrile (Rathburn Chemicals, Walkerburn, U.K.), methanol RPE-ACS (Carlo Erba, Milan, Italy), sodium acetate trihydrate RP Normapur (Prolabo, Paris, France), anhydrous 1-heptanesulphonic acid sodium salt (SHS) (99 %) (Interchim, Montluçon, France), acetic acid Purex (min. 99.8 %) (SDS, Peypin, France) were used. Water was deionized and doubly distilled from glass apparatus. The SHS reagent was prepared by dissolving 5.50 g of SHS, 3.0 mL of acetic acid and water up to 100 mL. Sodium acetate solution (0.05 M) was prepared in water.

Chromatographic Conditions

The mobile phase was obtained by mixing together 250 mL of acetonitrile with 350mL of methanol, 15 mL of the SHS reagent into 1000 mL volumetric flask and diluting to volume with 0.05 M sodium acetate. The mobile phase was then vacuum filtered through 0.2 μm Sartolon membranes (Sartorius, Göttingen, Germany) and degassed by ultrasonic vibration for ca. 5 min prior to use.

Chromatography was operated at ambient temperature and a flow-rate of 1.0mL/min was used. About 20 min were needed to get a stable baseline. The column was washed with a mobile phase consisting of acetonitrile-methanol-water (25:35:40) when not in use to ensure long column life. Retention data are expressed as capacity factors, k', which are defined by

$$k' = (t_R - t_o)/t_o$$

where t_R and t_o are the retention times of the sample peak and a non-retained compound, respectively. Injections of an aqueous solution of sodium nitrate were used to determine t_o. The retention times were determined in triplicate and the mean values were reported.

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Preparation of Standards

Individual stock solutions (100 mg/L) were prepared in methanol. For AMO, dissolution was facilitated by ultrasonication for ca. 5 min. The solutions were stored at 4°C in amber-colored bottles and were found to be stable for at least six months. The working composite standard was prepared by combining an aliquot of each stock solution and diluting the mixture with the mobile phase. Any of the other ten compounds could be used as internal standard for the analysis of one of the eleven tricyclic compounds. Working standard solutions were prepared by serial dilutions of the stock solution of the concerned compound with incorporation of constant volume of the stock solution of the internal standard and diluted with the mobile phase.

The calibration curves were constructed in order to encompass the expected concentrations in samples. The calibration equations were calculated by least-squares linear regression of the peak-height ratio of the drug/internal standard (y) versus drug concentration (x).

Preparation of Samples

Samples of pharmaceutical products were obtained from commercial sources and seven compounds of the studied series were analysed. Depending

on the theoretical amount of drug present in the sample, the volume of extraction solvent (methanol) and dilution solvent (mobile phase) has to be adjusted to obtain a final concentration near the median zone of the calibration solutions.

For tablets and capsules, ten tablets or the contents of ten capsules were accurately weighed and ground until just reduced to a fine powder. A portion of the powder equivalent to the average mass of each tablet or capsule was accurately weighed into a volumetric flask, extracted under magnetic stirring with methanol for 15 min, diluted to volume with methanol, and filtered with 0.45 μm membrane. An aliquot of the filtrate was transferred into a volumetric flask, internal standard solution was added and the volume was adjusted with the mobile phase. For oral solution, the sample was first diluted with methanol and final dilution was made with the mobile phase and the appropriate volume of internal standard solution was added.

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Precision and Recovery

The precision of the proposed method was evaluated by assaying repeated injections using the expected concentrations in the samples. The intra-day variation was investigated by five replicate analyses. The inter-day variation was determined by replicate analyses over a 3-day period.

Recovery was tested by comparing peak heights obtained following injection of standard solutions with those obtained from extracted sample containing equal amount of compound to be determined.

Method Development

A main drawback of the analysis of basic drugs by reversed-phase HPLC is frequently severe peak tailing shape, caused by ionic interactions of these bases with free silanol groups of the packing material. The addition of appropriate modifiers (buffer, alkylamine and/or long-chain ionic alkyl reagent) to the mobile phase represents a way to reduce peak asymmetry and effect selectivity changes.[22,23] Our investigations indicated that separation of all tricyclic drugs could not be achieved with only methanol or acetonitrile as organic modifiers in the mobile phase. Two organic modifiers proved to be necessary. When 0.01 M triethylamine was added to the mobile phase at pH compatible with the stationary phase, a remarkable decrease in retention was observed, the peak shape of all solutes improved greatly. Unfortunately, column lifetime was shortened; addition of amine was, therefore, left out of consideration. Among the buffer solutions which were tested, sodium acetate resulted in moderate

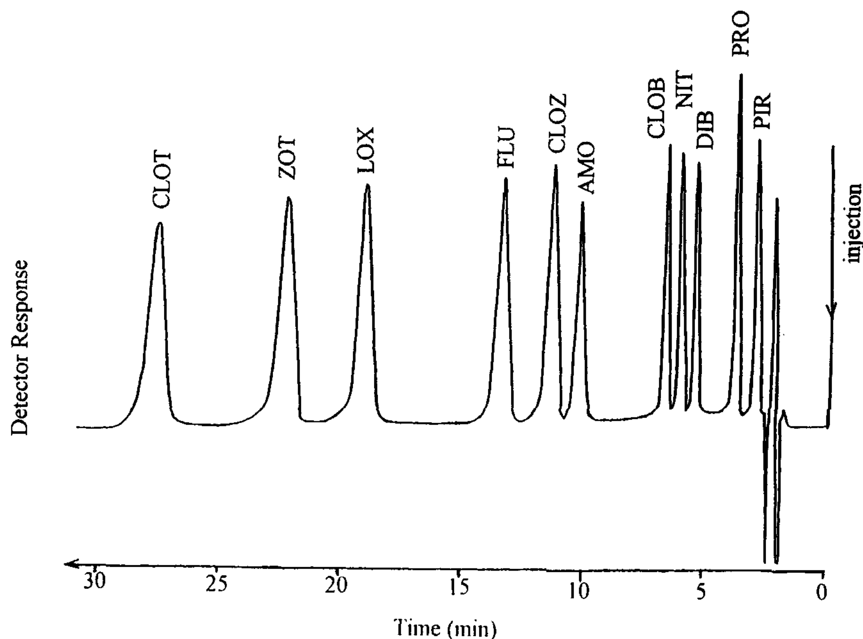

Figure 2. Chromatography of tricyclic imipramine-derived drugs. Column: Lichrosorb RP Select B-Hibar, 5μm (250x4.0mm I.D.); eluent: acetonitrile-methanol-SHS reagent-aqueous 0.05 M sodium acetate (25:35:1.5 up to 100 mL); flow-rate: 1.0 mL/min; detection: UV absorbance (230 nm).

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improvement in the peak shapes of the solutes and in the resolution but elution was time consuming. By adding to the eluent an acetic solution of sodium heptanesulphonate, an excellent separation was obtained with symmetrical peak shapes and baseline resolution.

The influence of the support on the retention and the selectivity of the chromatographic system was studied using RP Select B, Lichrosorb C8 and μBondapak C18 as supports.

Although similar chromatographic behaviours were observed, the Lichrosorb RP8 and μBondapak C18 columns did not give a good separation of DIB and CLOB because of a different position in the elution order of NIT and CLOB. The RP Select B column, however, gave good separations of all compounds investigated. It was therefore adopted for further investigations.

Table 1

HPLC Retention Data for the Eleven Tricyclic Drugs (Arranged in Order of Elution)*

Compound	k'
PIR	0.47
PRO	0.81
DIB	1.62
NIT	1.90
CLOB	2.24
AMO	3.86
CLOZ	4.43
FLU	5.38
LOX	8.09
ZOT	9.62
CLOT	12.19

* Chromatographic conditions as in Figure 2.

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RESULTS AND DISCUSSION

Figure 2 illustrates a typical chromatogram of the working standard mixture of the tricyclic drugs. The total run time for the separation of the eleven compounds was 30 min. Table 1 gives the retention data (k' values) for the eleven drugs arranged in their order of elution.

Structure and Separation

The bridging group in position 5 in the seven-membered ring has a strong influence on the k' values. CLOT and ZOT which have a sulphur atom as bridging group show high affinity for the stationary phase and have therefore a high k' value. Terminal piperazine ring in the side-chain results in high k' values especially if the distal nitrogen of the piperazine moiety is substituted. The bridging group in the seven-membered ring has, in that case, a marked effect: the k' values decrease in the order S, O, CH2, NH. For the compounds of this type the order of k' values is thus CLOT > LOX > FLU > CLOZ. CLOT is the latest eluting compound because of its sulphur atom as bridging group and because of its substituted distal nitrogen in the piperazine ring of the side-chain. AMO - despite the fact that an oxygen atom is present as bridging group - is the least retained compound among the five piperazinyl-dibenzo-azepine derivatives because the distal nitrogen of the piperazine moiety is unsubstituted. A carbonyl

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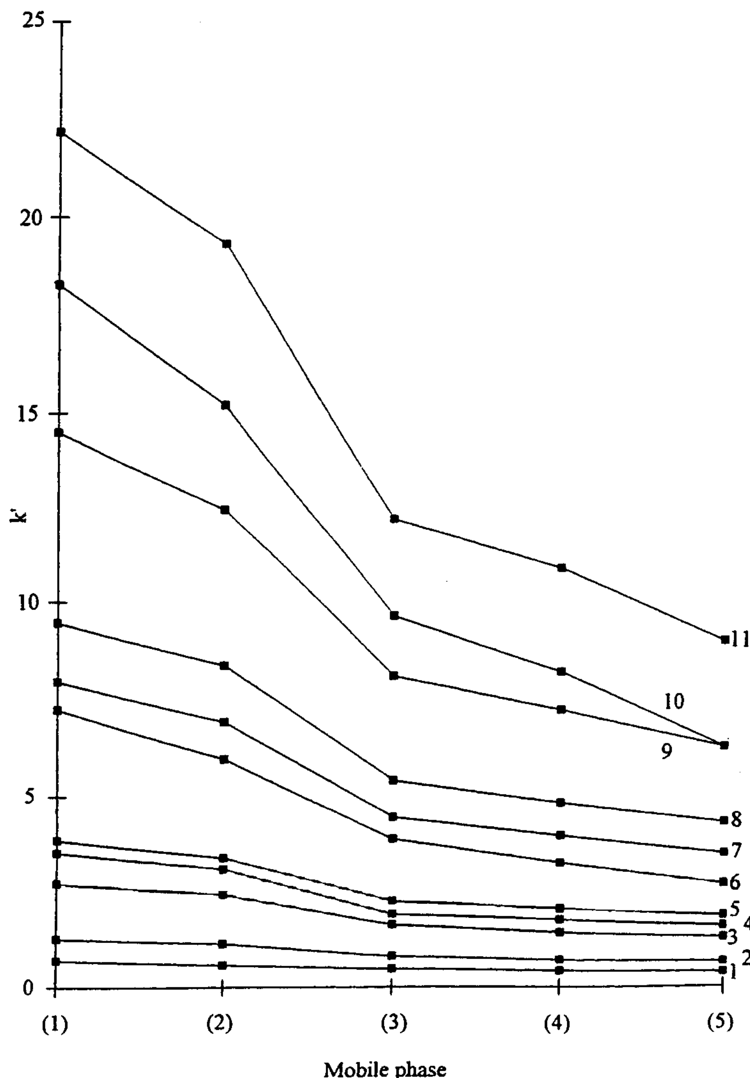

Figure 3. Effect of the volume percent of organic modifiers in the mobile phase on the k' values of the tricyclic compounds. Mobile phase: acetonitrile-methanol-SHS reagent-aqueous 0.05 M sodium acetate with the following proportions: (1)=20:35:1.5:up to 100; (2)=25:30:1.5:up to 100; (3)=25:35:1.5:up to 100*; (4)=40:1.5:up to 100; (5)=30:35:1.5:up to 100mL. Key: 1, PIR; 2, PRO; 3, DIB; 4, NIT; 5, CLOB; 6, AMO; 7, CLOZ; 8, FLU; 9, LOX; 10, ZOT; 11, CLOT. Operating conditions: column: Lichrosorb RP Select B (250x4.0mm I.D.); ambient temperature; flow-rate: 1.0 mL/min; detector UV 230 nm. *The effective mobile phase for separation.

group in the seven-membered ring reduces the retention of the compound as is the case with CLOB, NIT, DIB, PRO, and PIR. Replacing one benzene ring by a pyridine ring results in a low retention. This effect of lower retention is increased if a carbonyl group is present in the seven-membered ring as is the case with PIR and PRO which are the least retained compounds in the studied series. With its two carbonyl groups PIR is the most polar compound and is always the first eluted solute.

Composition of the Mobile Phase

Figure 3 illustrates the effects of altering the acetonitrile or/and methanol concentration of the mobile phase on the chromatographic behaviour of the eleven compounds. Altering the acetonitrile or methanol concentration in the mobile phase is effective in changing both k' and selectivity. The change in k' values with alterations in acetonitrile or methanol content is abrupt with the strongly retained drugs such as the five piperazinyl-dibenzo-azepines (AMO, CLOZ, FLU, LOX, CLOT) and ZOT, while the other compounds eluting with low capacity factors (PIR, PRO, DIB, NIT, CLOB) show only a gradual change in k'.

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A series of separations were carried out in which the molarity of sodium acetate solution was altered from 0.01 to 0.10 M (Figure 4). Two groups can be observed over this range: the first six eluted solutes viz., PIR, PRO, DIB, NIT, CLOB, and AMO, exhibit capacity factors which are virtually unaffected by the changes in buffer molarity. The overall selectivity of their separation undergoes a minor effect. Over the range 0.01-0.04 M the other five compounds viz., CLOZ, FLU, LOX, ZOT, and CLOT show a moderate increase in their capacity factors with increasing buffer molarity. A poor overall resolution of the eleven compounds is obtained in a short time. A change in elution order of AMO, CLOZ, FLU, ZOT, CLOT is observed. From 0.05 to 0.10 M there are considerable changes in the retention of CLOZ, FLU, LOX, ZOT, and especially CLOT with high k' values. As a result, analysis of the eleven compounds requires careful monitoring of the buffer molarity to maintain their excellent separation. Buffer molarity of 0.05 M was chosen to shorten the chromatographic run time and retain baseline separation of all compounds.

The k' values decrease for all the compounds when the volume of the acetic ion-pairing reagent in the mobile phase varies from 5 to 15 mL per liter: the decrease in retention is moderate for PIR, PRO, DIB, NIT, and CLOB, more important for AMO, CLOZ, FLU, and LOX, and drastic for ZOT and CLOT (Figure 5). 15 mL of the reagent in 1000 mL of mobile phase result in 4.08 mM sodium heptanesulphonate. At this concentration low k' values are obtained with considerable improvement of separation and baseline resolution of

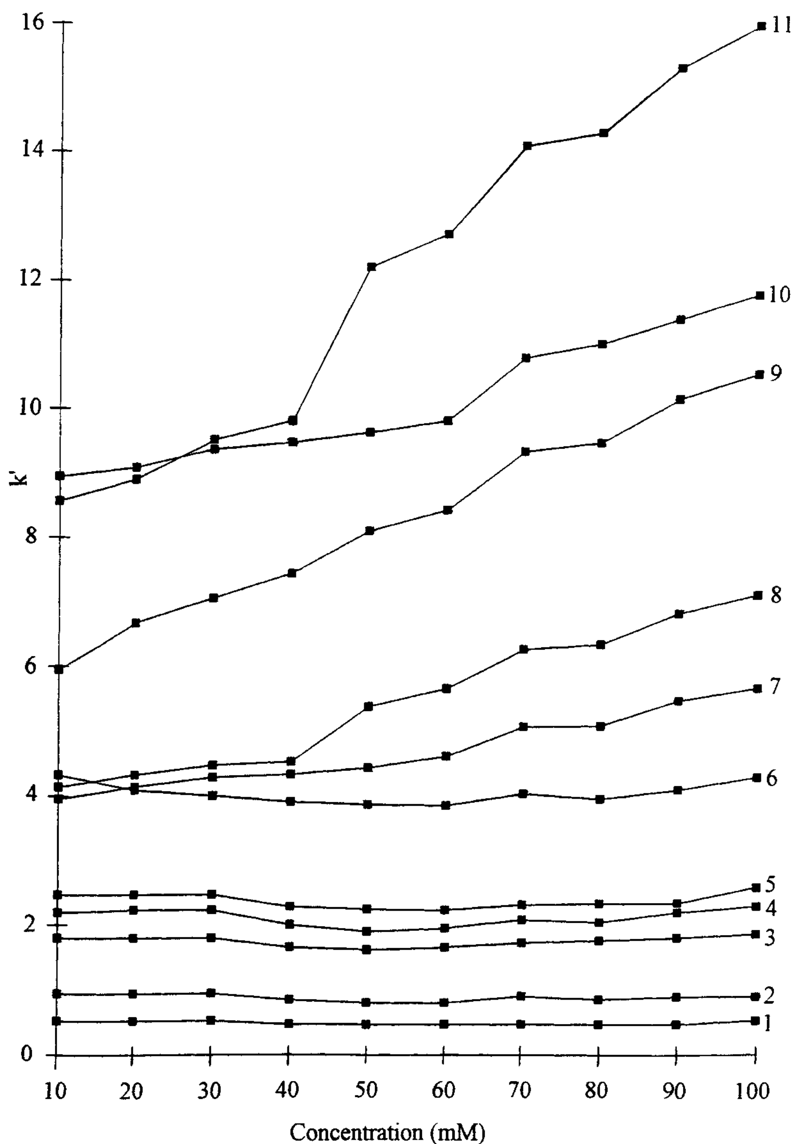

Figure 4. Effect of molarity of sodium acetate on the k' values of the tricyclic compounds. Mobile phase: acetonitrile-methanol-SHS reagent-water containing 0.01-0.10 M sodium acetate (25:35:1,5 : up to 100 mL). Other details as in Figure 3.

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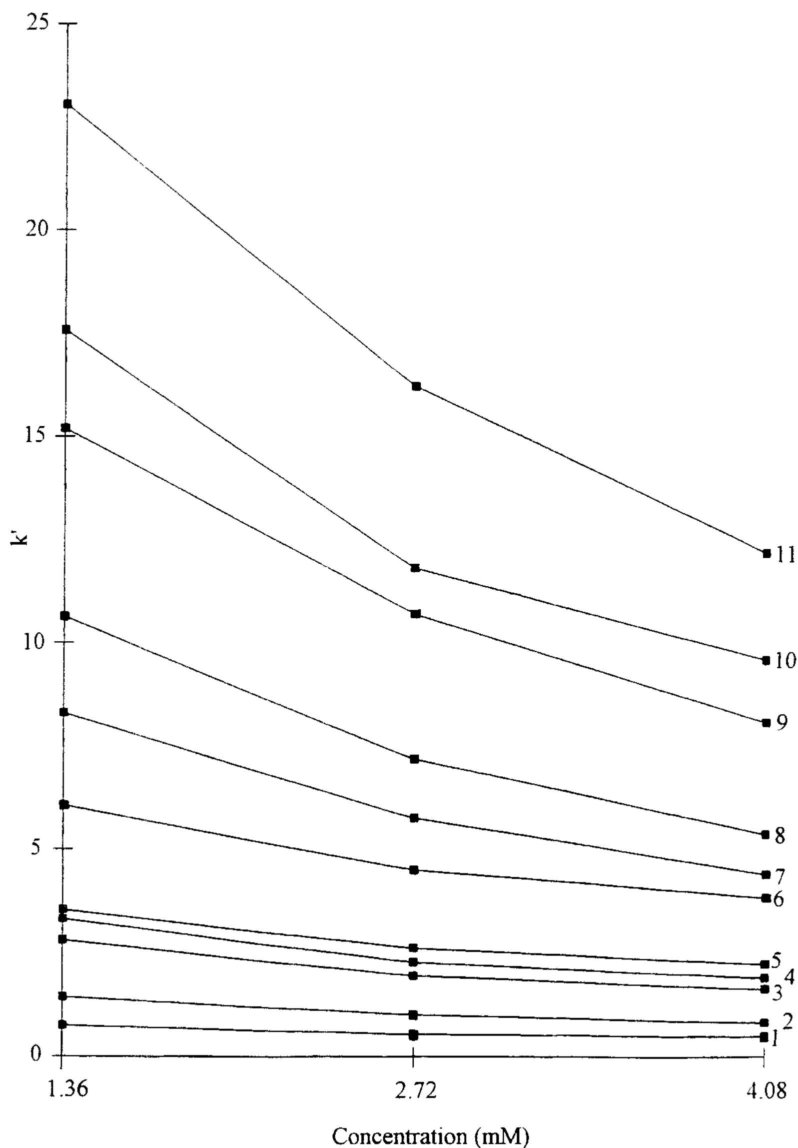

Figure 5. Effect of the concentration of sodium heptanesulphonate on the k' values of the tricyclic compounds. Mobile phase: acetonitrile-methanol-SHS reagent-aqueous 0.05 M sodium acetate (25:35:0.5-1.5:up to 100 mL). Other details as in Figure 3.

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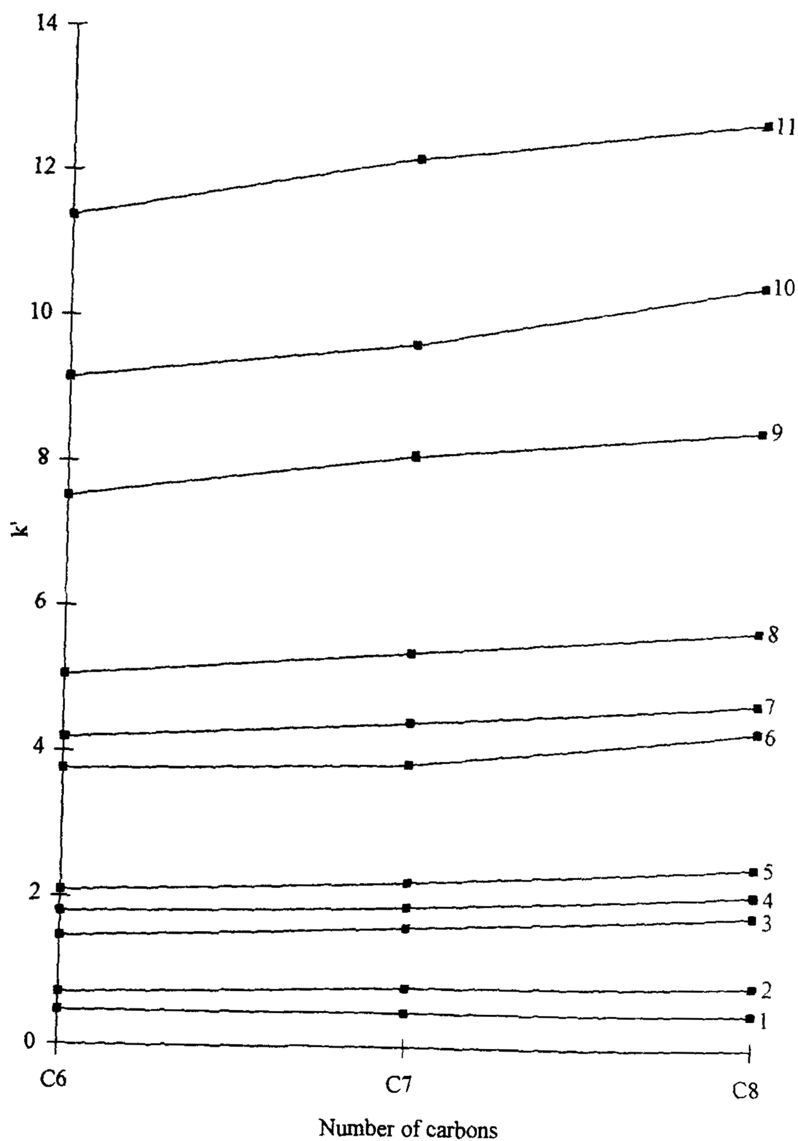

Figure 6. Effect of the alkylsulphonate chain length of the ion-air reagent on the k' values of the tricyclic compounds. Mobile phase: acetonitrile-methanol-alkyl (6-8 carbons) sulphonate reagent-aqueous 0.05 M sodium acetate (25:35:1.5:up to 100 mL). *Other details as in Figure 3.*

Table 2

Calibration Curves for Seven Tricyclic Derivatives: Linear Regression of the Peak-Height Ratio of Drug/Internal Standard (y) versus Drug Concentration (x)*

Compound	Internal Standard	Concentration Range	Slope	Intercept	Correlation Coefficient
PIR	PRO	2-10mg/L	0.256	0	0.9984
PRO	PIR	1-8mg/L	0.206	0.003	0.9977
DIB	NIT	1-8mg/L	0.251	0.005	0.9995
AMO	CLOB	0.5-5mg/L	0.475	0.007	0.9990
CLOZ	PRO	0.5-5mg/L	0.836	-0.014	0.9992
LOX	CLOZ	2-25mg/L	0.117	-0.016	0.9993
CLOT	NIT	2-8mg/L	0.537	-0.004	0.9984

* Calculations based on five points for each drug.

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the eleven compounds as sharp, symmetrical peaks in rather short run time. The chain length of alkylsulphonate pairing ion was varied from 6 to 8 (Figure 6). The k' value for PIR is constant, regardless of the ion-pair reagents, but the values for the other ten compounds increase with extending alkyl chain of the ion-pair reagent. With a chain length of 6 carbons, retention is insufficient for some compounds which prevents resolution especially for NIT and CLOB. Sodium octanesulphonate allows adequate resolution of all compounds but with slightly greater retention times than sodium heptanesulphonate.

These compounds, all positively charged at the acidic pH of the mobile phase, form neutral ion pairs with heptanesulphonate sodium salt, permitting efficient interactions between the analytes and the stationary phase. Elution strength of two organic modifiers (acetonitrile and methanol) and the buffering capacity of 0.05 M sodium acetate/sodium heptanesulphonate/acetic acid proved to be important to achieve the efficiency needed for this application.

Quantitative Aspects

The procedure was applied to the determination of active ingredients in seven commercial pharmaceutical formulations: six solid forms (tablets A, C, D, E and G, capsules B) and one liquid form (oral solution F). The linearity of the calibration graphs was evaluated for each drug. The equations obtained by the least-square regression fit are reported in Table 2.

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Table 3

Intra-Day Precision (n=5)

Compound	Concentration (mg/L)	Standard Deviation of Peak-Height Ratios	C.V. (%)
PIR	5	0.009	0.71
PRO	5	0.006	0.58
DIB	5	0.007	0.55
AMO	2	0.009	0.94
CLOZ	2.5	0.013	0.62
LOX	10	0.012	1.05
CLOT	5	0.021	0.78

Table 4

Inter-Day Precision Over a 3-Day Period

Compound	Concentration (mg/L)	C.V. (%)
PIR	5	0.31
PRO	5	0.48
DIB	5	0.39
AMO	2	0.41
CLOZ	2.5	0.36
LOX	10	0.42
CLOT	5	0.37

The confidence limits on the intercept (P=95 %) showed that each calibration graph passed through the origin. The linearity obtained allows only one standard solution to be used for the determinations. Intra-day precision of the method is illustrated in Table 3. Coefficient of variation was better than 1.5 %. Results for inter-day precision are shown in Table 4. The assays showed good precision.

Under the analytical conditions described, the limits of detection defined as the lowest mass of each substance (ng) resulting in a signal-to-noise ratio of 3 : 1 are reported in Table 5. The limits of quantitation defined as the lowest mass of substance (ng) that allows the determination with 5 % reliability are given in

Table 5

Detection Limit and Quantitation Limit for Seven Tricyclic Compounds

Compound	Detection Limit (ng injected)	Quantitation Limit (ng injected)
PIR	0.5	1.6
PRO	0.2	0.8
DIB	0.2	0.8
AMO	0.2	0.8
CLOZ	3	10
LOX	1.2	4
CLOT	1.2	4

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Table 6

Analysis of Pharmaceutical Formulations*

Formulation (active ingr.)	Manufacturer's Label Claim	Found (mg)±S.D.	Recovery (%)	C.V. (%)
Tablet A (PIR)	50mg/tablet	49.98±0.09	99.8	0.18
Capsule B (PRO)	50mg/capsule	48.80±1.60	97.6	3.27
Tablet C (DIB)	80 mg/tablet	78.24±1.14	97.8	1.46
Tablet D (AMO)	50mg/tablet	49.02±1.28	98.0	2.61
Tablet E (CLOZ)	25 mg/tablet	24.87±0.20	99.5	0.80
Oral Solution F (LOX)	25mg/mL	24.92±0.37	99.7	1.48
Tablet G (CLOT)	40mg/tablet	40.11±0.21	100.3	0.52

* Mean of five determinations.

Table 5. These limits could be enhanced for a specific analysis by using the appropriate absorption maximum, since the detector wavelength used - 230 nm - was a compromise made in order to detect all of the compounds concerned simultaneously, with a good sensitivity. The results obtained with the seven pharmaceutical formulations to be analysed are shown in Table 6. The quantities found were in conformity with the values claimed by the manufacturers.

CONCLUSION

The main aim of this paper was to develop an accurate, rapid method using an identical detection procedure for differentiating a class of tricyclic compounds. The proposed method may help to confirm or invalidate a difficult diagnosis of these structurally related compounds. It offers the possibility of monitoring drug levels with only a simple isocratic HPLC equipment and provides both the separation and determination steps, thereby eliminating interference problems. No attempt has been made in the present work to apply the HPLC system to the determination of these tricyclic drugs in biological fluids although such applications are feasible. That analytical system can be considered sufficiently reliable and specific to be applied in quality control laboratories for detection and quantitation of these drugs. For practical purposes it will be very rare for a sample to contain all of the eleven tricyclic drugs at the same time; therefore if an interfering drug is encountered when quantitating a particular tricyclic drug, it is possible to modify the ratio of mobile phase and change the resolution enough to avoid such interference.

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