Journal of Pharmaceutical & Biomedical Analysis
Vol. 6, Nos 6–8, pp. 859–866, 1988
Printed in Great Britain

0731–7085/88 $3.00 + 0.00
© 1988 Pergamon Press plc

Sensitive HPLC analysis of basic drugs of abuse having weak UV absorptivity using columns with low carbon loadings monitored at 205 nm*

S. O. BADIRU and T. M. JEFFERIES†

School of Pharmacy and Pharmacology, University of Bath, Bath, BA2 7AY, U.K.

Abstract: The selection of columns containing cyanopropyl-bonded silica (3.5% carbon loading) and their use under reversed-phase conditions has permitted short retention times with good resolution to be obtained for 16 basic drugs of abuse and three metabolites. Predominantly aqueous mobile phases enable the determination of these weakly UV-absorbing compounds over the concentration range 0.7–18 μg ml^{-1} with detection at 205 nm. Trimethylsilyl-bonded silica (2.6% carbon loading) also provided good resolution but required longer analysis times.

Keywords: *High-performance liquid chromatography; basic drugs of abuse; UV detection at 205 nm.*

Introduction

An essential requirement to combat the growth of drug abuse is the development of suitable analytical procedures for drug detection and determination. A difficult group of drugs for forensic science laboratories to analyse are those having weak UV absorbance, typically molar absorptivities of about 200, especially with the low levels that occur in biological fluids and tissues. In cooperation with the Central Research Establishment of the Home Office Forensic Science Service at Aldermaston, U.K. 16 basic drugs and three metabolites were selected for study because of their poor detectability in a routine method that uses a silica column with a methanol–aqueous ammonium nitrate (90:10, v/v) eluent and detection at 254 nm [1]. These compounds consisted of five primary, five secondary and nine tertiary amines. It is known that some tertiary amines can be detected by electrochemical (EC) means and an interesting recent study [2] has expanded the class of suitable compounds by using post-column photolytic derivatization followed by dual electrode EC detection. It is possible that some of the tertiary amines in this present report may be suitable for this technique, although it was found that it was not suitable for the amphetamines.

Following preliminary chromatographic studies the list of solutes was divided into two

* Presented at the "International Symposium on Pharmaceutical and Biomedical Analysis", September 1987, Barcelona, Spain.

† To whom correspondence should be addressed.

groups so that two isocratic systems could be developed that would permit the maximum resolution in each group. On the other hand, if all the solutes were studied as a single group, then gradient elution would be required for satisfactory chromatography. Listed in order of increasing capacity factors, Group A consisted of *p*-hydroxynorephedrine, *p*-hydroxyamphetamine, 2-phenylethylamine, *p*-hydroxymethylamphetamine, dex-amphetamine, phenmetrazine, methylamphetamine, mephentermine, phendimetrazine and chlorphentermine. Group B consisted of pethidine, pipradol, benzphetamine, fentanyl, normethadone, methadone, norpipanone, piritramide and dipipanone.

An earlier study had investigated pre-column derivatization reagents suitable for the primary and secondary amines amphetamine and methylamphetamine and some of their metabolites [3], and had found sodium naphthaquinone-4-sulphonate to be selective and sufficiently sensitive for the determination of these drugs in urine and plasma samples at the ng ml^{-1} level. Since it was not generally possible to derivatize tertiary amines, it was our intention to achieve a similar enhancement of sensitivity for Groups A and B drugs by using post-column ion-pair formation with sodium naphthalene-2-sulphonate, followed by extraction into an immiscible organic phase using solvent segmentation in a PTFE coil [4]. Preliminary studies on the stability of aqueous-immiscible solvent segmentation showed that when iso-octane was used as the organic phase, miscibility between individual segments began to occur when the percentage of organic modifier in the HPLC mobile phase reached 25% for methanol, 15% for acetonitrile, 10% for propan-2-ol and 5% for tetrahydrofuran.

It was therefore, essential to develop chromatographic systems that used predominantly aqueous mobile phases, and this was approached by considering columns (50 or 100 mm) packed with materials other than octadecyl-silica (ODS-silica), and optimized for selectivity by controlling mobile phase pH, buffer strength, temperature and choice of organic modifier. In order to achieve detection at 254 nm it was generally found that concentrations of 1 mg ml^{-1} were required and this produced poor chromatography. When detection was changed to 205 nm, sensitivity was greatly enhanced provided that the mobile phase did not generate a high background absorbance. This report describes an examination of cyanopropyl-derivatized silica, and trimethylsilyl-derivatized silica for the chromatography of the compounds in Groups A and B, and the performance of a commercially available cyanopropyl-silica cartridge column, of 2 mm i.d.

Experimental

Chemicals and solution

All the solvents were HPLC grade, buffer components were analytical grade, both from Fisons, Loughborough, U.K., and water was prepared by a Milli Q water purification system (Millipore, Harrow, U.K.). Phosphate buffer was prepared from potassium dihydrogen phosphate and disodium hydrogen phosphate.

Apparatus

Two systems were used:

(i) a modular HPLC system assembled from a Constametric III pump (Milton Roy, Stone, U.K.), a Rheodyne 7125 injection valve with 10 or 20 μl sample loop, a Cecil-212 UV detector (Cecil Instruments, Cambridge, U.K.) and BBC Servogor SE 120 recorder (Milton Roy, Stone, U.K.). The column, valve and solvent reservoir were immersed in a water bath maintained at 30°C by a thermostirrer (Gallenkamp, London, U.K.);

(ii) a Spectra Physics SP8100 liquid chromatograph with autosampler, oven and 20 μl sample loop, SP8440 UV-Vis detector and SP4200 computing integrator.

Chromatography

All solutes were dissolved in the current batch of mobile phase, and passed through a 0.2 μm Acro LC13 filter assembly (Anachem, Luton, U.K.) before analysis.

Columns were packed at 6000 p.s.i. using a Sat Rig column packer (Haskel Energy Systems, Sunderland, U.K.). Propan-2-ol was used as the slurry solvent and hexane as the packing solvent. Columns were rejected when the value for h, reduced plate height, obtained using a test mixture, exceeded a value of 5.0. No significant chromatographic difference was detected between the materials CPS-Hypersil and Spherisorb-CN. The Brownlee MPLC cartridge column (100 × 2.1 mm i.d.) containing 5-μm Spheri-5 Cyano was supplied by Anachem (Luton, U.K.).

Results and Discussion

Cyanopropyl-bonded silica

Cyanopropyl-bonded silicas are frequently marketed as normal-phase materials intended for the chromatography of polyfunctional aromatic compounds using organic solvent mixtures such as hexane and propan-2-ol. Under these conditions it is a weaker hydrogen-bonding adsorbent than underivatized silica and so produces narrower peaks for these compounds. However, they have properties that make them suitable for rapid chromatography using reversed-phase conditions. The percentage carbon loading is low, for example, for the Spherisorb range of −CN, −ODS 1 and −ODS 2 derivatized silicas the values are 3.5, 7.0 and 12.0% m/m respectively, whilst the surface coverage is relatively high, namely 0.6, 0.3 and 0.5 mM g^{-1} respectively. Additionally, the −CN group introduces polar properties [5] (log P = −1.07) on to the propyl chain (3× —CH_2—, log P = 1.59) that should reduce solute retention when compared to a propyl chain plus a terminal —CH_3 group (log P = 0.70). To further reduce solute retention a short column (50 × 4.6 mm i.d.) was packed and used at 30°C rather than at room temperature.

In view of the importance of mobile phase pH, its influence on the retention of Group A solutes was studied using 0.05 M phosphate buffer–methanol–acetonitrile (90:5:5, v/v/v). Retentions of all solutes, Fig. 1A, fell with pH as expected, but did not reach their minimum values over the pH range (5.4–7.2) for this particular buffer. It has been reported [6] that the pKa values of 30 basic drugs when measured in methanol–aqueous buffer (pH 4 or 7) were lower than aqueous pKa values reported in the literature. However, considering that four of the compounds in Fig. 1A have pKa values between 9.6 and 10.4 [7], it was not expected that a total of 10% organic modifier would have such a marked effect on pKa. The relationship between solute retention, solute pKa and mobile phase pH is being examined further. For practical purposes, this study showed that the k′ range could be varied between 0.5–2.0 at pH 5.4 and from 2–12 at pH 7.2. At a buffer pH of 7.2 and a constant 5% v/v acetonitrile content, the percentage of methanol in the mobile phase was increased stepwise up to 15% v/v. Solute log k′ values decreased linearly to a range of 0.5–3.0, without producing any selectivity changes. Buffer molarity also influences solute retention and was studied over the range of 0.005–0.05 M. Retention was reduced for all solutes with increase in buffer concentration. The range of 0.025–0.050 M was preferred because it would routinely produce more reproducible k′

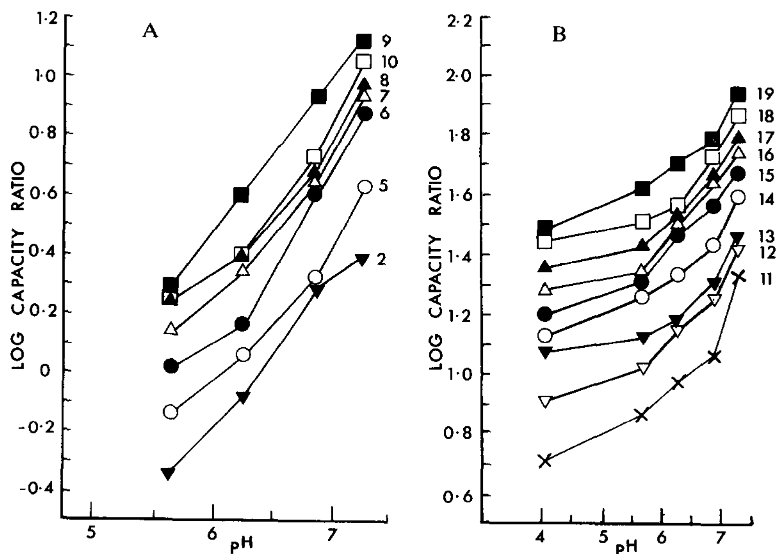

Figure 1
Influence of mobile phase pH on solute retention.
A. Chromatographic conditions for Group A solutes: 5-μm Spherisorb-CN column (50 × 4.6 mm i.d.) with 0.05 M phosphate buffer–methanol–acetonitrile (90:5:5, v/v/v) at 30°C. Injection volume, 10 μl, flowrate 1 ml min^{-1} monitored at 205 nm with 0.04 a.u.f.s. Solute code for all figures, with aqueous pKa values in brackets, where known: 1 = *p*-hydroxynorephedrine, 2 = *p*-hydroxyamphetamine (6.8), 3 = 2-phenylethylamine, 4 = *p*-hydroxymethylamphetamine, 5 = dexamphetamine (9.9), 6 = phenmetrazine (8.4), 7 = methylamphetamine (10.1), 8 = mephenterine (10.4), 9 = phendimetrazine (7.6), 10 = chlorphentermine (9.6). B. Chromatographic conditions for Group B solutes: Mobile phase, 0.005 M phosphate buffer-propan-2-ol-acetonitrile (65:17.5:17.5, v/v/v), other conditions as Fig. 1A. Solute code for all figures, with aqueous pKa values in brackets, where known: 11 = pethidine (8.7), 12 = pipradol (9.7), 13 = benzphetamine (6.6), 14 = fentanyl (7.3), 15 = normethadone (9.2), 16 = methadone (8.3), 17 = norpipanone, 18 = piritramide (8.5), 19 = dipipanone (8.5).

values due to the relatively small decrease in these values in this range. It is probable that phosphate ions form ion-pairs with protonated solutes, especially since at pH 7.2 HPO_4^{2-} ions, rather than $H_2PO_4^-$ ions, predominate. The uncertainty surrounding pKa values in the presence of organic modifier makes the estimation of this effect difficult, although the results are of practical value. A chromatogram of the ten compounds in Group A optimised for pH, buffer and organic solvents shows good resolution and sensitivity (6–12 ng each solute, on-column) within an analytical time of 8 min, Fig. 2A.

A similar optimization study was carried out for the Group B solutes. Figure 1B shows the same influence of mobile phase pH on solute retention as demonstrated in Fig. 1A. The pH range was extended to 4.0 using 0.005 M KH_2PO_4 solution adjusted to pH by the addition of orthophosphoric acid, and most solutes were eluted closer to their minimum k′ values, i.e. the values expected when mobile phase pH is less than solute pKa −2. At this pH, protonation of the amines should be complete. Since resolution was good at pH 4.0, this value was selected for further studies. The influence of buffer molarity on solute retention, showed the same relationship as for the Group A compounds. It is interesting to note that for six solutes in Group A, retentions at 0.005 M were in the range log k′ 1.0–1.4 and fell to log k′ 0.4–0.8 at 0.05 M, whilst the seven solutes in Group B having a

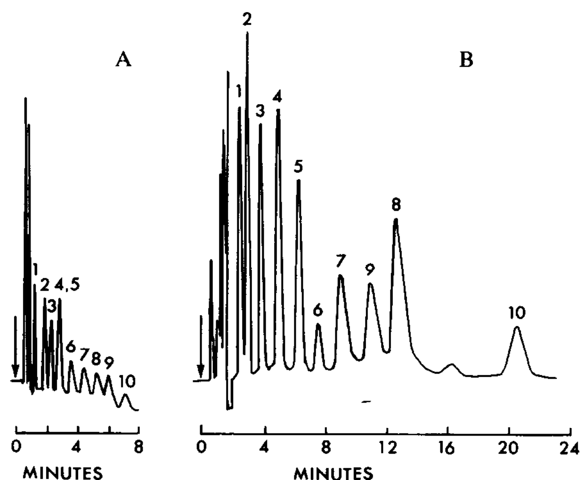

Figure 2
Chromatograms of Group A solutes.
A. 5-μm CPS-Hypersil column (50 × 4.6 mm i.d.) with 0.025 M phosphate buffer (pH 7.2)–methanol–acetonitrile (90:5:5, v/v/v) at 30°C, 1 ml min^{-1} flowrate monitored at 205 nm, 0.04 a.u.f.s. Injection volume (10 μl) contained 6–12 ng of each solute. Solute code as Fig. 1A. B. 5-μm SAS-Hypersil column (100 × 4.6 mm i.d.) used under conditions as in Fig. 2A, but using 0.02 M phosphate buffer at pH 5.4 and monitored at 0.02 a.u.f.s. Injection volume (20 μl) contained 25 ng of each solute. Solute code as Fig. 1A.

log k′ range of 1.05–1.48 at 0.005 M similarly fell to log k′ 0.4–0.8 at 0.05 M buffer. Considering the mobile phase pH was 7.2 for Group A and 4.0 for Group B, this suggests that ion-pair formation between protonated solute and phosphate anions cannot be the main factor in the effect that buffer has upon solute retention. The solute pKa range in Group A (6.8–10.4) is very similar to the seven solutes considered in Group B (6.6–9.2).

Considering the larger volumes of organic modifier required to achieve small k′ values for Group B compounds, the retentions obtained with propan-2-ol, acetonitrile and tetrahydrofuran were compared. For a mobile phase at pH 4.0 with 0.005 M KH_2PO_4, 35% v/v of any one of these solvents produced very similar retentions in the k′ range of 4–16, with few selectivity changes. When mixed in equal volumes propan-2-ol and acetonitrile reduced the k′ range to 4–10, whilst propan-2-ol and THF gave 4–15. A chromatogram of Group B compounds under conditions selected for minimum, reproducible k′ values is given in Fig. 3A, and shows good sensitivity (12 ng each solute, on-column).

Trimethylsilyl-bonded silica

This material was considered to be suitable for the rapid chromatography of the above solutes because it has a low carbon loading, the value for SAS-Hypersil for example, is 2.6%. A 100 × 4.6 mm i.d. column was packed and its performance examined using mobile phase compositions selected from the above study. The longer column was chosen to provide a greater column efficiency (*N*) and for Group A compounds the mobile phase pH was reduced from 7.2 (Fig. 2A) to 5.4 to reduce solute retention times. Figure 2B shows that good resolution was achieved for all compounds, although the time of analysis had increased to 22 min. Group B compounds were similarly chromato-

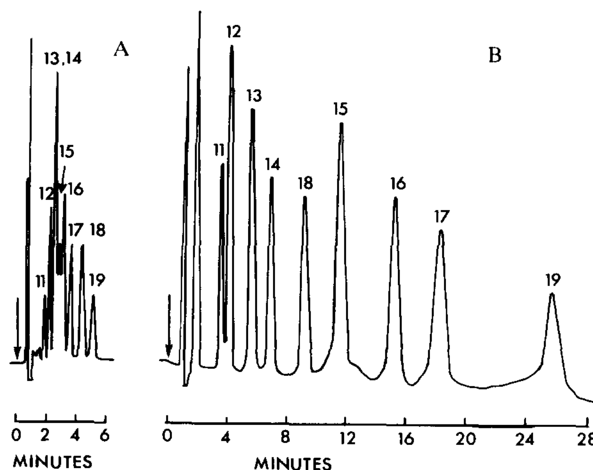

Figure 3
Chromatograms of Group B solutes.
A. Conditions as in Fig. 2A but with a mobile phase of 0.02 M phosphate buffer (pH 4.0)-propan-2-ol-acetonitrile (65:17.5:17.5, v/v/v). Injection volume (10 μl) contained 12 ng of each solute. Solute code as in Fig. 1B. B. Conditions as in Fig. 2B but using a 5-μm SAS Hypersil column (100 × 2.1 mm i.d.) with the 0.02 M phosphate buffer at pH 3.1. Injection volume (10 μl) contained 10 ng of each solute.

graphed using conditions similar to Fig. 3A, but with the pH reduced from 4.0 to 3.1. Good baseline resolution was obtained between all compounds, in an analysis time of 28 min. All solutes were readily detected at 25 ng on-column, but to reduce this level further, a 2 mm i.d. column was packed and chromatographed under identical conditions except for the flowrate, which was reduced to 0.5 ml min^{-1}. Figure 3B shows the good sensitivity obtained for 10 ng of each solute, on-column. Column efficiency (N) ranged from 860 (peak 12) to 3880 (peak 19).

Quantitative aspects

The suitability of the above procedures for quantitative determinations was examined in a number of ways. Using a 100 × 4.6 mm i.d. SAS-Hypersil column under conditions described in Fig. 2B, 5-point calibrations were made for a mixture containing dexamphetamine, methylamphetamine and three metabolites, namely *p*-hydroxynorephedrine, *p*-hydroxyamphetamine and *p*-hydroxymethylamphetamine. Linear relationships between solute concentrations and peak areas were obtained over the concentration range 10–100 μg ml^{-1} with correlation coefficients in the range of 0.998–0.999 and intercepts not significantly different from zero. Over the concentration range 2–20 μg ml^{-1} correlation coefficients were between 0.991 and 0.997, with small negative intercepts for three solutes. Using a 50 × 4.6 mm i.d. Spherisorb-CN column under conditions described in Fig. 2A, 6-point calibrations were made for a mixture of dexamphetamine, phenmetrazine, methylamphetamine and mephenthermine in mobile phase, over the concentration range 0.25–5 μg ml^{-1}. Linear relationships were obtained between peak height and concentration of solute for duplicate injections, with intercepts not significantly different from zero. Correlation coefficients for each calibration were >0.999 and relative standard deviations (RSD) for the slopes were

±0.32, 0.94, 1.9 and 0.41%, respectively. Identical calibrations were made by spiking plasma with the same stock calibration solutions, followed by their extraction as described in reference [3]. Linear relationships were obtained as before, with intercepts not significant; correlation coefficients were 0.996, 0.999, 0.977 and 0.999, respectively; RSD (slopes) were ±3.2, 1.4, 7.5, and 0.18% respectively. The percentage recovery of solutes from plasma, calculated as the spike calibration slope/control calibration slope, ×100 was found to be 80.8, 86.3, 81.1 and 98.7%, respectively. All these calibrations had been made without the benefit of an internal standard in order to test the quantitative behaviour of each solute. The calibrations show this to be satisfactory, permitting any one solute to be used as an internal standard for the other solutes.

Recommended procedure

Both column materials have been shown to be suitable for the qualitative and quantitative analysis of all the solutes examined in this study. However, cyanopropyl–silica was preferred because it would probably be more robust for routine use due to its higher carbon content and the mild range of pH (4.0–7.2) required for the mobile phase. A trimethyl–silica material routinely used at pH 3.1 would probably not have a long column life. In order to make the method more readily adopted by other workers, a commercially available 100 × 2 mm i.d. cartridge column containing 5-μm cyanopropyl–silica was tested for Group B compounds. The column temperature was raised to 40°C in order to reduce the organic modifier content from 35 to 24%, thus reducing the background absorbance at 205 nm. Figure 4 shows that good resolution was obtained with similar capacity factors (1.5–9.0) and column efficiency (N = 900–1350) to those in

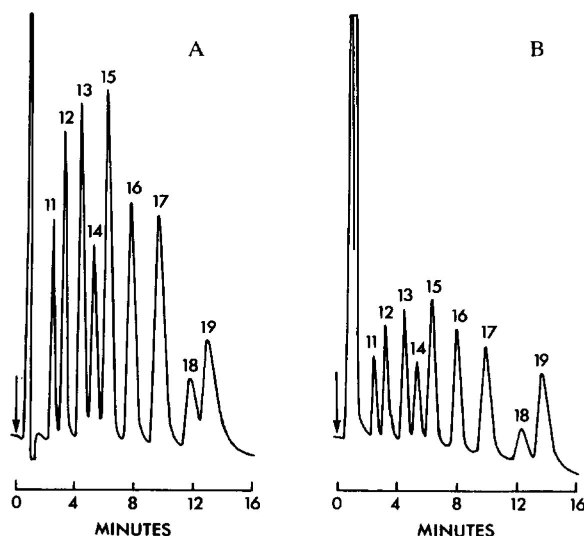

Figure 4
Chromatography of Group B solutes using a commercially available 2 mm i.d. cyanopropyl–silica column. A. 5-μm Spheri-Cyano cartridge column (100 × 2.1 mm i.d.), with a mobile phase of 0.025 M phosphate buffer–propan-2-ol–acetonitrile (76:12:12, v/v/v) at 40°C. Injection volume (10 μl) contained 80 ng of each solute, flowrate was 0.5 ml min^{-1}, monitored at 205 nm with 0.04 a.u.f.s. (attenuation = 4). B. As for Fig. 4A, but the injection volume (10 μl) contained 5 ng of each solute, at 0.01 a.u.f.s. (attenuation = 1).

Fig. 3A, although there was a slight tendency for some peak asymmetry (AS = 1.0–1.3). Six-point calibrations were made for a mixture of pethidine, benzphetamine, normethadone, methadone and dipipanone over the concentration range 0.7–18 $\mu g\ ml^{-1}$ using norpipanone as internal standard. Linear relationships between peak area ratios and solute concentration were obtained with correlation coefficients >0.998 and intercepts not significant. The precision (n = 7) for each solute solution gave RSDs of ±1.45, 1.27, 0.87, 0.55 and 4.7%, respectively, at the 9 $\mu g\ ml^{-1}$ level. Figure 4B shows good detection of solutes even at 0.5 $\mu g\ ml^{-1}$ (5 ng on-column).

Conclusions

Cyanopropyl-bonded silica when used under reversed-phase conditions produces short retention times with good resolution for 19 basic drugs of abuse, using predominantly aqueous mobile phases. This permits the determinations of these primary, secondary and tertiary amines at levels down to at least 10 ng on-column, at 205 nm, and 0.02 a.u.f.s. without the need to employ more complicated and expensive techniques such as post-column derivatization.

Acknowledgements — The authors wish to thank Dr R. Gill, Central Research Establishment, Home Office Forensic Science Service, Addermaston, U.K. for samples of all the drugs used in this study, and the following for additional supplies, Wellcome Foundation Ltd., Dartford for dipapanone and methadone, Janssen Pharmaceutical Ltd. for fentanyl and piritramide, and May and Baker Ltd., Dagenham for pethidine.

References

[1] R. Gill, M. D. Osselton, R. M. Smith and T. G. Hurdley, *J. Chromatogr.* **386**, 65–77 (1987).
[2] C. M. Selavka and I. S. Krull, *J. Liq. Chromatogr.* **10**, 345–375 (1987).
[3] B. M. Farrell and T. M. Jefferies, *J. Chromatogr.* **272**, 111–128 (1983).
[4] J. F. M. Kinkel and E. Tomlinson, *Int. J. Pharm.* **6**, 261–275 (1980).
[5] R. F. Rekker, *The Hydrophobic Fragmental Constant.* Elsevier Sci., New York (1977).
[6] T. L. Hafkenscheid and E. Tomlinson, *J. Chromatogr.* **292**, 305–317 (1984).
[7] *Clarke's Isolation and Identification of Drugs*, 2nd edn, (A. C. Moffat, Ed.). Pharmaceutical Press, London (1986).

[*Received for review 25 September 1987; revised manuscript received 23 October 1987*]