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APPLICATIONS OF COLUMN-SWITCHING TECHNIQUE IN BIOPHARMACEUTICAL ANALYSIS

I. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMITRIPTYLINE AND ITS METABOLITES IN HUMAN PLASMA

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SUMMARY

A new high-performance liquid chromatographic method for the determination of amitriptyline and its metabolites, nortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline, in plasma is described which uses direct injection and a column-switching valve. The method is based on the enrichment of drugs on a reversed-phase concentration column, packed with Corasil RP. The enriched drugs were then separated, using back-flush mode on a bonded-phase CN column using an isocratic acetonitrile–acetate buffer (60:40, v/v) mobile phase. The validation of the method showed excellent sensitivity, precision and reproducibility. The limit of detection, using a 250-μl direct injection of plasma, was between 5 and 10 ng/ml for each of the four drugs. The mean coefficient of variation for intra- and inter-assay was better than 5%. The method showed obvious advantages over conventional extraction procedures in terms of speed and ease of sample handling. The method has been successfully applied to the samples from patients receiving oral doses of amitriptyline.

INTRODUCTION

Tricyclic antidepressants, TCAs (Fig. 1), are widely used in the treatment of patients who are suffering from depression. The concentration of these drugs in plasma and the effect on depression symptoms is controversial [1] but it is very important that their therapeutic levels be monitored so that therapy can be made optimal. Several reports have shown that there are large individual variations in plasma concentrations among patients receiving the same dosage of these drugs [2–4].

Most existing methods for the determination of TCAs have been reviewed [5,6]. Gas chromatography (GC) using a variety of detection systems has been applied.

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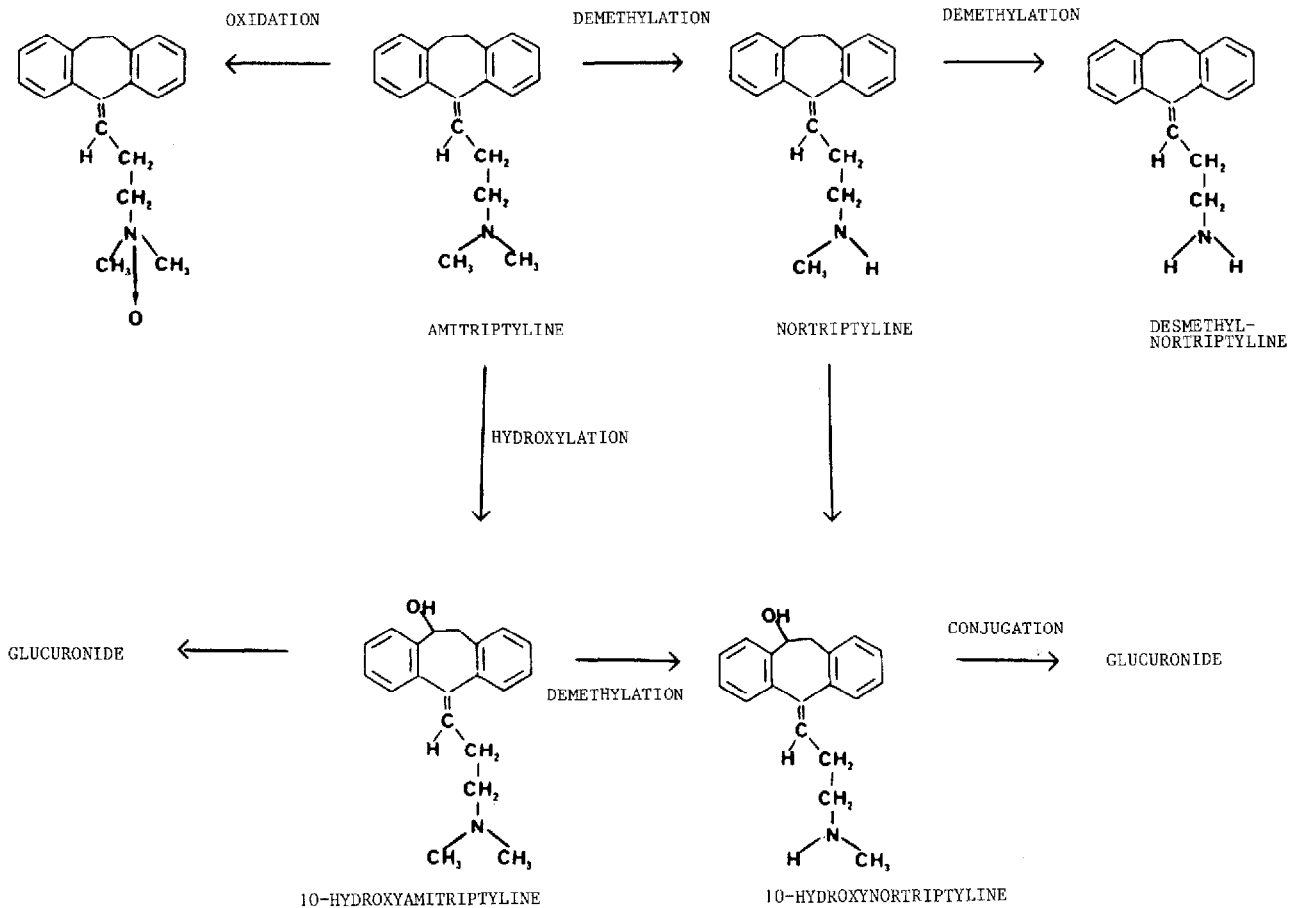

Fig. 1. Chemical structure of amitriptyline and its metabolic pathway.

These include flame-ionization detection (FID) [7–10], election-capture detection (ECD) [10–13], nitrogen-phosphorus detection (NPD) [14–16] and mass fragmentography [17]. Since 1975 there has been extensive development of high-performance liquid chromatography (HPLC) as a method of determination for plasma levels of tricyclics and currently rivals GC–NPD as the method of choice [18–26].

Common to all these methods is the need for the extraction of the drugs, which are lipophilic strong bases, from the biological medium at high pH. Although single-step extraction may be sufficient for some applications, usually a three-step extraction procedure is used with overall recovery of ca. 60–80% while the recovery for each individual step is around 90%.

Recently, sample clean-up using solid-phase extraction techniques has become popular. The approach, which overcomes the need for lengthy extraction techniques has been successfully applied to the TCAs [20,21,27,28]. Measurement of these drugs in plasma based on solid-phase extraction on disposable C_{18} bonded-phase columns yielded results in agreement with those obtained using a three-step extraction procedure [27,28].

This application involves the use of a switching valve which allows on-line sample loading and isolation of the analytes on a "concentration column", followed by rapid elution and direct analysis on the analytical column. The use of column-switching techniques has become a major area of interest and its application to drug analysis in biological samples has been the subject of a number of recent publications [29–34].

EXPERIMENTAL

Reagents

10-Hydroxynortriptyline, 10-hydroxyamitriptyline and desmethyldoxepin were obtained as a gift from the Laboratory of Clinical Pharmacology and Toxicology, Groot Ziekengasthuis, 's-Hertogenbosch, The Netherlands. Doxepin and protriptyline were obtained from Jervis Street Hospital (Dublin, Ireland) and the other members of the tricyclics were obtained as gifts from I.C.P. Dublin. Hexane, methanol, acetonitrile and isopropanol (all HPLC grade) were purchased from Fisons (Loughborough, U.K.). Ammonia solution, sodium hydroxide, sodium acetate and zinc sulphate were obtained from BDH (Poole, U.K.). Trichloroacetic acid was purchased from May and Baker (Dagenham, U.K.) and perchloric acid from Riedel-de-Haen (Hannover, F.R.G.).

For the preparation of the plasma samples, dried human plasma (from the Blood Transfusion Service Board, Dublin, Ireland) was dissolved in deionised water (obtained by the Milli-Q water purification system). The control plasma obtained was examined for the presence of endogenous components which might interfere with the tricyclic drugs in the assay system. The reconstituted plasma was stored frozen and used within two weeks of preparation.

Instrumentation

The HPLC system consisted of two Waters Assoc. (Milford, MA, U.S.A.) P-45 liquid chromatograph solvent delivery systems equipped with a Waters U6K

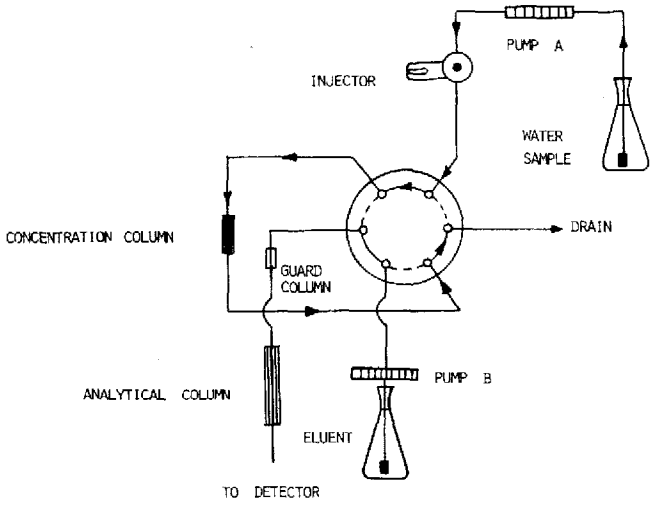

Fig. 2. Operation of column-switching valve for sample enrichment.

manual injector and fitted to a Shimadzu (Tokyo, Japan) SPD-6A variable-wavelength UV detector. The chromatograms were recorded on a Philips (Eindhoven, The Netherlands) PM 8251 single-pen recorder. A Rheodyne (CA, U.S.A.) 7000 six-port switching valve was used.

Chromatography

The instrument arrangement for the chromatography is shown in Fig. 2. The chromatographic conditions for the separation were as follows. Mobile phase pump A: water; concentration column (10×1.5 mm) dry-packed with Corasil (Waters Assoc.) RP C_{18} packing (37–50 μm) in our laboratory; wash time, 1.5 min; flow-rate, 0.8 ml/min. Mobile phase pump B: acetonitrile – 0.05 *M* acetate buffer (60:40, v/v) pH 7; stationary phase, Techsphere (HPLC Technology, Macclesfield, U.K.) 3CN (10 cm×4 mm); flow-rate, 0.9 ml/min; recorder chart-speed, 0.5 cm/min; detection wavelength, 215 nm; injection volume, 250 μl of plasma. Under the described chromatographic conditions the mean retention times (Fig. 3) were as follows: 10-hydroxynortriptyline, 6.0 min; 10-hydroxyamitriptyline, 7.5 min; nortriptyline, 10.0 min; amitriptyline, 12.5 min.

Preparation of standards

Amitriptyline hydrochloride (11.32 mg), nortriptyline hydrochloride (11.39 mg), 10-hydroxyamitriptyline hydrochloride (11.25 mg) and 10-hydroxynortriptyline hydrochloride (11.31 mg) were weighed and dissolved in 100 ml of methanol to yield a stock solution of 100 μg/ml amitriptyline, nortriptyline, 10-hydroxyamitriptyline and 10-hydroxynortriptyline. This stock solution was then diluted with water–methanol (1:1) to yield working standards ranging from 0.2 to 6 μg/ml.

Desmethyldoxepin (11.4 mg) was weighed and dissolved in 100 ml of methanol to yield a solution of 100 μg/ml. This was then diluted with a methanol–water

mixture to yield a solution of 10 μg/ml desmethyldoxepin. Spiked plasma standards ranging from 10 to 300 ng/ml of the drugs in plasma were then prepared in each assay day by spiking 1 ml of plasma with 50 μl of the working standards and 50 μl of the internal standards. Patient and blank plasma samples were spiked with 50 μl of methanol–water mixture to compensate for any changes in the composition of plasma.

Column-switching procedure

Fig. 2 shows a simple six-port switching valve. The spiked plasma sample is injected through the injector port and washed by the water from pump A onto the concentration column. The drugs are held on the concentration column while the other components in plasma are eluted to the drain. Meanwhile the eluent from pump B is passing through the analytical column and out to waste. On switching the valve, the eluent from pump B elutes the drugs, which have been held on the concentration column, in back-flush mode onto the analytical column where they are separated.

RESULTS AND DISCUSSION

In using the column-switching valve the following variations in sample handling were studied: sample pretreatment; concentration column packing; wash time; flow rates; and analytical column.

Sample pretreatment

Protein precipitation prior to injection was tried using (i) acid precipitation using perchloric acid or trichloroacetic acid and (ii) base precipitation of proteins with sodium hydroxide and zinc sulphate. The proteins were precipitated by addition of the reagent to plasma in tubes which were centrifuged for 15 min; the supernatant was injected for analysis. Direct injection with no sample pretreatment was also tried. For all concentrations, acid precipitation gave the lowest recovery, probably due to ionization of the basic tricyclic drugs in the acid solution which were then eluted with the water from pump A and not retained on the concentration column. Base precipitation also gave poor recovery probably due to co-precipitation of the drugs with plasma. The strong acids and base also dissolved the packing in the concentration column. Over the range of packings tried therefore, best recovery, sensitivity and reproducibility was achieved using a 250-μl direct injection of plasma. However, as it has been outlined in other publications [29], direct injection is not without its problems. These problems include (i) deterioration in the performance of the concentration column due to precipitation of plasma proteins when they come into contact with the solvents from pump B, i.e. the mobile phase, and (ii) build up of back-pressure due to accumulation of particulate matter in plasma which can be minimized by using the backflush mode.

Concentration column packings

The following packings were tried in the concentration column: LiChrosorb RP C_{18}, 10 μm; Hypersil phenyl, 10 μm; Vydak cyano, 30 μm; Corasil RP C_{18}, 37-

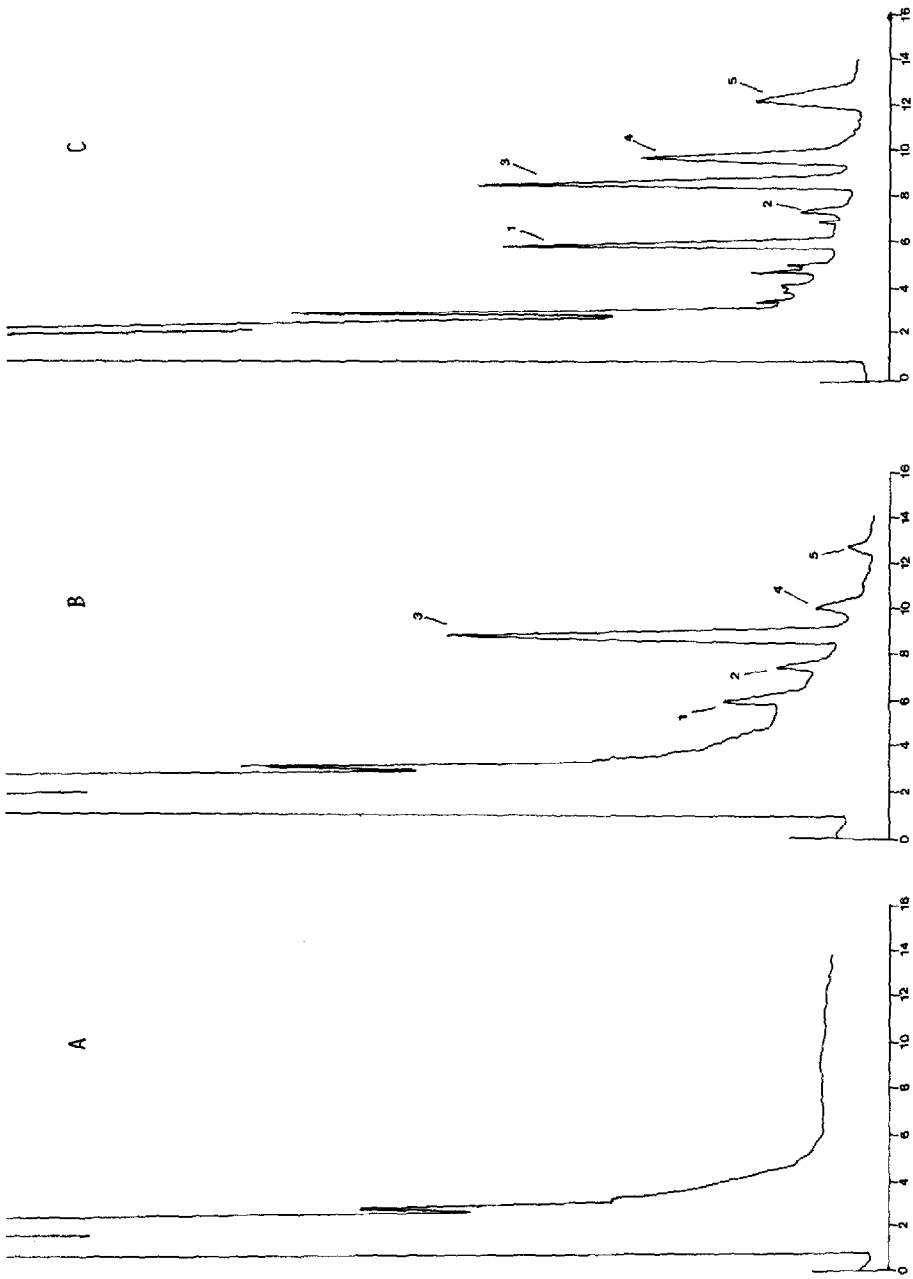

Fig. 3. Chromatograms of (A) a small pooled human drug-free plasma, (B) the same plasma spiked with 10 ng/ml of each of the drugs and (C) plasma of a patient receiving a 50-mg oral dose of amitriptyline. Peaks: 1 = 10-hydroxynortriptyline; 2 = 10-hydroxyamitriptyline; 3 = desmethyldoxepin (internal standard); 4 = nortriptyline; 5 = amitriptyline.

50 μm. The main factor is the size of the packing particles. Packings with particle size 10 μm do not allow direct injection since the small-size particles act as an efficient filter and after one to two injections become clogged with particulate matter from plasma.

Using 10-μm packings, acid or base precipitation must be used and, as outlined above, these gave poor recovery. A 30-μm Vydak cyano packing allowed direct injection; however, the column capacity was only 150 μl of plasma. No enhancement in sensitivity was achieved by using larger injection volumes.

Corasil RP packing, 37–50 μm particle size, proved to be the best among the packings tried. Although injection volumes of greater than 250 μl of plasma provided enhanced sensitivity, it limited the number of injections per concentration column. An injection volume of 250 μl plasma allowed for at least fifty consecutive injections.

Wash times and flow-rates

Wash times were varied from 1 to 10 min and flow-rates from 0.5 to 1.5 ml/min. Variation in these factors did not affect the chromatograms obtained to a large extent, but the sharpest peaks and cleanest chromatograms were obtained with a wash time of 90 s and a flow-rate of 0.8 ml/min. Shorter wash times result in very large plasma peaks and longer wash times give rise to band broadening.

Other CN columns

Separation of the drug and its metabolites may also be achieved using other CN columns. A μBondapak CN column (300×3.9 mm) was tried but it was necessary to vary both the composition of the mobile phase from 60:40 to 70:30 acetonitrile–acetate buffer and also to vary the molarity of the buffer from 0.05 to 0.03 *M* in order to obtain separation. However, the peaks obtained were somewhat broadened and not as well resolved.

Calibration and calculation

Evaluation of the assay was carried out using four-point calibration standards in the concentration range 10–300 ng/ml of drugs in plasma. The slope and intercept of the calibration curves were obtained by linear regression of the peak-height ratios of drug/internal standard versus the concentration of the drug (internal standard method).

The internal standard (I.S.) used was desmethyldoxepin which had a suitable retention time (t_R=8.5 min). These calibration curves were then used to interpolate the concentrations of drugs in patient plasma from the measured peak-height ratios.

Limit of detection

Using a 250-μl direct injection of plasma, and under the procedural conditions outlined, the limit of detection for each of the four drugs was between 5 and 10 ng/ml in plasma. The variation in detection limit was due to day-to-day changes in operational conditions and detection system. The detection limit was taken as the amount of compound giving a signal-to-noise ratio greater than 3:1.

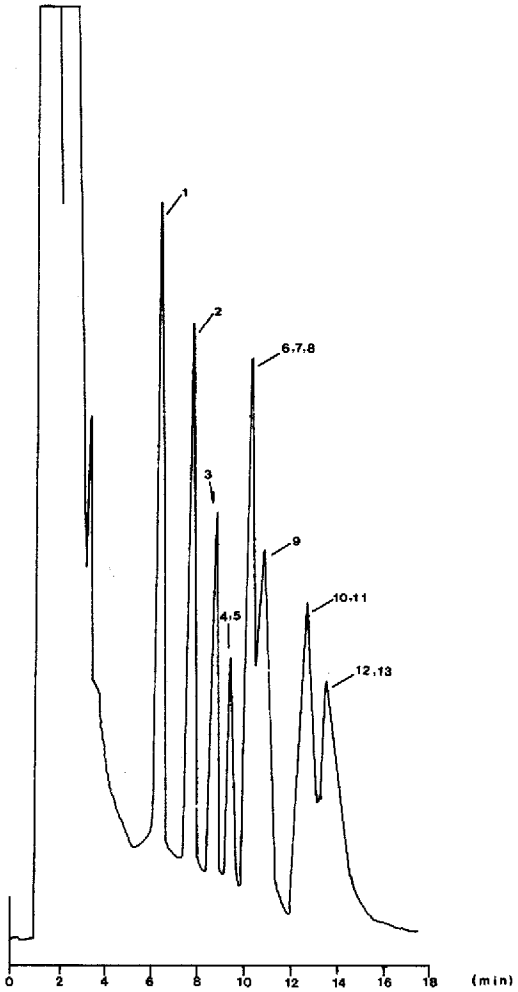

Fig. 4. Chromatogram of a small pooled human drug-free plasma spiked with tricyclic antidepressant drugs. Peaks: 1 = 10-hydroxynortriptyline; 2 = 10-hydroxyamitriptyline; 3 = desmethyldoxepin; 4 and 5 = protriptyline and desipramine; 6, 7 and 8 = trimipramine, cianoprimine and nortriptyline; 9 = doxepin; 10 and 11 = amitriptyline and imipramine; 12 and 13 = chlomiprimine and chloripramine.

Precision

Intra-assay variability was determined at four concentrations in quadruplicate at levels of 10, 50, 150 and 300 ng/ml of each drug in plasma. Inter-assay variability was determined singly and at the same four concentrations in four replicate runs. The precision of the method (mean coefficient of variation) for the values of the recovered determinate standards when calculated as "unknowns" against the linear regression lines for intra-assay and inter-assay were, respectively, 3.3 and 3.8% for amitriptyline, 4.5 and 3.3% for nortriptyline, 3.8 and 4.9% for 10-hydroxyamitriptyline and 3.1 and 5.0% for 10-hydroxynortriptyline.

TABLE I

PLASMA CONCENTRATIONS OF AMITRIPTYLINE AND ITS METABOLITES IN HUMAN SAMPLES

See text for dosage. Blood samples were taken 10 h after administration. N.D. = not detectable (below limit of detection).

Patient	Compound	Concentration (ng/ml)			
		Day 1	Day 14	Day 28	Day 42
1	Amitriptyline	31.9	54.5	106.7	74.0
	Nortriptyline	22.9	42.2	86.1	61.6
	10-Hydroxyamitriptyline	N.D.	N.D.	6.3	N.D.
	10-Hydroxynortriptyline	33.2	60.1	110.4	82.6
2	Amitriptyline	N.D.	36.7	50.1	47.2
	Nortriptyline	N.D.	55.7	47.3	56.7
	10-Hydroxyamitriptyline	N.D.	10.6	5.9	9.6
	10-Hydroxynortriptyline	N.D.	84.5	56.7	64.3
3	Amitriptyline	N.D.	N.D.	N.D.	N.D.
	Nortriptyline	N.D.	N.D.	N.D.	N.D.
	10-Hydroxyamitriptyline	N.D.	N.D.	N.D.	N.D.
	10-Hydroxynortriptyline	N.D.	N.D.	N.D.	N.D.
4	Amitriptyline	24.6	34.2	36.3	50.5
	Nortriptyline	7.2	30.4	50.4	49.2
	10-Hydroxyamitriptyline	N.D.	N.D.	7.4	6.9
	10-Hydroxynortriptyline	22.4	115.9	114.5	138.7

Linearity

Measures of linearity as defined by the correlation coefficient of the regression lines of intra-assays for all drugs were better than 0.999 and the intercepts did not differ greatly from the origin.

Recovery

The overall recovery was calculated in two different ways. First by comparing the peak heights of a series of spiked plasma samples after they had been taken through the entire procedure with a series of reference standards. Secondly, by comparing the slopes of the regression lines obtained from the two sets used in the first procedure. Using these methods in the concentration range 10–300 ng/ml, the mean overall recoveries were 90.74, 92.63, 83.85 and 90.50% for amitriptyline, nortriptyline, 10-hydroxynortriptyline and 10-hydroxyamitriptyline, respectively.

Interference study

Eight other members of the tricyclic drugs were tested for possible interferences with the measured substances. As can be seen from the chromatograms in Fig. 4, no other member of the tricyclics studied interfered with the determination of 10-hydroxyamitriptyline or 10-hydroxynortriptyline. Imipramine interfered

with amitriptyline and likewise cianopramine and trimipramine interfered with nortriptyline, but it is unlikely that any of these combinations would be administered simultaneously. Tranylcypromine which was given in combination with amitriptyline to some patients did not interfere and eluted with the plasma peak.

Plasma levels

The described method has been successfully applied to the measurement of amitriptyline, nortriptyline, 10-hydroxyamitriptyline and 10-hydroxynortriptyline in patients with neurotic and endogenous depression receiving oral doses of placebo, 50, 75, 100 or 150 mg of amitriptyline alone or in combination with tranylcypromine (Table I). The exact amount of dosage could not be revealed to us at this stage and, therefore, results in Table I are only indicative of the capability of the described method for the analysis of patient samples receiving the above doses of amitriptyline (Fig. 3C).

CONCLUSION

For routine analysis of the tricyclic drugs in plasma, a new method of analysis based on direct-injection column-switching technique was developed. The advantages of this method over conventional extraction methods is that it was less time-consuming, needed a smaller volume of plasma and gave better recovery. It has a comparable precision limit with conventional extraction methods. As compared to off-line solid-phase extraction [20,21] methods, it is less time-consuming and more selective, i.e. no unidentified peaks were observed with less running cost. The disadvantages are that the concentration column had to be changed after every fifty injections and it needed more elaborate instrumentation, i.e. two pumps and a column-switching valve.

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REFERENCES

1 B. Davis, Clin. Pharmacol. Ther., 16(1974) 637.
2 J. Fekete, P. Del Castilho and J.C. Kraak, J. Chromatogr., 204(1981) 319.
3 W. Hammer, S. Martens and F. Sjoquist, Clin. Pharmacol. Ther., 10(1969) 44.
4 A.H. Glassman and J.M. Perel, Clin. Pharmacol. Ther., 16(1974) 198.
5 B.A. Scoggins, K.P. Maguire, T.R. Norman and G.D. Burrows, Clin. Chem., 26(1980) 5.
6 T.R. Norman and K.P. Maguire, J. Chromatogr., 340(1985) 173.
7 J.E. O'Brien and O.N. Hinsvark, J. Pharm. Sci., 65(1976) 1068.
8 G.L. Corona and B. Bonferoni, J. Chromatogr., 124(1976) 401.
9 T.R. Norman, K.P. Maguire and G.D. Burrows, J. Chromatogr., 134(1977) 524.
10 T.R. Norman, G.D. Burrows, B.M. Davies and J.M.E. Wurm, Br. J. Clin. Pharmacol., 8(1979) 169.
11 P. Hartvig and B. Näslund, J. Chromatogr., 133(1977) 367.
12 O. Borga and M. Garle, J. Chromatogr., 68(1972) 167.

13 P. Hartvig, W. Handl, J. Vessman and C.M. Svahn, Anal. Chem., 48(1976) 390.
14 R.N. Gupta, G. Molnar, R.E. Hill and M.L. Gupta, Clin. Biochem., 9(1976) 247.
15 A. Jorgensen, Acta Pharmacol. Toxicol., 36(1975) 79.
16 D.N. Bailey and P.I. Jatlow, Clin. Chem., 22(1976) 777.
17 C.G. Hammar, B. Alexanderson, B. Holmstedt and F. Sjoquist, Clin. Pharmacol. Ther., 12(1971) 496.
18 S.M. Johnson, C. Chan, S. Cheng, J.L. Shimek, G. Nygard and J.K. Wihba Khall, J. Pharm. Sci., 71(1982) 1027.
19 M.W. Dong and J.L. Di Cesare, J. Chromatogr. Sci., 20(1982) 330.
20 F.A. Beierle and R.W. Hubbard, Ther. Drug Monit., 5(1983) 279.
21 A. Kobayashi, S. Sugita and K. Nakazawa, J. Chromatogr., 336(1984) 410.
22 P.M. Edelbroek, E.J. de Haas and F.A. de Wolff, Clin. Chem., 28(1982) 2143.
23 P.P. Rop, A. Viala and A. Durand, J. Chromatogr., 338(1985) 171.
24 T. Visser, M.C.J.M. Oostelbos and P.J.M.M. Toll, J. Chromatogr., 309(1984) 81.
25 T.A. Sutfin, R. D'Ambrosio and W.J. Justio, Clin. Chem., 30(1984) 471.
26 F.L. Vandemark, R.F. Adams and G.J. Schmidt, Clin. Chem., 24(1978) 87.
27 N. Narasimhachari, J. Chromatogr., 225(1981) 189.
28 T.C. Kwong, R. Martinez and J.M. Keller, Clin. Chim. Acta, 126(1982) 203.
29 R.D. McDowall, G.S. Murkitt and J.A. Walford, J. Chromatogr., 317(1984) 475.
30 M.W.F. Nielen, R.C.A. Koordes, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 330(1985) 113.
31 W. Roth and K. Beschke, J. Pharm. Biomed. Anal., 2(1984) 289.
32 W. Voelter, K. Zech, P. Arnold and G. Ludwig, J. Chromatogr., 199(1980) 345.
33 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, J. Chromatogr., 222(1981) 13.
34 W. Voelter, T. Kronbach, K. Zech and R. Huber, J. Chromatogr., 239(1982) 475.