Journal of Pharmaceutical & Biomedical Analysis,
Vol. 1, No. 1, pp. 73–82, 1983
Printed in Great Britain

0731–7085/83/010073–10$03.00/0
Pergamon Press Ltd.

The analysis of tricyclic antidepressant drugs at therapeutic blood levels by reversed-phase high-performance liquid chromatography using pairing and organic counter-ions

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Abstract: A serum assay method incorporating two internal standards suitable for routine clinical analysis of tricyclic antidepressant drugs at the therapeutic level is described. The method involves extraction and chromatography using a reversed-phase high-performance liquid chromatography system incorporating pairing and organic counter-ions. The quantitative characteristics of the method are reported and results are compared with consensus values for four commonly encountered drugs, amitriptyline, nortriptyline, imipramine and desipramine. The method is shown to be applicable to more recently developed antidepressants such as nomifensine and maprotiline and the flexibility of the chromatography is discussed. Sample chromatograms obtained from patient samples in the presence and absence of potentially interfering drugs and metabolites are discussed.

Keywords: *Ion-pairing HPLC: tricyclic antidepressants; clinical application; organic counter-ion.*

Introduction

The measurement of drug concentration in blood is becoming increasingly important as an aid to effective control of therapy. Because of this, a need exists for analytical methods which will allow specific, accurate and reproducible clinical analyses of drugs. Tricyclic antidepressant drugs constitute an important group for therapeutic drug monitoring. Although it has been claimed that there is no significant correlation between plasma antidepressant concentration and clinical response [1], it has also been suggested that, in common with other drugs, tricyclic antidepressants are most effective within defined therapeutic ranges [2–5]. The most widely used tricyclic antidepressants are amitriptyline and imipramine. These are known to be transformed *in vivo* to the clinically active metabolites nortriptyline and desipramine respectively [6].

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Other drugs in this class are known or suspected also to form active metabolites, often by demethylation. In practice it is found that patients may respond better to some antidepressant drugs than to others, for reasons that are still uncertain. Many so-called 'new generation' antidepressants have been introduced, such as, for example, maprotiline and nomifensine. While exhibiting generally similar antidepressant effects, these drugs differ markedly from conventional antidepressant drugs in chemical structure. They can thus be used as alternative therapy in those cases where the earlier, conventional drugs are considered unsuitable.

Many analytical techniques have been applied for the assay of these drugs in body fluids. Assays depending on fluorescence [7, 8] and radioimmunoassay [9], while having adequate sensitivity for the low plasma levels encountered, have dubious specificity. Although gas–liquid chromatography with electron capture detection [10] or nitrogen-specific detection [11, 12] has sufficient sensitivity and specificity, derivatization is required before chromatography.

High-performance liquid chromatography (HPLC) as a method of analysis is being increasingly exploited for the determination of drugs in serum. Several methods for the separation of tricyclic antidepressants have appeared in the literature using normal [13] and reversed-phase [14] techniques. It is known that such basic nitrogenous compounds are among the most difficult drug types to separate successfully by chromatography. It has been reported that the addition of fairly low molecular weight organic amines at low concentration to the eluent in reversed-phase ion-pair chromatography improves peak symmetry and thus resolution and sensitivity [14–19]. Different hypotheses have been advanced to explain this effect.

Attempts in our laboratories to separate the commonly used tricyclic antidepressants for clinical assay proved unsatisfactory, using any of the reversed-phase chromatographic methods published in the literature. Most of these depend upon the addition of higher alcohols to modify the octadecylsilane surface, or upon critical adjustment of pH and ionic strength. It was therefore found difficult to reproduce the chromatography obtained by other workers.

Recently, many drugs of the tricyclic antidepressant group have been used as solutes for a systematic examination of the effect on resolution of (i) concentration of pairing ion, (ii) concentration and type of added organic amine [20]. It is the purpose of the present investigation to report the quantitative clinical application of the separations achieved by this system of ion-pair chromatography, using representative members of this group, viz. amitriptyline, nortryptiline, imipramine and desipramine, and to compare the results obtained with consensus values obtained for plasma samples issued by a national quality control scheme.

In addition, it is intended to show that the method is equally applicable to other tricyclic antidepressants, including the recently introduced drugs maprotiline and nomifensine. A discussion of the pertinent factors affecting the separative and quantitative performance of the method is included, so that conditions can be adjusted as required to achieve separations desired by workers using different apparatus and stationary phases.

Experimental

Apparatus

Chromatography was carried out using a variety of equipment including Altex (M110A) and Waters Associates (M6OOOA) pumps. Detection was by Cecil

(CE20212), Pye Unicam (LC-3) or Altex (160) ultraviolet absorbance detectors. Manual injection using a Rheodyne 7125 valve fitted with a 100 μl loop was employed. Columns used were 100 × 4.6 mm i.d. (or 70 × 4.6 mm i.d.), slurry-packed [21] with 5 μm ODS-Hypersil (Shandon Southern Products). Retention time data were obtained directly from chromatograms as recorded on a potentiometric recorder. Peak height was used for quantitation.

Materials

Amitryptyline (Roche), nortriptyline (Lilly), imipramine, desipramine, maprotiline and clomipramine (Geigy), mianserine (Bencard), nomifensine (Hoechst), protriptyline (MSD), trimipramine (May & Baker), butriptyline (Ayerst), doxepin (Pfizer) and dothiepin (Boots) were all kindly donated by their manufacturers and used as their hydrochlorides. Sodium laurylsulphate (SLS) (Fisons), tetrabutylammonium bromide (TBA) (Aldrich) and acetonitrile (Rathburn or Fisons) were used as received. Water was glass-distilled and all other reagents were of AnalaR or equivalent grade.

Procedure

Extraction. To 2.00 ml of serum, 100 μl of the appropriate internal standards in 1 M hydrochloric acid was added. Details of the nature and final concentrations of internal standards are given in Table 1. After adding 0.5 ml of 1 M sodium hydroxide and 5 ml of diethylether and shaking, the organic layer was removed and saved. The extraction was repeated with a further 4 ml of ether and the combined organic extracts were acidified by the addition of 100 μl of 0.5 M sulphuric acid. After vigorous shaking and centrifuging, the organic layer was removed and discarded and the total aqueous extract was injected into the chromatograph. Details of the efficiency of extraction are given below.

Table 1
Internal standards and concentrations for the two detection wavelengths used in the analysis of tricyclic antidepressants

Detection wavelength (nm)	Drug	Internal standards	Concentration (ng/ml)
254	Amitriptyline Nortriptyline	Doxepin	100
	Imipramine Desipramine	Nomifensine	750
	Clomipramine		
230	Maprotiline	Doxepin	50
	Nomifensine	Butriptyline	500

Chromatography. A mobile phase of acetonitrile–aqueous 10 mM disodium hydrogen phosphate (pH 2) containing 80 mM SLS and 5 mM TBA (50:50 v/v) was employed. A flow rate of 2 ml/min and uv-detection at either 254 nm or 230 nm were employed. Details of modifications to the chromatography are given below.

Results and Discussion

Separation

Figure 1 shows representative chromatograms of the separations obtained for some of the drugs studied quantitatively. The mobile phase was selected on the basis of previously published data on the general retention behaviour of such compounds as a function of pairing ion in the presence and absence of organic counter ion [20], to which data on butriptyline had been added. This drug was required as an internal standard. This eluent was chosen to obtain resolution between the drug/metabolite pairs: imipramine/desipramine and amitryptyline/nortriptyline. Resolution between other antidepressants and their metabolites can be achieved by modifying the mobile phase as regards pairing ion concentration, or by omitting the organic counter-ion. Furthermore, such eluent modifications may be required to overcome interference due to concomitant drug therapy. The chromatographic system described, together with previously published data, appears to be sufficiently flexible to enable desired separations to be achieved relatively quickly.

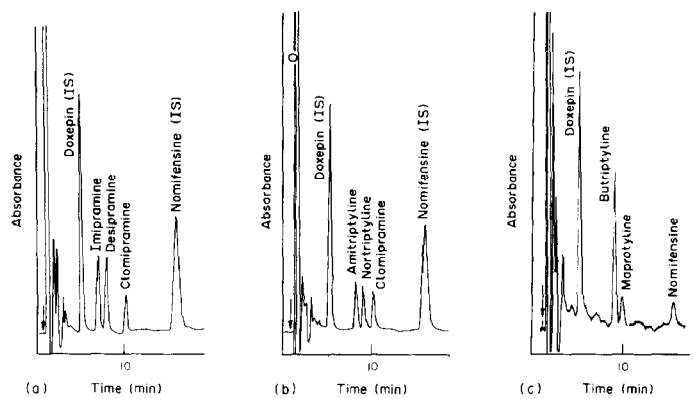

Figure 1
Representative chromatograms showing the separation obtained for tricyclic antidepressant drugs after extraction from bovine serum, including two internal standards. Chromatographic conditions: see text. (a) and (b): detection at 254 nm, 0.01 a.u.f.s.; (c) detection at 230 nm, 0.005 a.u.f.s. Serum drug concentration 50 ng/ml.

Quantitation

A detection of wavelength of 254 nm gave the desired sensitivity for the clinical assay of amitriptyline, nortripytyline, imipramine, desipramine and clomipramine. For the compounds nomifensine and maprotiline, 230 nm gave lower detection limits. As a check on possible metabolite or other drug interference, two internal standards were used for each drug assay [22]. The choice of internal standards and their respective concentrations depended upon the wavelength of detection selected for the drugs to be measured. Details are given in Table 1.

Calibration

Calibration curves in bovine serum were prepared over a concentration range of 50–500 ng/ml for the compounds indicated above, which represent examples of established and 'second generation' antidepressants. The regression data and correlation coefficients are listed in Table 2. In all cases good linearity is observed so that in the cases

shown either internal standard is adequate for any given analysis. Moreover, at the stated concentrations (Table 1), the peak height ratio of doxepin to nomifensine at 254 nm was found to be 2.19 (S.D. = 0.17, n = 28), while that of doxepin to butriptyline at 230 nm was 1.52 (S.D. = 0.08, n = 18). These data can provide an indication of interference by any drugs or metabolites, which coelute with one or both of the internal standards.

Table 2
Regression data for the calibration of tricyclic antidepressant drugs, using the respective internal standards

Drug*	Internal standards	Wavelength (nm)	Gradient $\times 10^3$	S.D. of gradient $\times 10^4$	Intercept $\times 10^2$	S.D. of intercept $\times 10^4$	r†
Amitriptyline	Doxepin	254	6.94	(5.0)	−6.0	(1.3)	0.9997
	Nomifensine	254	13.6	(1.6)	9.37	(4.5)	0.9991
Nortriptyline	Doxepin	254	6.16	(5.0)	−1.73	(1.4)	0.9986
	Nomifensine	254	12.60	(0.9)	1.95	(2.6)	0.9996
Imipramine	Doxepin	254	11.40	(1.7)	−6.55	(4.7)	0.9987
	Nomifensine	254	18.90	(2.5)	1.17	(6.9)	0.9989
Desipramine	Doxepin	254	10.90	(1.7)	−6.20	(4.9)	0.9985
	Nomifensine	254	17.90	(1.3)	8.51	(3.6)	0.9997
Clomipramine	Doxepin	254	3.32	(5.0)	−2.25	(1.4)	0.9987
	Nomifensine	254	8.20	(1.3)	−8.06	(3.2)	0.9988
Maprotiline	Doxepin	230	1.97	(0.1)	0.29	(0.37)	0.9997
	Butriptyline	230	1.50	(0.2)	−0.38	(0.61)	0.9987
Nomifensine	Doxepin	230	3.04	(0.2)	−1.05	(0.49)	0.9998
	Butriptyline	230	2.24	(0.3)	−0.67	(0.89)	0.9988

* Linear range: 50–500 ng/ml.
† Correlation coefficient (p = 0.95; n = 6).

Accuracy and precision

Blank bovine serum samples spiked with 50 and 250 ng/ml of the individual antidepressants were analysed by the above method, using the appropriate detection wavelength. The results, expressed as mean values of the concentrations found, are given in Table 3. The relative standard deviation values observed, both within-day and between-day, illustrate the precision of the method for routine purposes. The method is generally found to be more precise at higher concentration, due to the more complete recovery during extraction, as discussed below.

To examine the accuracy of the method further, the assay method was applied to specimens of pooled serum containing either amitriptyline and nortriptyline, or imipramine and desipramine, as distributed by a national test scheme [23]. The results obtained are shown in Table 4, together with the spiked value and the mean consensus value obtained by other laboratories using a variety of analytical procedures. For these two pairs of compounds, the proposed method is seen to compare favourably, especially when the large relative standard deviations associated with the quoted mean consensus values are taken into account [23–25].

Extraction recovery

The absolute recoveries from serum of eight drugs and internal standards at four different concentrations ranging from 50 to 400 ng ml^{-1} were measured by adding the required concentration of the drug to blank serum. The serum was extracted and after

Table 3
Accuracy and precision of analysis for two concentrations of tricyclic antidepressant drugs in bovine serum

	Within-day			Between-day		
	n	Mean (ng/ml)	RSD (%)	*n*	Mean (ng/ml)	RSD (%)
Serum spiked with 50 ng/ml						
Amitriptyline	6	54.10	3.9	9	52.08	6.8
Nortriptyline	6	51.75	3.6	9	49.25	5.4
Imipramine	4	47.58	5.3	8	50.12	6.3
Desipramine	4	46.72	5.4	8	47.28	7.2
Clomipramine	4	45.73	6.4	7	49.76	8.5
Maprotiline	4	47.21	6.2	9	46.83	8.8
Nomifensine	4	55.73	7.6	8	50.42	9.8
Serum spiked with 250 ng/ml						
Amitriptyline	5	253.12	1.9	10	251.50	4.5
Nortriptyline	5	248.31	2.6	10	249.32	5.6
Imipramine	4	247.26	3.3	8	250.53	5.6
Desipramine	4	255.30	3.5	8	256.41	5.2
Clomipramine	4	247.50	6.9	7	252.10	9.3
Maprotiline	4	250.565	7.3	9	248.95	9.9
Nomifensine	4	258.5	9.8	8	254.50	10.1

back-extraction was subjected to chromatography as described above. The peak height obtained for the drug of interest was compared with that obtained by injecting the calculated mass in acidic aqueous solution directly on to the column. The results shown in Table 5 represent the percentage recoveries, expressed as the mean of four determinations. The recoveries are seen to increase with increasing drug concentration, which is consistent with other findings [26–28].

It was found that the extraction time was not a critical factor in the assay, although very long extraction times did increase the concentration of metabolites detected.

Table 5
Mean percentage extraction recovery of antidepressant drugs from serum, as a function of concentration ($n = 4$).

	Spiked concentration (ng/ml)			
	50	100	200	400
Doxepin	72.6*	75.1	84.9	90.8
Amitriptyline	68.4	73.8	84.0	81.3
Nortriptyline	63.0	62.5	66.0	68.4
Imipramine	69.6	73.3	74.4	75.4
Desipramine	63.0	68.3	71.2	88.0
Clomipramine	68.0	68.5	71.5	77.6
Butriptyline	59.0	67.4	78.6	80.2
Maprotiline	67.1	70.9	72.8	82.0
Nomifensine	64.0	66.7	69.5	73.9

* Results expressed as % recovery.

Table 4
Comparison of analytical data for each of two pairs of tricyclic antidepressant drugs obtained by the present method (T), with the stated spiked values (S) and the mean consensus values by established methods (M)

Sample code no.	Amitriptyline*			Nortriptyline			Imipramine			Desipramine		
	S	M	T	S	M	T	S	M	T	S	M	T
1279	389	225	364	175	15.8	0	150	156.3	165.2	200	226.5	250.7
280	—	—	—	—	—	—	202	181	201.2	136.8	108.6	146.6
380	283	257.2	234	48.2	49.5	45	—	—	—	—	—	—
880	—	173.6	213	—	149.2	136	—	—	—	—	—	—
1080	249.2	233.2	226	188.2	217.3	166	99.6	105.1	106.3	351.2	387.6	344.5
1180	—	—	—	—	—	—	439	388	446	293	294.7	316
181	107.5	89.1	93	163.7	156.7	124	—	—	—	—	—	—
281	—	—	—	—	—	—	61.6	50.4	70.3	195.8	168.4	230.1
481	43.2	38	59	89.7	89.2	80	243	190	191.6	57.4	45.8	72.9

* Drug concentrations in ng/ml throughout.

Detection limits

Taking a signal-to-noise ratio of 3 as a criterion, the detection limits of the proposed method, using equipment described, were found to be 5 ng/ml for amitriptyline, nortriptyline, imipramine and desipramine at 254 nm; and 10 ng/ml for maprotiline and nomifensine at 230 nm.

Results from patients' samples

To indicate the usefulness of the method when applied to serum samples of patients, chromatograms were obtained using serum from patients receiving different tricyclic antidepressant drugs. To demonstrate the absence of metabolite interference, internal standards were omitted in these instances.

The extracts of patients' blank serum (Fig. 2) show no endogenous compound

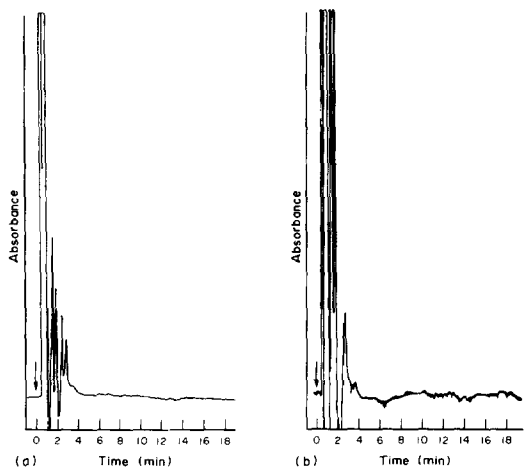

Figure 2
Representative chromatograms of extracts of blank serum from patients, showing endogenous components extracted. Chromatographic conditions: see text; A 254 nm, B 230 nm.

interference at either of the wavelengths used, after approximately 3 min extraction. Figure 3(a) shows that, after administration, the amitriptyline is clearly resolved from the nortriptyline and other unidentified metabolites; in this case either internal standard could be used. Figure 3(b) shows that administered imipramine is similarly well resolved from its metabolites. In this case, the patient was also receiving daily orphenadrine and chlorpromazine; neither of these drugs nor any metabolites appear to interfere with the imipramine determination. Figure 3(c) shows the results obtained during treatment with clomipramine. The peak appearing after the parent drug is considered to be a demethylated metabolite; once again, either internal standard could be used. Figure 3(d) shows the results obtained for serum from a patient receiving clomipramine together with flurazepam, L-tryptophan and chlorpromazine. In addition to the demethylated metabolite, numerous unidentified peaks are observed at short retention times, so that in this case, only the longer retained nomifensine would be appropriate for use as an internal standard for quantitation.

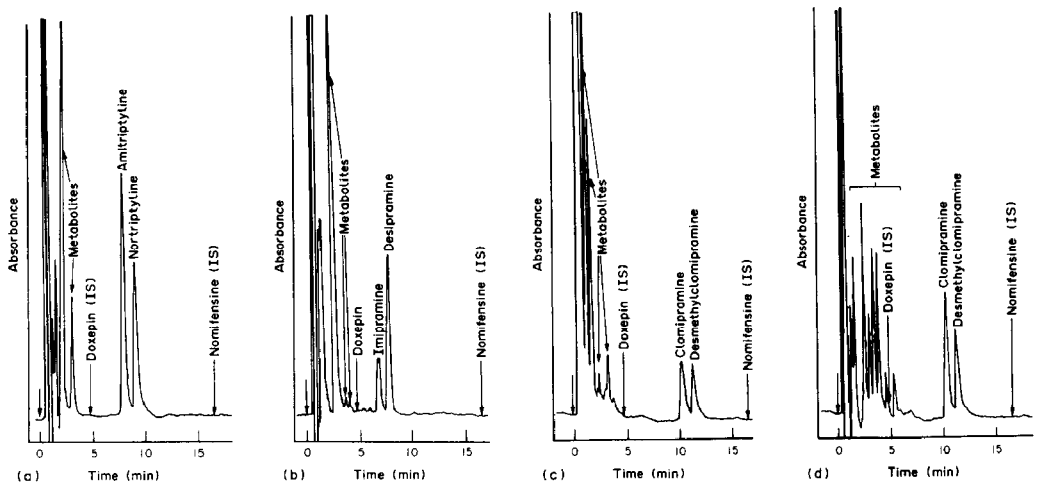

Figure 3
Representative chromatograms showing effect of metabolite interference. Chromatographic conditions: see text; detection at 254 nm.

The results illustrated in Fig. 3 indicate that the method described is capable of quantitating the drug levels found in blood samples from patients receiving clinical doses of antidepressants. The flexibility of the chromatographic system facilitates manipulation of retention times as a solution to the problem of interferences caused by concomitant drug therapy. It is suggested that these attributes, together with the simplicity of the extraction procedure and the satisfactory precision, make the proposed method appropriate for use both in pharmacokinetic studies and in therapeutic drug monitoring in routine hospital service laboratories.

Acknowledgements: One of us, CTH, gratefully acknowledges receipt of an SED research studentship. The receipt of an MRC Special Equipment Grant is also gratefully acknowledged.

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[*First received for review 9 June 1982; revised manuscript received 22 July 1982*]