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Quantitative Determination of Amitriptyline and Its Principal Metabolite, Nortriptyline, by GLC–Chemical Ionization Mass Spectrometry

W. A. GARLAND

Abstract □ A quantitative GLC–mass spectrometry assay was developed for the determination of the tricyclic antidepressant amitriptyline and its desmethyl metabolite (nortriptyline) in human plasma. The assay utilizes selective ion detection to monitor in a GLC effluent the MH^+ molecular ions of amitriptyline and nortriptyline generated by isobutane chemical ionization. The procedure, which utilizes deuterated analogs of amitriptyline and nortriptyline as internal standards, requires 1 ml of plasma and can measure 1 ng/ml of amitriptyline and 0.5 ng/ml of nortriptyline. The curves relating the amounts of amitriptyline and nortriptyline added *versus* the amounts found over a 100-fold range of amitriptyline and nortriptyline concentrations are straight lines with intercepts of approximately zero and slopes of unity. Analyses of plasma samples from three subjects receiving 50 mg of amitriptyline orally, three times a day, gave an average plasma concentration of 115 ± 42 ng/ml for amitriptyline and 109 ± 20 ng/ml for nortriptyline. Similar analyses of the plasma of three subjects who had received a single 50-mg oral dose of amitriptyline showed an average maximum plasma concentration of 25 ± 10 ng/ml for amitriptyline and 10 ± 4 ng/ml for nortriptyline. Seventy-two hours after administration, the average plasma amitriptyline and nortriptyline levels were 3 ± 1 and 3 ± 2 ng/ml, respectively.

Keyphrases □ Amitriptyline—GLC–mass spectrometric analysis, human plasma □ Nortriptyline—GLC–mass spectrometric analysis, human plasma □ GLC–mass spectrometry—analyses, amitriptyline and nortriptyline in human plasma □ Antidepressants—amitriptyline and nortriptyline, GLC–mass spectrometric analyses, human plasma

Amitriptyline and nortriptyline are used extensively for treating psychic depression (1). Nortriptyline is also generated metabolically in humans from amitriptyline (2, 3). Indeed, it has been suggested that the action of amitriptyline is mediated through its nortriptyline metabolite (4). Therapeutically, these compounds are reported to be effective over a relatively narrow range of plasma concentrations (5), although large interindividual variations in plasma concentrations are observed with similar dosing schedules (6–8). In addition, there are conflicting reports concerning the relative amelioration of depression as a function of plasma amitriptyline and/or nortriptyline levels (5, 8, 9).

Research on problems associated with amitriptyline therapy has been hindered somewhat by the lack of a sufficiently sensitive and specific assay for determining low levels of amitriptyline in plasma, *e.g.*, no single-dose amitriptyline pharmacokinetic data are available. Two flame-ionization GC assays suitable only for determining "steady-state" plasma concentrations of amitriptyline and nortriptyline were described (10, 11). Both assays require 3–5 ml of plasma and have a sensitivity limit of 25 ng/ml.

Recently, a GC procedure using a nitrogen detector was reported (12). It has a quoted sensitivity of 5 ng/ml for amitriptyline and 10–15 ng/ml for nortriptyline (2 ml of plasma extracted), but it cannot determine nortriptyline in humans following a therapeutic single dose of amitriptyline. Furthermore, it is only marginally suitable for amitriptyline, since the typical maximum concentration of amitriptyline after a 50-mg dose is only 15–25 ng/ml.

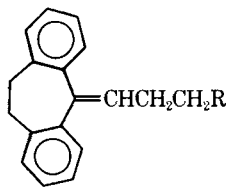
An electron-capture GC method for the determination of the heptafluorobutyrate derivatives of nortriptyline and some of its metabolites was reported (13). This procedure requires 4 ml of plasma and has a reported sensitivity of 10 ng/ml.

This report describes a GLC–mass spectrometric assay requiring 1 ml of plasma that is capable of determining 1 ng/ml of amitriptyline and 0.5 ng/ml of nortriptyline. The method, which utilizes chemical ionization GLC–mass spectrometry (14) with isobutane functioning both as a reagent gas and GLC carrier gas, is suitable for measuring amitriptyline and nortriptyline in humans following single-dose amitriptyline administration. To obtain sufficient sensitivity, selective ion detection is used to monitor the MH^+ ion of both amitriptyline and either nortriptyline or the trifluoroacetyl derivative of nortriptyline. Known quantities of deuterated analogs of both amitriptyline and nortriptyline are added to the plasma as internal standards.

EXPERIMENTAL

Apparatus—A quadrupole mass filter system[1] and data system[2] were used in conjunction with the gas chromatograph[3]. The GLC column, 1.21 m (4 ft) × 2 mm i.d., was silanized and packed with 3% OV-17 on 100–

[1] Finnigan model 1015D.
[2] Finnigan model 6000.
[3] Finnigan model 9500.



Ia: R = $N(CH_3)_2$—CH_3

Ib: R = $N(CH_3)_2$—CD_3

Ic: R = $N(CD_3)_2$—CD_3

IIa: R = $NHCH_3$

IIb: R = $NHCD_3$

IIc: R = $N(CH_3)$—$C(=O)CF_3$

IId: R = $N(CD_3)$—$C(=O)CF_3$

120-mesh Gas Chrom Q[4]. The column was conditioned at 325° with no flow for 3 hr and then at 275° overnight with a 20-ml/min helium flow.

Chromatographic conditions were: column oven, 230–240°; injection port, 275°; isobutane[5] gauge pressure, 1–3 lb/in.^{2}; and interface oven and glass-lined source reentrant tube, 250°. The column temperature and isobutane pressure were adjusted to provide an ion source pressure of 0.3–0.4 torr. Typical ion source settings were: ion volume + 8 v, ion repeller + 2 v, ion lens − 30 v, and filament − 150 v. The electron beam total emission was regulated at 800 μamp, while the source was operated without external heating (temperature 50°).

Materials—Nanograde quality *n*-hexane[6] and chloroform[6] and GLC quality trifluoroacetic anhydride[7] were used without further purification. Before each assay, hydrogen chloride gas was gently bubbled through 20 ml of chloroform in a 25-ml flask for 5 min. The flask was then capped, and this solution was used later to wash down the wall of the tubes used for evaporation of the plasma extraction solvent.

Stock solutions of amitriptyline (Ia), amitriptyline-d_3 (Ib), amitriptyline-d_6[8] (Ic), nortriptyline (IIa), and nortriptyline-d_3 (IIb) were made in distilled water using their respective hydrochloride salts, with all concentrations expressed in terms of the free base. Clinical control plasma[9] was used in the preparation of the amitriptyline and nortriptyline plasma standards.

Amitriptyline-d_3 and nortriptyline-d_3 were synthesized from nortriptyline and desmethylnortriptyline, respectively, using a published procedure (15). The relevant amine was treated with ethyl chlorocarbonate, and the resulting carbamate was reduced with lithium aluminum deuteride[10] at room temperature for no more than 8 hr. Both compounds showed satisfactory UV, IR, PMR, and mass spectral (electron and chemical ionization) properties. A selective ion detection analysis of amitriptyline-d_3 showed the presence of an ion equivalent to 3.03 ± 0.16% (n = 6) amitriptyline-d_0. A similar analysis of nortriptyline-d_3 showed the presence of an ion equivalent to 3.84 ± 0.17% (n = 6) nortriptyline-d_0. A selective ion detection analysis of amitriptyline-d_6 failed to detect any amitriptyline-d_0.

Tubes (15 ml)[11] with screw caps[12] were used for the plasma extractions; 5-ml glass-stoppered centrifuge tubes[13] were used for the final solvent evaporation. Pasteur pipets with hand-drawn constricted tips were utilized for all solution transfers. All glassware was soaked for 2 hr in a 0.1% solution of trimethylamine in methanol prior to use.

Assay Procedure—*Steady-State Plasma Concentrations*—One milliliter of plasma, 1.0 ml of internal standard solution (typically ~ 100 ng/ml of amitriptyline-d_3 and nortriptyline-d_3), 0.2 ml of 1 *M* H_2SO_4, and 5.0 ml of hexane were combined in a 15.0-ml test tube, and the mixture was shaken on a metabolic shaker–incubator[14] at 40 oscillations/min for 15 min. After centrifugation at 1000×*g*[15], the organic phase was removed and discarded. To the aqueous phase was then added 1.0 ml of 1.0 *M* NaOH and 5.0 ml of hexane. The mixture was then shaken and centrifuged, and the organic phase was transferred to a 5-ml centrifuge tube.

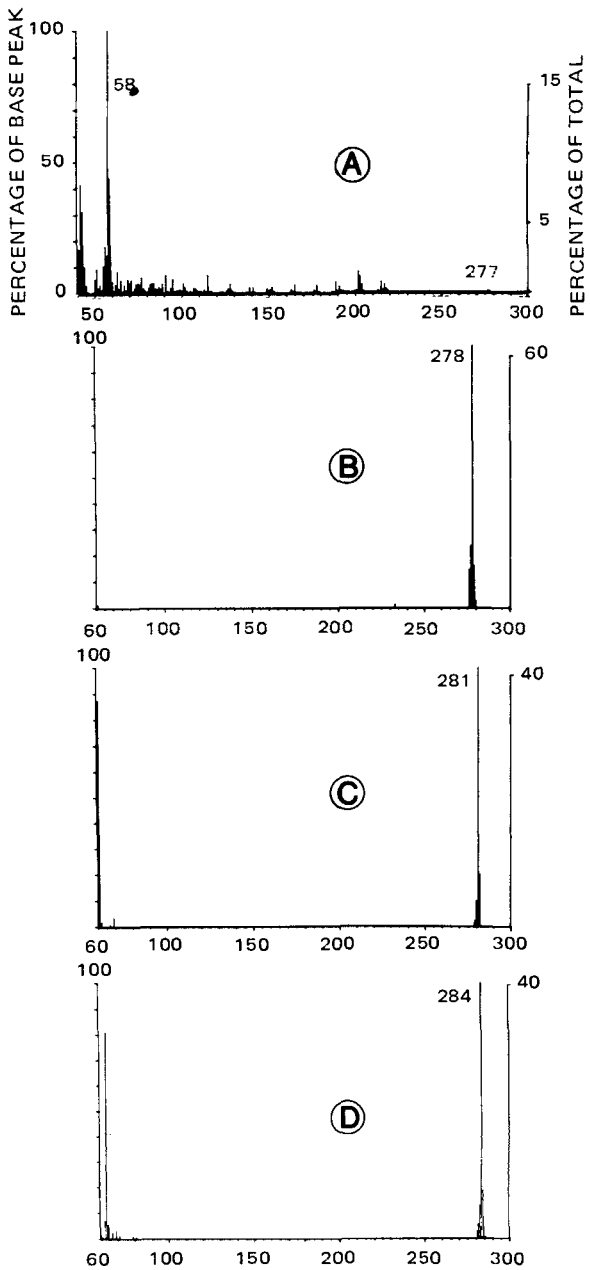


Figure 1—*Key: A, electron ionization (70 ev) mass spectrum of amitriptyline; B, isobutane (0.4 torr) chemical ionization mass spectrum of amitriptyline; C, isobutane (0.3 torr) mass spectrum of amitriptyline-*d_3*; and D, isobutane (0.6 torr) mass spectrum of amitriptyline-*d_6.

While the organic phase was being removed under a gentle stream of nitrogen at room temperature[16], another 5.0 ml of hexane was added to the aqueous phase, which was then shaken and centrifuged as before. This 5.0 ml was then transferred to the centrifuge tube, and the solvent was removed under nitrogen. The centrifuge tube wall was washed down first with 500 μl and then with 100 μl of hydrogen chloride (gas) treated chloroform. Following removal of this second chloroform wash, the residue was reconstituted in 30 μl of chloroform, and 10 μl was injected into the GLC–mass spectrometer.

Forty-five seconds after injection, the electron multiplier was turned on and the GLC flow divert valve was turned off, allowing the GLC effluent to enter the ion source. Fifty-five seconds after injection, the ion

[4] Applied Science Laboratories.
[5] Matheson, instrument purity.
[6] Mallinckrodt Chemicals.
[7] Pierce Chemicals.
[8] Synthesized by Dr. E. Kyburg, F. Hoffmann–La Roche & Co., Ltd., Basle, Switzerland.
[9] Hyland Laboratories.
[10] Stohler Isotopes, 99%D.
[11] Pyrex 9825.
[12] Lined with Teflon (du Pont).
[13] Pyrex 8084.
[14] Dubnoff, Scientific Glass Apparatus.
[15] Model HN-S, International Equipment Co.
[16] N-Evap, Organomation Associates.

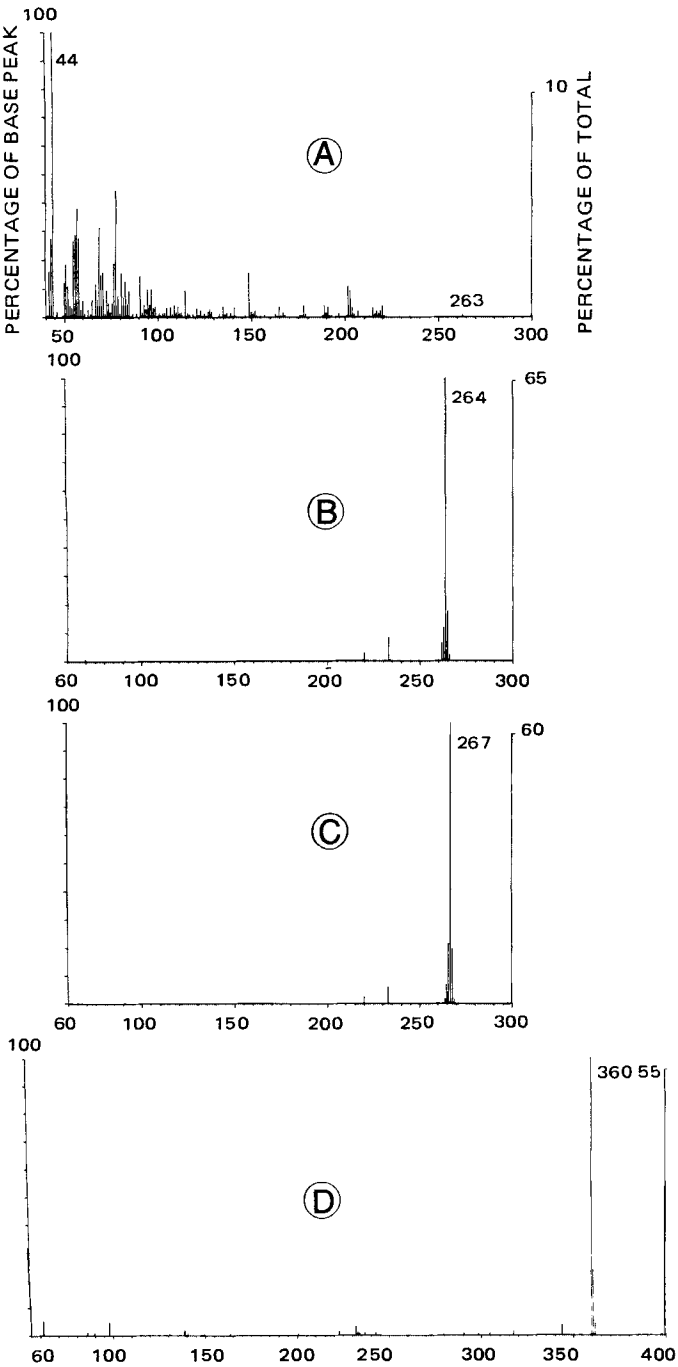


Figure 2—*Key: A, electron ionization (55 ev) mass spectrum of nortriptyline; B, isobutane (0.4 torr) chemical ionization mass spectrum of nortriptyline; C, isobutane (0.4 torr) chemical ionization mass spectrum of nortriptyline-*d_3*; and D, isobutane (0.6 torr) mass spectrum of the* N*-trifluoroacetyl derivative of nortriptyline.*

source supplies were turned on. Computer-controlled scanning and data collection were initiated another 5 sec later. Approximate retention times were 1.5 and 1.8 min for amitriptyline and nortriptyline, respectively. Three minutes after injection, the GLC flow divert valve was again turned on, the ion source supplies were turned off, and the computer data collection was halted.

Peak areas and preliminary concentrations were obtained using the software provided with the data system. Final concentrations were obtained by subtraction of the amount of either amitriptyline-d_0 or nortriptyline-d_0 present in the deuterated internal standards from the computer-calculated preliminary concentrations. The amounts of amitriptyline-d_0 and nortriptyline-d_0 in the d_3-labeled standards were periodically checked by selective ion analysis.

Single Amitriptyline Dose—The procedure followed was similar to

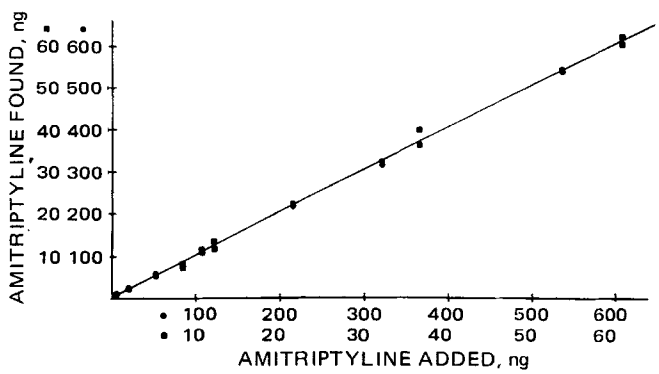


Figure 3—*Curve relating amount of amitriptyline added to clinical control plasma* versus *amount of amitriptyline found. Circles denote determinations using procedure described under* Experimental *for steady-state plasma amitriptyline* (m/e 278) *levels. For these samples, 122 ng/ml of amitriptyline-*d_3 (m/e 281) *was used as an internal standard. Squares denote determinations using the procedure described under* Experimental *for single-dose plasma amitriptyline levels. For these samples, 7.52 ng/ml of amitriptyline-*d_6 (m/e 284) *was used as an internal standard.*

that used with the steady-state plasma samples with the following three exceptions:

1. A lower internal standard concentration of approximately 20 ng/ml for both amitriptyline-d_6 and nortriptyline-d_3 was used. Amitriptyline-d_6 was usually used in preference to amitriptyline-d_3 in the single-dose assay, since the *m/e* 284 (amitriptyline-d_6) trace showed a significantly lower background signal at this concentration level as compared to that at *m/e* 281 (amitriptyline-d_3).
2. Fifty microliters of trifluoroacetic anhydride was added following the addition of the second hexane extract to the 5-ml centrifuge tube, and the solution was allowed to stand for 30 min before solvent evaporation.
3. The GLC flow divert valve was turned on again 4 min after sample injection. The retention time of *N*-trifluoroacetyl nortriptyline (IIc) and *N*-trifluoroacetyl nortriptyline-d_3 (II*d*) was 3.0 min.

Amitriptyline Single-Dose and Steady-State Human Plasma Samples—Plasma samples from normal adult volunteers were available from previous studies.

RESULTS AND DISCUSSION

A GLC–mass spectrometric assay for the heptafluorobutyrate derivative of nortriptyline was reported using conventional electron ionization and selective ion monitoring of the $M^+ - CH_3NHC(=O)C_3F_7$ fragment ion generated in the electron ionization mass spectrum (16). The quoted sensitivity for this assay is 1 ng/ml using 3 ml of plasma. Since amitriptyline cannot be easily derivatized, a similar electron ionization assay for this compound would require the monitoring of the relatively nonspecific *m/e* 58 ion found in the electron ionization mass spectrum of amitriptyline (Fig. 1A).

Isobutane chemical ionization, however, permits the monitoring of an ion (*m/e* 278) expressing information concerning amitriptyline's molecular weight (Fig. 1B). Similarly, this ionization technique also makes nortriptyline suitable for mass spectral analysis without prior derivatization (Figs. 2A and 2B). The maximum sensitivity obtainable, however, for the assay using underivatized nortriptyline was quite variable. Although this is of little consequence when determining steady-state plasma nortriptyline levels, it is significant when measuring the low nortriptyline levels found after single-dose amitriptyline administration. For low level determinations of nortriptyline, the *N*-trifluoroacetyl derivative had suitable chromatographic and mass spectral (Fig. 2D) properties.

Clinical control plasma subjected to the described procedure for steady-state plasma amitriptyline and nortriptyline levels showed no significant background ions at *m/e* 264, 267, 278, 281, or 284. If this same plasma is subjected to the procedure adopted for a single dose of amitriptyline, a noninterfering *m/e* 278 peak corresponding to a compound with a retention time longer than that of amitriptyline is sometimes observed.

Results obtained from the addition of known amounts of amitriptyline and nortriptyline to clinical control plasma followed by processing according to the described procedure can be seen in Figs. 3 and 4. A least-

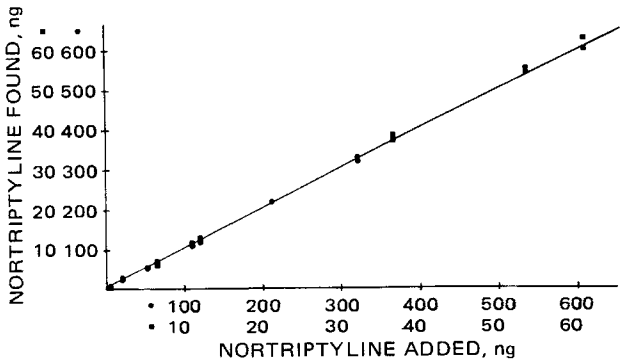


Figure 4—*Curve relating amount of nortriptyline added to clinical control plasma* versus *amount of nortriptyline found. Circles denote determinations using the procedure described under* Experimental *for steady-state plasma nortriptyline* (m/e *264*) *levels. For these samples, 108 ng/ml of nortriptyline-*d_3 (m/e *267*) *was used as an internal standard. Squares denote determinations using the procedure described under* Experimental *for single-dose plasma nortriptyline levels. For these samples, 4.61 ng/ml of nortriptyline-*d_3 *was used as an internal standard* [N-*trifluoroacetylnortriptyline-*d_3, m/e *363*].

squares analysis of the amitriptyline data (Fig. 3) gave a slope of 0.98 ± 0.01 and an intercept of 0.20 ± 0.23 ng. The corresponding curve (Fig. 4) for nortriptyline had a slope of 1.01 ± 0.01 and an intercept of 0.36 ± 0.20 ng. Thus, these data suggest that the extraction, mass spectrometry, and

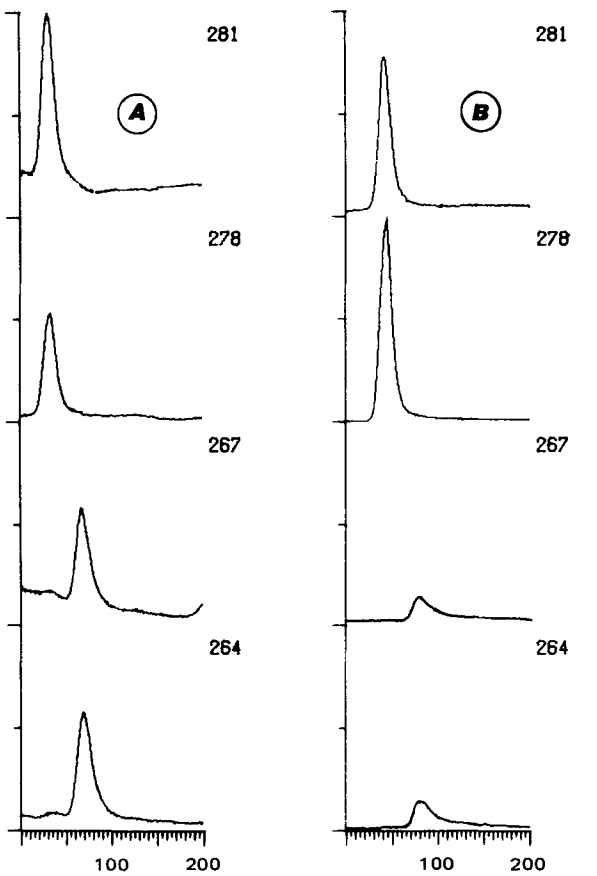


Figure 5—*Selective ion chromatograms for amitriptyline* (m/e *278*) *and nortriptyline* (m/e *264*). *Part A is for plasma obtained from Subject 1, 8 days after beginning oral 50-mg amitriptyline therapy three times a day. Internal standards amitriptyline-*d_3 (m/e *281*) *and nortriptyline-*d_3 (m/e *267*) *were present in concentrations of 122 and 104 ng/ml, respectively. The amitriptyline concentration was 64.2 ng/ml; the nortriptyline concentration was 100 ng/ml. Part B is the same as Part A except that the plasma is from Subject 3, 8 days after beginning amitriptyline therapy. The amitriptyline concentration was 124 ng/ml; the nortriptyline concentration was 87.1 ng/ml.*

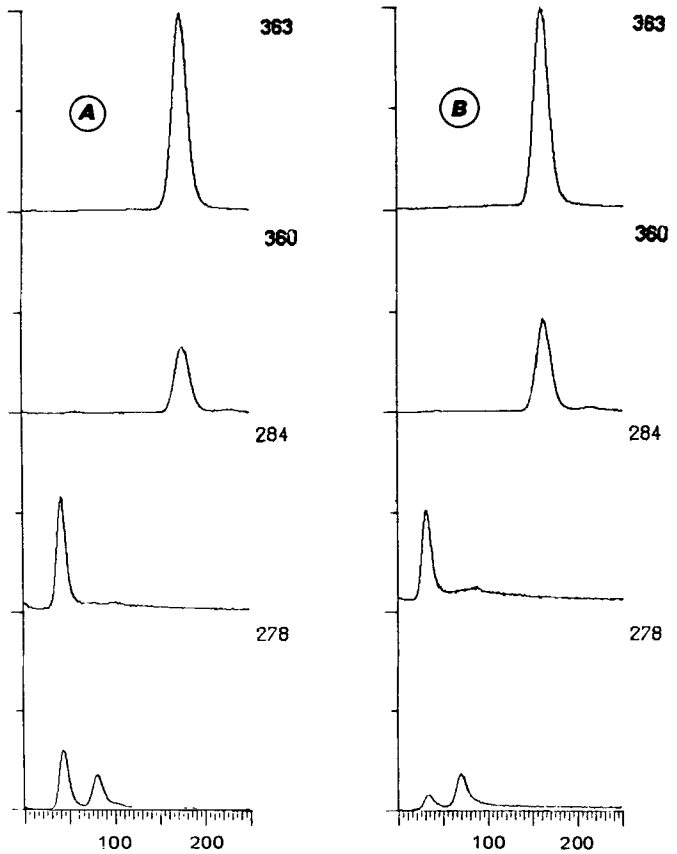


Figure 6—*Selective ion chromatograms for amitriptyline* (m/e *278*) *and* N-*trifluoroacetylnortriptyline* (m/e *360*). *Part A is for plasma obtained from Subject 6, 3 hr after oral administration of 50 mg of amitriptyline. Internal standards amitriptyline-*d_6 (m/e *284*) *and* N-*trifluoroacetylnortriptyline-*d_3 (m/e *360*) *were present in concentrations of 33.3 and 20.3 ng/ml, respectively. The amitriptyline concentration was 11.2 ng/ml; the nortriptyline concentration was 6.1 ng/ml. Part B is the same as Part A except 48 hr after amitriptyline administration. The amitriptyline concentration was 5.1 ng/ml; the nortriptyline concentration was 8.3 ng/ml.*

GLC characteristics of the labeled and unlabeled compounds are essentially identical.

Six samples containing 8.62 ng of amitriptyline plus 6.43 ng of nortriptyline were analyzed by the procedure for single-dose amitriptyline plasma levels using 7.52 ng/ml of amitriptyline-d_6 and 4.61 ng/ml of nortriptyline-d_3 as internal standards. The results ± *SD* for these samples were 8.03 ± 0.29 ng/ml for amitriptyline and 6.51 ± 0.23 ng/ml for nortriptyline. The recoveries ± *SD* for these samples, based on a comparison of the peak areas obtained by injecting known amounts of nortriptyline-d_3 and amitriptyline-d_6 into the GLC–mass spectrometer with those obtained from the internal standards carried through each assay, were 50 ± 26% for amitriptyline and 31 ± 10% for nortriptyline.

Six samples containing 53.9 ng of amitriptyline plus 53.6 ng of nortriptyline and six samples containing 539 ng of amitriptyline plus 536 ng of nortriptyline were analyzed by the procedure for steady-state levels, using 122 ng/ml of amitriptyline-d_3 and 104 ng/ml of nortriptyline-d_3 as internal standards. The results ± *SD* were 54.0 ± 0.5 ng/ml for amitriptyline and 52.8 ± 0.4 ng/ml for nortriptyline for the first set of samples and 547 ± 11 ng/ml for amitriptyline and 546 ± 10 ng/ml for nortriptyline for the second set. The recoveries ± *SD* for these 12 samples, based on a comparison of the peak areas obtained by injecting known amounts of amitriptyline-d_3 and nortriptyline-d_3 into the GLC–mass spectrometer with those obtained from the internal standards carried through each assay, were 80 ± 20% for amitriptyline and 65 ± 19% for nortriptyline.

The fairly wide range of recoveries observed for these 18 samples presumably is the result of considerable intersample variation in both extraction efficiencies and GLC column and glassware adsorption. These results further emphasize the importance of the stable isotope internal standards in ensuring accuracy.

Analyses for amitriptyline and nortriptyline in the plasma of three

Table I—Plasma Amitriptyline and Nortriptyline Levels (Nanograms per Milliliter) in Three Subjects Receiving Amitriptyline Therapy

Days after Beginning of Amitriptyline Therapy[a] (50 mg po Three Times a Day)	Subject 1		Subject 2		Subject 3	
	Amitriptyline	Nortriptyline	Amitriptyline	Nortriptyline	Amitriptyline	Nortriptyline
8	64.2 (63.0)[b]	100 (104)[b]	137	70.2	124	87.1
8.5	59.7 (56.1)	102 (99.4)	106	153	131	88.2
11	91.6 (86.2)	117 (116)	130	154	135	111
11.5	77.6 (74.3)	101 (102)	160	147	103	106
13	61.2 (59.8)	101 (102)	153	125	136	103
13.5	44.4 (40.4)	66.8 (67.3)	201	142	146	88.8
Average ± *SD*	66.5 ± 16.3 (63.3 ± 15.7)	98.1 ± 16.9 (98.3 ± 15.7)	148 ± 32	132 ± 32	129 ± 15	97.2 ± 12.7

[a] First blood sample on Days 8, 11, and 13 was taken immediately before the first dose of the day; the second blood sample was taken 1 hr after receiving the second dose. [b] Amitriptyline and nortriptyline levels obtained from a separate analysis of a 1-ml aliquot from the same plasma sample.

subjects receiving 50 mg of amitriptyline three times a day are presented in Table I. Excellent reproducibility of the duplicate analyses of the plasma samples from Subject 1 is seen, except for the amitriptyline levels calculated for the 13.5-day sample. The actual levels found are similar, if the difference in administered dose is considered, to those previously published (11, 17). Typical ion chromatograms from analyses of steady-state plasma samples can be seen in Fig. 5. In this figure, the substantial variation in nortriptyline and nortriptyline-d_3 recoveries from the two different samples (A and B) should be noted.

The results of single-dose administrations of 50 mg of amitriptyline can be seen in Table II. The maximum plasma amitriptyline levels ranged from 16 to 35 ng/ml in the three subjects. After 72 hr, the amitriptyline levels ranged from 2.3 to 4.4 ng/ml; nortriptyline levels ranged from 0.5 to 5.4 ng/ml. The drug half-lives for the three subjects, obtained using a least-squares analysis of the log amitriptyline concentration *versus* time data for the 8–72-hr plasma samples, were 35, 26, and 51 hr. These half-lives fall within the range (18–93 hr) previously found in 22 human subjects who had received a similar dose of nortriptyline (18). Typical ion chromatograms from an analysis of single-dose plasma samples can be seen in Fig. 6.

The sensitivity obtainable using this assay cannot be quoted in absolute terms but was found to be a function of both the condition of the ion source and quadrupole rod system and the amitriptyline and nortriptyline recoveries. With clean poles and ion source, an assay sensitivity of 1 ng/ml of amitriptyline and 0.5 ng/ml of nortriptyline is possible, provided the amitriptyline and nortriptyline recoveries for the particular samples are greater than 50%.

Table II—Plasma Amitriptyline and Nortriptyline Levels (Nanograms per Milliliter) in Three Subjects after Oral Administration of 50 mg of Amitriptyline

Hours after Administration	Subject 4		Subject 5		Subject 6	
	Amitriptyline	Nortriptyline	Amitriptyline	Nortriptyline	Amitriptyline	Nortriptyline
0	0	0	0	0	0	0
1	12.9	0.6	26.8	2.1	4.6	0
2	19.3	3.1	35.1	7.5	11.6	2.8
3	19.9	4.7	34.4	7.9	11.2	6.1
4	22.5	5.3	31.1	13.7	16.1	7.1
6	10.5	3.4	16.7	10.9	10.5	10.0
8	10.8	3.3	17.8	9.2	10.1	8.3
12	5.6	3.2	7.9	9.5	9.7	9.7
24	5.2	2.8	6.3	9.7	6.2	8.9
36	3.2	2.6	5.2	9.5	4.2	8.3
48	3.4	0.7	3.7	9.3	5.1	8.3
72	2.4	0.5	2.3	5.4	4.4	4.3

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