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SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR CHLORPROMAZINE AND THIRTEEN METABOLITES

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SUMMARY

A rapid and reliable procedure, based on a C_8 bonded phase extraction and reversed-phase isocratic high-performance liquid chromatographic separation with internal standard quantitation, has been developed for the determination of the antipsychotic drug chlorpromazine and thirteen common metabolites. The method allows quantitation of these analytes at the ng/ml concentration range in human plasma. An evaluation of recovery, detection limits, and reproducibility is presented along with application of the method to patient samples.

INTRODUCTION

The heterogeneity of human response to drug treatment has spurred the study of drug metabolism in humans and the search for appropriate animal models [1–3]. After more than thirty years of clinical use [4], the metabolism of the antipsychotic drug chlorpromazine (CPZ) is still of interest. CPZ has a wide and poorly understood therapeutic window [5,6], and its metabolites play an active role in clinical response [7]. CPZ therapy has also been associated with a variety of side-effects in patients, including acute dyskinesia [8].

Investigations into the relationship between metabolism and clinical effects of CPZ have been hampered by difficulties in the simultaneous analysis of all the CPZ metabolites commonly found in biological samples. Typical problems include instability, tendencies for adsorption loss, and low analyte concentrations in clinical samples [9]. Additional problems arise from the wide range of polarities exhibited by CPZ and its metabolites and from the need to remove matrix

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interferences [10]. Optimal analysis under these restrictions requires adequate sample clean-up, sensitivity, and short analysis time.

Previous studies of the clinical levels of CPZ and its metabolic fate have employed several different analytical techniques [9–26]. The most popular of these procedures use liquid–liquid extractions combined with either gas or liquid chromatography. In gas chromatographic assays of these compounds, derivatization of the extracted analytes is required to render volatility. The lack of thermal stability or volatility requirements for high-performance liquid chromatography (HPLC), however, confers the great advantage of minimal sample preparation.

Multi-stage extractions of single samples [10,13,16,18,20,21] or separate independent extractions of the same sample [11] are two methods used to recover a few of the more polar metabolites in addition to CPZ. Recently, bonded phase extraction procedures have been developed for rapid recovery of a number of drugs and their metabolites from clinical samples [27–29]. Speed and simplicity are among the potential advantages of this technique. In this paper we evaluate the use of bonded phase extraction coupled with an isocratic mobile phase developed by Kiel [30] for recovery and quantitation of CPZ simultaneously with thirteen common metabolites.

EXPERIMENTAL

Materials

Human plasma was obtained from the American Red Cross (Columbia, SC, U.S.A.). CPZ was donated by Smith Kline & French Labs. (Philadelphia, PA, U.S.A.). CPZ metabolite standards were the kind gift of Dr. A.A. Manian at the National Institute of Mental Health (Washington, DC, U.S.A.). Nortriptyline was obtained from Eli Lilly (Indianapolis, IN, U.S.A.). HPLC-grade methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). HPLC-grade phosphoric acid, reagent-grade sodium hydroxide, and reagent-grade sodium carbonate came from Fisher Scientific (Atlanta, GA, U.S.A.). Dibasic sodium phosphate and methylamine hydrochloride were obtained from Aldrich (Milwaukee, WI, U.S.A.). Analytichem International (Harbor City, CA, U.S.A.) supplied 1-ml, 100-μm, C_8 bonded silica extraction columns. Vacutainers were supplied by Becton-Dickinson (Rutherford, NJ, U.S.A.). The 250×4.6 mm analytical column packed with 5-μm C_8 bonded silica and 50×4.6 mm guard column packed with 40-μm C_8 bonded silica were both purchased from Supelco (Bellefonte, PA, U.S.A.).

Equipment

A Model CL centrifuge from International Equipment (Needham Heights, MA, U.S.A.) was used in the extraction procedure. HPLC instrumentation consisted of a Model 710B autosampler, two Model 6000A pumps, and a Model 440 detector from Waters Assoc. (Milford, MA, U.S.A.). Data analysis was performed with a Model 3390 integrator from Hewlett-Packard (Palo Alto, CA, U.S.A.) and an IBM PC (Boca Raton, FL, U.S.A.).

Standard preparation

Stock solutions of CPZ, each metabolite, and nortriptyline (NOR) were prepared in methanol at concentrations of 0.5 mg/ml. These solutions were stored in glass in the dark at −15°C. Working standard solutions of CPZ and metabolites were made by combining aliquots of each of these solutions, diluting to 20 000 ng/ml in 50% methanol–water, then serially diluting to desired concentrations with water. These working standards then contained a mixture of all the analytes at the same concentration level. NOR was diluted separately to a concentration of 1000 ng/ml for use as an internal standard. A small amount (0.1%) of triethanolamine was added to each standard to prevent adsorption of the analytes to the walls of the glassware. The stock solutions were stored in the dark at 4°C and allowed to reach room temperature before use.

Extraction column preparation

Extraction columns were prepared by sequential washing with column volumes of 0.2 *M* phosphoric acid, 0.25% sodium carbonate, methanol, and then water. The columns were centrifuged at a medium speed between washings and were not allowed to dry between preparation and use.

Sample preparation

Plasma calibration samples were prepared by mixing 1 ml of blank human plasma, a 30-μl aliquot of the 1000 ng/ml solution of NOR internal standard, and 100 μl of a working standard mixture of known concentration. This mixture was vortexed slightly before proceeding with the extraction.

Patient whole blood samples were collected in heparinized vacutainers which contained no interfering plasticizers. These samples were centrifuged within 2 h of sampling to obtain the plasma. The plasma was then stored at −15°C in clean vacutainers identical to those used for collection until analysis was performed. Patient plasma (1 ml) was spiked with 30 μl of 1000 ng/ml internal standard solution and vortexed slightly before extraction.

Extraction method

To the prepared plasma samples, 50 μl of 3 *M* orthophosphoric acid was added, and the solution was vortexed again briefly. The sample was pipetted into a prepared extraction column and centrifuged for 30 s at medium speed. The column was then washed with one column volume of water, centrifuged briefly, washed with 500 μl methanol, and centrifuged again. The column was further washed with a column volume of 0.25% sodium carbonate and centrifuged for approximately 30 s. The analytes were eluted with two 150-μl aliquots of methanol, centrifuged after each addition and collected in a clean conical centrifuge tube. After throughly mixing the two aliquots, a 50-μl sample from the combined extracts was injected onto the HPLC column.

HPLC conditions

The isocratic mobile phase was composed of 55% methanol, 45% water, 275 m*M* methylamine hydrochloride and 25 m*M* dibasic sodium phosphate, with the

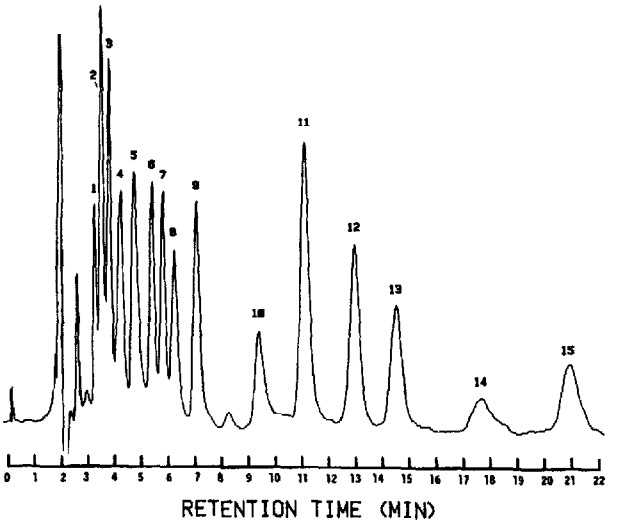


Fig. 1. Chromatogram of the extract of 1 ml of human plasma spiked with internal standard and 100 ng each of CPZ and thirteen common metabolites. Peaks: 1 = 7-hydroxychlorpromazine sulfoxide; 2 = nor$_2$chlorpromazine sulfoxide; 3 = nor$_1$chlorpromazine sulfoxide; 4 = N-S-chlorpromazine dioxide; 5 = chlorpromazine sulfoxide; 6 = 7-hydroxynor$_2$chlorpromazine; 7 = 7-hydroxynor$_1$chlorpromazine; 8 = 3-hydroxychlorpromazine; 9 = 7-hydroxychlorpromazine; 10 = 8-hydroxychlorpromazine; 11 = nortriptyline (internal standard); 12 = nor$_2$chlorpromazine; 13 = nor$_1$chlorpromazine; 14 = chlorpromazine-N-oxide; 15 = chlorpromazine.

pH adjusted to 6.75 as recommended by Kiel [30]. The C_8 analytical column was thermostatted to 40 °C, and a flow-rate of 2 ml/min was maintained. Absorbance was monitored at 254 nm with an attenuation of 0.005 a.u.f.s.

RESULTS AND DISCUSSION

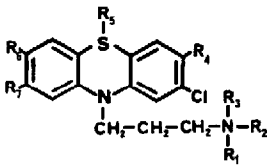
The metabolism of CPZ involves demethylation, hydroxylation, and oxidation. Moreover, secondary reactions also occur, and not necessarily in a unidirectional manner [31]. We have included all the common first- and second-pass unconjugated metabolites of CPZ in this assay to facilitate thorough studies of CPZ metabolism by the analysis of human plasma, and potentially in a variety of biological systems. Fig. 1 illustrates a representative chromatogram of 100 ng of CPZ and each metabolite following extraction from human plasma. The structures and elution order of the parent drug and metabolites are listed in Table I. It can be seen that the polarity range for this group of compounds is extremely wide. The internal standard, NOR, which elutes eleventh, was chosen for its structural similarity to CPZ: NOR contains nitrogen in a seven-member center ring and has an aliphatic side-chain, but contains no chlorine or sulfur. NOR also is easily separated from the other analytes in the chromatogram.

Chromatograms of standard mixtures of 25 ng each of CPZ and the thirteen metabolites plus the internal standard and a chromatogram of human plasma containing only internal standard are shown in Fig. 2. Fig. 2a shows a chroma-

TABLE I

STRUCTURES AND ELUTION ORDERS OF THE PARENT DRUG AND METABOLITES

The internal standard, NOR, elutes between CPZ-8-OH and NOR_2-CPZ.



Analyte	Elution order	R_1	R_2	R_3	R_4	R_5	R_6	R_7
CPZ	15	CH_3	CH_3	–	H	–	H	H
CPZ-N-oxide	14	CH_3	CH_3	O	H	–	H	H
NOR_1	13	CH_3	H	–	H	–	H	H
NOR_2	12	H	H	–	H	–	H	H
CPZ-8-OH	10	CH_3	CH_3	–	H	–	H	OH
CPZ-7-OH	9	CH_3	CH_3	–	H	–	OH	H
CPZ-3-OH	8	CH_3	CH_3	–	OH	–	H	H
7-OH-NOR_1	7	CH_3	H	–	H	–	OH	H
7-OH-NOR_2	6	H	H	–	H	–	OH	H
CPZ sulfoxide	5	CH_3	CH_3	–	H	O	H	H
N-S-dioxide	4	CH_3	CH_3	O	H	O	H	H
NOR_1 sulfoxide	3	CH_3	H	–	H	O	H	H
NOR_2 sulfoxide	2	H	H	–	H	O	H	H
7-OH-CPZ sulfoxide	1	CH_3	CH_3	–	H	O	OH	H

togram of an unextracted standard, while Fig. 2b shows a chromatogram of a standard that has been extracted from human plasma. The analyte peaks are well resolved, have good shape, and there is no interference in the chromatogram from the plasma as can be seen by comparing Fig. 2a and Fig. 2b with the plasma blank in Fig. 2c. Reagents used in the extraction procedure were chosen to avoid interconversion between analytes; for example, the use of sodium hydroxide to adjust the pH to a high level during a liquid–liquid extraction was found by Hubbard et al. [32] to reduce CPZ-N-oxide to CPZ.

It was critical to employ techniques which would reduce sample analysis time not only for high throughput but because of the sample degradation problems that complicate the analysis of CPZ and its metabolites [9]. The extraction procedure required only 8 min for a single sample, but many extractions could be performed simultaneously, depending only on centrifuge capacity. After the sample was applied to the extraction column, washing solutions could be applied rapidly since measuring exact volumes was not required. The total chromatographic run time was 22 min due to the use of an isocratic mobile phase. An isocratic approach avoided the lengthy equilibration time required with gradient elution procedures [33].

To compensate for any matrix effects on the quantitation of the analytes, calibration plots for each compound were constructed from chromatograms of hu-

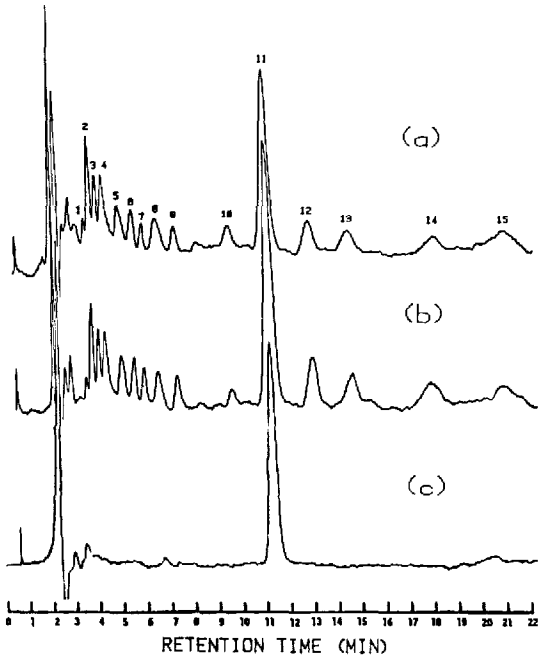


Fig. 2. Chromatograms of (a) 25 ng of each analyte extracted from 1 ml of human plasma, (b) 25 ng of each analyte diluted equivalently to (a) and injected directly into the HPLC system without extraction, and (c) extract from 1 ml of blank plasma spiked only with internal standard. For peak identification, see legend to Fig. 1.

man plasma which had been spiked with working standard mixtures and extracted by our method. The ratio of analyte peak height to that of the internal standard was calculated for each compound from fifteen chromatograms representing eight different concentration levels from 0 to 300 ng/ml. Regression analysis was used to fit a straight-line model with intercept to the data for each analyte. The coefficient of determination (r^2) in each of these regressions was never less than 0.93. The estimated values of the intercepts were not significantly different from zero at the 95% confidence level. A typical calibration plot for the five sulfoxide metabolites is shown in Fig. 3.

Detection limits for each analyte, estimated as the lowest concentration reproducibly discernible, are listed in Table II. Later-eluting metabolites exhibited higher detection limits, primarily due to band-broadening effects. A reversed-phase HPLC separation elutes the most polar analytes first, with less band-broadening, and thus places emphasis on those polar metabolites. In the case of the analysis of clinical samples of CPZ metabolites, this approach is desirable because the polar metabolites of CPZ are more likely to appear in small concentrations.

Mean recoveries for each analyte included in this assay are also shown in Table II. These measurements were made in triplicate at two concentration levels by comparing peak heights of a given analyte extracted from human plasma to those in unextracted standards of the same concentration (using experiments similar

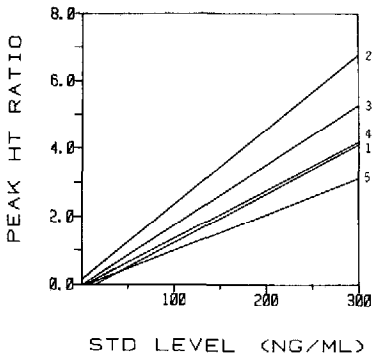


Fig. 3. Linear calibration plot for the sulfoxide metabolites. Similar results were obtained for the other analytes included in this extraction procedure. 1 = 7-Hydroxychlorpromazine sulfoxide; 2 = nor$_2$chlorpromazine sulfoxide; 3 = nor$_1$chlorpromazine sulfoxide; 4 = N-S-chlorpromazine dioxide; 5 = chlorpromazine sulfoxide.

to those displayed in Fig. 2). A *t*-test for the significance of the difference between two means showed that the recovery at the 25 ng/ml concentration level was not significantly different from recovery at the 100 ng/ml concentration level at the 95% confidence level. In addition to the bias produced by band-broadening in later-eluting compounds, this extraction procedure appears to impose a slight

TABLE II

DETECTION LIMITS, RECOVERY AND REPRODUCIBILITY OF THE METHOD

Blank human plasma (1 ml) was spiked with working standard mixture as described in the text to obtain concentrations of 25 and 100 ng/ml of each analyte.

Compound	Detection limit* (ng/ml)	Mean recovery ($n=3$) (%)		R.S.D.** (%)	
		25 ng/ml	100 ng/ml	25 ng/ml	100 ng/ml
7-OH-CPZ-SO	7.5	91.9	90.9	12.2	9.0
NOR_2-SO	5.0	96.7	101.6	23.1	9.3
NOR_1-SO	5.0	82.4	94.2	16.9	5.4
N-S-dioxide	5.0	73.5	87.4	27.7	18.8
CPZ-SO	10.0	88.3	93.5	13.5	8.6
7-OH-NOR_2	5.0	86.0	64.1	20.3	9.4
7-OH-NOR_1	5.0	71.0	59.7	17.8	12.0
CPZ-3-OH	7.5	79.6	76.6	27.8	8.5
CPZ-7-OH	7.5	68.4	70.3	10.3	15.8
CPZ-8-OH	10.0	77.6	72.8	24.5	11.6
NOR_2	7.5	73.0	69.0	14.0	13.5
NOR_1	10.0	76.7	78.8	17.9	15.2
CPZ-N-oxide	10.0	76.0	89.7	14.4	12.2
CPZ	10.0	83.0	74.1	23.1	15.6

*Concentration of lowest measurable standard.

**$n = 10$, pooled over a seven-day time period.

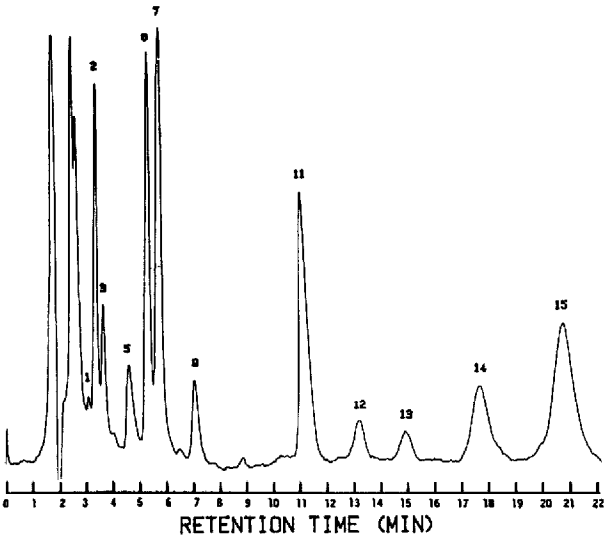


Fig. 4. Chromatogram of the extract of 1 ml of human plasma from a patient undergoing treatment with 350 mg CPZ per day for schizophrenia. For peak identification, see legend to Fig. 1.

bias toward recovery of the more polar metabolites. As stated previously, the influence on quantitation by this bias is removed by constructing the calibration curve with standards that have been extracted from the same matrix as the analytical samples.

The reproducibility of the extraction and HPLC method was determined separately for each analyte by comparing peak-height ratios (analyte to internal standard) from chromatograms of ten replicate samples processed randomly over a period of seven days. All samples were working standard mixtures extracted from a blank human plasma matrix. Replication included not only sample preparation, extraction, and chromatography, but also mobile phase, reagent, internal standard, and working standard preparation. Precision was studied at two analyte concentration levels, 25 and 100 ng/ml of each compound. The results for each compound are shown in Table II. The slightly higher reproducibility at the 100 ng/ml level is probably due to easier integration of the larger peaks.

This extraction and HPLC procedure was applied to fifteen patient plasma samples. While the results of this study will be presented in a separate paper [34], a typical chromatogram is presented in Fig. 4. The sample is from a patient who is undergoing treatment for chronic schizophrenia with CPZ only. The chromatogram shows extensive metabolism of the parent drug through demethylation, oxidation, and hydroxylation, as well as through combinations of these processes. Conspicuous is the absence of 3-hydroxy-CPZ, 8-hydroxy-CPZ, and N-S-dioxide metabolites in this particular patient sample.

CONCLUSIONS

A rapid and reliable procedure has been described for the simultaneous determination of CPZ and thirteen of its common metabolites in human plasma. Pre-

viously, several different assays were required to quantitate all of these compounds. The extraction method, using C_8 bonded phase columns, required less than 8 min, and chromatographic separation was accomplished within 22 min using an isocratic HPLC mobile phase. The versatility of this method is reflected in the narrow differences in detection limits (5–10 ng/ml) for fourteen compounds exhibiting a wide range of polarities and in the consistently high percentage of recovery at two concentration levels tested. This method is being used in our laboratory to study the metabolism of CPZ in humans as reflected by plasma levels of the parent drug and metabolites. We plan to evaluate the method for interference from other drugs to allow the analysis of plasma samples from patients undergoing multiple drug therapy. Also we plan to adapt the extraction procedure to a variety of biological matrices to better compare the metabolism of CPZ in animal models.

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REFERENCES

1 J.R. Gillette, in J.R. Mitchell and M.G. Horning (Editors), Drug Metabolism and Drug Toxicity, Raven Press, New York, 1984, p. 237.
2 J. Caldwell, Drug Metab. Rev., 12 (1981) 221.
3 E.J. Calabrese, Drug Metab. Rev., 15 (1984) 505.
4 S.J. Enna and J.T. Coyle, in S.J. Enna (Editor), Neuroleptics, Raven Press, New York, 1983, pp. 1–4.
5 A.V.P. Mackay, A.F. Healey and J. Baker, Br. J. Clin. Pharmacol., 1 (1974) 425.
6 T. Van Putten, P.R.A. May and D.J. Jenden, Psychol. Med., 11 (1981) 729.
7 J.P. Buckley, M.L. Steenberg, H. Barry III and A.A. Manian, J. Pharm. Sci., 62 (1973) 715.
8 F.J. Ayd, in S.J. Enna (Editor), Neuroleptics, Raven Press, New York, 1983, p. 75.
9 G. McKay, J. Geddes, M.J. Cowper and T.S. Gurnsey, Prog. Neuro-Psychopharmacol. Biol. Psychiatry, 7 (1983) 703.
10 T.L. Chan and S. Gershon, Mikrochim. Acta, (1973) 435.
11 P.N. Kaul, M.W. Conway, M.K. Ticku and M.L. Clark, J. Pharm. Sci., 61 (1972) 581.
12 J.W. Hubbard, K.K. Midha, I.J. McGilveray and J.K. Cooper, J. Pharm. Sci., 67 (1978) 1563.
13 S.H. Curry, Anal. Chem., 40 (1968) 1251.
14 C.M. Davis, Clin. Chim. Acta, 78 (1977) 71.
15 M. Linnoila and F. Dorrity, Acta Pharmacol. Toxicol., 42 (1978) 264.
16 D.N. Bailey and J.J. Guba, Clin. Chem., 25 (1979) 1211.
17 R.B. Patel and P.G. Welling, Clin. Chem., 27 (1981) 1781.
18 G. Alfredsson, B. Wode-Helgodt and G. Sedvall, Psychopharmacology, 48 (1976) 123.
19 G. McKay, K. Hall, J.K. Cooper, E.M. Hawes and K.K. Midha, J. Chromatogr., 232 (1982) 275.
20 F. Hartmann, L.D. Gruenke, J.C. Craig and D.M. Bissell, Drug Metab. Dispos., 11 (1983) 244.
21 L.D. Gruenke, J.C. Craig, F.D. Klein, T.L. Nguyen, B.A. Hitzemann, J.W. Holaday, H.H. Loh, L. Braff, A. Fischer, I.D. Glick, F. Hartmann and D.M. Bissell, Biomed. Mass Spectrom., 12 (1985) 707.
22 D.J. Smith, J. Chromatogr. Sci., 19 (1981) 65.
23 J.K. Cooper, G. McKay and K.K. Midha, J. Pharm. Sci., 72 (1983) 1259.

24 W.J. Allender and A.W. Archer, J. Anal. Toxicol., 7 (1983) 203.
25 C.N. Svendsen and E.D. Bird, Psychopharmacology, 90 (1986) 316.
26 R. Ninci, M.G. Giovanni, L. Della Corte and G. Sgaragli, J. Chromatogr., 381 (1986) 315.
27 R. Hartley, I.J. Smith and J.R. Cookman, J. Chromatogr., 342 (1985) 105.
28 P.M. Harrison, A.M. Tonkin, C.M. Cahill and A.J. McLean, J. Chromatogr., 343 (1985) 349.
29 G.S. Lensmeyer and B.L. Fields, Clin. Chem., 31 (1985) 196.
30 J.S. Kiel, Ph.D. Dissertation, The University of South Carolina, Columbia, SC, 1984.
31 E. Usdin, Psychopharmacol. Bull., 6 (1970) 3.
32 J.W. Hubbard, J.K. Cooper, E.M. Hawes, D.J. Jenden, P.R.A. May, M. Martin, G. McKay, T. Van Putten and K.K. Midha, Ther. Drug Monit., 7 (1985) 222.
33 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979, p. 691.
34 R.K. Abramson, C.S. Smith and R. Burns, in preparation.