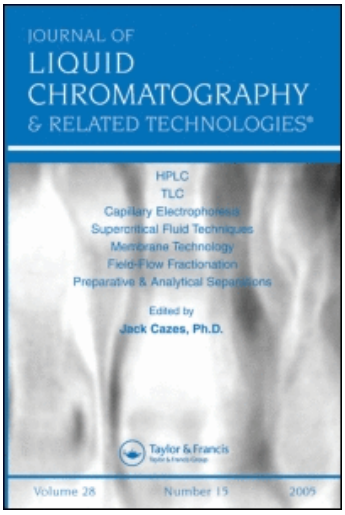

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DEVELOPMENT OF AN HPLC METHOD FOR THE TOXICOLOGICAL SCREENING OF CENTRAL NERVOUS SYSTEM DRUGS

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ABSTRACT

A simple and sensitive HPLC (high performance liquid chromatography) method has been developed for the qualitative and quantitative analysis of several CNS (central nervous system) drugs in clinical and forensic toxicology. The leading conditions were studied, namely parameters such as mobile phase pH, organic modifier percentage, and salt concentration. An isocratic HPLC elution, using a mobile phase composed of acetonitrile and pH 2.8 aqueous tetramethylammonium perchlorate and a C8 reversed phase column as the stationary phase, was found to be convenient for the separation of several CNS drugs, and for their detection and quantitation. The identification of the drugs was assured using their relative retention times, together with the peak area ratios at two different wavelengths (230 and 270 nm). A quick pre-treatment of the plasma samples, based on a SPE (solid phase extraction) procedure, with good extraction efficiency and satisfactory selectivity was developed. Under these conditions, a

mixture of fifteen CNS drugs (including antipsychotics, antidepressants and antiepileptics) and some selected active metabolites, was well separated for identification and quantitative determination purposes.

INTRODUCTION

Almost all of the drugs that act on the Central Nervous System (CNS) can have dangerous and potentially fatal side effects, even at therapeutical doses.[1] As an example, phenothiazine antipsychotic drugs can cause extrapyramidal effects such as tremor and rigidity;[2] tricyclic antidepressants can cause hypertension and arrhythmias;[3] several antiepileptic drugs and the antipsychotic drug clozapine can cause severe hematologic disorders.[4,5] Furthermore, patients who are hospitalized for drug overdose may not be able (or willing) to inform the physician as to what they have taken. In these cases, an analytical method that could identify and determine several CNS drugs would be very useful in order to characterize the damage to the patient and to direct the toxicological treatment. The method to be employed should be reliable and feasible and require small amounts of biological sample and simple, widely available instrumentation.

Most of the papers available in the literature regard the determination of drugs belonging to the same therapeutic class, or drugs that are structurally related.[6-8] The most studied drug classes include antidepressants,[9] neuroleptics,[10] benzodiazepines,[11,12] basic drugs.[13]

These studies are based on the use of complicated procedures (immunoabsorption,[11] gradient elution[14]) or sophisticated instrumentation, such as HPLC (high performance liquid chromatography) with diode array detection,[15,16] capillary electrophoresis,[7] and GC-MS (gas chromatography - mass spectrometry).[17]

Two interesting papers propose simple procedures based on the use of HPLC to identify and determine several antidepressant drugs.[9,18]

We have implemented a simple and fast isocratic HPLC procedure with single wavelength UV detection, which can detect at least 15 different CNS drugs, belonging to three different therapeutic categories, and can simultaneously quantify at least 12 of them. The tested substances were: antipsychotics (olanzapine, clozapine, loxapine, risperidone, haloperidol, fluphenazine and chlorpromazine), antiepileptics (carbamazepine, phenobarbital), and antidepressants (protriptiline, maprotiline, fluoxetine, paroxetine, amitriptiline, and imipramine). This last class is particularly important for toxicologists, because antidepressants are often involved in severe, sometimes fatal intoxications.[19,20] The incidence of antidepressants in sudden deaths submitted for toxicological analysis is reported[9] to be as high as 12%.

Some active metabolites of these drugs, which can contribute to intoxication or to prolonged toxic effects after discontinuation of the administration, were analyzed as well.

This work is a part of a broader study on the development of new analytical methods[21,22] for the clinical and forensic survey of drug toxicity.

EXPERIMENTAL

Chemicals

Eli Lilly Italia S.p.A. (Sesto Fiorentino, Florence, Italy) kindly donated fluoxetine hydrochloride, norfluoxetine hydrochloride, and olanzapine. Novartis Italia S.p.A (Origgio – MI, Italy) kindly donated clozapine, norclozapine, and clozapine N-oxide. Carbamazepine, triprolidine hydrochloride, protriptiline hydrochloride, haloperidol, maprotiline hydrochloride, imipramine hydrochloride, amitriptiline, fluphenazine hydrochloride, chlorpromazine hydrochloride were purchased from Sigma Chemicals. Loxapine succinate, paroxetine hydrochloride hemihydrate, risperidone and phenobarbital were gifts of Lederle Ldt. (Pearl River, N. Y., USA), SmithKline Beecham Pharmaceuticals, Janssen-Cilag Italia (Borgo S. Michele, Latina, Italy), and Rhône-Poulenc Rorer Italia (Milan, Italy), respectively. Carlo Erba (Milan, Italy) produced methanol (MeOH), acetonitrile, 25% (m/m) ammonia, and 65% (m/m) perchloric acid, all of analytical grade. Tetramethylammonium perchlorate and albumin of bovine serum were purchased from Sigma (St. Louis, MO, USA). Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Milford, Mass., USA).

Apparatus and Chromatographic Conditions

The chromatographic system for HPLC analysis was composed of a Jasco (Tokyo, Japan) model PU-980 chromatographic pump and a Jasco UV-975 detector.

For preliminary studies, a UV 6000 LP (TermoQuest, Italy) photodiode array detector was used.

Separations were obtained on a Varian (Harbor City, CA - USA) ResElut (C8 150 x 4.6mm, i.d. 5 μm) reversed phase column. The samples were injected into the column through a 20 μL loop. The mobile phase was prepared as follows: to about 100 mL of ultrapure water, 400 mg of tetramethylammonium perchlorate, and 0.2 mL of 7% (m/m) $HClO_4$ were added; the resulting solution was brought up to pH 2.8 with 2.5% (m/m) ammonia, then diluted to 200 mL with water. This aqueous solution was mixed with 117 mL of acetonitrile, fil-

tered through a Phenomenex membrane filter (47 mm membrane, 0.2 μm, NY), and degassed by an ultrasonic apparatus. The flow rate was kept at 1 mL/min. Data processing was handled by a Waters (Milford, Mass. - USA) model 745 integrator. The column was maintained at room temperature.

A Crison (Barcelona - Spain) MicropH 2000 pHmeter and an ALC (Milan, Italy) model 4225 centrifuge were used.

A Jasco UVIDEC-610 double-beam spectrophotometer was used for preliminary studies, in the choice of the detector wavelengths and for the stability control of the stock solutions.

Procedures

Individual stock solutions (1 mg/mL) of the various drugs were prepared in MeOH and stored at 4°C. They were stable for at least 3 months. The concentrations of the standard solutions, obtained by diluting the stock solutions with methanol, ranged from 10 to 5000 ng/mL, depending on the substance. Triprolidine hydrochloride was used as the internal standard (I. S.). Five separate mixtures were prepared. The first (mixture A) contained the following drugs: phenobarbital, olanzapine, clozapine, risperidone, loxapine, protriptiline, haloperidol, imipramine, amitriptiline, fluoxetine, and chlorpromazine, as well as the I. S.

The second (mixture A') contained the following drugs: phenobarbital, olanzapine, clozapine, risperidone, loxapine, paroxetine, haloperidol, imipramine, amitriptiline, fluoxetine, and chlorpromazine, as well as the I. S. The third one (mixture B) contained: carbamazepine, paroxetine, maprotiline, fluphenazine, and the I. S. The fourth one (mixture B') contained: carbamazepine, protriptiline, maprotiline, fluphenazine, and the I. S. The last one (mixture C) contained clozapine, norclozapine, clozapine N-oxide, fluoxetine, norfluoxetine and the I.S.

Relative retention times (RRT) were calculated by the comparison of each substance's retention time to that of the internal standard. This "tool" was used for the identification of the compounds as well as the absorbance ratio at two wavelengths (230 and 270 nm).

Calibration curves were generated, by means of the least square method, for each compound in different concentration ranges, after plotting each peak area (expressed as arbitrary units) against the respective drug concentration (expressed as ng/mL).

Spectrophotometric Assays

Each substance was subjected to spectrophotometric analysis. For this purpose, the standard solutions were prepared in mobile phase and were analyzed in the 200 - 400 nm range against an empty quartz cuvette as the "blank". These spectra were used for comparison with those obtained by the diode array detector, as well as for stability control.

Plasma Samples

The plasma samples were obtained as follows: blood samples from healthy volunteers and patients subjected to therapy with the described drugs, were drawn into test tubes, containing EDTA, and centrifuged at 3000 rpm for 20 minutes. The supernatant plasma was transferred into test tubes and frozen at -20°C until use.

Some preliminary experiments for the SPE procedure implementation were carried out (for the sake of practicality and simplicity), using samples of "reconstructed" plasma. This was prepared by dissolving 20 mg of KCl, 800 mg of NaCl, 20 mg of KH_2PO_4, 115 mg of Na_2HPO_4 and 4 g of bovine albumin in 100 mL of ultrapure water.

Solid-Phase Extraction (SPE)

For the SPE procedure, Waters Oasis HLB (30 mg - 1 mL) cartridges were used. Cartridges were conditioned with 1 mL of methanol and equilibrated with 1 mL of ultrapure water. Then they were loaded with 250 μL of blank plasma spiked with drugs and diluted with 1 mL of ultrapure water. The cartridges were washed twice with 1 mL of ultrapure water and dried before the final elution. The subsequent elution was carried out with 750 μL of methanol. After elution, the sample was injected into the HPLC.

For the extraction yield data the blank plasma samples were spiked with known amounts of the drug mixture standard solution, then subjected to the SPE procedure and HPLC analysis. The peak areas obtained for various drugs were compared to those obtained injecting the corresponding methanolic standard solution having the same concentration.

RESULTS AND DISCUSSION

In current psychiatric therapy, patients are often subjected to the co-administration of several drugs. As an example, patients under therapy with fluoxetine, an SSRI (selective serotonin re-uptake inhibitor) antidepressant, often take

antiepileptics (as mood stabilizers) and anxiolytics as well. Sometimes, even antipsychotics are used. In order to determine, simultaneously, several drugs in the plasma of patients, we started from our previous work on the HPLC determination of fluoxetine in pharmaceutical formulations[23] and in plasma.[24] We modified several leading conditions, in order to find those best suited for the separation, identification, and quantitation of several other CNS drugs.

Leading Conditions

In order to obtain good resolution of the various peaks, the effect of some parameters, such as the stationary phase lipophilicity and mobile phase pH, saline concentration and organic modifier percentage were investigated.

As the stationary phase, C8 and C18 columns were tried. Using the C18 column, peak tailing was much more of a problem than with the C8 column.

The starting mobile phase was a mixture of acetonitrile and pH 2.6 tetramethylammonium perchlorate.[24]

The pH values varied from 2.5 to 3.5. The influence of pH in this range was small, yet the best resolution was found at pH = 2.8.

The salt concentration was modified from 6.8 to 14.2 mM, and the resulting retention times were reported against the salt concentration (Figure 1). The best peak resolution was obtained with a 12.8 mM buffer concentration. Lower concentrations were not investigated, because the overall duration of the analysis would become too long.

The volume percentage of the organic modifier (acetonitrile), which has a strong effect on the peak resolution, varied from 30 to 55%, and the results were plotted as above, in Figure 2. The best peak resolution was obtained when acetonitrile was 37%.

Thus the leading conditions selected for all analyses were as follows: stationary phase - a C8 (150 x 4.6 mm) reversed phase column; mobile phase - a mixture of acetonitrile and 12.8 mM tetramethylammonium perchlorate (pH 2.8) in a 37:63 ratio, with a flow rate of 1 mL/min.; detection wavelength - 230 nm. The choice of 230 nm as the leading wavelength was made after preliminary spectrophotometric assays. The other λ, 270 nm, was selected from the same preliminary assays for the peak purity control.

These conditions are very different from those reported in the literature,[9] which use detection wavelengths of 220 and 254 nm, a C18 column as the sta-

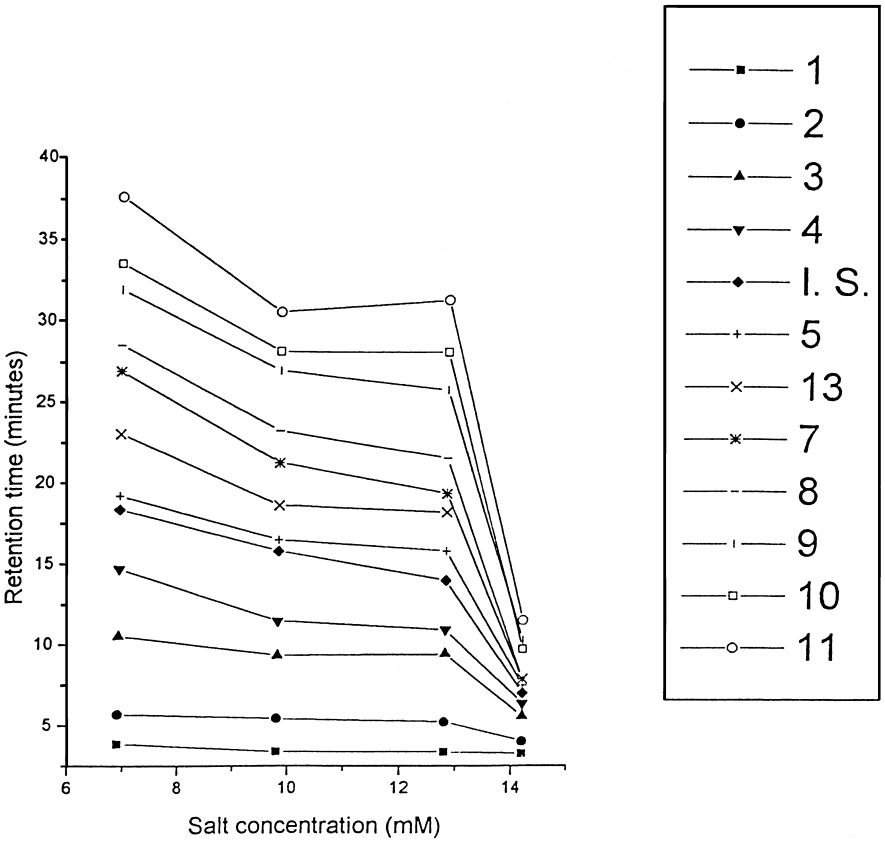


Figure 1. Effect of ammonium perchlorate concentration on analyte separation. The salt concentration varied between 6.9 and 14.2 mM. Numbers indicate: 1. Phenobarbital; 2. Olanzapine; 3. Clozapine; 4. Risperidone; 5. Loxapine; 7. Haloperidol; 8. Imipramine; 9. Amitriptyline; 10. Fluoxetine; 11. Chlorpromazine; 13. Paroxetine; I.S. Triprolidine.

tionary phase, a pH 8.0 acetonitrile-phosphate buffer mixture containing diethylamine as the mobile phase, and a flow rate of 2 mL/min.

Separation of CNS Drugs

Preliminary assays were performed using an HPLC apparatus with a diode array detector. The eleven CNS drugs and triprolidine as the internal standard (I.S.), dissolved in methanol (mixture A), were chromatographically well sepa-

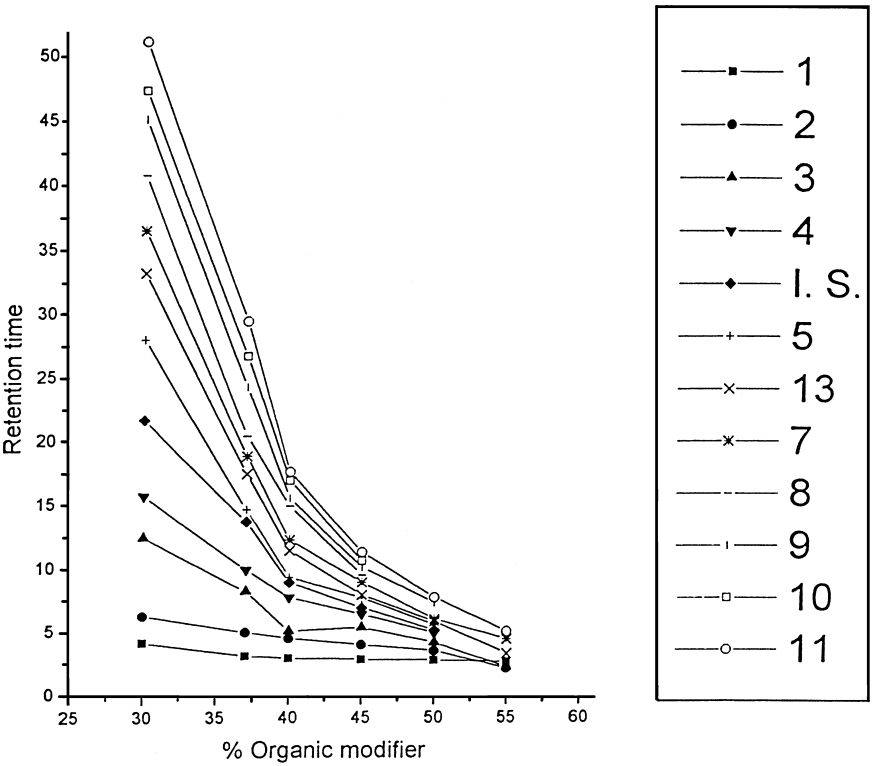
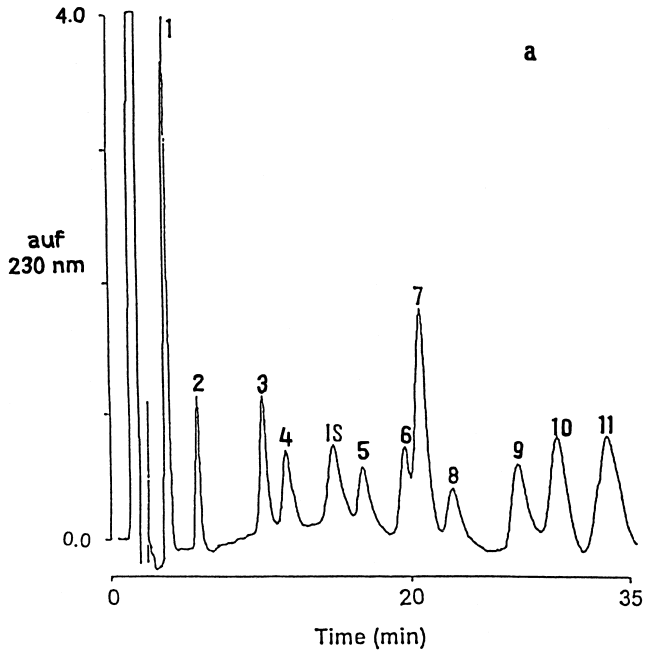


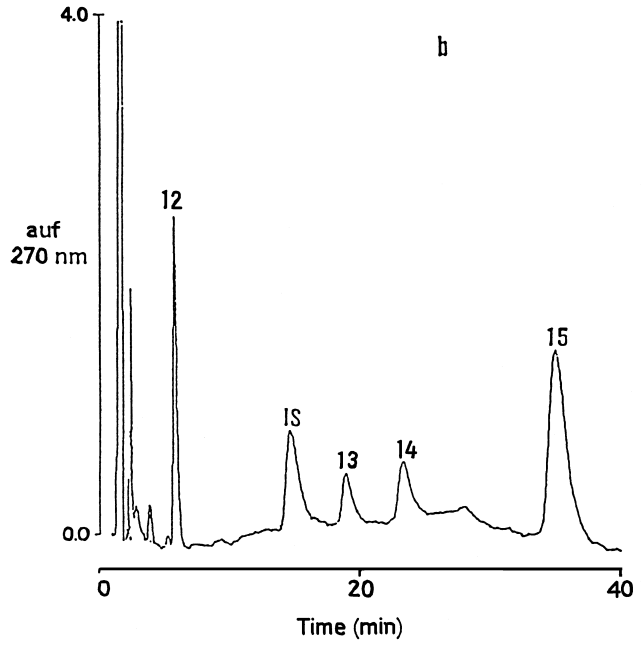
Figure 2. Effect of the percent volume of organic modifier in the mobile phase on analyte separation. The percentage of acetonitrile varied between 30 and 55%. Mobile phase: acetonitrile - aqueous solution of 12.8 mM ammonium perchlorate. Numbers indicate the different drugs, as in Figure 1.

rated and detected at 230 nm, as Figure 3a shows: only peak 6 (corresponding to protriptiline) was very near to peak 7 (corresponding to haloperidol).

For this reason, protriptiline was substituted with paroxetine in subsequent assays. The separation of four other drugs is shown in Figure 3b (mixture B), where the chromatogram was detected at 270 nm.

Figure 3. Chromatograms obtained from a diode array detection HPLC to show the separation and elution order of the analytes at 230 and 270 nm. (a) Mixture of eleven CNS drugs, where number 6 indicates Protriptyline and the other numbers are as in Figure 1. (b) Mixture of four CNS drugs, where number 12 indicates Carbamazepine; 13. Paroxetine; 14. Maprotiline; 15. Fluphenazine; I.S. Triprolidine.





It can be noted that some drugs in the two mixtures have similar retention times, but it is not a problem for the identification because, using the diode-array detector, different substances with the same retention time can often be identified from their spectra, as for example haloperidol and protriptiline. These two substances have similar retention times, but their spectra are morphologically different, with a λ_{max} = 245 nm for haloperidol and λ_{max} = 290 nm for protriptyline, thus they can be identified by the diode-array detector. The quantitation, however, is more difficult.

The availability of an HPLC diode-array apparatus is not very prevalent in clinical laboratories. Furthermore, the sensitivity is notably inferior (about 2-5 times) to a normal HPLC single wavelength detector. For this reason, we decided to use, for our study, an HPLC with single wavelength detector, which is more common, cheaper, and more sensitive. This last parameter is very important if a quantitative analysis of the drugs is also required.

The chromatogram of mixture A' substances, which can be simultaneously detected, is shown in Figure 4a. Every one of them is present in a different concentration, ranging from 100 ng/mL for olanzapine to 600 ng/mL for imipramine. All the substances were eluted between 3.3 minutes (phenobarbital) and 32 minutes (chlorpromazine).

Carbamazepine, maprotiline, protriptyline, and fluphenazine are also detected by this method, but they elute near other substances (olanzapine, imipramine, paroxetine, and chlorpromazine, respectively), therefore, they were analyzed separately (mixture B'). Figure 4b shows the chromatogram of these substances. Moreover, active metabolites of clozapine (namely norclozapine and clozapine N-oxide) and fluoxetine (namely norfluoxetine) were analyzed with this method. The chromatogram of Mixture C, containing the metabolites, their parent drugs, and the I.S., is shown in Figure 4c. In order to have a completely reliable identification of all detectable substances, assays were performed by injecting each standard solution twice, and by setting the detector the first time at 230 nm and the second time at 270 nm. Each substance is unequivocally identified using two parameters: the retention time (relative to the internal standard) and the ratio between the peak area obtained at 230 nm and the peak area obtained at 270 nm (as can be seen from Table 1). In this way, sub-

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Figure 4. Chromatograms obtained from a UV-detection HPLC to show the separation and elution order of the analytes at 230 nm. (a) Mixture of eleven CNS drugs, where the numbers indicate the different drugs as in Figure 1. (b) Mixture of four CNS drugs, where number 6 indicates Protriptyline and the other numbers as in Figure 3b. (c) Mixture of two CNS drugs and their active metabolites, where number 16 indicates Norclozapine; 3. Clozapine; 17. Clozapine N-oxide; 18. Norfluoxetine; 10. Fluoxetine; I.S. Triprolidine.

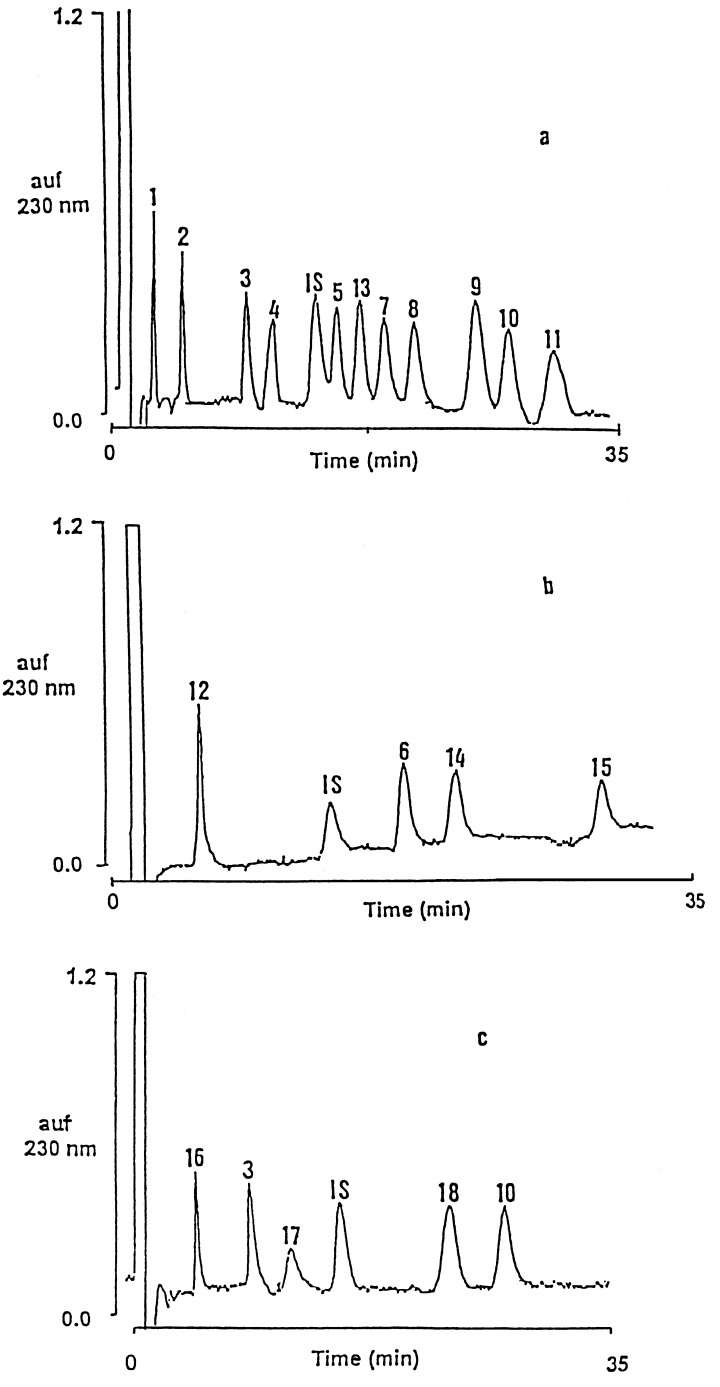

Table 1

Parameters for Drug Identification

Substance	Therap. Category	Retention Time (min.)	Relative Retention Time (RRT)	$Area_{230}/Area_{270}$ Ratio
1. Phenobarbital	antiepileptic	3.3	0.236	7.4
2. Olanzapine	antipsychotic	5.5	0.393	0.9
3. Clozapine	antipsychotic	9.2	0.657	2.1
4. Risperidone	antipsychotic	11.4	0.814	1.6
5. Loxapine	antipsychotic	15.8	1.129	5.0
6. Protriptiline	antidepressant	18.1	1.293	2.5
7. Haloperidol	antipsychotic	19.3	1.379	4.1
8. Imipramine	antidepressant	21.6	1.543	1.1
9. Amitriptiline	antidepressant	25.4	1.814	9.7
10. Fluoxetine	antidepressant	27.8	1.986	10.2
11. Chlorpromazine	antipsychotic	31.2	2.229	3.5
12. Carbamazepine	antiepileptic	5.3	0.379	2.2
13. Paroxetine	antidepressant	17.8	1.271	3.1
14. Maprotiline	antidepressant	21.5	1.536	3.2
15. Fluphenazine	antipsychotic	31.5	2.250	1.6
16. Norclozapine	antipsychotic metabolite	6.1	0.435	1.5
17. Clozapine N-oxide	antipsychotic metabolite	9.6	0.686	2.0
18. Norfluoxetine	antidepressant metabolite	17.0	1.213	16.4
I.S. Triprolidine	internal standard	14.0	1.000	2.8

stances, for example fluphenazine and chlorpromazine, which have very similar retention times and spectra (for which identification with the diode-array detector is rather difficult), can be identified by the different area ratio values.

Calibration curves were constructed for all the substances, by analyzing methanolic standard solutions; peak areas, expressed as arbitrary units, were plotted against analyte concentrations, expressed as ng/mL. The linearity ranges and the least square fitted lines for all the substances are reported in Table 2.

The method's precision was evaluated by injecting the drug mixture six times into the column. The repeatability (intraday) and intermediate precision

Table 2

Parameters for Drug Quantitation

Substance	Linearity (ng/mL)	Straight Line Equation* (y = a + bx) a	b	Rc	Detection Limit (ng/mL)
1. Phenobartital	250 - 1250	878	215	0.9964	100
2. Olanzapine	25 - 125	780	124	0.9956	10
3. Clozapine	25 - 500	-264	166	0.9997	10
4. Risperidone	25 - 250	-335	68	0.9900	20
5. Loxapine	25 - 250	-6.5	88	0.9958	20
6. Protriptiline	25 - 250	-219	178	0.9964	20
7. Haloperidol	62.5 - 625	287	86	0.9945	50
8. Imipramine	37.5 - 750	-213	54	0.9988	30
9. Amitriptiline	50 - 500	-541	126	0.9978	30
10. Fluoxetine	40 - 1000	-1090	168	0.9977	20
11. Chlorpromazine	100 - 500	-547	97	0.9993	50
12. Carbamazepine	100 - 1000	947	150	0.9998	20
13. Paroxetine	300 - 2500	-1045	25	0.9993	200
14. Maprotiline	500 - 5000	-407	24	0.9994	250
15. Fluphenazine	250 - 2500	-2328	92	0.9998	50
16. Norclozapine	25 - 500	-90	154	0.9973	15
17. Clozapine N-oxide	25 - 250	11	143	0.9984	15
18. Norfluoxetine	40 - 1000	-34	162	0.9995	20
I.S. Triprolidine	25 - 500	-809	195	0.9990	20

* **y** = peak area, arbitrary units; **x** = substance concentration, ng/mL; **Rc** = correlation coefficient.

(interday) assays gave good relative standard deviation (RSD%) values, which are shown in Table 3.

The quantitation limits (LOQ) ranged between 20 and 500 ng/mL for olanzapine and maprotiline, respectively, while the detection limits (LOD) were between 10 and 250 ng/mL. It should be noted that toxic plasma concentrations (e.g. in a suicide attempt) are generally very high, and these low quantitation limits are thus very rarely needed.

Table 3

HPLC Method Precision

Substance	Concentration (ng/mL)	RSD%* (Intraday)	RSD%* (Interday)	RSD%* on Plasma (Interday)
1. Phenobarbital	500	3.8	7.5	5.8
2. Olanzapine	100	3.8	8.7	9.6
3. Clozapine	100	3.9	6.1	6.5
4. Risperidone	200	6.5	3.8	5.9
5. Loxapine	200	8.4	6.7	8.7
6. Protriptiline	100	7.8	7.2	8.5
7. Haloperidol	500	5.2	6.2	8.4
8. Imipramine	300	6.4	8.0	8.1
9. Amitriptiline	400	3.6	3.0	4.7
10. Fluoxetine	400	4.1	2.3	4.8
11. Chlorpromazine	400	5.8	2.6	6.1
12. Carbamazepine	200	1.1	9.8	6.3
13. Paroxetine	500	1.3	7.0	8.5
14. Maprotiline	1000	3.0	7.9	8.2
15. Fluphenazine	500	3.0	5.8	6.0
16. Norclozapine	200	1.8	1.8	2.1
17. Clozapine N-oxide	100	4.3	1.4	3.9
18 Norfluoxetine	500	2.9	2.4	3.0
I.S. Triprolidine	400	1.9	6.0	6.2

* RSD% values were obtained from 6 independent assays.

Application to Plasma Samples

In order to apply the method to human plasma, several assays were performed using plasma taken from healthy volunteers. In order to eliminate the matrix interference as completely as possible a SPE procedure was implemented, using Oasis HLB (30 mg - 1 mL) cartridges. The cartridges were loaded with 250 μL of plasma. The elution was carried out with 750 μL of methanol.

The chromatogram of a "blank plasma" sample, subjected to the SPE procedure is reported in Figure 5a, while the chromatogram of the same plasma sample, spiked with mixture A', is reported in Figure 5b. As can be seen, no interfering peak is present in the blank plasma sample chromatogram, and the separation of the analyzed substances is always very good.

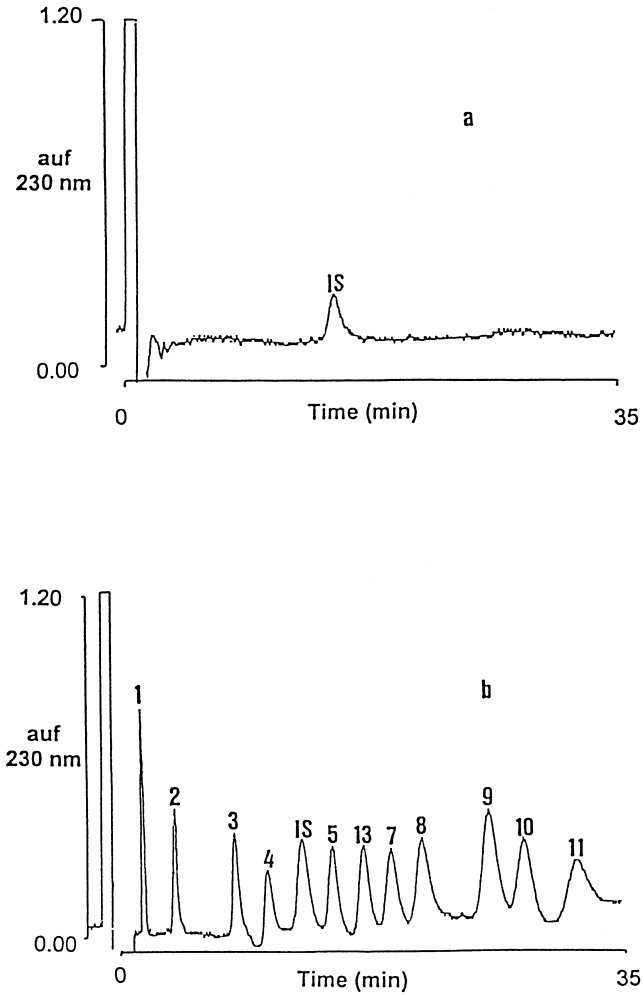

Figure 5. Chromatograms of plasma samples. (a) blank plasma containing triprolidine (I.S.). (b) blank sample, spiked with the same mixture as in Figure 4a.

The retention times and the 230/270 peak area ratios for "blank plasma" samples, have the same values as the standard solutions. Thus, Table 1 was also used for peak identification in plasma.

Extraction studies were performed for the various drugs. Absolute recovery (or extraction yield) was determined by comparing the peak areas of the

Table 4

Absolute Recovery Data

Substance	Concentration (ng/mL)	Percentual Recovery*
1. Phenobarbital	500	98.6
2. Olanzapine	100	94.9
3. Clozapine	100	91.9
4. Risperidone	200	75.1
5. Loxapine	200	100.5
6. Protriptiline	100	78.8
7. Haloperidol	500	83.4
8. Imipramine	600	97.3
9. Amitriptiline	400	90.6
10. Fluoxetine	500	95.8
11. Chlorpromazine	400	97.5
12. Carbamazepine	100	92.0
13. Paroxetine	500	83.1
14. Maprotiline	500	77.2
15. Fluphenazine	200	99.6
16. Norclozapine	200	78.6
17. Clozapine N-oxide	100	96.0
18. Norfluoxetine	500	77.1
I.S. Triprolidine	400	90.0

* Percentual recovery values are the mean of 3 independent assays.

extracted plasma with the corresponding peak areas of standard methanolic solutions. The values of the extraction yields of all analyzed drugs extracted from "blank plasma" resulted to be in the 75% - 99% range, and were constant for each drug (Table 4). These results are highly satisfactory.

The data of the linearity ranges and the least square fitted lines of the calibration curves in plasma are reported in Table 5.

The accuracy and intraday variation of the assays were assessed for each drug by analyzing six "spiked" plasma samples at concentrations corresponding to the upper limit, lower limit, and middle value of the calibration curves.

All results of percent recovery are in the 90 – 101% range, with the respective RSD% (intraday) included between 1.2% (for carbamazepine) and 8.6%

Table 5

Parameters for Drug Quantitation in Plasma

Substance	Linearity (ng/mL)	Straight Line Equation* (y = a + bx) a	b	Rc	LOD (ng/mL)	LOQ (ng/mL)
1. Phenobarbital	250 - 1250	-51.4	1.73	0.999	100	250
2. Olanzapine	25 - 125	-25.6	5.57	0.997	10	25
3. Clozapine	25 - 500	-16.0	6.46	0.999	10	25
4. Risperidone	40 - 250	-27.2	3.03	0.991	10	25
5. Loxapine	40 - 250	-6.5	4.97	0.995	20	40
6. Protriptiline	40 - 250	-17.1	8.15	0.996	20	40
7. Haloperidol	75 - 625	-25.7	2.04	0.994	50	75
8. Imipramine	75 - 750	-31.5	1.83	0.995	30	75
9. Amitriptiline	50 - 500	-14.3	4.52	0.999	30	50
10. Fluoxetine	40 - 1000	0.7	3.03	0.999	20	40
11. Chlorpromazine	100 - 500	-0.6	2.90	0.999	50	100
12. Carbamazepine	100 - 1000	-60.2	1.85	0.000	25	50
13. Paroxetine	300 - 2500	0.7	0.73	0.998	200	300
14. Maprotiline	500 - 5000	-0.5	0.67	0.999	250	500
15. Fluphenazine	250 - 2500	-1.4	1.54	0.998	100	200
16. Norclozapine	25 - 500	-8.9	3.15	0.998	15	25
17. Clozapine N-oxide	25 - 250	1.2	3.0	0.997	15	25

***** **y** = peak area, arbitray units; **x** = substance concentration, ng/mL; **Rc** = correlation coefficient. Note that these equation parameters were obtained from data analyses performed by a Varian Star Chromatography software, and are different from those of the standard calibrations,f or which data analyses were carried out by means of a Waters integrator.

(for loxapine). These intermediate precision values (n = 6) resulted to be very close to those found for standard solutions of the same drugs (Table 3, column 3).

Regarding the quantitation limits of the method, Figure 6 shows an HPLC chromatogram of a sample of extracted plasma showing the peak response at the quantitation limit of each drug. These LOQ values (Table 5) are about the same as standard methanolic solutions.

Analysis of plasma samples of some patients were carried out. The identification of the drugs was performed using data in Table 1. The quantitative determination of drug levels were obtained by interpolating on the respective calibration curves taking into account the absolute recovery data for the necessary mathematical correction. These preliminary data seem to be satisfactory.

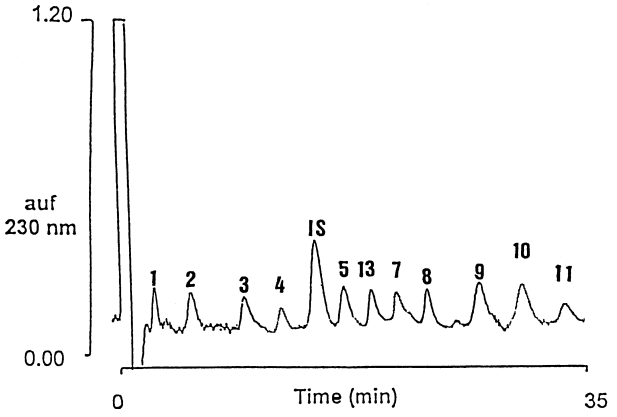

Figure 6. Chromatogram of plasma sample spiked with mixture A' at the quantitation limit.

Stability Studies

Presently stability studies on these CNS drugs are in progress, which have until now demonstrated that all the drugs are stable in stock methanolic solution (1 mg/mL) for at least three months; until now it has been found that the drugs are stable in plasma at the concentrations examined for at least 24 hours.

CONCLUSIONS

The goal of this paper was to develop a simple and reliable method using the same analytical procedure for the separation and identification of several CNS drugs.

The proposed method, based on reversed-phase HPLC with ultraviolet detection, seems to be suitable for the rapid and feasible identification and determination of different CNS drugs in human plasma.

The pre-treatment of the plasma, based on an accurate SPE procedure, needs very small amounts of sample and gives very good extraction yield data while minimizing interference due to the biological matrix.

This method has the advantage of satisfactory sensitivity and precision, and can identify and determine at least fifteen different drugs and some select-

ed metabolites in a short time using a simple and widely available HPLC apparatus.

It may help in confirming or invalidating a difficult diagnosis of intoxication from CNS drugs. Moreover, using this method, the plasma levels of numerous drugs including antidepressants, antipsychotics, and antiepileptics can be monitored.

Assays are in progress, in order to extend the method's application to a wide number of patient plasma samples and to make the method suitable for the identification and determination of other CNS drugs (such as benzodiazepines) often involved in intoxication and poisoning cases.

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