ELSEVIER

Journal of Chromatography B, 673 (1995) 311–315

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Column liquid chromatographic determination of clozapine and N-desmethylclozapine in human serum using solid-phase extraction

Ram N. Gupta

Department of Laboratory Medicine, St. Joseph's Hospital, 50 Charlton Ave. East, Hamilton, Ont. L8N 4A6, Canada

First received 25 April 1995; revised manuscript received 13 June 1995; accepted 13 June 1995

Abstract

A 0.5-ml aliquot of a serum sample, after the addition of a 50-μl aliquot of a 5 μg/ml solution of amoxapine as the internal standard, is vortex-mixed with 0.5 ml of acetonitrile and centrifuged. The supernatant is applied to a 1-ml BondElut C_{18} silica extraction column-conditioned with subsequent washings with 1 *M* HCl, methanol and water. After passing the sample at a slow rate, the column is washed twice with water and once with acetonitrile. The desired compounds are then eluted with a 0.25-ml aliquot of 35% perchloric acid–methanol (1:100, v/v). A 15-μl aliquot of the eluate is injected onto a 150 × 4.6 mm I.D. column packed with 5-μm C_8 silica particles and eluted at ambient temperature with a mobile phase of 0.1% tetramethylammonium perchlorate–acetonitrile (73:27, v/v) adjusted to pH 4.2 with 10% perchloric acid. The peaks are detected with an absorbance detector at 245 nm.

1. Introduction

Clozapine, an atypical neuroleptic is being increasingly used in Canada for the treatment of patients who do not respond to classical neuroleptics. In spite of its high efficacy and absence of extrapyramidal side effects, use of clozapine is restricted due to its potentially high haematological toxicity. Patients receiving clozapine are monitored weekly for their haematological parameters. No clear relationship between serum drug concentrations and toxicity, as yet, has been established although a minimum effective drug concentration of 350 ng/ml has been suggested [1]. However, monitoring serum clozapine concentration is now a common practice in most of the psychiatric clinics.

Clozapine is mainly metabolized to N-desmethylclozapine and clozapine-N-oxide. Pharmacological potency of these metabolites has not been determined. However, N-desmethylclozapine which is present in significant amounts in the serum of patients receiving clozapine, is determined and reported along with the concentration of clozapine. On the other hand, clozapine-N-oxide is present in patient's serum in relatively low amounts, and generally not measured.

Column liquid chromatography (LC) is the most popular technique for the determination of clozapine and N-desmethylclozapine. A number of LC procedures using liquid–liquid extraction [2–8] or solid-phase extraction (SPE) [9–12] have been described. Off-line SPE is now the preferred technique to isolate analytes from biological fluids prior to their determination by

0378-4347/95/$09.50
SSDI 0378-4347(95)00262-6

LC. The present study describes an alternative LC procedure with the use of a convenient SPE procedure recently described for the determination of antidepressants in serum [13] for the simultaneous determination of clozapine and N-desmethylclozapine. In the previously described SPE procedures, the extract was evaporated to dryness to be reconstituted in a small volume of solvent for adequate sensitivity. In the present report, the SPE procedure and the detection of the analytes have been optimized and a sensitive determination of clozapine and N-desmethylclozapine has been achieved without prior evaporation of the extract. Olesen and Poulsen [14] have described an on-line fully automated procedure for the assay of clozapine and N-desmethylclozapine.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Deionized water was distilled in an all-glass still.

Clozapine, N-desmethylclozapine and clozapine-N-oxide were obtained as a gift from Sandoz Canada (Dorval, Que., Canada). Clozapine and N-desmethylclozapine bases were also obtained from Sigma (St. Louis, MO, USA). Amoxapine base was obtained from Lederle Laboratories (American Cyanamid, Pearl River, NY, USA). Stock solutions of clozapine, of its metabolites and amoxapine were prepared separately in methanol at a concentration of 1 mg/ml.

A serum standard of clozapine and N-desmethylclozapine (2000 ng/ml) each was prepared by mixing 100 μl each of clozapine and N-desmethylclozapine stock solutions in a 50-ml volumetric flask and making up to volume with drug-free serum. This standard was serially diluted to prepare eight serum standards. The concentration of the most dilute standard was 15.6 ng/ml.

Working internal standard solution was prepared by mixing 50 μl of stock amoxapine solution with 10 ml of 1% potassium bicarbonate solution. The solution was stored at 4°C for one week and then discarded.

2.2. Extraction

A 0.5-ml aliquot of the sample was mixed with 50 μl of the working internal standard solution and 0.5 ml of acetonitrile in a 12×75 mm glass disposable tube. After centrifugation at 1500 *g* for 3 min, the supernatant was applied to a 1-ml BondElut C_{18} extraction column (Varian Associates, Harbor City, CA, USA) which had been previously activated by washing successively once (1 column volume) with 1 *M* HCl, twice with methanol and once with water. The sample was passed slowly through the column by mild suction. The column was then washed successively twice with water and once with acetonitrile, making sure that each column was drained completely after every wash. The tips of the columns were wiped with tissue and placed on 16×100-mm glass tubes containing correspondingly labelled 1.5-ml plastic sample cups. An aliquot of 0.25 ml of methanol containing 1 ml/100 ml of 35% perchloric acid was applied to each column. The liquid was allowed to pass through the column bed by gravity and finally drained completely by centrifugation at 1000 *g* for 30 s. The cups were covered with aluminium foil and loaded into the autosampler. A 15-μl aliquot of the eluate was injected onto the chromatographic system.

2.3. Chromatography

A modular chromatographic system consisting of a Model LC-6A pump, a Model SPD-10A absorbance detector, a Model Sil-9A autosampler and a Model CR501 integrator plotter (all from Shimadzu, Columbia, MD, USA) was used. A 150×4.6 mm I.D. Ultrasphere Octyl reversed-phase column packed with 5-μm bonded silica particles (Beckman Instruments, San Ramon, CA, USA) protected by a 15×3.2 mm I.D. guard cartridge packed with 7-μm C_8-silica particles (Applied Biosystems, San Jose, CA, USA) was used as the analytical column. The mobile phase of 0.1% aqueous tetramethylammonium perchlorate–acetonitrile (73:27) adjusted to pH 4.2 with 10% perchloric acid was pumped at a flow-rate of 2 ml/min with an operating pressure of 14

MPa. Chromatography was performed at ambient temperature. The peaks were monitored at 245 nm, the wavelength of absorption maximum of clozapine at acidic pH [15].

3. Results and discussion

3.1. Internal standard

A number of compounds were screened for the selection of an optimal internal standard for the determination of clozapine. Amoxapine has a ring structure and absorption spectrum similar to that of clozapine. Amoxapine is not lost during extraction by the described procedure. Some of the compounds, e.g. clonazepam [8] or flurazepam [10] used as internal standards in the previously described solid-phase extraction procedures could not be used as the internal standard in the present procedure because these compounds are weakly basic and are poorly retained by the extraction column in the presence of acetonitrile and further removed during acetonitrile wash step.

3.2. Method validation

The recovery of extraction as determined by comparing peak areas of serum extracts with those of unextracted standards prepared in the elution reagent was ca. 90% (range = 86–96%) for each of the four compounds, i.e. clozapine, desmethylclozapine, clozapine-N-oxide and amoxapine. There was no change in the ratio of peak areas of drug or metabolite/internal standard after extraction. The extracts are stable for at least 8 h when stored at room temperature and for three days when stored at 4°C.

The standard curves are linear for the range tested for clozapine and N-desmethylclozapine from 15.8 to 2000 ng/ml. The linear regression of peak-area ratios of drug/internal standard (y) vs drug concentration (x) are excellent: for clozapine, $y = -0.03 + 1.36x$ ($r = 0.999$) and for N-desmethylclozapine, $y = -0.004 + 0.747x$ ($r = 1.0$).

The chromatogram of an extract of drug-free

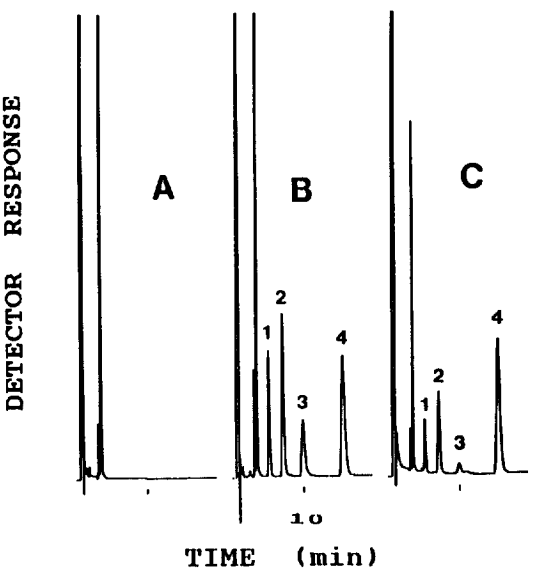

Fig 1. Chromatograms of extracts obtained from: (A) drug-free serum; (B) serum supplemented with 200 ng/ml of N-desmethylclozapine, 300 ng/ml of clozapine and 150 ng/ml of clozapine-N-oxide; (C) serum of patients receiving clozapine. Peaks: 1 = N-desmethylclozapine (5.3 min); 2 = clozapine (7.3 min); 3 = clozapine-N-oxide (10.2 min) and 4 = amoxapine (15.8 min). Detector sensitivity = 0.01 AUFS. Integrator: attenuation = 2; chart speed = 2 mm/min.

serum sample (Fig. 1A) shows an extremely stable baseline. The chromatogram of an extract of a serum standard supplemented with N-desmethylclozapine, clozapine and clozapine-N-oxide (Fig. 1B) shows good separation of all the compounds from one another and from the solvent and the serum peaks. A chromatogram of an extract of serum obtained from a patient receiving 300 mg of clozapine per day does not show any additional peak which could interfere with the quantitation of clozapine or desmethylclozapine. The blood of the patient collected in the morning prior to the administration of the drug showed a trough concentration of 134 ng/ml of clozapine and 72 ng/ml of N-desmethylclozapine.

Analysis of serum supplemented with low (50 ng/ml) and high (1000 ng/ml) amounts of each of clozapine and N-desmethylclozapine showed acceptable precision (Table 1).

The limit of quantitation under the described conditions is 15 ng/ml for both clozapine and

Table 1
Precision and accuracy of the method

	N-Desmethylclozapine			Clozapine		
	Mean (ng/ml)	C.V. (%)	Dev.[a] (%)	Mean (ng/ml)	C.V. (%)	Dev. (%)
Within batch (n = 8)						
Low[b]	55	4	+10	53	4.5	+6
High[c]	1064	2.1	+6.4	1040	1.5	+4
Between batch (n = 10)						
Low	50	7.5	0	50	9.4	0
High	988	3.1	−1.2	1016	1.3	+1.6

[a] Bias from the spiked value.
[b] Spiked concentration = 50 ng/ml.
[c] Spiked concentration = 1000 ng/ml.

N-desmethylclozapine which is quite adequate for therapeutic monitoring. However, the sensitivity of quantitation can be reduced to half by injecting 30 μl of the extract. There is some distortion of peaks when more than 30 μl of the extract is injected.

3.3. Selectivity of the assay

The described procedure shows a high degree of selectivity. Acidic, neutral and weakly basic compounds are eliminated during wash steps with acetonitrile. However, strongly basic drugs, e.g. antidepressants, antihistamines, phenothiazines, antiarrhythmic drugs are co-extracted with clozapine. However, the chromatographic system used in the described procedure is quite selective and the commonly prescribed basic drugs do not interfere with the drug, metabolite or the internal standard peaks. An extract of Ciba-Corning TDM III high-level control (Ciba-Corning Diagnostics, Irvine, CA, USA) which contains 32 drugs at toxic concentrations did not show any peak between 3.5 and 20 min.

4. Conclusion

The described procedure is a convenient alternative LC procedure suitable for monitoring therapeutic concentrations of clozapine and N-desmethylclozapine in routine clinical laboratories.

Acknowledgements

I thank Ms. Nicole Verret of Sandoz Canada (Dorval, Que., Canada) for arranging a gift of pure drug compounds (clozapine, N-desmethylclozapine and clozapine-N-oxide). Technical assistance of Mrs. Maria Stefanec is highly appreciated.

References

[1] P.J. Perry, D.D. Miller, S.V. Arndt and R.J. Cadoret, Am. J. Psychiatry, 148 (1991) 231.
[2] W. Zeren, L. Minglian, X. Peipei and Z. Yanlin, Biomed. Chromatogr., 1 (1986) 53.
[3] C. Haring, C. Humpel, B. Auer, A. Saria, C. Barnas, W. Fleishhacker and H. Hinterhuber, J. Chromatogr., 428 (1988) 160.
[4] C. Humpel, C. Haring and A. Saria, J. Chromatogr., 491 (1989) 235.
[5] M.J. Lovdahl, P.J. Perry and D.D. Miller, Ther. Drug Monit., 13 (1991) 69.
[6] M.-C. Chung, S.-K. Lin, W.-H. Chang and M.W. Jann, J. Chromatogr., 613 (1993) 168.
[7] S.A. Volpicelli, F. Centorrino, P.R. Puopolo, J. Kando, F.R. Frankenburg, R.J. Baldessarini and J.G. Flood, Clin. Chem., 39 (1993) 1656.

[8] P.T. McCarthy, S. Hughes and C. Paton, Biomed. Chromatogr., 9 (1995) 36.
[9] D. Wilhelm and A. Kemper, J. Chromatogr., 525 (1990) 218.
[10] H. Weigmann and C. Hiemke, J. Chromatogr., 583 (1992) 209.
[11] D.J. Freeman, M. Li and K. Oyewumi, Ther. Drug Monit., 15 (1993) 147.
[12] E.O. Fadiran, J. Leslie, M. Fossler and D. Young. J. Pharm. Biomed. Anal. 13 (1995) 185.
[13] R.N. Gupta, J. Liq. Chromatogr., 16 (1993) 2751.
[14] O.V. Olesen and B. Poulsen, J. Chromatogr., 622 (1993) 39.
[15] Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids, and Post-Mortem Material, Pharmaceutical Press, London, 1986, 2nd ed., p. 488.