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Simultaneous quantitation of loxapine, amoxapine and their 7- and 8-hydroxy metabolites in plasma by high-performance liquid chromatography

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ABSTRACT

Loxapine, its N-demethylated metabolite amoxapine, and their 7- and 8-hydroxy metabolites were determined simultaneously in plasma by a simple two-step extraction procedure followed by reversed-phase liquid chromatography. Baseline separation was achieved by a 5-μm Spherisorb C_6 column. The mobile phase consisted of 5 m*M* phosphate buffer (with 14 m*M* orthophosphoric acid)–acetonitrile (with 105 μ*M* nonylamine) (77:23, v/v). Assays of the steady-state plasma samples obtained from seventeen patients on loxapine showed substantial amounts of 8-hydroxy metabolites, lesser amounts of loxapine, amoxapine and 7-hydroxyloxapine and trace amounts of 7-hydroxyamoxapine. As 8-hydroxy metabolites possess only weak dopamine-D_2 blocking activity, the final neuroleptic property of loxapine may be affected significantly by metabolic polymorphism.

INTRODUCTION

Loxapine (LOX) is a dibenzoxazepine neuroleptic, commonly used in the treatment of schizophrenia. Its N-demethylated metabolite, amoxapine (AMX), is an antidepressant by itself and is also widely used in psychiatry. Both compounds are rapidly absorbed and 7- and 8-hydroxy metabolites have been reported [1–4]. *In vitro*, some, but not all of these hydroxylated metabolites possess affinities to serotonin-S_2 and dopamine-D_2 receptors as high as those of their parents [5–10]. Both AMX and LOX also down-regulate serotonin-S_2 receptors acutely [11–13]. As both neuroleptic and antidepressant compounds exhibit metabolic polymorphism, simultaneous determination of LOX, AMX and their metabolites is important in studies of bioavailability, as well as in the evaluation of clinical responses.

Many chromatographic methods for the quantitation of AMX and its metabolites have been reported [1,14–19]. Of these methods, gas chromatographic (GC) or gas chromatographic–mass spectrometric (GC–MS) methods usually involve

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derivatization to improve detector sensitivity and chromatographic separation among metabolites [3]. On the other hand, use of high-performance liquid chromatography (HPLC) is particularly well suited for non-volatile or thermally unstable compounds, and derivatization is not required [20]. Very few articles described the simultaneous quantitation of LOX and its active metabolites. One HPLC methodology was in abstract form without patient plasma data [21]. Two GC papers included patient serum data on some metabolites [3] and the identification of plasma and urinary metabolites [22].

We describe a reversed-phase liquid chromatographic (RP-LC) procedure which can be used to quantitate simultaneously LOX, AMX and their hydroxylated metabolites. We also report plasma drug levels obtained from patients under LOX treatment to illustrate the metabolic patterns one may encounter in clinical practice.

EXPERIMENTAL

Chemicals and reagents

LOX, AMX, 7-hydroxyloxapine (7-OH-LOX), 8-hydroxyloxapine (8-OH-LOX), 7-hydroxyamoxapine (7-OH-AMX) and 8-hydroxyamoxapine (8-OH-AMX) were gifts from American Cyanamid, Lederle Labs. (Pearl River, NY, U.S.A.). 2-Hydroxydesmethylimipramine (2-OH-DMI) was a gift from Ciba-Geigy (Summit, NJ, U.S.A.). [^{3}H]Spiroperidol (S.A., 23.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). All chemicals used were of analytical grade and the chromatographic solvents used were of HPLC grade. Membrane filters (0.2 μm, Pall Ultrafine Filtration, New York, NY, U.S.A.) were used for filtering the mobile phase components. Disposable polypropylene centrifuge tubes (Sarstedt, Quebec, Canada) were used for sample extractions.

Stock solutions of reference standards (equivalent to 1 mg/ml free base) were prepared in ethanol–0.1 *M* hydrochloric acid (1:1, v/v). All solutions were stored below 0°C. Dilutions were made fresh daily for each analysis.

Chromatography

A Waters HPLC system (Waters, Milford, MA, U.S.A.) was used. A multisolvent delivery module (Model 600) was connected to a stainless-steel column (15 cm × 0.46 cm I.D.) packed with 5-μm Spherisorb C_6 material (Chromatography Sciences, Montreal, Canada). Samples were injected through an autosampler (WISP 710B). Peaks were monitored at 210 nm using a variable-wavelength UV detector (Model 481) set at 0.001 a.u.f.s. A Data Module 740 integrator set at a range of 10 mV and a chart speed of 0.2 cm/min was used to record all chromatograms. The mobile phase consisted of 5 m*M* KH_2PO_4 containing 14 m*M* orthophosphoric acid–acetonitrile containing 105 μM nonylamine (77:23, v/v). The flow-rate was at 2.2 ml/min and chromatography was carried out at 35°C.

Extraction

Plasma (2.0 ml) and 0.1 ml of internal standard solution (100 ng of 2-OH-DMI) were added to a polypropylene tube. After mixing, sample was basified with 0.3 ml of 2 *M* carbonate buffer (pH 9.7). A 4.0-ml volume of the extraction solvent (heptane–isopentyl alcohol, 93:7, v/v) was added and the sample was mechanically shaken for 20 min. After centrifugation (5 min at 1600 *g*), the upper organic layer was transferred to a polypropylene tube containing 0.25 ml of 7 m*M* orthophosphoric acid. After vigorous vortexing, the sample was centrifuged for 10 min at 1600 *g*. The top organic layer was carefully aspirated. An acidic aliquot of 0.1 ml or more was injected into the HPLC system.

Clinical samples

Subjects were patients on various doses of LOX at the Clinical Psychopharmacology Unit of the Clarke Institute. Blood samples were obtained by venipuncture with heparinized vacutainers before the morning dosage of medication. All plasma samples were frozen at −70°C until assayed. One patient was switched to AMX after four months of treatment with LOX. Single and serial samples were obtained from seventeen patients (Table III) over different duration of time (four weeks to fourteen months). All samples reported were obtained from subjects after being on a constant dosage for at least seven days.

Binding assays

Rat striatal [^{3}H]spiroperidol (0.2 n*M*) binding was performed as previously described [12], using (+)-butaclamol (1 μM) to define baseline. IC_{50}s, the concentrations of each drug needed to half-inhibit the specific binding of spiropheridol in the radioreceptor assay, for LOX, AMX and their 7-and 8-hydroxy metabolites were determined.

RESULTS

Chromatography

Precision and recovery experiments were carried out by spiking 2-ml drug-free plasma samples. The within-run and between-day coefficients of variation (C.V.s) for LOX and its metabolites are listed in Table I. For all compounds, peak heights varied linearly with concentration to at least 200 ng/ml, with correlation coefficients >0.995. Day-to-day reproducibilities were assessed by analysis of the slopes of the calibration curves for each day. C.V.s for six sets of slopes, ($n = 4$) were all within 10% range. Detection limits (signal-to-noise ratio = 4) ranged from 1 ng (all hydroxy metabolites) to 2 ng (LOX and AMX) injected on-column. Absolute recoveries ranged from 65 to 85% with the exception of 7-OH-AMX and 8-OH-AMX, which were between 45 and 50%. Relative retention times (RRT) of commonly used tricyclic antidepressants and neuroleptics are listed in Table II.

TABLE I

WITHIN-RUN AND BETWEEN-DAY VARIATIONS IN DETERMINATION OF LOX AND ITS METABOLITES (n = 4)

Compound	Mean concentration (ng/ml)	C.V. (%)	Mean concentration (ng/ml)	C.V. (%)
Within-run				
7-OH-AMX	10.3	5.7	48.7	15.0
7-OH-LOX	10.0	2.5	48.1	2.6
8-OH-AMX	20.0	3.1	102.6	3.1
8-OH-LOX	20.2	2.4	101.6	2.1
AMX	9.3	8.2	48.9	4.1
LOX	9.9	12.1	52.4	9.7
Between-day				
7-OH-AMX	10.7	18.0	48.5	5.8
7-OH-LOX	9.7	12.0	49.0	7.1
8-OH-AMX	19.6	7.7	101.5	3.2
8-OH-LOX	19.1	12.0	100.0	7.5
AMX	9.2	6.5	49.3	4.7
LOX	10.6	10.4	52.0	9.0

TABLE II

RELATIVE RETENTION TIMES (RRT) OF COMMON ANTIDEPRESSANTS, NEUROLEPTICS AND THEIR METABOLITES TESTED FOR INTERFERENCE

HPLC conditions were as described in Experimental. RRT values are retention times relative to AMX.

Drug	RRT	Drug	RRT
7-OH-AMX	0.31	Doxepine	1.58
7-OH-LOX	0.38	OH-Clomipramine	1.80
8-OH-AMX	0.42	Desmethylimipramine	2.11
E-OH-Amitriptyline	0.45	Haloperidol	2.16
8-OH-LOX	0.53	Protriptyline	2.46
E-OH-Nortriptyline	0.54	Imipramine	2.50
OH-DMI	0.64	Amitriptyline	2.87
Z-OH-Amitriptyline	0.71	Chlorpromazine	>3.0
OH-Imipramine	0.75	Clomipramine	>3.0
Propranolol	0.83	Desmethylclomipramine	>3.0
Z-OH-Nortriptyline	0.85	Maprotiline	>3.0
AMX	1.00	Nortriptyline	>3.0
OH-Haloperidol	1.35	Thioridazine	>3.0
LOX	1.39	Trimipramine	>3.0
OH-Desmethylclomipramine	1.55	Trifluoperazine	>3.0

TABLE III

SINGLE AND MULTIPLE SAMPLING PLASMA LOXAPINE AND METABOLITES LEVEL

All samples were taken 9–12 h after the last dose. N.D. = Not detected, below 2 ng/ml detection limit.

Patient	Dosage (mg/day)	Concentration (ng/ml)					
		7-OH-AMX	7-OH-LOX	8-OH-AMX	8-OH-LOX	AMX	LOX
1	10	N.D.	N.D.	2.4	3.8	N.D.	N.D.
2	40	N.D.	3.3	10.9	15.1	4.8	12.1
3	50	N.D.	N.D.	8.1	3.5	N.D.	N.D.
4	75	N.D.	8.2	44.6	87.0	N.D.	9.5
5	100	N.D.	13.2	39.0	61.5	6.7	30.5
6	150	N.D.	10.8	102.3	167.3	6.1	43.2
7	150	N.D.	11.1	96.6	185.5	N.D.	23.5
8	150	N.D.	14.9	36.0	118.7	6.8	40.1
9	50	N.D.	22.7	28.5	92.1	2.9	31.2
	55	N.D.	14.4	19.5	69.0	3.1	44.0
10	250	N.D.	56.2	148.6	166.2	16.7	55.6
	300	N.D.	52.1	182.5	136.2	11.6	36.8
11	30	N.D.	4.1	11.1	13.3	2.2	12.0
	45	N.D.	8.2	25.3	41.0	3.5	15.1
12	50	N.D.	4.3	12.8	31.5	2.3	7.0
	75	N.D.	10.1	20.4	59.3	3.2	21.8
13	125	N.D.	8.5	29.5	74.1	2.8	20.2
14	20	N.D.	2.2	3.6	13.8	N.D.	2.7
	30	N.D.	N.D.	8.2	20.9	N.D.	N.D.
	40	N.D.	3.2	8.3	26.7	N.D.	7.5
15	45	N.D.	5.0	17.6	19.5	N.D.	N.D.
	125	N.D.	16.4	42.2	67.5	3.1	14.4
16	50	N.D.	N.D.	21.2	9.5	N.D.	N.D.
	100	N.D.	12.4	39.0	77.3	N.D.	15.2
	125	N.D.	21.4	64.5	104.2	5.1	20.8
	150	N.D.	20.8	64.7	107.5	6.0	9.7
17	10	N.D.	N.D.	6.2	5.3	N.D.	N.D.
	15	N.D.	N.D.	7.1	9.7	N.D.	14.6
	25	N.D.	2.9	11.2	15.5	N.D.	18.0
	80	N.D.	7.2	33.2	39.6	3.7	11.5
	175[a]	12.0	–	196.0	–	18.1	–

[a] Drug given was amoxapine.

Plasma drug and metabolite levels

Plasma drug and metabolite levels of the patients are listed in Table III. 7-OH-AMX was not detected in any of the samples, even when the daily dosage of LOX reached 300 mg. It was, however, detectable when AMX was given at 175 mg daily. 8-Hydroxylation appears to be the predominant metabolic pathway for both AMX and LOX. When LOX was given, both 8-OH-AMX and 8-OH-LOX

predominated in the plasma, with 8-OH-LOX levels exceeding those of 8-OH-AMX for most samples, and also substantially exceeding LOX levels almost in all samples measured. Amoxapine levels ranged roughly between one half to one third of the LOX levels in all samples.

[^{3}H]Spiroperidol displacement

The IC_{50}s ($n = 2$) were 1.6 μM for 8-OH-AMX, 1.3 μM for 8-OH-LOX, 295 nM for AMX, 140 nM for LOX, 13 nM for 7-OH-AMX and 9 nM for 7-OH-LOX.

DISCUSSION

Methodology

A classical liquid–liquid extraction procedure [23] was chosen over solid-phase extraction. The latter usually requires a vacuum-dependent elution device with a limited capacity and pre-packed cartridges. Successful elution of all hydroxy metabolites and parent compound would require meticulous manipulation of elution solvent composition and different types of cartridges. On the other hand, modification of the liquid–liquid extraction can be carried out more easily.

Plasma was basified with carbonate buffer (pH 9.7) instead of sodium hydroxide which has been reported to reduce chlorpromazine N-oxide to chlorpromazine [24]. The presence of loxapine N-oxide in human serum has been reported by Cooper and Kelly [3]. Since compounds of interest are weakly basic, extraction into heptane and back-extraction into dilute orthophosphoric acid removed most acidic and neutral interfering substances. Addition of isopentyl alcohol to heptane minimized emulsion and increased the extraction recovery of hydroxylated metabolites. A second heptane extraction increased the recovery by less than 10% and was, therefore, omitted. Polypropylene tubes were used to eliminate glass adsorption problems.

RP-LC was used because of its great versatility, resistance to contamination and shorter equilibration time. Normal-phase chromatography is less suitable, due to the high affinity of endogenous hydrophilic plasma constituents and hydroxylated metabolites for the polar silica packing material, which leads to long retention. Interactions between the hydroxylated metabolites and residual free silanol groups of the RP stationary phases, such as peak tailing, was not observed (Fig. 1A). Another advantage is that the acid back-extract can be injected directly into the chromatograph.

In our laboratory, several types of RP columns (LC-1, C_{18} and CN) were used with different mobile phases containing varying concentrations (0.001–0.3%) of alkylamines (propylamine, triethylamine, hexylamine, nonylamine and trihexylamine) for ionization suppression. None gave a baseline separation of all compounds and a sufficient separation of 7-OH-AMX from polar endogenous plasma interferences. The combination of a Spherisorb C_6 column with the addition of a

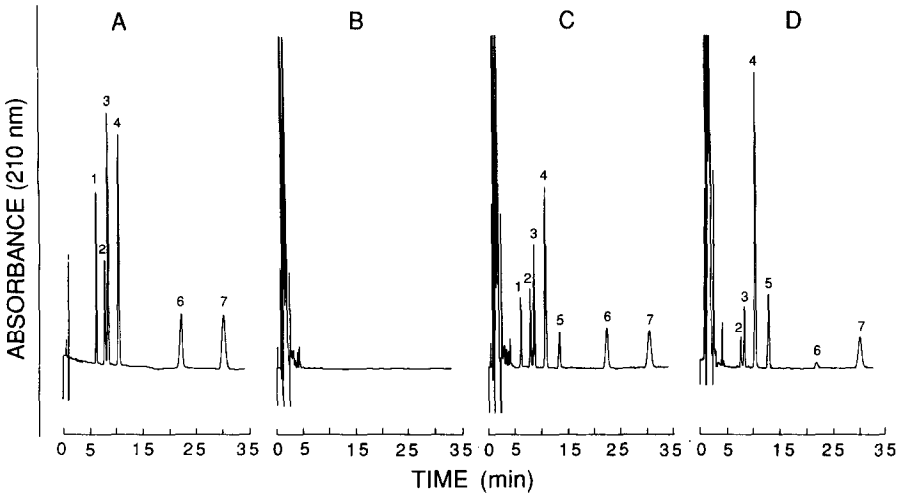

Fig. 1. Sample chromatograms of 0.1-ml injections of: (A) unextracted reference standards: 50 ng 7-OH-AMX (1), 7-OH-LOX (2), AMX (6) and LOX (7) and 100 ng 8-OH-AMX (3) and 8-OH-LOX (4); (B) a drug-free plasma blank; (c) a spiked 2-ml plasma sample containing 100 ng 7-OH-AMX (1), 7-OH-LOX (2), 2-OH-DMI (5), AMX (6) and LOX (7) and 200 mg 8-OH-AMX (3) and 8-OH-LOX (4); (D) a 2-ml plasma sample from a patient receiving 125 mg LOX per day: 6.9 ng 7-OH-LOX (2), 29.0 ng 8-OH-AMX (3), 70.8 ng 8-OH-LOX (4), 3.3 ng AMX (6) and 21.6 ng LOX (7). Details of HPLC conditions are listed in text.

trace amount of nonylamine (24 μM final) in the mobile phase (5 mM KH_2PO_4–acetonitrile, 77:23, v/v) enhanced peak symmetry and resolution among peaks (Fig. 1C). Higher concentration of nonylamine would reduce the retention of all analytes, resulting in fused peaks. Column temperature was raised to 35°C to shorten retention and increase peak response.

Ultraviolet absorbance was monitored at 210 nm which is about three times higher than in the 240–260 nm region. As shown in Fig. 1B, no interfering endogenous peaks were observed from the plasma blank extract at such a low wavelength.

Fig. 2 shows a typical set of six calibration curves based on the peak-height ratios of the six sets of reference standards *versus* internal standard. Concentrations of the 8-hydroxy metabolites were doubled to cover the much higher concentration ranges in plasma specimens. Since a non-weighted linear regression curve was used, the number of low standards was increased to improve quantitation accuracy on low values.

Patient data

Our data show that patients given the neuroleptic LOX metabolized the parent drug predominantly into the 8-hydroxylated metabolites. 7-OH-LOX also appeared to be a significant metabolite and its level at times reached that of its

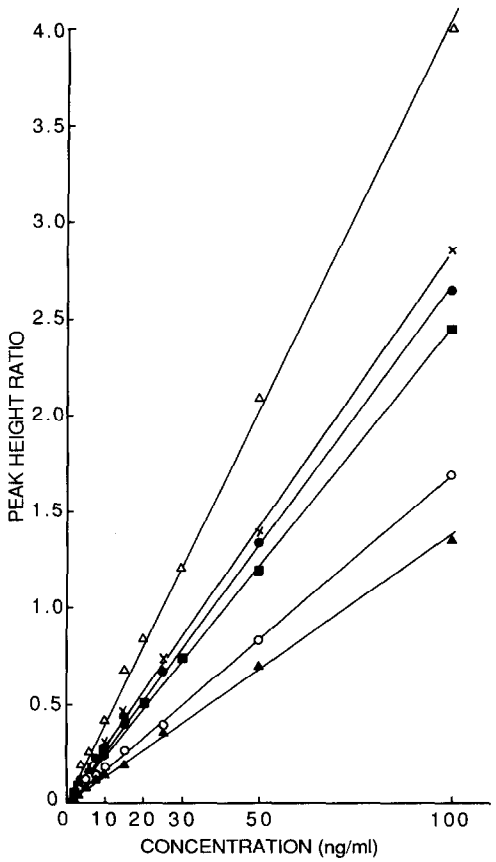

Fig. 2. Peak-height ratio as a function of drug concentrations for 7-OH-AMX (●), 7-OH-LOX (×), 8-OH-AMX (■), 8-OH-LOX (△), AMX (○) and LOX (▲).

parent LOX. Amoxapine appears to be a minor metabolite. Both 8-hydroxylated metabolites were measurable in samples even when the parent compound and the other metabolites were undetectable. Apparently, 7-OH-AMX represents a minor metabolite of both LOX and AMX [4]. A similar conclusion was reached when data from amoxapine overdoses were examined [25]. As 7-OH-AMX was reported in the urine samples from these overdosed patients, there is also the unlikely possibility that the kidney is the site of 7-hydroxylation. Most likely, however, it represents only an accumulative effect.

Previous studies, employing rat striatal [^{3}H]spiroperidol binding displacement and dopamine-stimulated adenylate cyclase assays, clearly demonstrated that hydroxylation of both AMX and LOX at the 7 position enhances its potency as a dopamine receptor blocker. However, the dopaminergic blockade properties of the 8-hydroxylated metabolites are not as clear. They were shown to be weak in one study [7], but strong in another [10]. Since 8-OH-LOX and 8-OH-AMX

together represent the major metabolites of loxapine, it was an important point to clarify. In our laboratory, we were able to confirm the findings of Coupet and Rauh [7]. Since the 8-hydroxylated metabolites have low affinities for the dopamine-D_2 receptors, metabolic polymorphism may affect the net neuroleptic activity of LOX in some subjects. On the other hand, down regulation of serotonin-S_2 receptors, inhibition of norepinephrine and serotonin uptake and [^{3}H]imipramine binding are indicators of possible antidepressant properties. LOX, AMX and their hydroxylated products all seem to possess moderate to high potency on most of these parameters [5–7,10,11,26]. Thus, metabolic polymorphism should not affect the antidepressant property of LOX to a significant extent.

In summary it appears that metabolic polymorphism of LOX may alter its net dopaminergic properties, while affecting its antidepressant properties to a lesser extent. Our HPLC method enables the simultaneous quantitation of all the major metabolites of LOX. Monitoring the plasma drug levels of schizophrenic patients during the course of treatment should contribute to a better titration of the LOX dosage or assist in the decision of switching to another neuroleptic.

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