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Short Communication

A high-performance liquid chromatographic method for the analysis of amoxapine in human plasma

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Introduction

Amoxapine, like its methylated analogue, loxapine, is a dibenzoxazepine compound that exhibits anti-depressant activities similar to those of classical tricyclic antidepressants; amitryptyline and imipramine [1, 2]. Therapeutic doses of anoxapine range from 150 to 300 mg day^{-1}. The drug is extensively metabolized and undergoes a significant first pass effect.

Two major metabolites are found in the blood of subjects: 8-hydroxyamoxapine, which is pharmacologically active, and 7-hydroxyamoxapine, to which most of the side effects are attributed [3, 4]. There are reports pointing to the wide interindividual variations in concentration levels of the drug and its metabolites [5]. The drug itself has raised significant analytical interest. A GC method used by Calvo *et al.* [6] provided most of the useful pharmacokinetic data, and also provided the lowest detection limits, 0.2 ng ml^{-1}, for all three analytes.

A number of HPLC methods have been developed, mostly for therapeutic drug monitoring purposes and usually they have been characterized by rather poor sensitivity for amoxapine, achieving detection limits of only 10 to 50 ng ml^{-1} [7–9]. One of the published methods [10] reported 3 ng ml^{-1} as the limit of quantification, while another HPLC method employing an electrochemical detector has a limit of quantification of 5 ng ml^{-1} [11].

The goal of this study was two-fold. The first objective was to develop a quick and sensitive amoxapine assay in human plasma, which could be used for pharmacokinetic and relative bioavailability purposes. The second objective was to use this assay to

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evaluate inter- and intra-subject variability of amoxapine after administration of a single dose of the drug.

Experimental

Reagents

Amoxapine was obtained from Chelsea Laboratories (Lakeview, NY, USA). Nortryptyline hydrochloride (internal standard) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were HPLC grade and were used without further purification.

Apparatus

The chromatographic system consisted of a Waters Model 590 Programmable Solvent delivery module, a Waters WISP 710B autosampler, Model 481 UV detector; all from Waters (Milford, MA, USA). The column (5 μm, 4.6 mm i.d. × 150 mm) was packed with ODS-2 Spherisorb, obtained from Phase Sep (Norfolk, CT, USA). Chromatograms were recorded by SP 4270 integrator (Spectra-Physics, San Jose, CA, USA) and data were collected and reduced on a Spectra-Physics Chrom Station. In one part of the project, a photo diode-array detector (Polychrom 9060; Varian, Walnut Creek, CA, USA) was also used.

Chromatographic conditions

The mobile phase was prepared by mixing 2000 ml of 0.15% triethylamine (adjusted to pH 3.0 with 85% H_3PO_4) with 2000 ml of acetonitrile. The mobile phase was filtered through a 0.45-μm nylon filter. The flow rate was 1.5 ml min^{-1}, which resulted in a back-pressure of approximately 1400 psi (96 bar). The detector was set to 212 nm and 0.005 AUFS. Retention times were 4.6 and 7.3 min for the amoxapine and the internal standard, respectively. The total chromatographic run time was 9 min and the chromatographic system was maintained at room temperature (22 ± 3°C).

Standard and quality control preparation

The standards and quality control (QC) samples were prepared by adding appropriate volumes of an aqueous solution of amoxapine to human plasma containing EDTA as an anti-coagulant. The volume added was ≤2% of total volume of the sample so that the integrity of plasma was maintained. Quality control samples were prepared using separately weighed stock solutions. After aliquoting, 1 ml samples were stored at −80°C until analysed.

Clinical pharmacokinetic study

Six healthy male volunteers, 18–45 years of age, weighing at least 60 kg and not receiving any medication for the 7 days preceding the study participated in the project after giving a written informed consent. A single dose of 150 mg of amoxapine (Asendin®, Lederle, USA) was administered orally with 250 ml of water, and blood samples were collected 0.0, 0.33, 0.67, 1.0, 1.5, 2, 3, 4, 6, 8, 12, 16, 24 and 32 h after dosing. The same panel of volunteers took part in the second period of the project after 14 days of wash-out. The second period was conducted exactly the same way as the first. Ethical aspects of this study were considered and approved by the Institutional Review Board.

Method of extraction

Internal standard was added (200 μl of nortryptyline hydrochloride, 1 μg ml^{-1}) to a standard, QC sample or clinical sample, followed by 200 μl of 5 M NaOH, and the mixture was vortexed. After adding 6 ml of 1-chlorobutane, the tubes were shaken for 20 min on a reciprocal shaker at 150 rpm and then centrifuged for 10 min at 1000 *g*. The upper organic layer was transferred to conical tubes containing 300 μl of 0.1 M hydrochloric acid. The tubes were shaken and centrifuged again. The upper organic layer was aspirated, and the aqueous phase (150 μl) was injected into the chromatographic system.

Results and Discussion

Method development and assay validation

Precision and accuracy. A set of seven calibration standards, a set of three duplicate QC samples, a zero and a blank were analysed with every batch of clinical samples. The between-run precision and accuracy of the assay are shown in Table 1. Linear response of amoxapine and internal standard peak height ratio was observed over the concentration range 2.0–202.4 ng ml^{-1}. A linear-regression analysis using a least-squares fit was performed with the data weighted according to the reciprocal of the drug concentration. The correlation coefficients, an indication of linearity, were ≥ 0.9985 (for six separate curves). The RSD $\leq 7.2\%$ ($n = 6$) and the deviation from nominal concentration, a measure of accuracy, was $\leq 10.8\%$ at the lower limit of quantification (2.0 ng ml^{-1}).

Table 1
Between-run precision and accuracy for amoxapine in human plasma

Nominal concentration (ng ml^{-1})	Mean (ng ml^{-1})	SD	RSD (%)	% Nominal	*n*
2.0	2.22	0.160	7.2	110.8	6
10.1	9.30	0.352	3.8	92.1	6
25.3	24.78	1.665	6.7	97.9	5
50.6	51.43	2.486	4.8	101.6	6
75.9	72.06	1.577	2.2	94.9	5
101	100.2	3.94	3.9	99.0	5
202	206.6	4.74	2.3	102.1	6

Recovery. Recovery was calculated by comparing the QC with a separately prepared calibration curve, which allowed a correction to be made for all the losses in volume due to sample transfer. The recovery of amoxapine at 15 ng ml^{-1} was 89% (RSD = 3.7%, $n = 6$).

Chromatography. Chromatograms obtained while using the described procedure are shown in Fig. 1. Panel A shows a blank plasma, panel B shows a subject plasma sample, 4.0 h after drug administration, and panel C represents a plasma spiked at a concentration of 10.1 ng ml^{-1}. Plasma was collected from 10 healthy donors and screened for interference at the retention times of amoxapine and internal standard. No significant interference was observed in drug-free plasma samples.

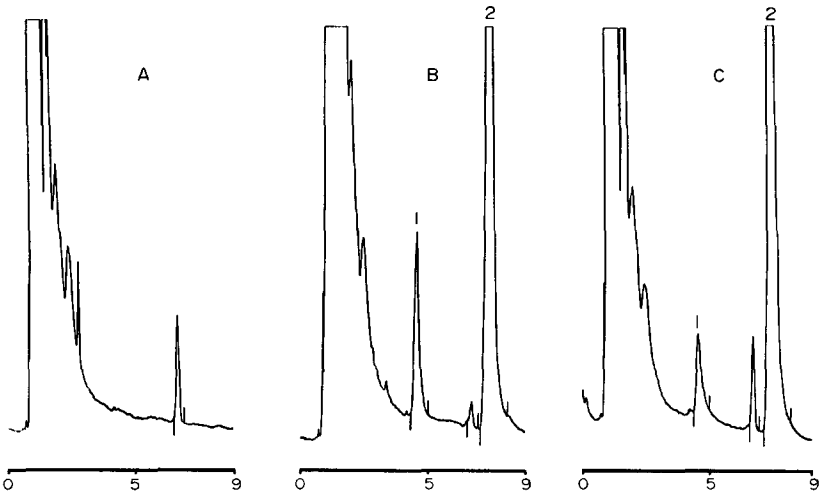

Figure 1
Representative chromatograms obtained from a blank plasma (A), a volunteer plasma 4.0 h after the 150 mg amoxapine dose (B), a standard spiked at concentration 10.1 ng ml^{-1} (C). Retention times: 1, amoxapine — 4.6 min; 2, nortryptyline (off-scale) — 7.3 min. Chromatographic conditions: wavelength, 212 nm, 0.005 AUFS. Mobile phase: 50% acetonitrile, 50% triethylamine phosphate buffer, pH 3.0. Flow rate, 1.5 ml min^{-1}.

Comparison with other amoxapine HPLC methods

Most of the previous amoxapine HPLC methods were developed for therapeutic drug monitoring, offered poor sensitivity and were not without weaknesses. The method by Tasset [7] reported a limit of quantification (LOQ) of 50 ng ml^{-1}, involved a very elaborate sample workout (solid-phase extraction, back extraction, organic phase evaporation) and was characterized by a short column life. The separation between the drug and the metabolites was very good, and run time was 7–8 min. The method by Ketchum [8] offered 10 ng ml^{-1} as the LOQ, no baseline separation between peaks of interest, problems with durability of columns and a long retention time of about 24 min. The assay described by Kobayashi [9] had certain advantages, such as a very good separation, simple extraction procedure and a short run time of 10 min. The only disadvantage was the LOQ of 10 ng ml^{-1}.

Beierle's method [10] reported an LOQ of 3 ng ml^{-1}, however, the separation of the drug and metabolites was very poor. The method involved rather simple solid-phase extraction and had a very short run time of 6 min. The amperometric detection system used by Suckow [11] allowed a reduction in LOQ (5 ng ml^{-1}), as well as simple sample preparation (back extraction) and an acceptable run time of ca. 15 min. Unfortunately, amperometric detection is not universally available and the object of this study was to develop an HPLC method with UV detection.

The present study focused on the parent drug only in order to optimize the conditions and achieve the greatest sensitivity, which would render the method suitable for pharmacokinetic purposes. To obtain meaningful pharmacokinetic data, the concentration of a drug has to be monitored for the period of time equal to at least 3–4 half-lives. This meant that an LOQ of 5–10% of the peak concentration (C_{max}) was needed.

Figure 2 shows that C_{max} values of 50–80 ng ml^{-1} were achieved following oral dosing with 150 mg of amoxapine. Therefore, the LOQ of 2 ng ml^{-1} (s/n = 14; RSD $\leq$ 7.2%)

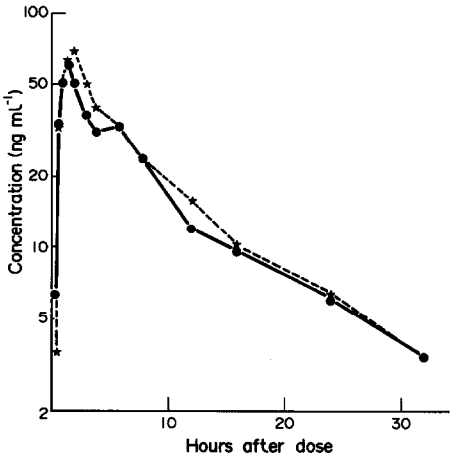

Figure 2
Mean plasma profiles after administration of 150 mg of amoxapine to six volunteers: –●–, period 1; --★--, period 2.

obtained with the present method was adequate for the analysis of pharmacokinetic samples.

During the course of this project, 244 samples (standards, quality controls and clinical samples) were analysed. Only seven samples (<3%) were rejected for reasons of poor chromatography, or standards or QCs outside of acceptance criteria. The method is rapid and, with automation, about 100 samples a day can be analysed.

Identification of amoxapine

Identification of analytes in chromatography may be accomplished by comparing retention times of standards with retention times in an unknown sample. In this present study an additional test was needed to prove that the metabolites of amoxapine did not co-elute with amoxapine because authentic samples were not available. In order to do this, a number of standards and clinical samples were extracted and injected into the system, equipped with a diode-array detector. This detector provided a "purity parameter", which is a mathematical reduction of all the spectral information to a single number and described as a type of weighted average wavelength [12]. Beierle [10] has shown that the UV spectra of the amoxapine and 8-hydroxyamoxapine differ significantly; the parent drug shows maxima at ca. 209, 252 and 298 nm, while the metabolite shows maxima at 213 and 267 nm and lack of maximum at ca. 300 nm.

The purity parameter at the peak apex was taken into consideration. Standards provided the mean value of purity parameter; 215.29 (SD = 1.786, n = 6), while clinical samples 214.45 (SD = 1.519, n = 18). The difference between them is not statistically significant (t-test, P = 0.05) and there was no reason to believe that either of the metabolites co-eluted with amoxapine. Moreover, these metabolites are much more polar than the parent drug and can be expected to elute before the parent drug in reversed-phase HPLC systems [7–9, 11].

Pharmacokinetic application

Figure 2 shows that the present method is applicable to pharmacokinetic studies in man and from the data the following parameters were determined: area under the curve (AUC), area under the curve extrapolated (AUC_∞), peak concentration (C_{max}), time to

Table 2
Pharmacokinetic parameters for amoxapine in human plasma

Subject	Period	AUC 0–32 (ng h ml^{-1})	AUC$_\infty$ (ng h ml^{-1})	C_{max} (ng ml^{-1})	t_{max} (h)	k_{el}	$t_{0.5}$
1	1	805.67	880.83	62.2	4.00	0.084	8.27
	2	1010.04	1092.34	111.3	2.00	0.084	8.27
2	1	419.43	452.08	50.8	1.50	0.070	9.84
	2	319.74	357.24	36.8	1.50	0.080	8.70
3	1	465.71	503.94	72.6	1.50	0.076	9.14
	2	437.57	469.32	45.2	2.00	0.082	8.46
4	1	211.88	224.70	73.6	0.67	0.096	7.21
	2	222.57	234.61	61.9	1.00	0.103	6.74
5	1	677.59	806.11	104.41	1.50	0.051	13.70
	2	878.94	959.85	138.51	2.00	0.070	9.84
6	1	378.46	420.43	104.31	0.67	0.064	10.77
	2	447.52	488.97	81.40	1.00	0.068	10.26
Mean (±SD)	1	493.12 (214.54)	548.01 (248.73)	78.02 (22.02)	1.64 (1.23)	0.074 (0.016)	9.43
	2	552.73 (317.23)	600.39 (344.60)	79.18 (39.51)	1.58 (0.49)	0.081 (0.013)	8.56

the peak (t_{max}), elimination rate constant (k_{el}) and elimination half-life ($t_{0.5}$). The trapezoidal method was used to calculate the AUC until the final detectable plasma concentration. The residual area, extrapolated to infinity, was added to the AUC, calculated by the dividing of the final concentration by k_{el}. The results are summarized in Table 2, while the mean plasma profiles are shown in Fig. 2.

Intra-subject variations in AUC$_\infty$, C_{max}, t_{max} and $t_{0.5}$ were 126, 179, 200 and 139%, respectively. Inter-subject variation was much greater, being 485, 376, 597 and 203%, respectively. On the other hand, the study showed that there was no statistically significant difference (*t*-test, $P = 0.05$) in the main pharmacokinetic parameters determined in the first period compared with the second one. Moreover, there was a good agreement between these data and those published by Calvo *et al.* [6] after administration of 100 mg amoxapine ($t_{0.5}$ = 9.80 h, t_{max} = 1.55 h, C_{max} = 51.50 ng ml^{-1}, AUC$_\infty$ = 654.00 ng h ml^{-1}).

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