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REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF LOW CONCENTRATIONS OF HALOPERIDOL IN PLASMA

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SUMMARY

A sensitive liquid chromatographic method for the determination of haloperidol in plasma is described. The efficient and simple extraction procedure, followed by reversed-phase ion-pair liquid chromatography on a 3-μm octadecylsilica column and UV absorbance detection, makes it possible to determine concentrations down to 0.5 nmol/l with acceptable precision. In a pharmacokinetic study, in which 5 mg of haloperidol were given orally, the plasma levels were followed for 48 h.

INTRODUCTION

A number of methods for the determination of haloperidol in plasma or serum have been published. The techniques used are gas chromatography with electron-capture [1] or nitrogen detection [2–4], gas chromatography in combination with mass spectrometry [5] and liquid chromatographic methods with UV absorption [6–13] or electrochemical detection [14]. Also, non-selective methods such as radioimmunoassay [15] and radioreceptor assay [16] have been used.

Haloperidol appears to have a therapeutic window between roughly 15 and 50 nmol/l [17,18]. The limit of quantitation for most of the selective methods is in the range 5–20 nmol/l, thus making the determination of steady-state levels uncertain. For calculation of pharmacokinetic parameters, it is necessary to be able to determine concentrations as low as 1–2 nmol/l. Of the published chromatographic methods, at present only gas chromatographic methods offer this sensitivity [3,5]. Further, the published methods are generally laborious, utilizing extraction procedures with up to ten steps, making the sample capacity low.

This paper describes a very sensitive, yet simple, method based on reversed-phase liquid chromatography. The high sensitivity was accomplished by a combination of an efficient extraction procedure, a short column packed with small

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(3 μm) particles and the use of modern instrumentation. The simple sample work-up was favourable for the sample capacity. It was possible to determine concentrations down to 0.5 nmol/l with acceptable precision [coefficient of variation (C.V.) 10%], and at 30 nmol/l the C.V. was 1.0%. The method was primarily used in a pharmacokinetic study in which a single dose of 5 mg of haloperidol was administered and the haloperidol levels were followed for 48 h.

EXPERIMENTAL

Materials and reagents

Haloperidol was obtained from Janssen Pharmaceutica (Beerse, Belgium). The internal standard, chlorohaloperidol, was a gift from Huddinge Hospital (Huddinge, Sweden). N,N-Dimethyloctylamine (DMOA) was purchased from ICN Biomedicals (Plainview, NY, U.S.A.) and sodium octyl sulphate from Research Plus (Bayonne and Denville, NJ, U.S.A.). Other chemicals were of HPLC or analytical-reagent grade obtained from the usual commercial sources and used as received.

Standard solutions of haloperidol and chlorohaloperidol were prepared in phosphate buffer (pH 2) and stored in a refrigerator. New standard solutions were prepared every month.

Blood samples were taken in Venoject tubes and plasma was separated from red blood cells within 30 min. The samples were stored at −20 °C until the time of analysis.

Extraction procedure

To each sample tube, containing 2 ml plasma, the internal standard was added followed by sodium hydroxide solution and an organic phase. After agitating the tubes in a rotating mixer they were centrifuged and then placed in a freezer at −20 °C for 1–2 h to break the emulsions that were usually formed. After centrifugation, the organic layer was transferred into a glass tube and evaporated to dryness under a gentle stream of air at 40 °C. The residue was dissolved in phosphate buffer (pH 2) and organic phase was added. After brief (5–10 s) mixing on a Vortex mixer, the aqueous layer was transferred into the autosampler vial and injected into the liquid chromatographic system. The details of the extraction procedure are shown in Fig. 1.

The absolute recoveries were obtained by comparing the slopes of the calibration graphs for directly injected standard solutions and extracted plasma standard samples.

Liquid chromatographic system

The solvent delivery system was an LKB 2150 pump. Sample injection was performed with a Perkin-Elmer ISS-100 autosampler equipped with a 265-μl sample loop. The column (100 mm × 4.6 mm I.D.) was factory-packed with Nucleosil 120-3 C_{18} (Macherey & Nagel, Düren, F.R.G.). A Perkin-Elmer LC-95 UV absorbance detector was used. The detector was equipped with a 4.5-μl flow-cell and operated at 248 nm with a 0.1-s response time. The chromatograms were

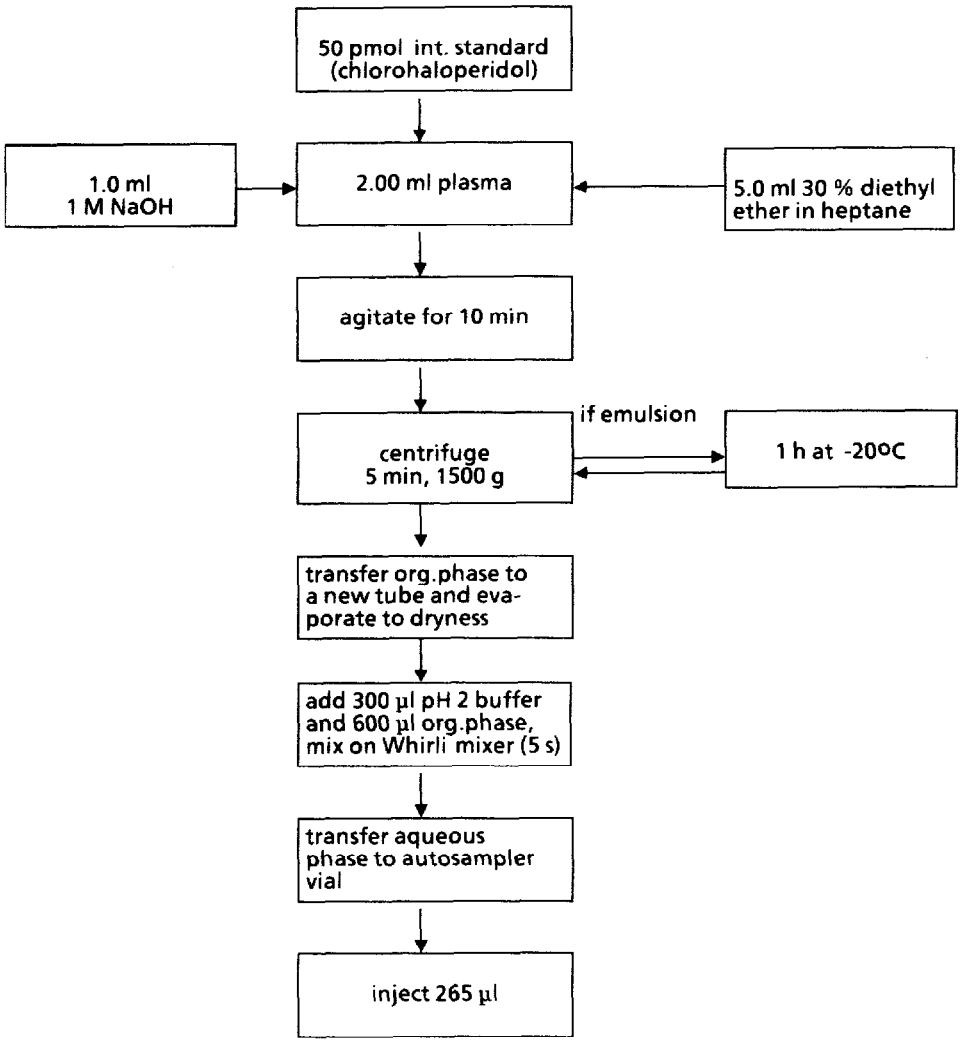

Fig. 1. Scheme of the extraction procedure.

recorded and analysed with a Hewlett-Packard 3390A integrator where peak-height measurements were used. For the determinations of the instrumental limit of detection (ILOD), the signal and the noise (peak-to-peak noise) were measured with the integrator in the plot mode.

The mobile phase was acetonitrile–phosphate buffer (pH 2) (0.05 *M* sodium dihydrogenphosphate adjusted to pH 2.0 with orthophosphoric acid) (46:54, v/v). To the mobile phase were added 0.5 m*M* DMOA and 4 m*M* sodium octyl sulphate. The flow-rate was 1.3 ml/min.

The linearity of the calibration graph was initially established by a number of conventional calibration runs. Quantitations were thereafter performed from a number ($n=6$) of standard samples with the same concentration. Blank standards were also run on each occasion to confirm the origin as fixed position for the calibration graph.

RESULTS AND DISCUSSION

Extraction

Extraction of haloperidol has been performed by liquid–solid extraction [11,12], but is usually performed by liquid–liquid extraction at alkaline pH with an organic phase consisting of an alkane, such as hexane or heptane, modified with some percentage of an alcohol, e.g., isoamyl alcohol. The choice of a modifier with proton-donating properties is not obvious as haloperidol contains both proton-donating and -accepting groups. In Table I, five different organic phases are compared in which the alkane is modified with both proton donors (alcohols) and a proton acceptor (diethyl ether). All five organic phases showed acceptable absolute recoveries (Table I), but the recoveries with the alcohol-modified organic phases were comparatively lower. More important, however, was that the appearance of the chromatograms for plasma blanks, extracted with these five organic phases, did not allow low-concentration determinations. Organic phase 3 gave more disturbing peaks than the other four. In this experiment, the evaporation residues were dissolved in pH 2 buffer. By carrying out a simple clean-up extraction, it was possible to remove virtually all blank disturbances. This was achieved by adding twice the volume or organic phase after redissolution in pH 2 buffer. This extraction, in which the interfering endogenous compounds are extracted back into the organic phase, leaving haloperidol and internal standard in the aqueous layer, did not significantly change the absolute recoveries (Table I). The most favourable blank chromatograms resulted from organic phases 4 and 5. Owing to the higher recovery, organic phase 4 (30% diethyl ether in heptane) was preferred for further development of the method. The absolute recovery of chlorohaloperidol, the internal standard, was 95% using this organic phase. Fig. 2 shows the chromatograms for blank plasma without the clean-up extraction after evaporation and redissolution (Fig. 2a) and with the clean-up extraction step according to Fig. 1 (Fig. 2b).

A problem with organic phases 4 and 5 was a tendency for emulsion formation

TABLE I

ABSOLUTE RECOVERIES OF HALOPERIDOL FROM PLASMA AFTER EXTRACTION WITH DIFFERENT ORGANIC PHASES

No.	Organic phase	Absolute recovery of haloperidol (%)	
		Without clean-up extraction*	With clean-up extraction**
1	1.5% Isoamyl alcohol in heptane	89	90
2	4% Isopropanol in octane	86	80
3	80% Diethyl ether in hexane	98	Not determined
4	30% Diethyl ether in heptane	103	98
5	20% Diethyl ether in octane	92	92

*Redissolution of the evaporation residue in pH 2 buffer.

**Redissolution in pH 2 buffer; twice the volume of organic phase added.

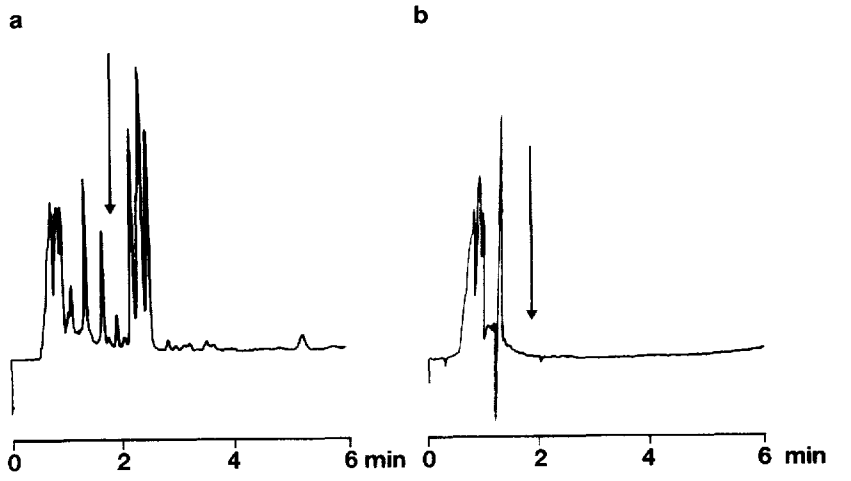

Fig. 2. Chromatograms of blank plasma: (a) without the clean-up extraction after evaporation and redissolution; (b) with clean-up extraction, i.e., the residue was dissolved in pH 2 buffer and extracted with twice the volume of organic phase, the aqueous phase was injected after brief mixing and phase separation. The arrow indicates the position of haloperidol.

to occur during the plasma extraction. By freezing the samples after the centrifugation and then including one final centrifugation, the emulsion was broken and the organic layer could be transferred into another tube for evaporation.

Detection

Some different detection techniques have been used in liquid chromatographic determinations of haloperidol in biological materials. Haloperidol has a UV absorbance maximum at 248 nm, a wavelength at which endogenous disturbances are usually manageable. UV absorbance detection around this wavelength has been used in most studies. Haloperidol also shows a high molar absorptivity at 195 nm [11,13]. At this low wavelength, problems with endogenous interferences in the chromatogram can be expected. Electrochemical detection can be used [14], but only at a high potential (+0.90 V) where most of the required selectivity is lost. Fluorescence is another detection technique with inherent selectivity. As haloperidol lacks natural fluorescence, a post-column derivatization procedure must be used. A system in which N-methylnicotinamide was used for the fluorogenic reaction has been described [19]. The reported sensitivity was not adequate but could probably be improved.

For the actual assay, UV absorbance detection at 248 nm was chosen as the alternative detection techniques did not seem to offer any advantages in terms of sensitivity and/or selectivity. Further, modern UV absorbance detectors are sensitive, reliable and uncomplicated. UV absorbance detection at 195 nm was tried but resulted in a higher limit of quantitation owing to endogenous interferences.

Chromatographic conditions

Published methods make use of 5- or 10-μm ODS particles packed in 250- or 300-m long tubes. As low detection limits are imperative for haloperidol assay, it

is essential to minimize the dilution in the column. The dilution is proportional to the retention volume and inversely proportional to the square root of the column efficiency [20]. Therefore, a 3-μm particle ODS column (100 mm $\times$ 4.6 mm I.D.) was used in this method, giving a high column efficiency and a low retention volume. An additional advantage with 3-μm particles over 5- and 10-μm particles is that higher flow-rates can be used without a decrease in column efficiency, giving shorter analysis times [21].

The mobile phase chosen was acetonitrile–phosphate buffer (pH 2) (46:54, v/v) with the addition of 0.5 m*M* DMOA and 4 m*M* sodium octyl sulphate. The amine modifier, DMOA, was added to the mobile phase in order to reduce the influence of residual silanol groups on the peak shape [22–24]. Sodium octyl sulphate was added to affect the selectivity between the endogenous compounds and haloperidol. This lipophilic anion gives a strongly retained ion pair with haloperidol which is a cation at pH 2, whereas the neutral endogenous compounds are largely unaffected. The high concentration of octyl sulphate, 4 m*M*, retains haloperidol strongly and allows an increase in the acetonitrile concentration of the mobile phase, which means that the non-ionized endogenous compounds are eluted earlier.

The loop volume was large, 265 μl, making the relative volume loss during redissolution and injection small. This large volume can be injected without a decrease in column efficiency as the sample is injected dissolved in pH 2 buffer, in which the high capacity factors for haloperidol and chlorohaloperidol give an efficient enrichment in the injection zone on the top of the column [25].

The retention times for haloperidol and chlorohaloperidol were 2.9 and 3.9 min, respectively, under the chosen conditions. Fig. 3a shows a chromatogram of a sample taken before dosing, demonstrating the absence of interfering endogenous

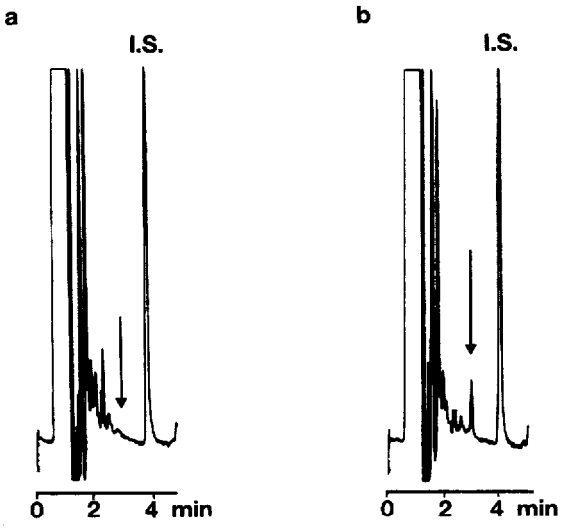

Fig. 3. Chromatograms of extracted plasma from the same subject: (a) before dose; (b) 32 h after dose; haloperidol concentration found, 2.2 nmol/l. The arrow indicates the position of haloperidol.

compounds. Fig. 3b shows a chromatogram of a sample from the same subject taken 32 h after a 5-mg dose of haloperidol.

Sensitivity and repeatability

The sensitivity of an analytical method or instrument is often expressed as the limit of detection (LOD). This term is generally defined as the smallest amount of analyte that can be detected with reasonsable certainty for a given procedure. The IUPAC definition for LOD, originally for spectrometric analysis [26], is LOD = kS_b/m, where S_b = standard deviation of the blank signal, m = the analytical sensitivity (the slope of the signal versus concentration graph) and k is a numerical factor chosen depending on the confidence level required. Usually $k = 3$ is recommended, giving a confidence level of 99.8–89% depending on the probability distribution of the blank signal [26].

The spectrometric definition of LOD has been adopted in chromatography [27], S_b now being the standard deviation of the baseline noise. The measurement of the baseline noise is not unambiguous, and different models have been proposed [27,28]. These models are only applicable, however, if the influence of the matrix is the same for all samples. If, for example, plasma samples are analysed, there are usually many more or less detectable peaks from endogenous compounds, the number and amounts varying between each sample. This results in poor repeatability at amounts near the LOD. The term "limit of detection" is therefore often inappropriate when the sensitivity of a bioanalytical method is being discussed and should be used only for measurements with no matrix interference. A more useful way to describe the sensitivity could be to give two parameters, the ILOD and the limit of quantitation (LOQ). The ILOD is the amount of analyte, dissolved in mobile phase or equivalent, giving a peak height that is three times the standard deviation of the baseline noise under the given chromatographic conditions. The LOQ is the concentration of analyte in the matrix that could be quantitated with a given, reasonable precision using the given analytical procedure. If an LOQ is claimed, the within-run variation at this concentration, or even better the between-run variation, should always be reported. The set of two parameters might also be helpful for external laboratories not being successful in reproducing the method, indicating whether the instruments or the procedures are not working properly. From the regulatory viewpoint, a similar way of reporting the sensitivity of a bioanalytical method has been proposed [29].

In this work, the ILOD was found to be about 350 fmol. To reach this value,

TABLE II

REPEATABILITY

Added concentration (nmol/l)	Determined concentration (nmol/l)	C.V. (%)	n
0.5	0.6	10.3	10
3.0	2.9	3.7	8
30.0	29.4	1.0	8

the condition of the UV absorbance detector, i.e., the flow cell and the lamp, must be very good. The limit of quantitation was 0.5 nmol/l, where the within-day variation was 10% (C.V.). This concentration corresponds to an amount injected of about 850 fmol when the volume losses during the sample work-up and injection are taken into consideration. The repeatability was studied at three concentration levels, 0.5, 3 and 30 nmol/l, and was found to be very good with regard to both accuracy and precision at the higher levels (Table II).

Chromatographic selectivity

The capacity factors for some other psychoactive drug are compared in Table III to give an appreciation of the selectivity of the chromatographic system. As

TABLE III

CAPACITY FACTORS (k') FOR SOME PSYCHOTROPIC DRUGS

Chromatographic system as in Experimental; compounds dissolved in pH 2 buffer.

Compound	k'	Compound	k'
Haloperidol	2.1	Sulpiride	0.2*
Chlorohaloperidol	3.2	Remoxipride	0.6
Chlorpromazine	3.5	Perphenazine	1.1
Thioridazine	6.0	Biperiden	2.5

*Split peak.

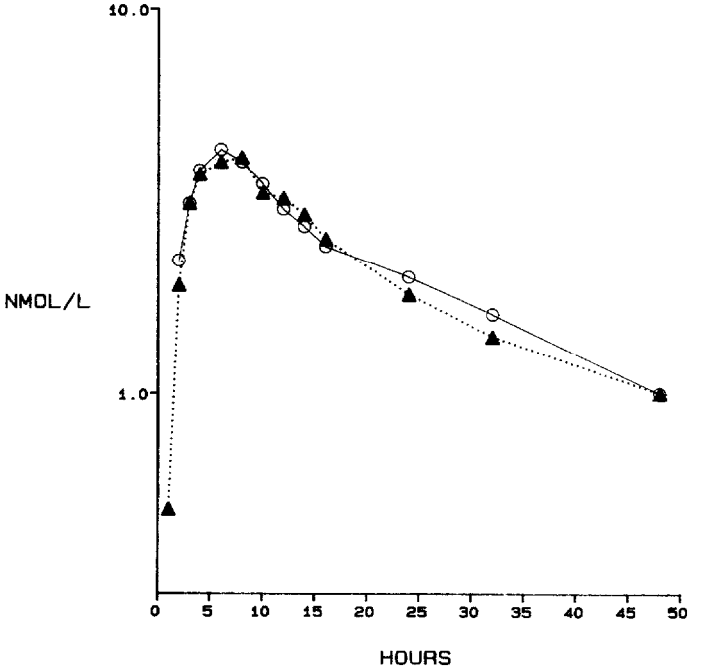

Fig. 4. Mean plasma concentrations of haloperidol for ten subjects given 5 mg as a tablet (○) and ten subjects given 5 mg as a capsule (▲).

can be seen, none of the drugs except chlorpromazine interferes with the haloperidol or chlorohaloperidol peak, but it must be emphasized that the metabolites of these drugs are also potential interferences.

When chronic therapy plasma samples were assayed, a peak interfering with haloperidol was frequently found. Owing to the side effects of haloperidol, anticholinergics are often used in combination with haloperidol in chronic therapy. Biperiden is one of the most commonly used anticholinergics, and it was found that, when present in high concentrations, it did interfere with the haloperidol peak. The interference could be minimized by reducing the acetonitrile concentration in the mobile phase from 46 to 42%.

Haloperidol is reported to be converted in man into a slightly more hydrophilic metabolite, possibly with some neuroleptic activity [30]. Unfortunately, this metabolite was not available and the possibilities of co-determine it could not be investigated.

Application

The method was developed for use in a pharmacokinetic study in which 5 mg of haloperidol were given orally, as a tablet or capsule, in a single dose to twenty healthy subjects. Fourteen samples were taken from each subject between 0 and 48 h after administration. The resulting mean plasma concentration curves are shown in Fig. 4. The maximum plasma concentrations (C_{max}) of haloperidol were between 1.9 and 10.0 nmol/l. The method has also been used for determinations of plasma concentrations of haloperidol during chronic therapy.

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