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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS QUANTITATIVE ANALYSIS OF PROPRANOLOL AND 4-HYDROXYPROPRANOLOL IN PLASMA

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SUMMARY

A simple and rapid high-pressure liquid chromatographic procedure is reported for the simultaneous quantitative determination of propranolol and 4-hydroxypropranolol in plasma. Following an extraction the samples are chromatographed on a reversed-phase column and the components in the column effluent are detected by fluorescence monitoring. Using 1-ml plasma samples propranolol and 4-hydroxypropranolol concentrations at least as low as 1 ng/ml and 5 ng/ml, respectively, can be quantitated. The reproducibility of the method is satisfactory and no interference from endogenous plasma components or other drugs has been observed. A single plasma sample can be analyzed in approximately 20 min.

INTRODUCTION

Propranolol, a β-adrenergic blocking drug, has found wide application for the treatment of cardiac arrhythmia, sinus tachycardia, angina pectoris and hypertension [1], but its use has also been suggested for a number of other conditions, including dysfunctional labour [2], migraine [3], and anxiety [4]. Evidence has accumulated which suggests that the effectiveness of propranolol in the prophylaxis or treatment of certain conditions is related to the plasma concentration of the drug. Propranolol plasma levels in the range of 50—100 ng/ml at the end of a dosing interval are usually considered to be necessary for the suppression of ventricular ectopic beats [5] and inhibition of the tachycardia of strenuous exercise [6], while plasma levels of about 30 ng/ml and above are associated with attenuation of the symptoms of angina pectoris [7, 8]. However, it is possible that any such relationship between propranolol plasma level and therapeutic response is fortuitous in view of the fact that a metab-

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olite of propranolol, namely 4-hydroxypropranolol, has been reported to be approximately equipotent as the unchanged drug in producing β-blockade [9] and in addition the available data indicates that this metabolite is formed to a much greater extent following oral administration compared to intravenous administration of propranolol [10]. It would, therefore, appear to be desirable that an analytical procedure be available for the simultaneous quantitative determination of propranolol and its active metabolite in plasma. Such a method would permit re-evaluation of the question of whether or not relationships actually exist between propranolol plasma level and therapeutic response and an assessment of the role played by 4-hydroxypropranolol in a patient's response to propranolol therapy. Additionally, the analytical method may be valuable for the routine clinical monitoring of plasma concentrations of drug and active metabolite in patients undergoing therapy with propranolol.

Several methods have been reported for the determination of propranolol in plasma. The most widely used procedure is the fluorometric method of Black et al. [11] as modified by Shand et al. [12] which uses 4 ml of plasma and can be used for propranolol concentrations above about 5 ng/ml. This method may sometimes result in high background values which may be variable from day to day in the same individual [13]. Di Salle et al. [14] have described a gas chromatographic (GC) method for the determination of propranolol in plasma which involved a lengthy and laborious sequence of extractions followed by derivitization, GC and detection with an electron capture detector. Using plasma volumes of 0.5—2 ml it was possible to detect 1—5 ng/ml [13, 14]. Another GC method has been reported [15] in which the drug was extracted from 1 ml plasma following either a single or a back extraction procedure, derivatization and GC separation of the di-trifluoroacetyl derivatives of the drug and internal standard (oxprenolol) with subsequent electron capture detection. The total analysis time for each sample using the single extraction method (which could be used for propranolol levels greater than 5 ng/ml) was 45 min, while for the analysis of lower concentrations, when it was necessary to use the back extraction work-up technique, the assay turn around time was 70 min per sample.

All of the procedures discussed above suffer from the disadvantage that they only quantitate the unchanged drug. More recently the description of a quantitative method for the simultaneous determination of propranolol and its active metabolite, 4-hydroxypropranolol, has appeared in the literature [16]. In that method 1 ml of plasma, with sodium hydrogen sulphite, internal standard and buffer added, was extracted with 10 ml of ethyl acetate for 10 min followed by centrifugation for 5 min. After transfer to another tube the ethyl acetate layer was evaporated to dryness at 60° under a stream of nitrogen. At that stage trifluoroacetylation was carried out using a 15 min reaction time. The derivatized compounds were analyzed using the technique of selective ion monitoring by injection of an aliquot of the reaction mixture into a gas chromatograph—mass spectrometer. The minimum detectable concentration of propranolol and 4-hydroxypropranolol in plasma was 1 ng/ml and 5 ng/ml, respectively.

The purpose of this paper is to describe a rapid, sensitive and specific high-

pressure liquid chromatographic (HPLC) assay for the simultaneous determination of propranolol and 4-hydroxypropranolol concentrations in plasma.

EXPERIMENTAL

Reagents and standards

Propranolol hydrochloride, 4-methylpropranolol hydrochloride and 4-hydroxypropranolol hydrochloride were kindly supplied by Ayerst Labs. (New York, N.Y., U.S.A.). Standard solutions of the salts of propranolol and 4-hydroxypropranolol in methanol were prepared for spiking of blank plasma with known amounts of the two compounds; these solutions were stored at −20° when not in use. Fresh methanolic solutions were prepared periodically. An aqueous solution of 4-methylpropranolol hydrochloride which contained 150 ng/ml was prepared and stored at 4°.

Glass-distilled methanol, ethyl acetate and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Phosphoric acid, sulphuric acid, sodium bicarbonate, sodium carbonate, and sodium hydrogen sulphite were ACS reagent grade and were obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). An 0.06% solution of phosphoric acid in distilled water was prepared (final pH 2.6) and passed through a membrane filter for subsequent use in the preparation of the HPLC mobile phase. An aqueous solution with a pH of 2.2 was prepared by dilution of sulphuric acid with distilled water. Finally, a carbonate buffer (pH 9.5) was prepared by making a solution in distilled water which contained both sodium bicarbonate (0.5 *N*) and sodium carbonate (0.5 *N*).

Other drugs which were tested for potential interference of the assay procedure had, in most cases, been donated by pharmaceutical manufacturing companies.

HPLC instrumentation and conditions

The HPLC system consisted of a model M-6000A pump for delivery of mobile phase, a model U6K injection loop and a 30-cm μBondapak alkyl phenyl column (particle size 10 μm) obtained from Waters Assoc. (Milford, Mass., U.S.A.) together with a model FS 970 fluorescence detector of Schoeffel (Westwood, N.J., U.S.A.). The output from the detector was connected to a 10-mV potentiometric 10-in. recorder (Linear Instr. Corp., Irvine, Calif., U.S.A.). Chromatography was carried out at ambient temperature.

The mobile phase for the chromatography was prepared by mixing 27 parts of acetonitrile with 73 parts of 0.06% phosphoric acid solution, and this passed through the HPLC system at a rate of 2 ml/min (approximate operating pressure was 1700 p.s.i.g.). The fluorescence detector was operated with the wavelength of excitation set at 205 nm and an emission filter (KV 340) was used to select the fluorescence emission for detection. The recorder chart speed was 8 in/h.

Plasma sample preparation

Aliquots of plasma (1 ml) were pipetted into 13 × 100 mm screw-capped

culture tubes which contained 20 mg of sodium hydrogen sulphite. After the addition of internal standard solution (0.1 ml of 150 ng/ml 4-methylpropranolol hydrochloride solution), 1 ml of carbonate buffer (pH 9.5) and 3 ml of ethyl acetate, each tube was vortexed for 1 min to promote mixing of the immiscible phases. Following centrifugation at 800 *g* for about 2 or 3 min most of the ethyl acetate layer was transferred by Pasteur pipette to a 12-ml tube which had a tapered base and contained 0.1 ml of a dilute sulphuric acid solution (pH 2.2). Each tube was closed with a screw cap and vortex mixed for 1 min followed by brief centrifugation as described above. A 50-μl aliquot of the lower aqueous phase was injected into the HPLC.

Standard curves were prepared by spiking blank pooled human plasma with propranolol hydrochloride and 4-hydroxypropranolol hydrochloride such that the concentrations of each compound in plasma were 1, 2, 5, 10, 25, 50, 100, and 150 ng/ml, followed by extraction and chromatography as described above. The peak height ratios of propranolol and 4-hydroxypropranolol to 4-methylpropranolol were plotted against the concentration of propranolol and 4-hydroxypropranolol, respectively, in order to provide standard curves. A programmable calculator (Hewlett Packard model 97) was used to fit equations to the data.

Reproducibility studies

Reproducibility studies were performed at two concentrations for both compounds by doing six replicate analyses of plasma samples which had been spiked such that the concentrations were 10 ng/ml for one series and 50 ng/ml for the second series.

Drug interference studies

A number of other drugs and drug metabolites were tested for potential interference of the assay by injecting aliquots of stock solutions of the compounds being tested into the HPLC.

RESULTS AND DISCUSSION

A number of HPLC columns and mobile phases were tested during preliminary studies, but the μBondapak alkyl phenyl column with a mobile phase of acetonitrile—0.06% phosphoric acid (27:73) was the only system which afforded suitable resolution of the unchanged drug, metabolite and internal standard from each other and endogenous components from plasma. Chromatograms resulting from the analysis of blank human plasma together with similarly treated plasma which had been spiked with propranol and 4-hydroxypropranolol (10 ng/ml of hydrochloride salt of each in plasma) are shown in Fig. 1. The compounds eluted from the reversed-phase column as symmetrical peaks with retention times of 3.9, 7.9, and 11.6 min for 4-hydroxypropranolol, propranolol and 4-methylpropranolol, respectively. No interference in blank plasma was observed at the retention times of the three compounds of interest, although a very small endogenous peak eluted after the 4-hydroxypropranolol peak. The presence of this small peak does not appear to jeopardize the analysis of the metabolite since it eluted on the tail of the 4-hydroxypropranolol

peak. Analysis of other batches of blank plasma resulted in chromatograms which were qualitatively and quantitatively similar to that which is illustrated in Fig. 1.

The standard curve for propranolol in plasma was linear ($y = 0.0549x + 0.0026$, $r = 0.9994$) over the range of plasma concentrations from 1 to 150 ng/ml (based on the hydrochloride salt). Those lower and upper limits of the standard curve correspond to 0.875 and 131 ng of propranolol base per ml of plasma, respectively. The mean response factor (peak height ratio divided by propranolol concentration) for the eight point standard curve was 0.0555; the individual response factor values fell within a narrow range from 0.0505 to 0.0580 which indicates good linearity over the concentration range studied. The signal to noise ratio at the 1 ng/ml concentration was approximately 10 to 1, which suggests that quantitation and/or detection of considerably lower concentrations would be possible.

The standard curve for 4-hydroxypropranolol hydrochloride in plasma was constructed over the concentration range of 5—150 ng/ml (4.41—132 ng of the base per ml). This standard curve had a small inflection at about 40—50 ng/ml and was apparently linear above that value. This phenomenon was reproducible in that other standard curves prepared for the metabolite behaved in the same manner. The exact reason for such a behavior is unknown. A power equation provided a good fit ($y = 0.0130x^{1.0987}$, $r = 0.9992$) to the experimental data which enabled plasma concentrations to be determined from peak height ratio information by use of the equation.

The precision of the method was determined by six replicate assays at two concentrations for both the unchanged drug and metabolite. At the 10 ng/ml level the coefficient of variation for 4-hydroxypropranolol and propranolol was 15.4% and 9.6%, respectively, while at the 50 ng/ml level the corresponding values were 8.8% and 6.0%, respectively. These reproducibility data com-

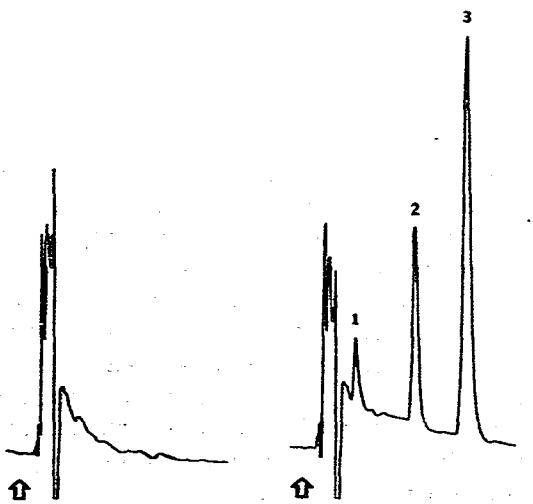

Fig. 1. Chromatograms of extracts of blank human plasma (left) and plasma spiked with 10 ng/ml of propranolol and 4-hydroxypropranolol (right). Peaks 1, 2 and 3 are 4-hydroxypropranolol, propranolol and 4-methylpropranolol, respectively. The arrow marks the point of injection.

pare favorably with those reported for the simultaneous mass fragmentographic assay for propranolol and its metabolite [16].

The metabolite, 4-hydroxypropranolol, is stable in plasma for at least one week if frozen [16]. However, in agreement with Walle et al. [16], it was found to be necessary that sodium hydrogen sulphite be added to the plasma prior to the first extraction in order to limit the extent of decomposition of the metabolite at the elevated pH values employed in that step, because stability decreases with increasing pH. In the absence of sodium hydrogen sulphite erratic results sometimes occurred, particularly at low concentrations of 4-hydroxypropranolol. Methanolic stock solutions of the metabolite stored at −20° appear to be stable for at least several days as assessed by quantitatively comparing chromatograms from freshly prepared solutions with those of older solutions. Additionally, 4-hydroxypropranolol appears to be adequately stable in the dilute sulphuric acid solution (pH 2.2) which was used for the back extraction and injection into the HPLC because no systematic trends toward decreasing peak height ratios were observed during the reproducibility studies in which all samples (six for each concentration) were extracted at the same time but injected over a period of hours.

The extraction work-up procedures in which the plasma is extracted with organic solvent followed by back extraction into a small volume of aqueous acid are relatively rapid and simple, and they serve several important functions. Firstly, the extraction sequence used provides a degree of sample clean-up because acidic and neutral compounds (endogenous and other drugs) would be separated from the compounds of interest. Secondly, the procedures used isolate propranolol, its metabolite and internal standard in a solvent which is suitable for direct injection into the HPLC, since aqueous sulphuric acid is miscible with the HPLC mobile phase. Finally, adequate concentration of the sample occurs by the back extraction into a small volume of acid so that time consuming evaporation steps are not necessary.

It should be pointed out that volumes of plasma smaller than 1 ml could be analyzed at the expense of assay sensitivity. For example, the analysis of 200-μl aliquots of plasma would permit quantitation of propranolol and 4-hydroxypropranolol levels of at least 5 and 25 ng/ml, respectively, which may be adequate for routine clinical monitoring of plasma levels.

A number of other basic drugs and drug metabolites were tested for potential interference of the assay but none of the compounds tested were found to interfere with the analysis of propranolol and its metabolite. The compounds investigated in this experiment were the other cardioactive agents and their metabolites, namely lignocaine, monoethylglycinexylidide, glycinexylidide, procainamide, N-acetylprocainamide, and quinidine. The other compounds which were also shown not to impair the usefulness of the assay were chlordiazepoxide, chlorpromazine, fluphenazine, perphenazine, prochlorperazine, trifluoperazine, and trimeprazine. In addition, chromatograms resulting from the analysis of various batches of blank plasma and plasma obtained from patients on other drug therapy showed no interfering peaks.

The method has been used for analysis of plasma samples collected from patients receiving propranolol. Fig. 2 shows chromatograms resulting from the extraction and chromatography of plasma samples obtained from patients

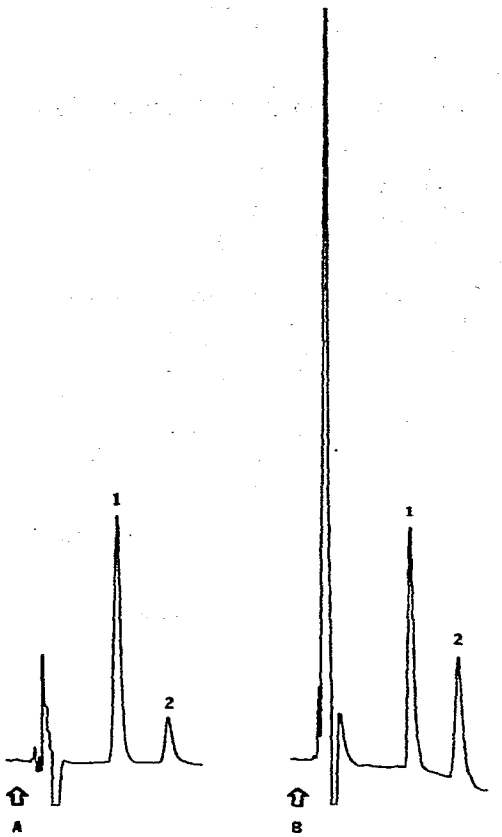

Fig. 2. Chromatograms of extracts of plasma obtained from two patients who had received propranolol intravenously. Peaks 1 and 2 are propranolol and 4-methylpropranolol, respectively. The arrow marks the point of injection. Concentration of propranolol in sample A and B is 101.0 ng/ml and 35.5 ng/ml, respectively.

who had received propranolol by the intravenous route. In these cases the 4-hydroxypropranolol peak is absent because that metabolite is not formed to any significant extent following intravenous administration of propranolol [10].

CONCLUSIONS

The HPLC method described permits the rapid simultaneous determination of plasma concentrations of propranolol and its active metabolite, 4-hydroxypropranolol. The preparation of plasma samples prior to chromatography is relatively simple and no evaporation or derivatization steps are necessary. The total turn around time for the analysis of a single sample is approximately 20 min but this would be shortened considerably when many samples are extracted simultaneously. The sensitivity and precision of the method are good and no interference is observed from plasma samples or from a number of other commonly used drugs. It is concluded that the analytical method described is suitable for routine clinical monitoring of plasma levels in patients or for use in research studies in pharmacokinetics.

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