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Note

Assay of nadolol in serum by reversed-phase high-performance liquid chromatography with fluorometric detection

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Nadolol, 2,3-*cis*-1,2,3,4-tetrahydro-5-[2-hydroxy-3-(*tert.*-butylamine)propoxy]-2,3-naphthalenediol (I), is a relatively new, non-selective, water-soluble, long-acting β-adrenergic blocking drug with a serum half-life of 16—20 h and an oral potency 1.5 times that of atenolol and propranolol [1].

Because of its large volume of distribution and 30—40% oral availability [2], therapeutic doses of I yield low serum concentrations. If the pharmacokinetics of I and intra-individual variations in response to it are to be investigated, a simple, sensitive and rapid assay method for this drug in serum is necessary.

Several methods for the assay of I have been published [3—10]. Patel et al. [3] used reversed-phase high-performance liquid chromatography (RP-HPLC) with spectrophotometric detection but this method is too insensitive for serum assay and is applicable only to bulk materials. The fluorometric determination evolved by Ivashkiv [4] is applicable to both serum and urine, but lacks accuracy and selectivity due to the extraction and derivatization steps involved and the variable background fluorescence from fluctuating serum components in the extract.

Funke et al. [5] used mass spectrometry for the determination of serum I after extraction by Ivashkiv's method [4] and gas chromatographic (GC) separation. Whilst this method is more accurate than the fluorometric one, it is even more tedious and requires the use of a mass spectrometer for detection. The use of GC with nitrogen—phosphorus detection [6] yields the required sensitivity of 10 ng/ml in serum, but still needs extraction and derivatization steps.

Schäfer-Korting and Mutschler [7] have used thin-layer chromatography

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with fluorodensitometric scanning of the plates at an excitation wavelength (λ_{ex}) of 265 nm with a 313-nm filter for fluorescence emission (em). The detectability was 5 ng per spot after extraction from 2 ml plasma.

The simplest method is that of Surmann [8] involving protein precipitation followed by RP-HPLC separation, but requires electrochemical detection which is not yet in common use. Gupta et al. [10] used fluorometric detection at λ_{ex} 265 nm and λ_{em} 305 nm, but the method does not exploit optimal excitation conditions. It also involves an initial column extraction, evaporation and redilution of 1 ml of plasma before injection onto the column for counter-ion elution with tetramethylammonium hydroxide in 17.5% acetonitrile.

Cation-exchange columns have recently [11] been utilized for HPLC determination of nadolol with fluorometric detection at λ_{ex} 205 nm and filterless emission. Using 1 ml serum and a two-stage extraction procedure a minimum detectable level of 1 ng/ml nadolol in serum was achieved.

This paper describes a rapid, extractionless method for the assay of I in serum using RP-HPLC separation and fluorometric detection without pre- or post-column derivatization with a fluorophor. The method is accurate to 5 ng/ml using only 100 μl serum.

EXPERIMENTAL

Reagents

All solvents used were spectroscopic grade and the water was purified by the Milli Q system (Millipore). All other reagents were analytical-reagent grade.

Standards

Nadolol was obtained from Squibb Labs. and 10 mg were dissolved in 50 ml mobile phase. A stock solution of 10 μg/ml was prepared from this and kept at 4°C for up to one month. The other standards were made by further dilution of this with mobile phase to yield 10, 20, 50, 100, 200 and 500 ng/ml, and 1 μg/ml I. All standards were made double the final strength for addition of 100 μl standard to 200 μl serum in the preparation step.

Internal standard

Pindolol was found to give a satisfactory retention time at pH 4.6 for internal standard purposes, but because of its poor detectability at the assay conditions a concentration of 2 μg/ml was found to be necessary for low level assays whilst 20 μg/ml was used for the higher levels, for purposes of strip chart recording.

Sample preparation

A 50-μl volume of mobile phase was added to a 100-μl serum sample in a centrifuge tube and the tube was shaken by hand for 5 sec; then 50 μl of 20% perchloric acid in mobile phase were added and the tube was again shaken by hand for 5 sec allowing the protein to precipitate. To this, 50 μl internal standard solution were added and the whole was shaken for 30 sec on a vortex mixer and then spun at 3000 *g* for 3 min on a bench top centrifuge. A 100-μl aliquot of the clear supernatant was injected onto the column.

Preparation of standards

A 50-μl volume of standard solution was added to 100 μl blank serum in a centrifuge tube, the tube was shaken by hand for 5 sec and allowed to stand at least 5 min; then 50 μl of 30% perchloric acid were added, the tube was shaken for 5 sec and then 50 μl internal standard solution were added. The tube was then shaken for 30 sec on a vortex mixer, spun at 3000 *g* for 3 min and 100 μl of the supernatant were injected onto the column.

Chromatography

The HPLC separation was performed on a Spectra Physics 8100 liquid chromatograph with an autoinjector and 100-μl sampling loop. A 250 mm × 4.5 mm stainless-steel column with 5 μm ODS (Spherisorb) packing preceded by a 40 mm × 4.5 mm guard column with the same packing was used. All connections were minimum volume.

The mobile phase was methanol—0.05 *M* ammonium dihydrogen phosphate buffer, pH 4.6 (30:70). The flow-rate was 2 ml/min and column temperature was 36°C.

Detection

A Perkin-Elmer 650-10 dual-monochromator fluorescence detector was used. The best response to I in mobile phase at pH 4.6 was at λ_{ex} = 227 nm and λ_{em} = 299 nm.

The detector output was recorded simultaneously on a Perkin-Elmer 56 strip-chart recorder and a Spectra Physics SP 4200 integrator. The concentrations of nadolol in serum were estimated on the basis of peak height ratio from a standards calibration curve.

RESULTS AND DISCUSSION

The initial investigation of the RP-HPLC retention characteristics of nadolol yielded a satisfactory baseline resolution in serum and an acceptable retention time of about 6 min with a 30% methanol, pH 4.6 mobile phase at 2 ml/min on a 25-cm 5-μm ODS column.

The UV/Vis absorption spectrum of I, in this mobile phase, showed an absorption maximum at 227 nm (Fig. 1). The fluorescence properties of I in mobile phase were thus investigated at λ_{ex} = 227 nm and showed an emission maximum at λ_{em} = 299 nm (Fig. 2). Mobile phase alone gave a flat response in the region scanned.

The response ratio of peak height standard to peak height internal standard was found to be linear over the range of three orders tested. To maintain accuracy when using a strip-chart recorder, it was found necessary to assay in two ranges: 5—250 ng/ml I with 2 μg/ml pindolol as internal standard; and 50 ng/ml—2 μg/ml I with 20 μg/ml pindolol as internal standard.

The chromatograms for blank serum and blank serum spiked with 10 and 100 ng/ml I and 2 μg/ml pindolol as internal standard, as well as a volunteer sample taken 2 h after dosing with 160 mg nadolol and with 20 μg/ml pindolol as internal standard are shown in Fig. 3. The serum concentration of nadolol after 2 h was found to be 758 ng/ml.

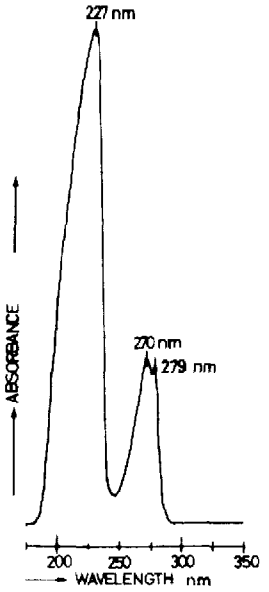

Fig. 1. UV absorption spectrum of nadolol scanned on a Beckman Acta M IV dual-beam spectrophotometer with solvent as reference. The solvent was methanol—0.05 *M* ammonium dihydrogen phosphate buffer, pH 4.6 (30:70).

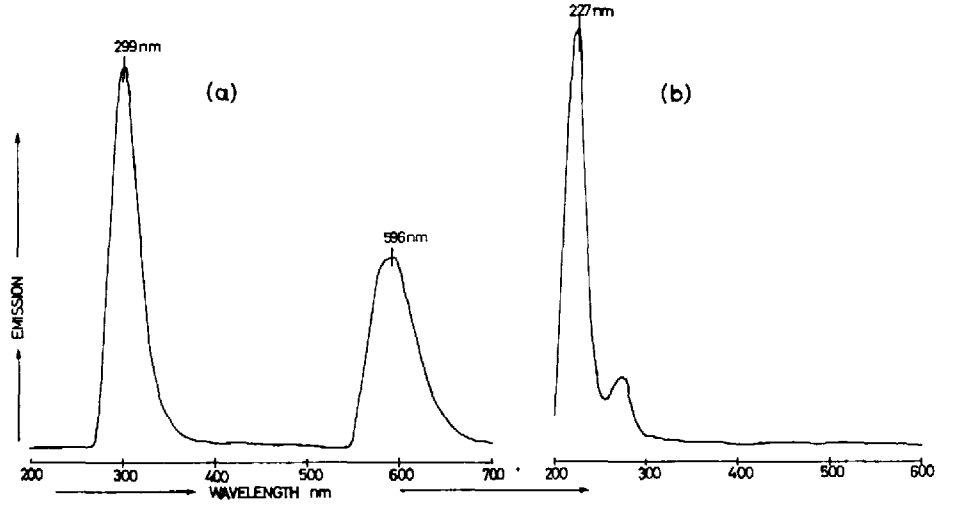

Fig. 2. Emission spectra of nadolol in mobile phase. (a) Emission spectrum with λ_{ex} constant at 227 nm and emission wavelength scanned. (b) Emission spectrum with λ_{em} constant at 299 nm and excitation wavelength scanned.

Initially the internal standard was added to the serum sample before denaturation and precipitation of the protein with perchloric acid, but a large inter-sample variance for the pindolol peak height was found. Addition of the internal standard after denaturation gave a variance similar to that for I. This was probably due to the protein binding ability of pindolol [12] and its co-precipitation with the protein. The intra-sample variance over ten injections is 1.5 and the inter-sample variance over ten assays is 4.1 at the 50 ng/ml level.

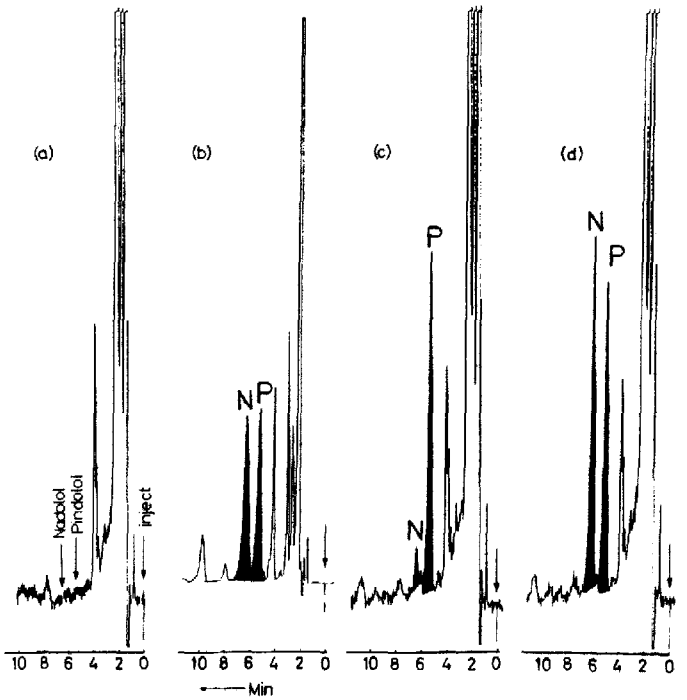

Fig. 3. Chromatograms of 100-μl serum samples. (a) Blank serum: fluorometer range = 30; recorder range = 10 mV. (b) Volunteer sample 2 h after administration of 160 mg nadolol; internal standard 20 μg/ml pindolol. Fluorometer range = 3; recorder range = 20 mV. (c and d) A 100-μg blank serum spiked with 1 ng (10 ng/ml) and 10 ng (100 ng/ml) nadolol, respectively, with 200 mg (2 μg/ml) pindolol as internal standard. Fluorometer range = 30; recorder range = 10 mV. Peaks: N = nadolol; P = pindolol.

The results obtained indicate that this simple, rapid and accurate method for the assay of nadolol in serum is sufficiently sensitive to follow the serum concentrations of I for several days after single dosage, and rapid enough for routine determination of therapeutic concentrations in serum.

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