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Note

Estimation of nadolol levels in plasma using high-performance liquid chromatography with recirculating eluent flow

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In order to measure plasma levels of nadolol, a long-acting beta-adrenoceptor antagonist drug [1], a reliable and uncomplicated assay procedure is required. Other assays have been described but these have their own problems and disadvantages. The method of Ivashkiv [2] requires several millilitres of plasma and uses a time-consuming acid back-extraction and derivatization procedure, followed by fluorometric quantitation. The assay of Patel et al. [3] does not present an extraction procedure from plasma. Others, Schäfer-Korting and Mutschler [4], Surmann [5] and Funke et al. [6] employ instrumentation which is unavailable to many analysts (fluorodensitometry, high-performance liquid chromatography (HPLC) with electrochemical detection and gas chromatography—mass spectrometry, respectively).

However, most laboratories using HPLC will have the facility of UV detection, and the advantages of simple extraction and recirculated eluent flow make the assay presented a useful tool in the analysis of plasma samples for nadolol. Table I shows the results of some recent work carried out in this department with nadolol.

MATERIALS AND METHODS

Reagents

Nadolol was supplied by Squibb (Middlesex, U.K.) and acebutolol, internal standard, by May and Baker (Dagenham, U.K.). The extraction solvent was diethyl ether anaesthetic grade and the methanol used was Pronalysis 'Ar', both obtained from May and Baker. Octane sulphonic acid was from Aldrich (Gillingham, U.K.). The water used was double-glass-distilled.

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TABLE I

PLASMA NADOLOL LEVELS OF PATIENT D.O. FOLLOWING ONCE-DAILY ADMINISTRATION OF A 80-mg DOSE

	Time (h)	Nadolol level (ng/ml)
Day 1	0	0
	2	79
	3	70
	5	30
	8	19
Day 2	0	9
	3	47
Day 3	0	16
	3	30
Day 5	0	5
	3	67
Day 6	0	12
	2	33
	3	52
	5	48
	8	40

The following precautions are taken: (1) ensure that all glassware tubes etc. are scrupulously clean, by soaking in methanol overnight; (2) each new bottle of diethyl ether is checked for purity be evaporating a 5-ml aliquot to dryness, and chromatographing a reconstituted fraction.

Chromatography

A Shimadzu LC4A liquid chromatograph with a Shimadzu SPD 2AS variable-wavelength UV detector was used with a wavelength of 220 nm and a range of 0.01 a.u.f.s. The recorder was a Perkin-Elmer Model 056. The column used was a 10-cm Hypersil, 5-μm particle size, ODS column. The eluent was methanol—water (57:43) containing 0.1% octane sulphonic acid, flow-rate 1.0 ml/min. The injector used was either a manual, Rheodyne Model 7125 or an Automatic-Shimadzu Model SIL 2AS, the injection volume used was 10 μl.

Calculation

In order to calculate the amount of nadolol in the test samples, a calibration curve of peak height ratio (nadolol/acebutolol) versus concentration of known

TABLE II

PERCENT EXTRACTION AT CONTROL CONCENTRATIONS OF NADOLOL

Added (ng/ml)	Percent extraction
20	94
50	88
100	106
200	97
Mean $\bar{X}$	96

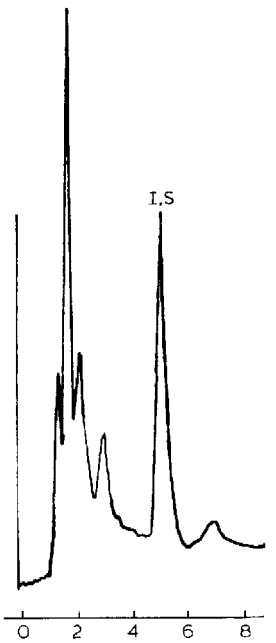
control was drawn. The graph was linear in the range 0—400 ng/ml ($r = 0.998$) and passed through the origin. From this graph the concentration of the test samples can be derived. Percent extraction at control concentrations can be seen in Table II.

Extraction

To 1.0 ml of control or test plasma, were added 100 µl of acebutolol, the internal standard. To this was added 1.0 ml of a 10 *M* sodium hydroxide solution, followed by 4.0 ml of diethyl ether, the extraction solvent. The extraction tube was then capped and very briefly vortexed, prior to mixing on a rotary mixer for 15 min. After mixing, the sample was then centrifuged for 5 min at 2000 *g* and 3.2 ml of supernatant were removed to conical-bottom glass centrifuge tubes and allowed to evaporate to dryness in a water bath at 40°C. The sample was then reconstituted (see Discussion) in 100 µl of methanol—water (57:43) and 10 µl were injected into the liquid chromatograph.

Standards

The nadolol stock solution was 1 mg/ml in a methanol—water (50:50) solution. From this stock solution was prepared a 20 µg/ml working standard, which was added to 1 ml of control plasma to give concentrations of 0, 20, 50, 100, 200 ng/ml nadolol (i.e. 0, 1, 2.5, 5, 10 µl of working standard). These control samples are treated in a similar manner to those of test patient samples.



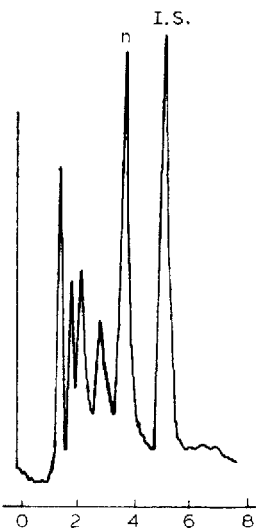


Fig. 1. Chromatogram of a blank patient sample containing internal standard (I.S.), retention time 5.1 min.

Fig. 2. Chromatogram of a patient sample containing 182 ng/ml nadolol (n), retention time 3.8 min, and internal standard (I.S.)

The acebutolol stock solution (internal standard) was 1 mg/ml in a methanol—water (50:50) solution.

From this was prepared a 2.0 μg/ml working standard. A 100-μl aliquot of this solution was added to all tubes (patient and control samples) to give an approximate concentration of 200 ng/ml in plasma.

Typical chromatograms from patient plasma are seen in Figs. 1 and 2.

RESULTS

In order to ascertain the precision and reproducibility of the assay, three sets of six tubes each containing 1.0 ml of plasma were prepared. In the first set, the concentration of spiked nadolol was 190 ng/ml; second set 95 ng/ml and third 35 ng/ml. The results obtained on analysis of each concentration were as follows: at 190 ng/ml level: coefficient of variation (C.V.) 4.3% (mean = 190.8 ng/ml, S.D. = 8.2 ng/ml); at 95 ng/ml level: C.V. 4.2% (mean = 93.2 ng/ml, S.D. = 3.9 ng/ml); at 35 ng/ml level: C.V. 6.8% (mean = 32.8 ng/ml, S.D. = 2.2 ng/ml).

DISCUSSION

Extraction procedure

Percentage extraction versus pH (15 min mixing and 5 min centrifugation); see Table III. From the results shown in Table III the best extraction was obtained using 1.0 ml of 10 *M* sodium hydroxide. Therefore, in order to prevent tube gelling during extraction, the initial vortex must be very brief and also the laboratory temperature* should not be allowed to rise unduly otherwise gelling becomes a problem. Furthermore, any increased mixing time will cause gelling. Also, reduction of mixing time to 5—10 min resulted in a recovery of only 64—83% as opposed to 96% for 15 min mixing time.

Extraction cleanliness. (a) In many patient samples a large interfering peak was present (Fig. 3) which appeared just after the nadolol peak. In general this peak was not a problem, but should it be desirable to reduce this peak size, it can be achieved by employing a neutral pH wash, i.e. after adding the internal standard solution to the test or control plasma, 2 ml of diethyl ether were added, the tube was vortexed for 10 sec and centrifuged for 1 min and then the supernatant layer aspirated off to waste. The normal extraction may then proceed by adding the sodium hydroxide solution and diethyl ether etc. Figs. 3 and 4 show one patient sample without and with a neutral pH wash, respectively. With this step, little or no nadolol is lost to waste, and there is also no detriment to the accuracy of the assay.

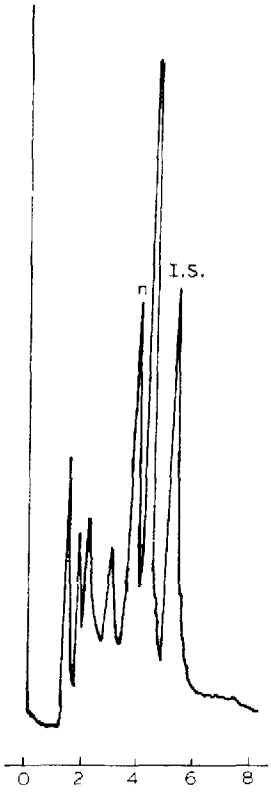
(b) One further point with regard to this peak, is that it would appear from analysing patient plasma samples, that there is a tendency for this peak to increase in magnitude and frequency of occurrence with increased sample storage time. With this point observed, the analysis of samples should be carried out as soon as possible after patient sampling. However, this factor has not

*If the laboratory temperature is very high (> 23°C) chilling of tubes in a freezer (—18°C) prior to centrifugation will alleviate problems with gelling.

TABLE III

PERCENTAGE EXTRACTION VERSUS pH (15 min MIXING AND 5 min CENTRIFUGATION)

pH	Volume of 10 *M* sodium hydroxide added (μl)	Percent extraction
1.0	—	Dirty extract
4.0	—	Dirty extract
7.0	—	8
9.0	—	21
11	—	38
14	100	53
14	500	68
14	700	78
14	800	94
14	1000	96



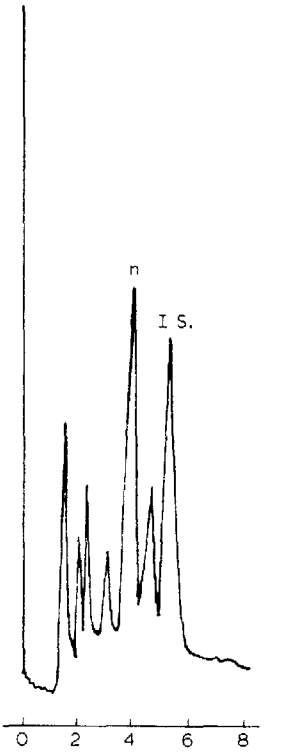


Fig. 3. Chromatogram of a patient sample containing nadolol (n) and internal standard (I.S.). A large peak appears at 4.6 min (without neutral pH diethyl ether wash).

Fig. 4. Chromatogram of a patient sample (as Fig. 3) after neutral pH diethyl ether wash.

been fully investigated as yet, and, as with all plasma sample analyses, it is good policy to analyse as soon as possible.

Recirculating eluent

The facilty of recirculated eluent is simply obtained by allowing the effluent from the detector to pass back to the eluent reservoir by means of a piece of tubing. The eluent reservoir should be fairly tightly sealed, as is usual, to prevent any evaporation of constituents which would effect the retention times of the sample components. In general, we used one new batch of 300 ml of eluent for every 100 samples which were chromatographed. In practice this meant making up this volume of eluent every three days and allowing the pump to operate continuously over this period. With this system the baseline drift was never greater than 10% full scale deflection per day and the baseline can be corrected to recorder zero once daily. If the eluent was used for greater than this period of time or number of samples, there was a tendency for the baseline noise to increase which would effect the signal-to-noise ratio of the chromatogram. However, larger volumes of eluent may allow a greater number of samples and longer eluent batch-life to be used. However, the batch of 300 ml suited our particular application needs quite adequately. As already stated the advantages of this system are many, especially with respect to labour saving where it is not necessary to make up a new batch of eluent every day and then wait for the system to equilibrate. This recirculating system has also been used very successfully for other drug analysis procedures within this department. Furthermore, this can be a considerable economy measure, as the amount of materials used is greatly reduced.

Reconstitution

Samples should not be reconstituted in eluent as nadolol is not stable in methanol—water containing octane sulphonic acid. Indeed all trace of nadolol had disappeared from the extracts within five days when reconstituted in eluent, whereas no change was seen even after eight days when reconstituted in methanol—water alone.

Column life

Although we did not use a guard column, in order to preserve the efficiency of the column, it would possibly be beneficial to employ one if greater number of samples were to be analysed.

CONCLUSION

From this we may conclude that the assay presented provides a useful method for the analysis of nadolol in patient plasma samples and allows the turnover of many samples per day in a simple and uncomplicated procedure.

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